Connective Tissue and Its Heritable Disorders

Molecular, Genetic, and Medical Aspects

second edition

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Preface

We were gratified by the reception accorded the first edition of this book, and delighted that it should have been found useful by so many. Nevertheless, given the passage of time and the intensity of advances in the field it was bound to become outdated sooner or later, and the need for a second edition became clear some time ago. With the encouragement of our colleagues, which we should, perhaps, have resisted had we appreciated at the time that to produce a second edition involved no less effort than to produce a first, and the support of John Wiley & Sons, we embarked upon our planning for this new edition some four years ago. Given the developments that occur almost weekly in some area or other of connective tissue research there is no "perfect moment" at which to produce a volume of the scope of this, but we have striven to ensure that it is as up to date as possible by giving all contributors the opportunity to add a section, "Recent Developments," to their respective chapters at the proofs stage to take account of the most recently made available information only shortly before publication.

The structure of the first edition is maintained in the second, although the content has considerably expanded. Part One, "Biology of Extracellular Matrix," addresses the morphological, physiological, and molecular biological aspects of the connective tissues and their constituents under normal conditions, while in Part Two, "Heritable Disorders of Connective Tissue," specific disorders, or groups of disorders, are extensively covered in dedicated chapters. A number of disorders have been added to those considered in the first edition, some of the less common or only recently defined of which are included in an additional chapter, "Miscellaneous Disorders," towards the end of the book. In all cases, all aspects of each disorder, whether scientific or clinical, are described in detail, and it is hoped that the reviews will prove useful to both researchers and medical practitioners alike. A new Appendix includes a chapter on collagen metabolites in body fluids, this being occasioned by the commercial availability of kits that may be diagnostically useful that were not on the market at the time of the first edition. Finally, we have been pleased to be able to add the reminiscences of Karl Piez in the collagen area to those of Victor McKusick as they relate to heritable connective tissue disorders. These two contributions not only make pleasant reading in themselves, but also help to provide a contextual foundation for the chapters that follow, and serve to emphasise how significant the progress that has been made subsequently in a relatively short space of time.

We would close by thanking all the contributors and expressing our hope that this second edition will not be found wanting.

Peter Royce
Beat Steinmann

January 2002

Acknowledgments

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The Editors would thank the Marfan Foundation of Switzerland for further financial support and encouragement. For a number of years, the Foundation has supported research into Marfan syndrome and its delineation from related disorders such as congenital contractural arachnodactyly, Ehlers-Danlos syndrome, and cutis laxa, and promoted the exchange of knowledge between experts and lay persons.

Despite our innate reticence, it would be less than fair not to acknowledge our respective families for their tolerance during the preparation of this book, given the disruption it has occasioned to their lives.

Preface to the First Edition

Although it has become something of a cliché to talk in terms of an explosion of knowledge having occurred in a particular field, it is the case, nevertheless, that there have been dramatic advances in our understanding of the extracellular matrix and its components over the past two decades or so, and these have, in turn, enhanced our perception of the molecular and genetic basis of several of the heritable disorders of connective tissue. Despite this, however, certain conditions have stubbornly resisted the yielding up of their secrets, and there is still some way to go before we can claim full comprehension of them.

In 1988, when this volume was conceived, no comprehensive text covering the heritable disorders of connective tissue had appeared since the 4th edition of McKusick's *Heritable Disorders of Connective Tissue* in 1972, then long out of print, and the time seemed appropriate to begin planning a new book which would take account of developments that had occurred since then. Conscious of our illustrious predecessor, we were gratified to receive from Victor McKusick a personal view of the development of a field to which he has contributed so greatly, and this forms an introductory chapter to the current volume.

In selecting conditions for inclusion, we experienced some difficulty in deciding just where the line should be drawn. All those diseases classically regarded as inherited disorders of connective tissue, such as osteogenesis imperfecta, the Ehlers-Danlos syndrome, Marfan syndrome, the chondrodysplasias, cutis laxa, and defects in mineralization, are covered. We have also included a number of conditions in which connective tissues are only secondarily affected, such as homocystinuria, alcaptonuria, α1-antitrypsin deficiency, Menkes disease, and prolidase deficiency. On the other hand, common diseases such as osteoporosis or arthritis, for which there may be a genetic predisposition, are not included. This is partly to avoid encountering semantic problems, and partly because of the difficulty of their phenotypes overlapping with those of mildly expressed forms of osteogenesis imperfecta and chondrodysplasia. We have also excluded rheumatic diseases that do have a genetic component, on the grounds that they are not generally regarded as inherited disorders of connective tissue *sensu strictu*. One feature which might well be considered curious is the inclusion of disorders of keratinization and the forms of epidermolysis bullosa simplex, which was on the grounds of practicality and interest, rather than strict accuracy. Despite the different embryological origins of dermis and epidermis, we feel that an interest in skin is more likely to embrace the whole tissue than merely one or other part of it. Wherever possible, throughout the book, disorders have also been identified by their MIM numbers, these being the numbers assigned to them by McKusick in his *Mendelian Inheritance in Man: Catalogs of Autosomal Dominant, Autosomal Recessive, and X-Linked Phenotypes* (popularly referred to as "McKusick Index Medicus"), published by the Johns Hopkins University Press, Baltimore and London.

When drawing up an outline for the book, it very quickly became apparent that its usefulness would be greatly enhanced by the inclusion of a detailed coverage of the composition and biology of the extracellular matrix, and a series of appropriate reviews occupy a large part of its first half. With the addition of morphological descriptions of specific connective tissues, and of the principles of medical genetics, without which the disorders cannot be appreciated, either in terms of their behavior in populations, or at a molecular level, we should like to believe that we have succeeded in compiling a text which will be of benefit not only to clinical and associated personnel, but also to basic scientists, for whom the juxtaposition of basic and clinical reviews within one set of covers might aid an enhanced awareness of the functional significance of the molecules they are studying.

Despite the appeal of the ancient maxim that to be happy in one's work one should not do too much of it, we found during the editing of this book that such an option did not arise. Indeed, the task was far more arduous than ever we could have conceived.

Nevertheless, our dealings with all those various contributors, to whom we are deeply grateful, remained pleasant, and any modest success the book may enjoy is a tribute to all their hard work and amiability throughout.

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August 1992

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Introduction, Part I

Research on Collagen in the Author's Laboratory, 1952–1982[1]

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INTRODUCTION

I was trained as a chemist, receiving a Ph.D. from Northwestern University in 1952. The school had an excellent chemistry department. Irving Klotz, a professor there, was one of my heroes because of his rigorous science and clarity of thought. My thesis had two parts: a synthetic organic chemistry problem and paper chromatography of amino acids in some biological samples. I did not discover anything important. However, I found that I was at ease in the laboratory. Biochemistry was a rudimentary science that was poised to take off. Chromatographic methods, amino acid sequencing, protein crystallography, electron microscopy, and cell culture were the leading technologies. Protein structure, nucleic acid structure, and molecular interactions were the driving concepts. Enzymology was alive and well but soon to pass its zenith. Immunology was still an art. Molecular biology studied chemistry and structure of proteins and nucleic acids but did not include gene isolation and manipulation, which were 20 years away.

I must admit that I did not appreciate the biological implications of what was to happen, but I have never regretted my chemical bias. Chemistry is the hard part of biological chemistry. What good is it to find a mutation in DNA and not understand what this does to the three-dimensional structure of the coded protein? Or worse, not to have the basis for understanding when someone else explains it?

Before graduate school, I had worked briefly as a technician in the National Institute of Dental Research (NIDR, now the National Institute of Dental and Craniofacial Research) at the National Institutes of Health (NIH). It was natural that I return. I was one of the first scientists hired there with interests beyond applied dental problems. I did not serve a postdoctoral fellowship in the usual sense but was told to do what I thought was important. With a continuing interest in chromatography of amino acids, I showed that diastereoisomers of amino acids could be separated by ion exchange chromatography [2]. I built an amino acid analyzer following the model developed by Stanford Moore and William Stein, but with a single column and the ability to analyze hydroxyproline and hydroxylysine [3]. Because two of the diastereoisomeric amino acids are hydroxyproline and hydroxylysine, and both are essentially unique to collagen, I developed an early interest in collagen. Because collagen is the major protein of skin, tendon, bone, and tooth, this interest was also consistent with the mission of NIDR.

In those early years, I also entered into some collaborative studies with Harry Eagle in another institute at NIH. He was largely responsible for making a science out of what was then the art of cell culture. We studied some aspects of amino acid metabolism in cell culture. In the course of this collaboration, we unexpectedly found that ^{14}C-labeled amino acids traveled slightly faster on ion-exchange columns than the same nonlabeled species [4]. This was important because a cut from the center of a peak would have a lower specific activity than the whole peak, giving false values in labeling studies. I learned from Harry that technology was all well and good, indeed necessary, but to apply it to a significant problem was what really counted. Perhaps this experience served the role of a postdoctoral fellowship. I did not myself ever grow a cell in culture but I learned what was involved and how to interpret data.

My interest in collagen was cemented when I met Jerome Gross in the mid-1950s. Jerry had served a postdoctoral fellowship in the laboratory of F.O. Schmitt at Massachusetts Institute of Technology. By electron microscopy, the group had made major findings about collagen to which I will return. My point here is that this early meeting was the start of a long-term scientific collaboration and friendship. I well remember telling Jerry something like, "A good problem for me would be to determine the complete structure and amino acid sequence of collagen." Sanger had recently sequenced the first protein, insulin, but of course collagen was very much larger and even its chain structure was not known.

That is what I set out to do. It was a chemical problem with many biological consequences. Among them were the heritable diseases of connective tissue, some of which were already thought to be a consequence of mutations in collagen. Thus, this chapter in this book. What I propose to do is recount events in which my laboratory played a role in reaching this goal. In some cases, I will go farther afield for the sake of completion, but this should not be considered

[1] Some of the events summarized here have been recounted in a brief review [1] written from a somewhat different point of view.

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a comprehensive review or, for that matter, a complete personal history. Contributions made by other laboratories can be found in the original references. I will stop at 1982, when I left full-time academic research. Molecular biology in its modern form had by then taken over and others are more competent than I to pick up the story there.

The reader will see that my approach was systematic. The problem was researched from the bottom up. Flashes of insight did occur but they always arose from the data, not the imagination. This may be viewed as a limitation, but it was my style. Surprises were common, as they should be in advancing into the unknown; the unexpected was what we welcomed to give us new leads.

TYPE I COLLAGEN — CHEMISTRY AND STRUCTURE, 1956

In 1956, only one collagen type was known, now type I collagen. The following was known about it at that time:

1. It had been discovered by French scientists in the early 1930s (see [5]) that collagen in some tissues could be dissolved in acid and reconstituted into fibers. This finding was later repeated and expanded by Orekhovitch and Shpikiter in Moscow [6].
2. The collagen fiber was shown by x-ray diffraction [7] and by electron microscopy [8] to be periodic, the period being about 64 nm and denoted the *D* period. Figure 1 shows a wide-angle x-ray diffraction pattern.
3. The total amino acid composition of collagen was determined by Bowes and Kenton in England [9]. The one-third mole percent glycine, the presence of about 10 mole percent each of proline and hydroxyproline, and the presence of hydroxylysine, unique to collagen, were remarkable.
4. Segment long-spacing (SLS) crystallites of collagen in which rod-like collagen molecules occurred in bundles

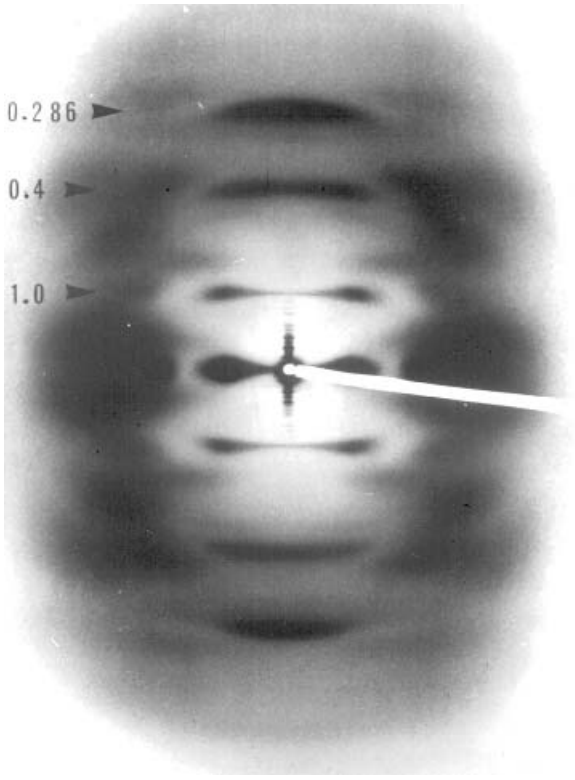

Figure 1. Wide-angle x-ray diffraction pattern of a collagen fiber. The short lines on the meridian near the center of the pattern arise from the *D* periodicity. These can be seen expanded on the medium-angle diffraction pattern in Figure 11. The layer lines at 1.0 and 0.4 nm arise from structural helices in the molecule. The reflection at 0.286 nm arises from the residue spacing. Photograph courtesy R.D.B. Fraser.

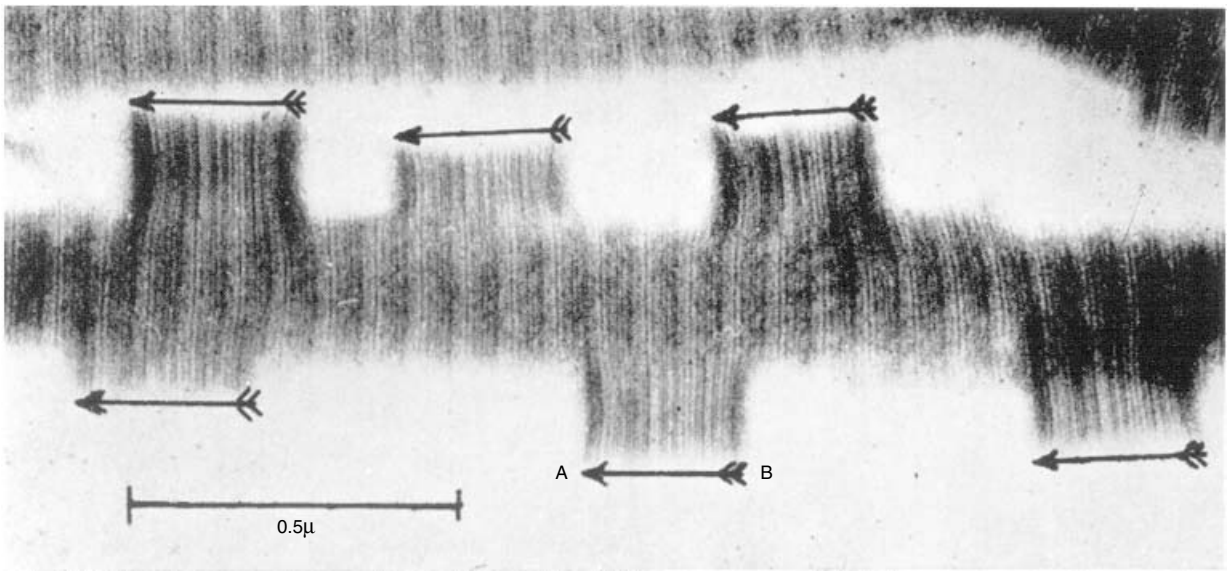

Figure 2. An electron micrograph of SLS crystallites formed on a collagen fibril. The crystallites are bundles of collagen molecules (arrows) with their ends aligned. They span about four periods in the fibril showing that molecules in the fibril must be parallel but staggered in a regular way. The A and B ends are now known to be the C- and N-terminal ends, respectively, of the three α chains comprising molecules. (From Hodge and Schmitt [11] with permission.)

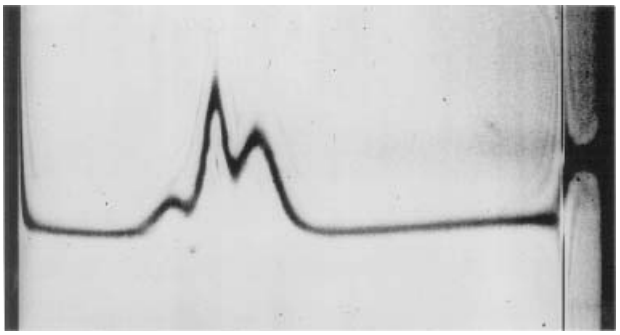

Figure 3. An ultracentrifuge pattern of denatured collagen extracted from shark skin [14]. Sedimentation right to left. Schlieren patterns formed by the α-, β-, and γ-components (right to left) can be seen. They have been shown to be single, double, and triple chains, the latter two containing cross-links.

with their ends aligned were observed by Schmitt et al. [10]. Because these bundles were about four times as long as the *D* period, it was concluded that collagen molecules must be overlapped in an ordered array in the fibril. A remarkable electron micrograph illustrating this result is shown in Figure 2. This led to the hypothesis by Gross et al. of a "tropocollagen particle" about 4*D* long and very thin [12]. Later refinement gave a value of 4.4*D*.

5. Orekhovitch and Shpikiter showed that denatured collagen contained two components of different sizes in the ultracentrifuge [13]. They called them the α- and β-components and believed them to be stoichiometrically related. Somewhat later, a still larger component, the γ-component, was reported by several groups. An ultracentrifuge pattern of these components is shown in Figure 3.
6. Rich and Crick [15] and Ramachandran and Kartha [16] independently showed that the wide-angle x-ray diffraction pattern of fibers of collagen (Fig. 1) could be explained by aggregates of molecules each a supercoiled triple helix with glycine residues in every third position in the center of the molecule. The other amino acid residues were on the outside of the molecule, where their side chains could direct the specific interactions between molecules to form fibrils and fibers. This structure is illustrated in Figure 4.
7. The first detailed physical chemical characterization of collagen in solution was done by Boedtker and Doty [17]. The dimensions of the molecule were correctly found to be 300 × 1.4 nm and the molecular weight was 345,000, somewhat higher than the correct value of about 300,000. These results were entirely consistent with the proposed "tropocollagen particle" of Schmitt and Gross. For the first time, the collagen molecule and its relationship to the fibril were defined.

CHAIN STRUCTURE OF THE COLLAGEN MOLECULE

My entry into collagen research, beginning in the middle 1950s, was to determine the chain structure of the type I molecule. Although x-ray diffraction indicated three polypeptide chains, this information applies only to local structure because the coherence of diffraction patterns of collagen is only about 10–20 nm whereas the molecule is about 300 nm long. Whether the chains were parallel or antiparallel, all or partly triple helix, folded or straight, and identical or nonidentical was not known. Complicating the picture was the presence of cross-linked components in all extractable collagens. The chemistry and location of these cross-links were unknown.

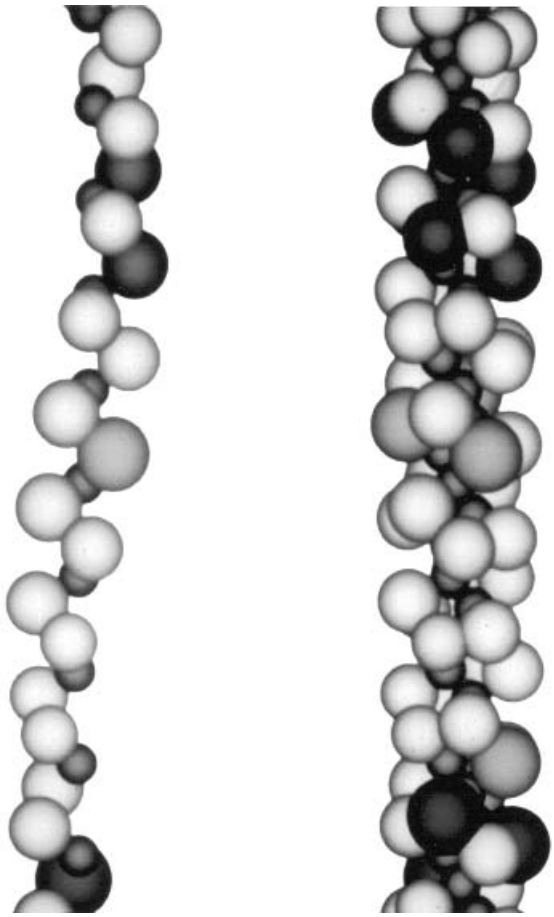

Figure 4. Ball models of segments of a collagen molecule (right) and of a single α chain (left) taken from the molecule. The small balls in the center of the molecule represent glycine residues occurring in every third position in each α chain. The side chains of other amino acid residues point outward. (Computer-drawn model by Benes Trus.)

The first surprise was that the ratio of α- to β-components varied with the type of sample, a result published with George Martin[2] and Jerome Gross[3] [18]. They were not stoichiometric subunits of the molecule as previously thought. Denatured salt-extracted collagen from vertebrate skin contained very little β-component whereas acid-extracted collagen contained a large amount. It was known

[2] George Martin had come into the laboratory, not under my auspices, somewhat before this time and we had begun to collaborate. He provided skills in cell culture and animal studies that I lacked. We have continued to collaborate, on and off, over the years.

[3] Jerome Gross provided major stimulus to me as a beginning scientist and helped immeasurably in the interpretation of data.

that salt-extracted collagen was recently synthesized whereas, acid-extracted collagen was older. In some samples, γ-components were found (Fig. 3). These results suggested that α-components were the basic polypeptide chains of the collagen molecule — now referred to as α chains — and that β- and γ-components were two and three, respectively, cross-linked α chains.

At that point we needed a way to separate α chains. Peterson and Sober [19] had recently invented the modified cellulose exchange resins that could be used to chromatograph proteins.[4] As these materials were not yet available commercially, we made our own carboxymethyl (CM)-cellulose and found that denatured collagen could be chromatographed [18,20].[5] To keep the components from reassociating, we kept the columns at 40°C. We monitored the effluent by ultraviolet absorption and analyzed fractions by ultracentrifugation and amino acid composition. We found that denatured vertebrate skin collagen from several species and collagen from fish swim bladder contained two types of α chains, named α1 and α2, in a 2:1 ratio, and two types of dimers of α chains, named β_{11} and β_{12}, the subscripts indicating the type(s) of α chain making up the dimer. An example of a CM-cellulose chromatogram of denatured collagen is shown in Figure 5.

The finding of two types of α chains in a 2:1 ratio was a major surprise. The unsymmetrical nature of type I collagen was bothersome. From time to time, we thought we had data for three different chains, but that turned out not to be true.

The molecular weights of the α chains were found to be about 95,000 and of the β-components twice that [21].[6] These values were consistent with three α chains, or one α chain and one β-component, per molecule in extractable collagen. The simplest model for the collagen molecule was then two identical chains and one similar but nonidentical chain, each extending the full length of the molecule. Of course, x-ray diffraction had shown that each chain was a helix with their axes probably parallel and supercoiled. This was shown by Andrew Kang[7] and Yutaka Nagai[8] to be correct [22] by cleaving the native molecule with the vertebrate collagenase of Gross and Lapière [23], separating the still helical one-quarter and three-quarter pieces, and characterizing the α chain fragments in them. The one-quarter piece of the molecule contained the C-terminal one-quarter of two α1 chains and one α2 chain and the three-quarter piece of the molecule contained the N-terminal three-quarters of the same chains. Molecular weights were as predicted by the model.

This story is not complete without mentioning the later discovery that collagen is synthesized and secreted as a precursor protein with propeptides at the N- and C-terminal ends. This was shown independently in the laboratories of Paul Bornstein [24] and George Martin [25].

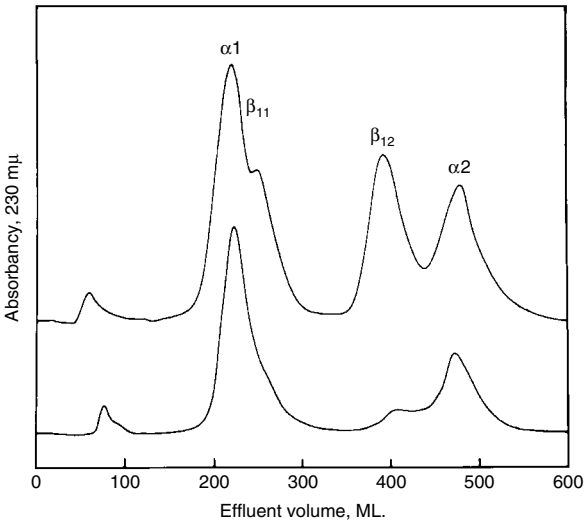

Figure 5. Chromatograms of denatured collagen fractionated on CM-cellulose at 40°C. The acid-extracted collagen (top) can be seen to have a greater proportion of β-components than the salt-extracted collagen (bottom). (Chromatograms by K.A. Piez.)

COLLAGEN CROSS-LINKS

With the chain structure of collagen known, we turned our attention to the characterization of cross-links in collagen, specifically in the β-components that we could isolate. Three discoveries came together to make this possible. First, Gross and Witkop [26] introduced the technique of cyanogen bromide (CNBr) cleavage of polypeptide chains. This chemical method cleaves at methionine residues yielding peptides with C-terminal homoserine the size of the distance between methionine residues in the original chain. Because the α chains of mammalian collagens contain 8–10 methionine residues, the number of peptides to be separated was manageable using ion-exchange chromatography.

The second discovery, made by Paul Gallop and his colleagues [27], was that collagen contained aldehydes. Although they did not correctly identify or locate them, they devised the methods to assay them. The third discovery, made in the laboratory of Miles Partridge [28], was that elastin contained pyridinium cross-links that, from their chemistry, probably arose from the condensation of four lysine residues through aldehyde intermediates, as shown

[4]The availability of this technology is an example of the early NIH where openness was the rule and collaboration easily obtained. Under modern pressures, that attitude has been largely lost.

[5]The first commercial samples of CM-cellulose were unreliable, which gave us an advantage over our competitors who could not repeat our results for some time. I have been reminded of this by Klaus Kühn whose group at the Max Planck Institut für Biochemie in München was making major contributions to our knowledge of collagen during this same period. At one point when I visited Klaus, he and Jürgen Engel brought out copies of my publications and grilled me on every fine point. I believe I passed that test.

[6]Marc Lewis and I taught ourselves ultracentrifugation with some help from colleagues such as William Carroll. Marc was much better at it than I, and became one of its strongest proponents.

[7]Andrew Kang was a physician choosing NIH over service in the armed forces. I remember Andy stating on his application that the only laboratory in NIDR that he would consider joining was mine. Many bright young physicians came to NIH as research associates during this period and went on to become leaders in medical science and education.

[8]Yutaka Nagai had come from a postdoctoral fellowship in Jerome Gross' laboratory where he had participated in collagenase characterization. He returned to Japan where he became a well-known professor. He recently retired but is active as a consultant.

later in my laboratory [29].[9] So we were on the lookout for lysine-derived aldehydes and cross-links arising from them.

At this point, Paul Bornstein came into the laboratory.[10] We devised chromatographic methods to separate the CNBr peptides of the α1 and α2 chains and the β_{12}-component of extracted rat skin collagen and examined them for aldehydes.[11] From the α1 chain, we found a small peptide in two forms, one containing a lysine residue and the other a simple aldehyde but no lysine. A similar situation existed in the α2 chain. From β_{12} these peptides were not found, but another that contained an α, β-unsaturated aldehyde was identified. It had the amino acid composition of the sum of the two aldehyde-containing peptides from the α1 and α2 chains. Schematics of the crucial chromatograms are reproduced in Figure 6. It could be deduced that the cross-link was the aldol condensation product of two lysine-derived aldehydes from the α1 and α2 chains [30,31]. The lysine-derived aldehyde has the chemical name δ-semialdehyde of α-aminoadipic acid. It has been given the trivial name allysine. Its hydroxylated counterpart, arising from hydroxylysine and named hydroxyallysine, also participates in cross-links.

This was the first cross-link identified in collagen.[12] Of course, the collagen fraction that we studied represented a small percentage of the total collagen and was not representative of mature collagen. Later studies from the laboratories of Bailey and Tanzer showed that aldimine cross-links and cross-links derived from other condensations are also present in more mature collagen [32,33]. Allysine and hydroxyallysine seem to be present only in the terminal nontriplet regions of the molecule. They condense with lysine, hydroxylysine, and histidine residues at sites in the helical region of adjacent molecules. More than two chains may be involved. It can be imagined that, in highly cross-linked collagens, multiple condensations tie together all the molecules in a fibril at periodic sites along the fibril.

The identification of aldehydes in collagen, and earlier evidence that collagen from lathyritic animals was deficient in cross-links, provided the basis for understanding this disease [31,34]. Lathyrism, characterized by fragile connective tissues, occurs in animals that eat the sweat pea, *Lathyrus odoratus*, and in experimental animals fed β-aminopropionitrile, the active component. This and related compounds are inhibitors of the enzyme lysyl oxidase, which converts lysine and hydroxylysine in peptide linkage to allysine and hydroxyallysine.

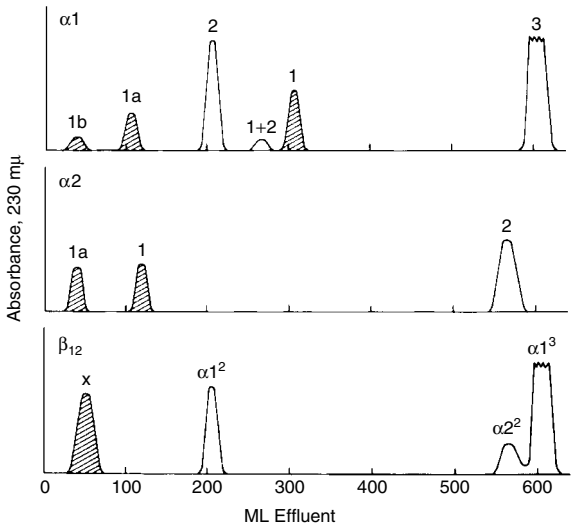

Figure 6. Schematics of the chromatograms on phosphocellulose of the small CNBr peptides from the α1, α2, and β_{12} chains of type I collagen [31]. Peaks 1 from α1 and α2 are lysine-containing peptides from the N-terminal ends of the α chains. Peaks 1a are aldehyde-containing peptides without lysine derived from peaks 1. Peak 1b is a small amount of peak 1a that did not bind to the column. Peak X on the β_{12} chromatogram is the sum of peaks 1a from α1 and α2 and contains an unsaturated aldol cross-link. The nonshaded peaks are other CNBr peptides not involved in cross-linking.

COLLAGEN TYPES

The question of whether the collagen from skin, tendon, and bone was the only collagen or whether there were other collagens derived from different genes was a matter of discussion in my laboratory and, I am sure, other laboratories. Edward J. Miller, in my laboratory but working on his own, gave the question a biological twist. If there are other collagens, they must have different biological roles. So the place to look is in a tissue with a unique function. He chose cartilage and found the first new collagen, designated type II [35]. It had only one kind of α chain, named α1(II), which had a different amino acid composition, and thus a different gene, from the chains of type I collagen, now known as α1(I) and α2(I).[13]

At about the same time, Erwin Epstein, working with me, did a CNBr digestion of whole skin. He isolated a CNBr peptide with a triplet sequence but not from type I or type II collagen [36]. We named the new collagen type III. When isolated later, it was found to have three identical α chains, named α1(III).

The question about different collagens was now answered. Types I, II, and III were shown to be fibrillar collagens with

[9]The British group was somewhat dismayed when, in a matter of weeks, we did the obvious experiment that showed that lysine was indeed the precursor of the cross-links in elastin. It just happened that we had the systems in place to do this. Elastin is an interesting protein and from time to time we did some experiments on it when we could think of an interesting one.

[10]Paul Bornstein had come into my laboratory as a physician on leave from his residency, having been deserted by Chris Anfinsen who had gone to Harvard for a year. Paul expressed ignorance about biochemistry and how to do research. I like to think I replied, "What is known you can read about. What is unknown you will participate in discovering." I was less eloquent, but that is what I meant and Paul did just that.

[11]I urged that we look first in the large CNBr peptides because statistically it was more likely that the cross-links would be there. Paul Bornstein, who was doing most of the experiments, opted for the small peptides because they would be easier to characterize if the cross-links were there. He was right even if illogical!

[12]During this period, there was considerable controversy among the several groups working on collagen cross-links. In the end, it was clear that everyone had made important contributions to the solution of a difficult chemical problem. However, there has been some confusion about credit, which I hope is clarified here.

[13]Many people were not happy about this nomenclature. Several informal committees were appointed at various times to come up with a better nomenclature. However, none was ever devised and the original has persisted.

similar structures. Other collagens have been found that are quite different in structure, as detailed elsewhere in this book. There are now about 19 collagens plus several other proteins with some triple-helical structure that might have been named collagens if they had been discovered by collagen chemists.

AMINO ACID SEQUENCING OF COLLAGEN

With the ability to isolate α chains and fragment them into CNBr peptides, it was possible to systematically sequence the various α chains. Some of this began in my laboratory [37] but was limited largely to the N-terminal nonhelical end where we had found the cross-link. The work was continued elsewhere by Paul Bornstein, Andrew Kang (with Jerome Gross), and the group in Klaus Kühn's laboratory. Eventually, it was possible to assemble a complete sequence of the α1(I) chain [38], although not all of it was derived from the same species.

About this time, a group from my laboratory made a trip to Johns Hopkins to talk to Victor McKusick. We felt that we were close to looking for mutations in collagen that would explain some of the heritable diseases of connective tissue. My laboratory never did this but we had helped to set the stage. This book makes it evident that this effort has gone far beyond the original dream.

During this period our interactions with Israeli scientists were a major factor in our progress.[14] Trips back and forth and collaborative projects were common. In 1971, Wolfie Traub and I published a review that brought into focus the current research at a critical time [39].

Of course, we were on the brink of the era of molecular biology. Manual and even automatic sequencing of polypeptide chains was about as tedious a task as one can imagine. It beggars the mind to think how we have gone from that technology to the DNA sequencing of today that has now given us the whole human genome.

PHYSICAL CHEMICAL STUDIES

Some people have thought that I was basically a physical chemist. That is not true, although the technology appealed to me and I have frequently used it, as has already been noted. One such study arose when Merry Sherman came into my laboratory about 1967. She brought with her a partially completed problem on mechanochemistry begun with Aharon Katchalsky[15] at the Weizmann Institute in Israel. Aharon had built a machine in which a cross-linked collagen tape was wound over several wheels and passed through two baths, one containing water and the other concentrated lithium bromide (LiBr). On entering the LiBr, which is a strong denaturing agent, the collagen triple helix would collapse and a section of tape would shrink. On reaching the water, the LiBr would be washed out, helix would reform, and the tape would return to its original length. Properly connected in the machine, the reversible shrinkage and stretching would make the wheels move continuously. To a casual observer, it would seem to be a perpetual motion machine, but of course the mechanical force was derived from the chemical energy of dilution of the LiBr as it was carried by the tape bound to denatured collagen from one bath to the other. It was a mechanochemical engine. Merry's task was to show that when collagen denatured it bound LiBr and released it when force was used to restore the triple helix. It was a neat problem successfully completed with complex thermodynamics that I did not fully understand. However, it made me a proud coauthor with Aharon and Merry [40].

Merry and I went on to study helix formation in a small CNBr peptide from α1(I). It was shown that this process could be treated as a reversible third-order chemical reaction [41,42] as opposed to the phase transition applied to the melting of whole collagen. The application of this result to biological questions is perhaps not obvious, but it brought a great deal of understanding to the problem in general.

FIBRIL STRUCTURE OF COLLAGEN

The period from about 1960 to 1970 was highly productive and exciting. It seemed as though each experiment suggested another in a never-ending stream — and most of them worked. It was the scientific method (hypothesis, experiment, proof) at its best. Having reached the original goal of characterization of the type I collagen molecule, it was natural to keep going and study fibril structure and assembly. To do this, I felt I needed to know more about the three-dimensional structure of proteins and the associated technologies.[16]

To accomplish this, I spent the academic year 1972–1973 in the laboratory of David Phillips (now Sir David Phillips) in Oxford, England, working with Andrew Miller and his students.[17] Andrew was analyzing in more detail than was previously possible the packing of collagen molecules in the fibril. He had been able to obtain improved x-ray diffraction patterns. I will return to these studies later. There were also extensive computational facilities and electron microscopy available. Most important, there were people who thought about and worked with proteins in three dimensions.

I had the composite sequence of the α1(I) chain with me. We discussed within Andrew's group the idea that there must be some evidence in the sequence of the *D* stagger between molecules. That is, because side chains interact between molecules to determine the *D* stagger, there must be complementarity (as opposed to homology, which was absent). We did what amounted to an autocorrelation analysis using, in one case, the sequence of charged amino acids and, in another, the sequence of amino acids with large hydrophobic side chains. These are the side chains that would interact. We called it an interaction analysis. The sequence

[14] I had many friends and colleagues at the Weizmann Institute in Rehovot and at the Hadassah Medical Center in Jerusalem, including Wolfie Traub, Ephraim Katchalsky, Shmuel Shoshan, and Jacob Menczel. These kinds of relationships are what make science a personal as well as an intellectual experience. My experiences in Israel could be the subject of another chapter.

[15] Aharon Katchalsky (later Katzir) was a well-known physical chemist specializing in irreversible thermodynamics. He was killed several years later in a random attack by Japanese terrorists at the airport near Tel Aviv. His brother, Ephraim Katchalsky-Katzir, was an equally well-known chemist (proteins), who later became president of Israel.

[16] I toyed with the idea of going to a laboratory where I could learn the techniques of cell and molecular biology. I decided that three-dimensional protein structure was closer to what I already knew and better for an old dog learning new tricks. I often wonder what would have happened if I had chosen the other course.

[17] This year was one of the most pleasant in my life. I need not state that scientists in the United Kingdom have every bit the same intellectual drive and ability as anywhere else, but they are more relaxed and do fewer experiments. The secret is that with more thoughtful planning, their success rate is higher than elsewhere.

was compared with itself as one chain was slid past itself one residue at a time. Wherever an amino acid of one charge on one chain was close to an amino acid of opposite charge on the other, a positive score was recorded and summed to give a total score for that stagger. The same was done for hydrophobic amino acids, but of course they are only of one kind. If at any stagger a maximum in the total score was seen, this would be evidence of complementarity and perhaps indicative of a real set of interactions.

The result is shown in Figure 7 [38].[18] There were indeed maxima in both analyses at staggers of 234, 468, 702, and 936 residues that were more prominent in the sum of the two curves. The *D* stagger must then be 234 amino acid residues. Because the residue spacing along the axis of the collagen fiber is known to be 0.286 nm and 0.286 × 234 is 66.9 nm, 234 amino acid residues is entirely consistent with x-ray and electron micrographic measurements of *D* but expressed in structural units. It was noted, and can be seen in Figure 7, that within the hydrophobic interaction curve there was additional structure. Analysis showed this to be based on an approximate 2 *D*/11 (42.5 residues) repeat of hydrophobic residues in the α1(I) sequence but its relationship to structure was unknown at that time. I will return to this surprising point later. This one-dimensional solution of fibril structure allowed us to reconstruct from the sequence the band pattern of the collagen fibril seen by electron microscopy [43]. This is illustrated in Figure 8.

At Oxford, we also prepared and analyzed electron micrographs of some polymorphic collagen fibrils prepared *in vitro*. These included centrosymmetric fibrils, diagonally banded fibrils, and checkerboard fibrils [43]. The result was that these fibrils could all be explained if there were a *D* periodic filamentous substructure arranged in various ways. The limit diameter could be calculated as about 4.0 nm. It has also been clear from electron micrographs of normal collagen fibrils that they appear to be composed of narrow filaments or microfibrils, as can be seen in Figure 9.

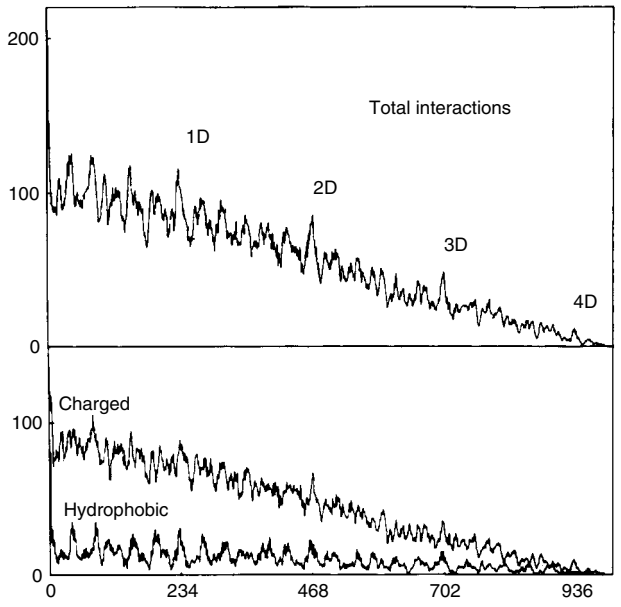

Figure 7. An interaction analysis of charged and hydrophobic side chains in the α1(I) chain [38]. The y-axis is the interaction score and the x-axis is the stagger. See text for explanation.

[18]It was the practice in many British laboratories in their search for equality to list authors alphabetically. I complained about this to David Phillips, feeling that I had been the major contributor to this project and should be first author. He was polite but obdurate. In any case, the group was very much a congenial team.

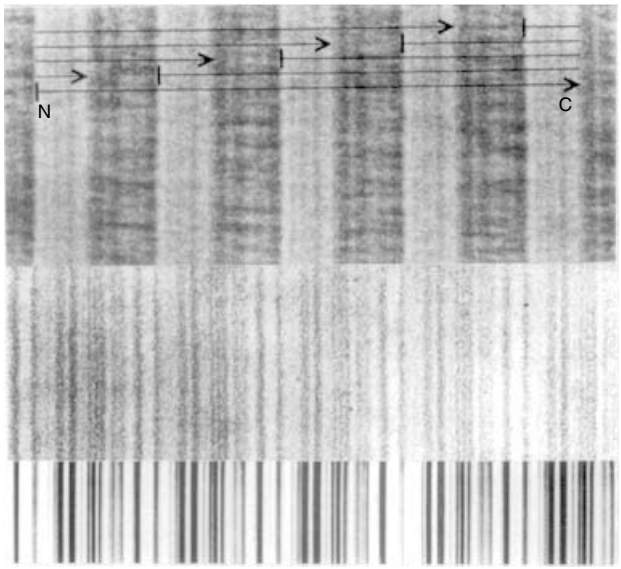

Figure 8. Reconstruction of the staining pattern seen in electron micrographs of a collagen fibril from the amino acid sequence using $D = 234$ amino acid residues [43]. **Top**: An electron micrograph of a fibril negatively stained with phosphotungstic acid. Superimposed is a representation of *D*-staggered collagen molecules showing gap and overlap regions. The gap regions take up more stain (darker) than the overlap regions and there is some positive staining. **Middle**: An electron micrograph of a fibril positively stained with phosphotungstic acid; only charged residues retain stain. **Bottom**: A print of charged residues in the sequence overlapped photographically by *D*. The correspondence among the patterns can best be seen by tilting the page.

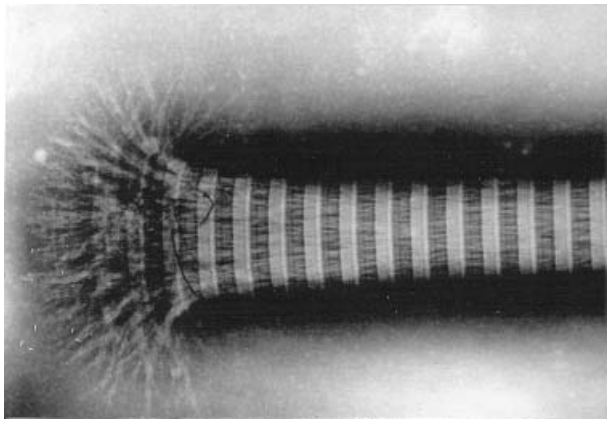

Figure 9. An electron micrograph of a fibril teased from rat tail tendon and cut across in a microtome. The fibril has flared at the end revealing a filamentous substructure that is continuous with the normal *D* periodicity. (Micrograph by K.A. Piez.)

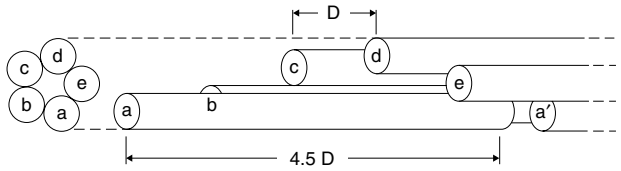

Figure 10. The Smith microfibril [44]. Collagen molecules are represented by rods much shortened for representation. Each molecule is staggered by 1 *D* and rotated by one-fifth of a circle so that the helix is closed after five molecules and the sixth molecule is in line with the first with a gap.

A possible explanation of these microfibrils lies in the structure proposed by Smith [44]. He reasoned that in as much as collagen molecules are 4.4 *D* long, if staggered by 1 *D* and rotated by one-fifth of a circle, they could form a closed five-fold helix of equivalent molecules. Overlap regions would have a cross-section of five molecules while gap regions had four. Such a helix is illustrated in Figure 10. This helix has a calculated diameter of about 4.0 nm and could be the microfibril deduced from electron micrographic evidence. That is, a fibril is a bundle of microfibrils, the number determining the diameter. I will return to this model in a moment, but must keep to chronological order to make clear the reasoning involved.

On returning to the NIH, my research focused first on *in vitro* fibril formation. The identification of an early intermediate was the goal. One was found both by light scattering [45] and by electron microscopy [46]. It contained up to about 100 molecules, but its structure was never determined in detail. It may be that it was not a true intermediate but rather a by-product of *in vitro* conditions. In any case, these studies were not very revealing.

When Benes Trus, an x-ray crystallographer, model builder, and computer scientist, came into the laboratory in about 1975, we returned to the sequence analysis begun in Oxford. That was a one-dimensional analysis. With both the α1(I) and α2(I) sequences now available, we could build molecules in the computer in three dimensions. We also reevaluated conclusions made from the x-ray diffraction data that others had obtained.

Our approach was to evaluate, by interaction curves and Fourier analysis, edges along a collagen molecule that could interact with edges on adjacent molecules. To do this, the supercoil pitch of the α chains had to be known. This can be understood if it is recognized that the amino acids along an edge arise from amino acids in the linear sequence of the α chains spaced apart by the number of residues in the supercoil. X-ray diffraction data at that time gave a value of the supercoil pitch in the range 30–40 residues. We tried many values and found that at 39 residues (*D*/6) the molecule became two-sided. One side was dominated by alternating charged and hydrophobic patches, each kind spaced *D*/6 apart. The other side was dominated by hydrophobic patches *D*/11 apart [47]. Thus, the earlier observation of a 2 *D*/11 repeat in the α1(I) sequence was apparently related to intermicrofibrillar interactions.

This could hardly be a coincidence, or could it? In any case, we proposed that this data fit a microfibril that was a right-handed helix of molecules given a left-handed twist. The *D*/6 sides of molecules would face into the center of the microfibril and define intramicrofibrillar interactions, whereas the *D*/11 hydrophobic sides would be on the outside where interactions between microfibrils would occur. If the twist was 30 *D*/11, the microfibril would have six-fold screw symmetry that would be consistent with hexagonal packing of microfibrils as would be expected [48]. In the microfibril model, there are, then, three levels of coiling, the three-residue left-handed helix of individual α chains, the 39-residue right-handed supercoil of the three α chains in the molecule, and the proposed 30 *D*/11 (638-residue) left-handed twist of the microfibril. These coils have opposite handedness — left, right, left — which is how a rope is made to prevent uncoiling. There may be a fourth level of coiling of microfibrils in the fibril.

The x-ray diffraction data were being refined by Andrew Miller and his colleagues [49,50] in Oxford and Grenoble and by Fraser and MacRae [51] in Melbourne. An example of the kind of diffraction patterns that were obtained is shown in Figure 11. The crystal model proposed by them consisted of molecules in cross-section placed on a near-hexagonal lattice in which the hexagonal planes were spaced by slightly different values. Molecules were related axially by *D* staggers and were straight-tilted by about 5°. The unit cell contained one collagen molecule.

This model certainly fit the diffraction data, but it had no provision for any substructure such as the microfibril that other data favored.[19] I felt that this was a fatal flaw. Furthermore, the supercoil pitch of α chains required by the diffraction data was 30 residues rather than the 39 residues suggested by sequence analysis. Remembering that the data on x-ray diffraction patterns show that coherence is only 10–20 nm — that is, regions of crystalline order may not be large — we proposed what we called a compressed microfibril model [48,52]. In this model, portions of molecules, specifically the overlap regions, were induced to be crystalline as a result of lateral compression arising from stretching or internal crystal energy. A near-hexagonal lattice of molecules in cross-section would be obtained, with systematic displacements explaining intensities of reflections on the x-ray diffraction pattern. Furthermore, the supercoil helix could be distorted by the compression to force ridges into grooves and give a value in crystalline regions of 30 residues but in a relaxed fibril would average 39 residues. The induced order would then be compensated for in the disordered gap regions of the microfibril. The model that we proposed is shown in Figure 12. It has the same unit cell as the refined model of Miller and Fraser [49–51] and fits the diffraction data at least as well. A final feature is that nuclear magnetic resonance spectroscopy (NMR) of relaxed fibrils has shown that, contrary to crystalline order, molecules are in continual motion rotating segmentally about their axes by as much as 30° [53].

We could not be sure that this model was correct, but it was consistent with all the data obtained by electron microscopy, x-ray diffraction, and sequence analysis. Today, 20 years later, there is very little additional data and no better model.

EPILOG

In 1982, I left the NIH and academic research and entered the biotechnology world, working first at Collagen

[19] It is my experience that x-ray crystallographers, with some notable exceptions, treat their data and models very narrowly. Protein crystals are artifacts, including crystalline order in collagen fibers, and may distort reality.

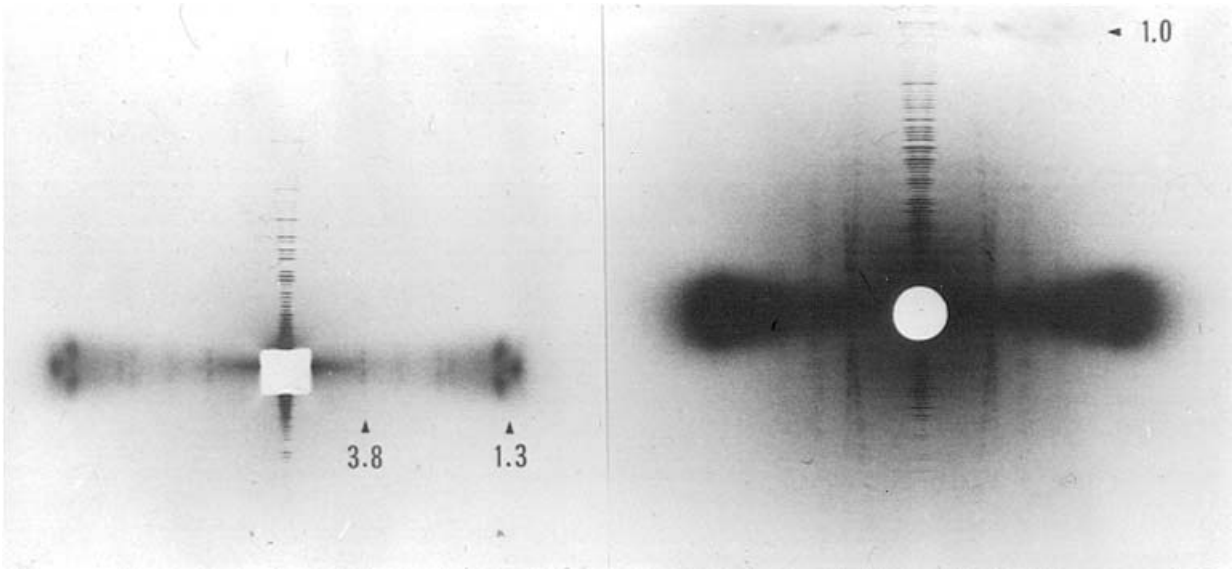

Figure 11. Medium-angle x-ray diffraction patterns of slightly stretched rat tail tendon collagen. **Left**: Unstained. **Right**: Lightly stained with phosphotungstic acid. The reflections along the meridian relate to molecular features sampled by the *D* periodicity. Reflections on and near the equator relate to lateral packing of molecules. Row lines can be seen to be split and sampled on the 1-nm layer line. (Photographs courtesy R.D.B. Fraser.)

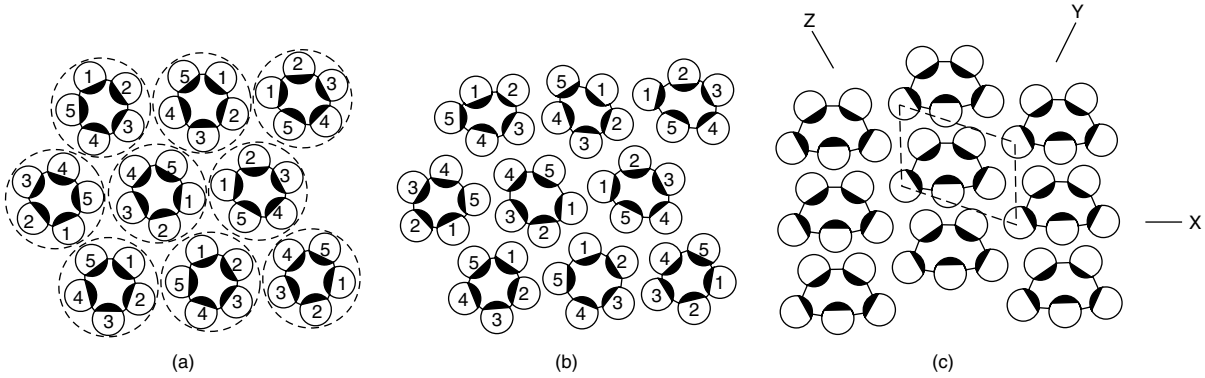

Figure 12. Proposed molecular packing of collagen molecules in cross section cut through the overlap region [48]. **a**: Hexagonally-packed five-fold microfibrils. One side of all the molecules faces to the inside of the microfibrils. **b**: The same microfibrils loosely packed to represent molecular motion in relaxed fibrils. **c**: The same microfibrils under tension (and compression) forcing overlap segments of molecules onto a near-hexagonal lattice with planes X, Y, and Z giving rise to the crystallographic unit cell indicated by dash lines.

Corporation[20] and then at Celtrix Pharmaceuticals[21] in California. We did many interesting things there including discovering transforming growth factor β2. I later played a small role in determining its three-dimensional structure by x-ray diffraction [54]. In 1991, I returned to the east coast as a Fogarty Scholar-in-Residence at the NIH and a part-time professor in Darwin Prockop's department at Thomas Jefferson University in Philadelphia. In 1996, I became scientist emeritus at NIH and remained an advisor to FibroGen, a biotechnology company in California and Europe whose scientific founders were Darwin Prockop, Kari Kivirikko, Sheldon Pinnell, and I. George Martin is Senior Vice President for Scientific Affairs. One of FibroGen's projects is to develop, and one day hopefully manufacture, recombinant human collagen to replace animal-source collagen in medical devices.

The story of collagen chemistry and structure has come a very long way. I can claim to have been a witness to much of it and sometimes to have contributed to its advance. Collagen, and connective tissue, has progressed from being viewed as an inert residence for cells, to an active player in development and disease, such as the heritable disorders of connective tissue discussed in this book.

[20]Collagen Corporation was founded by a group of physicians at Stanford, including John Daniels, who had worked with me as a research associate. John was particularly adept at methodology, which he was able to translate into manufacturing processes. He also had a good sense of entrepreneurship.

[21]Celtrix was a spin-off of the Research and Development Department from Collagen Corporation.

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Introduction, Part II

Heritable Disorders of Connective Tissue: A Personal Account of the Origins, Evolution, Validation, and Expansion of a Concept

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INTRODUCTION

The concept that certain disorders represent generalized, single-gene-determined defects of one or another element of connective tissue is a simple one — especially when viewed from the perspective of 2001. The concept was heuristically powerful because it predicted the practicability of identifying a unitary "cause" at a biochemical, or, as we now say, a molecular level. (This is, in general, a strength of Mendelism.)

Weve, writing in Utrecht in 1931 [1], must have had the concept in mind when he referred to the Marfan syndrome as dystrophia mesodermalis congenita typus Marfan. Furthermore, Archibald Garrod in the same year, in his work *The Inborn Factors in Disease* [2], wrote as follows concerning osteogenesis imperfecta:

> ... the hereditary form of brittleness of the bones which has been observed in large numbers of the members of successive generations of some affected families. The fragility is apparently due to a defect of the mesenchyme, leading to an imperfect formation of connective tissues, rather than to any imperfect laying down of calcium salts in the bones; for the blue colour of the sclerotics of the eyes of those members of a family in which the condition occurs, is certainly due to imperfection of the fibrous tissue of the sclerotics, which, being less opaque than it should be, allows the black pigment of the choroid to show through it.

These writings were not the source of the concept for me, however. Rather, I can trace it to two colleagues at Johns Hopkins, Bentley Glass, who schooled me in the principle of pleiotropism, and Richard Follis, who, in essence, had the concept in relation to osteogenesis imperfecta.

My first published piece of clinical investigation concerned the syndrome of polyps and spots, which became known as the Peutz-Jeghers syndrome [3]. In the study of the five cases I collected and reported with the five that Harold Jeghers had observed in Boston, I sought the advice of Professor Glass in the Department of Biology at Johns Hopkins. He made it clear to me that genetic linkage was an unlikely cause of the syndrome and that pleiotropic expression of a single mutant gene was the probable explanation, even for such an improbable association as polyps and spots. That was in 1949 [4].

In a pathology conference at Johns Hopkins in 1950, Richard Follis, an associate professor of pathology, presented a case of lethal osteogenesis imperfecta congenita that he had studied in great detail because of his interest in bone disease. Histologically, he showed that not only the organic matrix of bone [5] but also connective tissue of the skin and sclera (and perhaps other structures) was defective [6,7].

It was soon thereafter that I began studies of the Marfan syndrome, when patients with this disorder were encountered in my experience as a cardiologist beginning in 1948. The designation "heritable disorder of connective tissue", and a formal statement of the concept, probably first appeared in print in March 1955 in an article in *Circulation* entitled, "The Cardiovascular Aspects of Marfan's Syndrome: A Heritable Disorder of Connective Tissue" [8].

Parenthetically, "heritable" was chosen, rather than "genetic" or "inherited", to convey the notion that the disorders are capable of being inherited, although in the individual instance they may have arisen by new mutation — another concept that Bentley Glass taught me.

Note that in March 1955 I wrote "Marfan's syndrome." Perhaps the editors of *Circulation* changed my usage. At any rate, when my full-dress article on the Marfan syndrome appeared in the *Journal of Chronic Diseases* in December 1955, the nonpossessive form of the eponym was used. Indeed, it is probably to the then-editor of this journal, J. Earle Moore,[1] that I owe my devotion to nonpossessives. He, in turn, got it from Morris Fishbein, longtime editor

[1] J. Earle Moore (1892–1957), a distinguished Johns Hopkins syphilologist, founded a multifaceted chronic disease clinic at Johns Hopkins as an outgrowth of his venereal disease clinic. It was that clinic I took over on July 1,1957, renamed the Moore Clinic, and developed into a medical genetics clinic that, at the same time, studied chronic diseases such as hypertension, systemic lupus erythematosus, sarcoidosis, and even cancer (the beginning of medical oncology at Johns Hopkins). In 1989, with the creation

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of the *Journal of the American Medical Association* (AMA) who, in his manual on medical writing (and in his editing of AMA journals), insisted on the nonpossessive form. Amusingly, later editions of the *AMA Manual of Style* favored the possessive form. Over the years, I have had a difficult time over this issue with editors, who seem to have problems realizing that eponyms are convenient "handles," but not statements of property rights. However, the ninth and latest edition of the *AMA Manual of Style* (1998) recommends that the possessive form be avoided in eponymous terms.

The concept of heritable disorders of connective tissue, developed around the Marfan syndrome and osteogenesis imperfecta, logically suggested that there must be other disorders that fitted the category. The Ehlers-Danlos syndrome was an obvious one and pseudoxanthoma elasticum another. I undertook studies of these and of the Hurler syndrome in 1954 and 1955. The chapters of my monograph *Heritable Disorders of Connective Tissue* were first published serially in monthly issues of the *Journal of Chronic Diseases* from November 1955 to May 1956, inclusive. The collected chapters, with preface, acknowledgments, index, and a portrait of Archibald Garrod, to whom the book was dedicated, appeared in hardback in June 1956 [9]. J. Earle Moore advised the publisher that the book would be of limited interest, mainly to geneticists, for which reason only 1,000 copies were printed. All 1,000 were sold by Christmas of 1956. That was the only error of judgment in which I ever caught Dr. Moore.

The inclusion of the mucopolysaccharidoses among the heritable disorders of connective tissue was prompted mainly by a seminal paper by Brante [10], published in 1952, in which he pointed out that the storage material is mucopolysaccharide, not lipid. The disorder had been called lipochondrodystrophy by Washington [11] in the 1930s, on the basis of the mistaken impression that the storage material was lipid. The designation was, admittedly, an improvement on "gargoylism," which was used for a long time, especially in publications in English, on the basis of the clinical appearance of patients with the Hurler syndrome. Lipochondrodystrophy was the term adopted for the *Index Medicus*, which persisted in using it until well into the 1970s — several years after the nature of the disorders as lysosomal degradation defects had been thoroughly established.

ADDITIONAL HERITABLE DISORDERS OF CONNECTIVE TISSUE

From the first edition of *Heritable Disorders of Connective Tissue* (1956) [9], fibrodysplasia ossificans progressiva and some other disorders have been discussed as such conditions. Skeletal dysplasias, i.e., the "chondrodystrophies," were discussed mainly in relation to their differentiation from the Morquio syndrome and only to a limited extent in their own right, until the fourth edition (1972), when a fairly full discussion with abundant illustrations of individual types was given and the international classification was presented. (In fact, the Morquio syndrome was discussed only as part of the differentiation from the Hurler syndrome until the third edition [1966], when it became part of a six-way classification of the mucopolysaccharidoses.)

During the period 1962 to 1965, discovery of the simulation of the Marfan syndrome by the inborn error of metabolism, homocystinuria, drew attention to the fact that heritable disorders of connective tissue can be secondary in nature, the connective tissue becoming damaged as a result of a primary defect that leads to an accumulation of metabolites, a deficiency of metabolites, or both. A separate chapter on homocystinuria appeared in the third edition (1966). At the same time, a chapter was devoted to alkaptonuria, which, in its disabling clinical feature, namely the characteristic spondyloarthropathy, and perhaps valvular heart lesions, is a heritable disorder of connective tissue. Menkes disease is also a heritable disorder of connective tissue, although not a primary one; it found its way into the fourth edition (1972).

Cutis laxa, which should have had a separate chapter from the beginning, was elevated from being merely part of the differential diagnosis of the Ehlers-Danlos syndrome to separate-chapter status in the fourth edition (1972). Like the chondrodystrophies, epidermolysis bullosa also came to be recognized as a heritable disorder of connective tissue. Work so indicating was discussed as early as the first edition (p. 315).

The 1972 edition also had an extensive discussion of skeletal dysplasias as a separate chapter, but no other major chapters were added. With the molecular delineation of genetic disorders at an increasing pace during the last decade, the kinship of many other disorders to the classic heritable disorders of connective tissue has become evident, as reviewed in this volume.

THE IDENTIFICATION OF GENETIC HETEROGENEITY

The three main principles of clinical genetics relate to pleiotropism, genetic heterogeneity, and variability. The first has already been referred to. Repeatedly, in recent decades, when a particular phenotype ("disorder") has been studied in detail, two or more genetically separate, although clinically similar, entities have been found. Thus, the separation of homocystinuria from the Marfan syndrome was an example of the identification of genetic heterogeneity. Establishing subtypes of the "true" Marfan syndrome, in a convincing manner, has been difficult. In part, this is because of the third principle listed above, namely, variability. We have shown, for example, that even brothers can differ widely in the severity and even the nature of the manifestations. But, of course, the main reason it was difficult to establish heterogeneity was the lack of knowledge of the basic biochemical defect in the Marfan syndrome until 1991 (see below).

Genetic heterogeneity has been studied extensively in relation to the Ehlers-Danlos syndrome, osteogenesis imperfecta, the mucopolysaccharidoses, and the skeletal dysplasias. Of these categories, the genetic nosology (the delineation and classification of genetic disorders) of the mucopolysaccharidoses has been most satisfactory, simply because cell physiology and enzymology have provided methods for defining the basic defect in each. I have reviewed the nosology of these disorders elsewhere [12]. Two forms, the autosomal recessive Hurler syndrome and the X-linked Hunter syndrome, were recognized in 1956, although the chapter was entitled "The Hurler Syndrome." By the third edition of *Heritable Disorders of Connective Tissue* (1966), a six-way classification was possible. By the fourth edition

of the Center for Medical Genetics at Johns Hopkins in another site and the pressing problems of the AIDS epidemic, the Moore Clinic was restored in large part to its original function as a clinic for sexually transmitted diseases. In 1999, the Center for Medical Genetics became the Institute of Genetic Medicine.

(1972), mucopolysaccharidosis (MPS) V (Scheie syndrome) of the six-way classification had been recognized as an allelic variant of the Hurler syndrome, and presumed genetic compounds were identified; at least two subtypes of the Hunter syndrome (MPS II) and the Maroteaux-Lamy syndrome (MPS VI) were characterized on the basis of clinical severity; three clinically indistinguishable subtypes of MPS III (Sanfilippo syndrome) were identified on the basis of specific enzyme deficiencies; and MPS VII (Sly syndrome; β-glucuronidase deficiency) had been characterized. A fourth form of Sanfilippo syndrome (MPS IIID) and a form of Morquio syndrome due to β-galactosidase deficiency (MPS IVB) were found later in the 1970s.

The Ehlers-Danlos syndrome (EDS), defined as hyperextensible skin and joints, has been found to have extensive heterogeneity. For the most part, differentiation has been on clinical grounds, beginning with the five-way classification advanced by Beighton (1970) in his monograph [13]. As biochemical defects were identified, separate forms could be recognized. The first molecular defect to be found in a heritable disorder of connective tissue was deficiency of lysyl hydroxylase [14], in a form of the EDS that I called the ocular-scoliotic type (or type VI). Characterization of the clinical phenotype — marked fragility of the eyeball, leading to rupture from even minimal trauma, and severe scoliosis — was useful to the nosology, even though the enzyme deficiency could not be demonstrated in some clinically typical cases [15], indicating that heterogeneity exists even within one type. The identification of apparently persistent procollagen in a clinically characteristic form of EDS that had also been called arthrochalasis multiplex congenita strengthened the case for a further separate form, type VII [16]. With the proliferation of other forms, the classification reached a total of about a dozen types of EDS. EDS XI was called familial joint instability syndrome by Horton et al. [17] and some would question the necessity or desirability of classifying it as a form of EDS. All classifications need a category for cases (and their families) that defy precise pigeonholing; that is, type XII in some classifications of EDS. Some families with mitral valve prolapse as a predominant feature in multiple members in a dominant pedigree pattern have joint and skin changes that might qualify them either for the diagnosis of a form of EDS or for labeling as MASS (mitral, aorta, skin, skeleton) syndrome, an overlap disorder [18].

Over time, it became apparent that the diagnostic criteria established in 1986 and published in 1988 [19] failed to discriminate adequately between different types of EDS, and between different types of EDS and other phenotypically related conditions. In addition, elucidation of the molecular basis of several types of EDS has added a new dimension to the characterization of this group of disorders, and thus a revised classification of EDS, "the Villefranche nosology, 1997" was proposed, based primarily on the cause of each type [20]. This simplified classification into six major types, the guiding principle in the formulation of which was their usefulness to the generalist, and defines major and minor diagnostic criteria for each, complemented wherever possible with laboratory findings. It should aid the accurate diagnosis of EDS, thereby facilitating improvement in the following areas: (1) diagnostic uniformity for clinical and research purposes, (2) natural history, (3) management, (4) genetic counseling, and (5) potential areas of research.

From an early stage, osteogenesis imperfecta (OI) was recognized as occurring in two forms, congenita and tarda. The two forms were separately described in the nineteenth century, and based thereon were given separate eponyms, Vrolik and Lobstein, respectively. The most widely used classification in recent years has been that of Sillence [21–24], which recognizes four main types, with subtypes of type II, osteogenesis imperfecta congenita [22]. This clinically based classification, however, was shaken when it turned out that different (allelic) mutations in one and the same gene, i.e., that for the α1 chain of type I collagen, can result in any of several types of OI, from the mildest tarda to the most severe congenita and even result not in OI but in EDS. This should not have surprised us, because the experience with that other heteromeric protein, hemoglobin, had taught us precisely the same lesson.

Subclassification of pseudoxanthoma elasticum (PXE) was long unsatisfactory, again mainly because of lack of biochemical understanding. Pope [25,26] classified PXE into two autosomal dominant and two autosomal recessive forms, based on mode of inheritance and clinical characteristics. With the demonstration that the basic defect in PXE resides in the gene, *MRP6*, for the ATP-binding cassette protein, ABCC6 (MIM 603234), it has been found, however, that various mutations cause either autosomal recessive or autosomal dominant forms of the disorder; there is no evidence of nonallelic heterogeneity (see Chapter 11, this volume).

THE SEARCH FOR BASIC DEFECTS IN HERITABLE DISORDERS OF CONNECTIVE TISSUE

Fundamental to the concept of heritable disorders of connective tissue was, of course, the notion that a unitary defect involved primarily one element of connective tissue. Collagen was fairly clearly that element in the case of OI. Further pinpointing of the defects has had to await the development of methods and knowledge in the years since Follis's publications [5–7]; since that time, the specific collagen involved has been found to be "collagen vulgaris," or type I collagen, the predominant collagen of skin, tendon, and bone. Identification of the basic defect has come more slowly in the case of the other disorders of the fibrous elements of connective tissue.

If anyone had asked me in 1960, when the second edition of *Heritable Disorders of Connective Tissue* was published, how long it would take before the basic defect in the Marfan syndrome was known, I am sure I would have predicted a much shorter time than the 31 years it did in fact require to establish that it resided in *FBN1*, the gene for fibrillin-1 [27]. Because the 1957 work of Vernon Ingram had identified a single amino acid substitution in the β-globin molecule as the basis of sickle cell anemia [28], and because of the support this finding provided for the Pauling concept of molecular disease, we were thinking that sequencing of the collagen molecule(s) or the protein(s) of the elastic fiber would reveal a similar simple change in heritable disorders of connective tissue. Indeed, that proved to be the case, but demonstration thereof had to await methodologic advances for the study of genes and proteins that, in the case of the connective tissues, were many times larger than the globin genes and proteins.

> The demonstration that the anomaly in certain hemoglobin variants consists of substitution of one amino acid out of many making up the hemoglobin protein stimulates speculation about the nature of the fibrous proteins in heritable disorders of connective

> tissue. It is to be hoped that studies in the near future will explore the possibility, for example, that in osteogenesis imperfecta an anomalous species of collagen is produced which differs in only one amino acid from the collagen in persons free of the disease. (McKusick, *Heritable Disorders of Connective Tissue*, 1960, p. 317).

As stated earlier, lysyl hydroxylase deficiency in type VI EDS was the first specific defect to be found — the work of Krane, Pinnell, and Erbe [14]. Underhydroxylation of lysyl residues in collagen was the tip-off. Discovery of other defects in collagen in EDS followed thereafter: the demonstration of deficiency of type III collagen in type IV EDS [29] and the demonstration of structural defects in type I procollagen preventing its normal conversion to collagen in type VII EDS [30,31], rather than this disorder being caused by procollagen N-proteinase deficiency as originally suggested [16]. Elaboration of the molecular lesions present in these disorders is discussed elsewhere in this volume.

Molecular methods have permitted the proof of *de novo* dominant mutation as the basis of the overwhelming majority of cases of OI congenita. In those instances of more than one affected sib, gonadal mosaicism is suspected and, indeed, has been proved by analysis of DNA of the sperm of the father [32]. The curious fact that complete loss of one of the two types of polypeptide chain that make up the type I collagen molecule can be much less disruptive than deletion of a segment of one chain has been explained as a consequence of what Darwin Prockop has called "protein suicide" [33]: Because of the deletion, that chain does not associate appropriately into the triple-helical molecule and those molecules that are formed are unstable. Thus, even though only half of the chains are of the mutant type, a majority of the heteromeric molecules will be affected and the clinical picture will be severe.

Elucidation of the basic defects has been aided greatly by cell culture techniques. In the first edition of *Heritable Disorders of Connective Tissue* (1956), I suggested the usefulness of tissue culture to the study of this group of disorders, particularly to the study of the mucopolysaccharidoses.

> In connection with the heritable disorders of connective tissue, the first objective of tissue culture studies should be the *in vitro* replication of the morphological abnormalities. In osteogenesis imperfecta and in the Hurler syndrome one can with justification anticipate success in demonstrating morphologic abnormality, in the fibers formed in the first instance, possibly in the fibroblast itself in the second, i.e., the "gargoyle cell" may be demonstrable in culture (McKusick, *Heritable Disorders of Connective Tissue*, 1956, p. 210).

Danes and Bearn [34] demonstrated a cellular phenotype of the mucopolysaccharidoses, specifically cytoplasmic metachromasia, and concluded that heterozygotes for the Hunter syndrome show in culture two classes of cells: those with metachromasia (like that in the hemizygous affected male) and those without cytoplasmic metachromasia. But it remained for Elizabeth Neufeld and her colleagues to apply to cell cultures more refined measures of abnormality, namely the accumulation of ^{35}S and its impaired "washout" [35]. Their creative next step was to "nail down" the basic distinctiveness of several types of mucopolysaccharidosis by mixing experiments, which also served the purpose of demonstrating "correction factors" that are secreted from cells. The identification of a unique correction factor in each disorder was the basis for identifying the specific enzyme deficiencies.

The concept of lysosomal storage diseases was not advanced until 1964 [36]. In the third edition of *Heritable Disorders of Connective Tissue* (1966), the thinking that had prevailed up to that time — that there was an overproduction of mucopolysaccharides rather than defective degradation of them — was still echoed. It was even suggested by Dorfman at one time that there might be a change in the protein core of the mucopolysaccharides such that turnover did not occur properly, and regulation was disturbed [37].

In the late 1950s, the study of mucopolysaccharides excreted in the urine was a main method for identifying these disorders as mucopolysaccharidoses, and in the 1960s a main method for distinguishing them — on the basis of the specific pattern of mucopolysaccharides in the urine. It was puzzling that in both the Hurler and Hunter syndromes, clearly distinct entities, the same mucopolysaccharides were excreted in excess, namely dermatan sulfate and heparan sulfate. But the predominant excretion of heparan sulfate in the Sanfilippo syndrome was a strongly differentiating feature, as was also the excretion of keratan sulfate in the Morquio syndrome and the predominant excretion of dermatan sulfate in the Maroteaux-Lamy syndrome (MPS VI).

This volume discusses a number of newly elucidated diseases that any new edition of *Heritable Disorders of Connective Tissue* would need to discuss, even in separate chapters. One such condition is the Stickler syndrome. Demonstration that the defect in at least one form of the Stickler syndrome resides in type II collagen was established essentially by a reverse genetics approach; it was shown by Francomano et al. [38] that the clinical phenotype is linked (with no recombination) to the type II collagen gene. Because type II collagen, or cartilage collagen, occurs in cartilage, including the nucleus pulposus, and also in the vitreous of the eye and in the inner ear, the joint, spinal, ocular, and auditory features of the Stickler syndrome find ready explanation. Another example is Alport syndrome, the X-linked form of which results from defects in the $\alpha 5$ chain of type IV collagen [39]. Another form of Stickler syndrome has a defect in the $\alpha 1$ or $\alpha 2$ chains of type XI collagen, and an autosomal recessive form of Alport syndrome has a defect in the $\alpha 3$ chain of type IV collagen.

In the case of cutis laxa, mutations have been identified in two different genes, the elastin gene (which when deleted causes a different disorder, supravalvular aortic stenosis) and the X-linked gene, *ATP7A*, which causes neonatal cutis laxa and a special form of cutis laxa, the occipital horn syndrome. (Other mutations in *ATP7A* cause Menkes syndrome.)

The search for basic defects illustrates the evolution of methods in the last 45 years. Cell culture methods have already been noted; biochemical methods for the characterization of the several forms of collagen and other connective tissue proteins came along in the 1960s and 1970s, and still continue. Since 1980, methods for studying the genes themselves provided by recombinant DNA technology have made it easier in most instances to study the gene or the messenger RNA than to study the protein gene product.

Since 1989, in *Mendelian Inheritance in Man* (12th print edition in 1998) and in the continuously updated online version thereof (OMIM; www.ncbi.nlm.nih.gov/omim), we have been cataloging the intragenic lesions identified as the fundamental "causes" of various genetic diseases [40]. Called

allelic variants, these include point mutations and length mutations. The point mutations include nonsense mutations, missense mutations, initiator codon mutations, terminator codon mutations, promoter mutations, and splicing mutations. The length mutations include deletions, insertions, duplications, and rearrangements. The information now available reflects the extent to which methods for identifying mutations have advanced. Cataloging the information is considered important for the design of DNA diagnosis, as well as for theoretical analysis.

In summary, I have outlined the origin of the concept of heritable disorders of connective tissue, its evolution with the delineation of genetic heterogeneity and primary and secondary forms, and finally its validation by the definition of molecular defects. The concept is undergoing continuing expansion as susceptibility to common disorders of connective tissue, e.g., osteoporosis, osteoarthritis, and aneurysm, is being related to genetic differences in type I, type II, and type III collagen, respectively.

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Chapter 1, Part I

Morphology and Chemical Composition of Connective Tissue: Structure of the Skin and Tendon

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INTRODUCTION

In this chapter we focus on the organization of the connective tissue and the structure of other components in skin and tendon. Both have similar matrix composition, but they have dissimilar morphologies reflecting their very different functions. The skin is an organ that forms the "covering" of the body. It contains all of the tissues of the body and maintains a structural and functional barrier between the internal environment of the organism and the external environment, and thus has well-developed mechanisms to protect against radiant energy, mechanical trauma, microbial invasion and water loss. In contrast, the tendon is an internal, cylindrical or ribbonlike **structure** composed of fibrous connective tissue and cells organized to serve the specific function of mediating and sensing tension between muscle and bone. The organization of the connective tissue matrix in the skin and tendon illustrates the diversity of connective tissue matrix assemblies and their specialization to subserve specific functions. It is important to recognize, however, that these macro-matrix assemblies reflect tissue-specific matrix hierarchies and cell-matrix microenvironments, and that these contribute to the regulation of matrix organization as a whole. In the skin, for example, matrix molecules that exist on the surfaces of fibroblastic cells interact with elements of the cytoskeleton, thus influencing their shape, attachment, and migration, and determining their role in matrix synthesis and deposition. Our discussion of skin goes beyond the structure and composition of the matrix by reviewing the other tissues and structures which are part of the skin and which influence and interact with the dermal connective tissue.

SKIN

Epidermis

The Keratinocyte and Epidermal Layers

The epidermis is a 0.06–1.0 mm-thick, keratinized, stratified squamous epithelium that covers and is attached to the dermis (Fig. 1). It consists of viable (basal, spinous, granular) and nonviable (cornified) keratinocytes organized into layers. Other resident cell types migrate into the epidermis during embryogenesis or differentiate *in situ* in a pathway that is distinct from that of the keratinocyte. The basal layer of keratinocytes includes dividing cells. Some spinous cells may still belong to the proliferative pool of cells, but the majority of them, like the granular cells, are postmitotic. The cornified layer is comprised of dead, keratinized cells that form the protective outer layer of the skin. The various cells of the epidermal layers differ (at least) in size, shape, position, kinetic properties, mechanical strength, hydration, biochemical composition, and cytoplasmic and cell surface antigens. Many of these differences reflect the state of differentiation of the cells in their respective layers.

The basal cell layer. Basal keratinocytes of the stratum germinativum are cuboidal to columnar in shape (Fig. 1). They join with each other by desmosomal junctions and attach to the underlying basal lamina by hemidesmosomes and integrin receptors [1,2]. Both junctions are well characterized in terms of their structure and protein composition [3,4], although how they assemble and interact

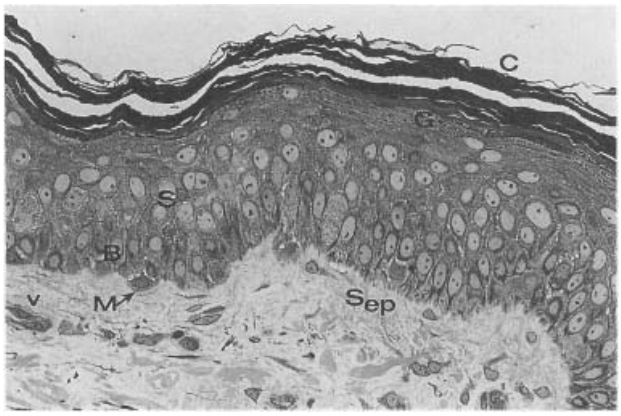

Figure 1. Histologic section of the full thickness adult upper arm epidermis showing basal (B), spinous (S), granular (G), and cornified (C) cell layers. A melanocyte (M) is indicated. Note the fine connective tissue of the subepidermal zone (S_{ep}), the vessels (v), and the abundance of cells in this portion of the papillary dermis. ×435.

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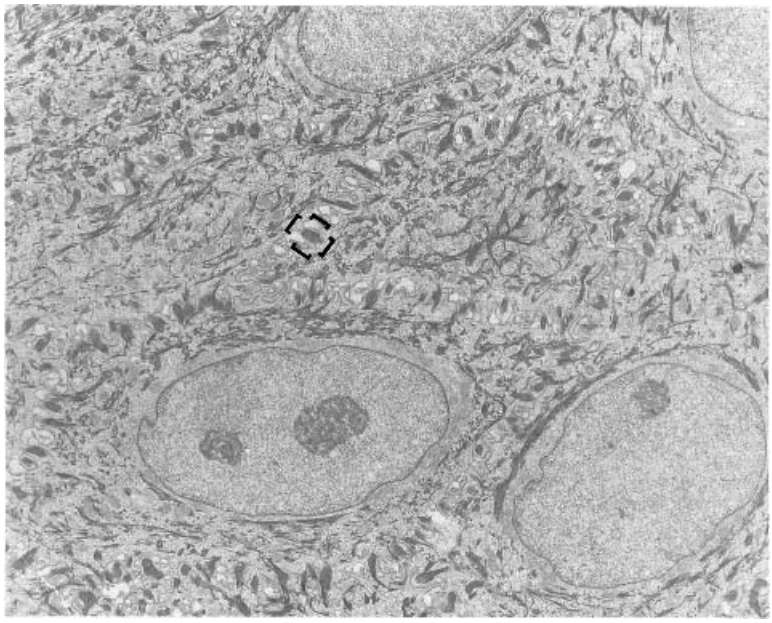


Figure 2. Electron micrograph of spinous cells of the epidermis showing the density of dark-appearing keratin filaments within the cytoplasm and associated with the desmosomes (in brackets). ×6, 270.

(at the molecular level) with elements of the cytoskeleton is yet to be resolved. Fine bundles of low molecular weight, keratin intermediate filaments (50 and 58 kDa keratins, or K14 and K5 [5,6]) extend throughout the cytoplasm and insert into the attachment plaques of the desmosomes and hemidesmosomes at the cell periphery (Fig. 2). The keratin filaments, actin microfilaments, and microtubules form an integrated cytoskeleton that preserves the shape and structural integrity of the keratinocyte, yet which is sufficiently flexible to permit the cell to move out of the basal layer into the suprabasal compartment. The keratins account for approximately 30% of the total protein in basal cells [7].

Although all basal cells in normal human epidermis are similar morphologically, they are heterogeneous in function. Stem cells, transient amplifying cells, and postmitotic cells have been identified within the basal layer of monkey palm epidermis [8,9]. In this tissue, deep rete ridges alternate with thinner epidermal regions. Basal cells that are located in the deep rete ridges and have a smooth basal border are thought to be stem cells. They contain fewer keratin filaments, are heavily melaninized, divide slowly, and give rise to highly proliferative, suprabasal, transient amplifying cells. The latter cells become postmitotic in the differentiating cell compartment as they move toward the epidermal surface. In contrast, basal cells located in the shallower portions of the epidermis have serrated basal borders and a more extensively developed network of keratin filaments. They contain less pigment and have limited incorporation of ^{3}[H] thymidine and are suggested to attach the epidermis to the dermis. The marked differences between these two populations of basal cells in monkey palm are less apparent in palmar and other epidermal tissue of the human, but nonetheless can be demonstrated [8,9]. Other studies have also documented the presence of stem cells in human adult and fetal epidermis [10–12].

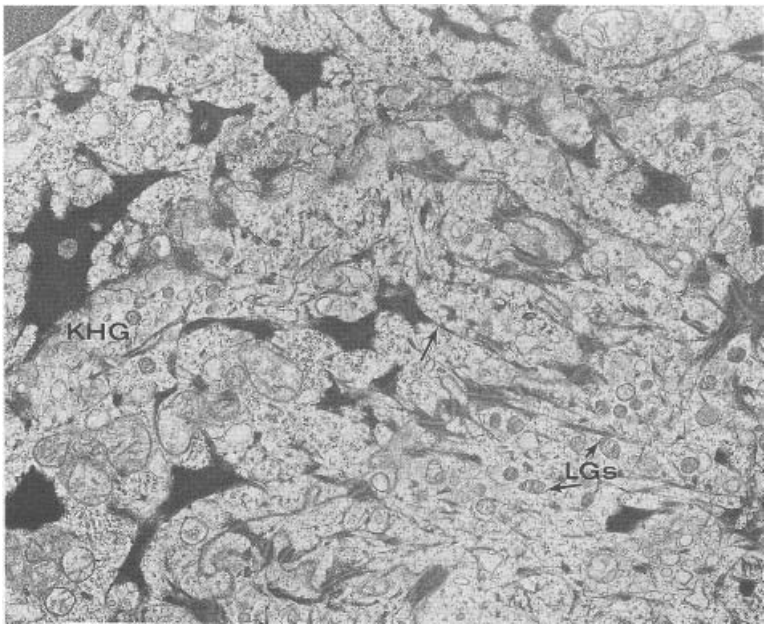
The spinous cell layer. Three to four layers of suprabasal keratinocytes constitute the stratum spinosum (Figs. 1 and 2). The name of this region is derived from the image of spines that is created when the cytoplasm between desmosomal attachments shrinks toward the nucleus as a result of chemical fixation and dehydration during histological preparation. The morphology and composition of spinous cells change between the lowermost (suprabasal) and uppermost (subgranular) layers. The cells immediately above the basal layer are polyhedral; those adjacent to the granular layer are flattened and appear rectangular in sectioned specimens. All spinous cells contain large, well-formed bundles of keratin filaments (Fig. 2) that include the K5 and K14 keratins synthesized in basal cells plus the pair of high molecular weight (56.5 and 67 kDa, or K1 and K10), differentiation-specific keratins [13] expressed as new gene products by these cells. Keratin accounts for a greater percentage of the total protein in the cell than it does in the basal keratinocyte (up to 85%) and is presumed to form a more rigid cytoskeleton.

Cells of the uppermost spinous layer contain other proteins characteristic of a more differentiated epidermal phenotype and organelles which participate in the events of terminal differentiation. Involucrin [14,15], keratolinin [16], loricrin [17], and the pancornulins [18] are proteins that will contribute to the cornified cell envelope (see below). They are first synthesized in the uppermost spinous cells. Small (100–500 μm diameter), membrane-bound organelles called lamellar granules are present in the cytoplasm. The contents of lamellar granules are arranged in alternating thick and thin lamellae that are thought to represent packing of either a series of flattened membrane disks or liposomelike structures [19]. The lipid-rich composition of lamellar granules — phospholipids, glycolipids, and free sterol — is accounted for by the membranes. Lamellar granules also contain proteolytic enzymes (lipases, proteases, glycosidases) which are thought to remodel the lipids once they are released into the extra-cellular space (see below) [20–22]. Other differentiation markers expressed by spinous layer

cells include the surface-associated pemphigus antigen, blood group antigens [23], various receptors and carbohydrate moieties [24], and membranous and intercellular lipids.

The granular cell layer. Keratin filaments form even larger bundles of filaments than in the spinous cells in cells of the two or three granular cell layers and they associate with an electron-dense protein, profilaggrin, to form keratohyalin granules (Fig. 3a). Profilaggrin, synthesized in the granular cell layer, is a high molecular weight (>400 kDa), histidine-rich, phosphorylated polymer of filaggrin monomers joined by link proteins [25,26]. It is synthesized only in keratinizing cells that express the high molecular weight keratin pair [27]. It is unclear whether, or how, the keratohyalin granules function in the granular cell layer; rather, their role appears to be related to organization of the cornified cell. Alterations in the synthesis and processing of profilaggrin with concomitant alterations in keratohyalin structure (or absence of keratohyalin granules altogether) occur in different forms of ichthyosis [28,29].

The protein constituents of the cornified cell envelope, identifiable in spinous cells, become cross-linked by calcium-dependent, epidermal transglutaminase enzymes within granular cells, even though the cornified cell envelope



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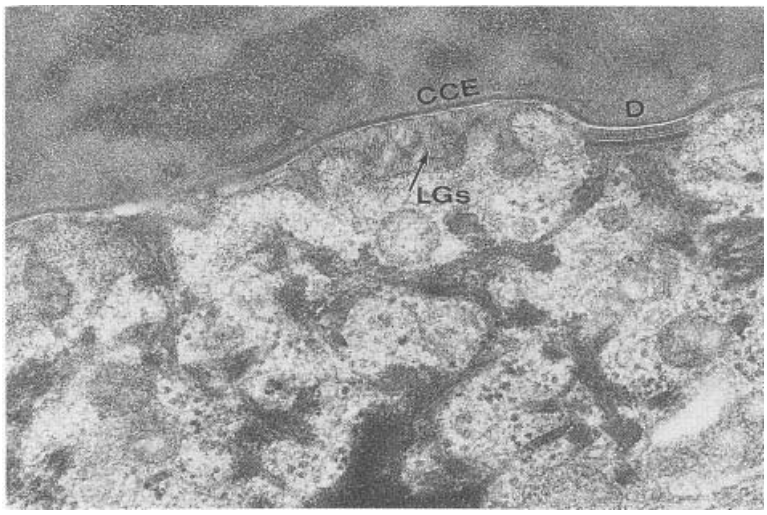


b

Figure 3. Electron micrographs of the granular (**a**) and uppermost granular and first cornified cell layers (**b**). In (**a**), note the large, stellate keratohyalin granules (KHG) associated with intermediate filaments (arrow) and lamellar granules (LGs) in the cytoplasm. The lamellar granules in (**b**) are evident in clusters within the extracellular space between the granular and cornified cells. Note the cornified cell envelope (CCE) in the first cornified cell and the modified desmosome (D) present between the two cell layers. (a) ×16, 500; (b) ×61, 300.

itself is evident morphologically only in the cornified cell (Fig. 3b). A cytosolic form of epidermal transglutaminase present in granular layer cells and a particulate membrane-bound form present in spinous and granular layer cells [14,30], are believed to form ε (γ-glutamyl) lysine cross-links between envelope protein precursors in a sequential and reinforcing manner [reviewed in 31].

The lamellar granules are present within the cytoplasm and cluster at the periphery of the granular cell, where their membranes fuse with the plasma membrane for the release of their contents into the intercellular space (Fig. 3a,b). The corelease of lipids and enzymes is presumed to accomplish the remodeling of "probarrier" polar lipids from within the granules into the neutral lipids which organize into lamellae [22]. The lamellae form a hydrophobic seal at the granular layer–cornified layer interface that impedes the transport of polar substances across the epidermis and retards water loss [32–35]. Animals that are deficient in essential fatty acids and/or have reduced quantities of lamellar granules exhibit increased water loss through the skin [36]. The contents of the granule are also thought to contribute to the unique lipid surface composition of the cornified cell envelopes [31] and to be important in desquamation of the cornified cell [34]. Individuals affected with harlequin ichthyosis uniformly have abnormal lamellar granules in which the internal lamellae are disorganized or absent [29].

Granular cells are an interesting contradiction in that they are involved concurrently in the synthesis of new molecules and in the degradation of their nucleus, cytoplasmic organelles, and molecules, as they prepare to undergo terminal differentiation to the cornified cell layer. A viable, synthetically and metabolically active cell is transformed into a dense, dehydrated, nonviable cell that contains little other than proteinaceous material and that is bounded by a cornified cell envelope. This transition is poorly understood. Enzymes that are involved in the breakdown of DNA and RNA have been demonstrated biochemically and various morphologic stages of nuclear degradation have been described [37], but there is as yet no explanation for the abrupt and "clean" transition, i.e., how organelles and cytoplasm are remodeled and recycled without evidence of cell debris in either layer of the normal epidermis. Intermediate stages in the breakdown of granular cells, recognizable as "transitional cells," are seen occasionally in normal human epidermis, although they are a regular feature of certain other keratinizing epithelia [38]. Transitional cells retain some of the organelles characteristic of the granular cell and, like the cornified cell, they have a cornified cell envelope; the cytoplasm is typically dense and the nucleus is pyknotic or shows structural evidence of degradation.

The cornified cell layer. The cornified layer shows great variability in thickness, ranging from 15–25 cell layers in regions of the limbs and trunk (Fig. 1), to hundreds of cell layers in the thick skin of the palms and soles [39]. Individual cells of the stratum corneum are thin, flattened, and polyhedral in shape. They are nonviable and filled with disulfide-bonded, keratin intermediate filaments embedded in an electron-dense matrix. A thick (12–15 nm), rigid, resistant, cornified cell envelope protects the cell from degradation by solvents, keratinolytic agents, and other potentially damaging substances (Fig. 3b). The cornified cell envelope is the most insoluble component of the epidermal cells. Lipids unique to the stratum corneum are covalently bound to the outer surface of stratum corneum cells [31,40].

The organization of the contents of cornified cells varies with their position within the stratum corneum. The matrix between the filaments is filaggrin; the 37 kDa protein subunits result from dephosphorylation and proteolysis of profilaggrin during the granular-cornified cell transition [41]. Filaggrin is thought to facilitate alignment of the keratin filaments [42,43], thus resulting in a structure referred to by Brody [44] as the "keratin pattern." The presumed function of filaggrin is called into question, however, because patients affected with ichthyosis vulgaris lack profilaggrin–filaggrin, yet demonstrate a normal keratin pattern in the stratum corneum [28]. Filaments are compact and aligned in the layers most proximal to the granular cell layer, but as the cells are pushed further toward the epidermal surface their contents decrease in density and the filaments become more random within the cell. This may occur as a result of further breakdown of filaggrin into pyrrolidone-carboxylic acid and urocanic acid, and, ultimately, into component amino acids [45]. The dissociation of filaggrin may be important in the water-binding capacity of cornified cells that are present on the outer surface of the skin.

Desquamation of the cell layers of the stratum corneum is essential for the epidermis to maintain a consistent thickness. The enzymatic contents of the lamellar granules and membrane-bound enzymes of the stratum corneum are thought to promote desquamation by remodeling the lipids in the intercellular spaces at the granular cornified cell interface and within the stratum corneum. Lipases and proteases degrade adhesive lipids and proteins, including those related to the modified desmosomal junctions [46]. Certain polar lipids are nonetheless present within some regions of the stratum corneum and have been demonstrated to act as another mechanism for water binding within these layers [47].

Although we have described unique phenotypes and functions of keratinocytes in accord with their positions within the epidermal strata, and the strata as separate compartments, it should be emphasized that the cells are a continuum and that the links are not only structural but also physiological. The cells communicate by gap junctions [48]. The number and position of cells in a vertically stratified, physiologically integrated unit have been recognized by microinjecting intracellularly the fluorescent, membrane-impermeable, gap-junction permeable Lucifer Yellow dye, and following its distribution into adjacent cells. Communication among cells of varying stages of differentiation is likely to be important in the integrated control of cell activities [49].

Melanocytes, Langerhans cells, and Merkel cells. Cells other than keratinocytes are also found in the epidermis, either as resident populations or in response to a transient condition. Melanocytes, Langerhans cells, and Merkel cells are components of normal epidermis. They migrate into the epidermis early in embryogenesis (melanocytes and Langerhans cells) or differentiate *in situ*, probably from an ectodermal/keratinocyte progenitor cell (Merkel cell). Lymphocytes and polymorphonuclear leukocytes are also recognized in the epidermis under abnormal conditions.

Melanocytes are dendritic cells which typically reside in the basal epidermal layer (Figs. 1,4). They migrate into the epidermis from the neural crest at around 50 days estimated gestation age and increase in number during the first trimester to a density that is approximately the same as in adult epidermis. Melanin synthesis begins in the third month of fetal development and melanosomes

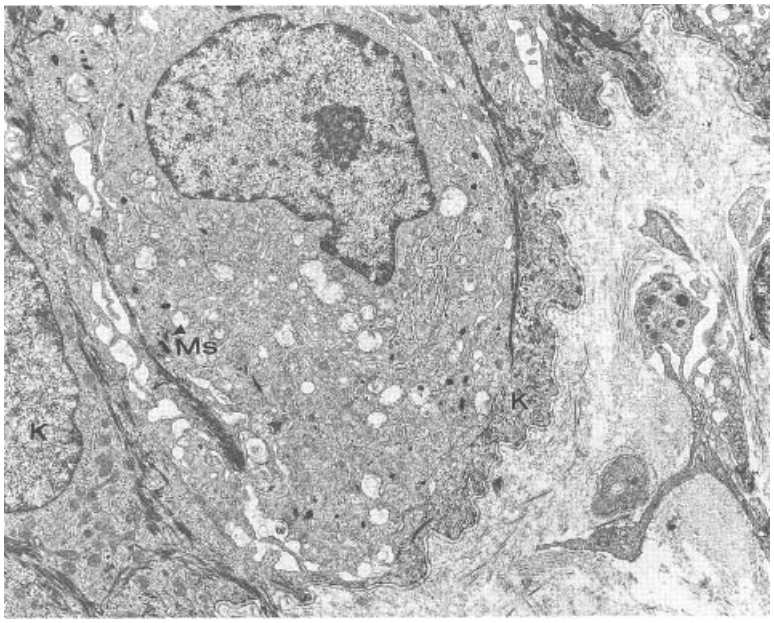


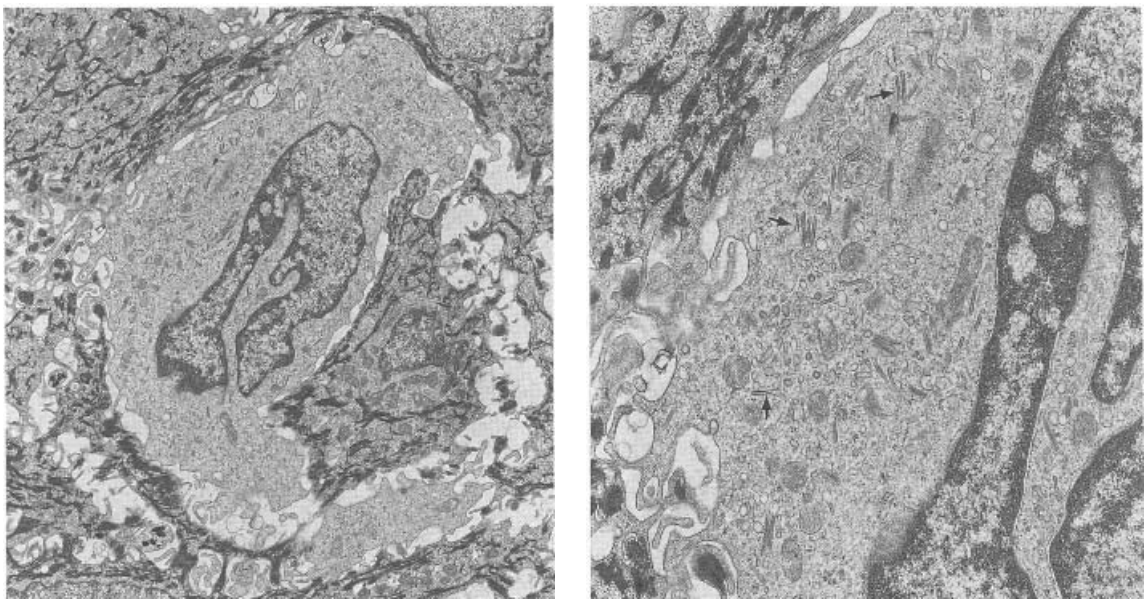
Figure 4. Electron micrograph of a mature melanocyte within the adult epidermis. Surrounded by keratinocytes, the melanocyte shows abundant mitochondria, rough endoplasmic reticulum, and melanosomes (Ms) (arrowhead) in varying stages of maturation. Keratin filaments are present in adjacent keratinocytes (K). ×8, 330.

are transferred by phagocytosis to basal keratinocytes in the fifth month [50]. Melanocytes in adult skin are easily seen at the light microscopic level as more lightly stained cells that often "protrude" from the basal surface of the epidermis toward the dermis (Fig. 1). They do not form junctions with other cells of the epidermis nor with the basal lamina, although melanocytes and keratinocytes interact via soluble mediators. Melanocytes contain fine, vimentin intermediate filaments that occur individually within the cytoplasm (compared with bundles of keratin filaments seen in keratinocytes) and the characteristic membrane-bound melanosome in which melanin pigment is synthesized. Immature melanosomes are recognized by a lattice like protein scaffolding within the organelles. This structure is obliterated when the melanosomes become actively involved in melanin synthesis. Mature melanosomes often occur in greater density in keratinocytes than in melanocytes because the phagocytosed melanosomes are collected in membrane-bound vacuoles known as melanosome complexes. Thus melanin pigment becomes distributed throughout cells of the basal epidermal layer, providing the skin reasonably uniform protection from ultraviolet radiation. Pigmentation is under endocrine control and varies with the type of melanin produced and the amount and distribution of melanosomes. As keratinocytes move into the first suprabasal layers, the melanosomes they contain are degraded by lysosomes, although occasionally melanosomes can still be recognized in stratum corneum cells. There is a growing body of work that defines paracrine and even autocrine regulation of melanocyte activities in the epidermis [51].

Langerhans cells are bone marrow-derived, antigen-presenting cells of the immune system. They migrate into the embryonic epidermis early in development, where they are recognizable, typically, in suprabasal layers by a few of their characteristic surface and cytoplasmic antigens (HLA-DR and ATPase reactivity) [52]. In adult epidermis, Langerhans cells account for about 2–6% of the total epidermal cell population [53]. Like melanocytes, Langerhans cells are dendritic, but they are easily distinguished from them by their suprabasal location, pale cytoplasm, and lobulated nucleus (Fig. 5a). Langerhans cells are also recognized by a number of characteristic surface markers (e.g., CD1, CD4, CD45, S100, T200, Fc-lgG and C3b receptors, HLA-DR, HLA-DQ, and HLA-DP [for detailed summary, see 54]) that can be demonstrated by immunohistochemical staining of epidermal sheets or sections of skin. Langerhans cells present soluble antigens and haptens to sensitized T-cells.

Langerhans cells also contain short, rodlike Langerhans cell granules (Fig. 5b). The ultrastructure of the granule gives the impression of a racquet-shaped organelle in sectioned tissue, but when the granules are reconstructed in three dimensions they are found to be flattened disks with the two sides of the disk spaced widely apart at one site. An appropriate section through both the parallel and expanded regions gives the image seen in section. The granule has been shown to form by internalization (endocytosis) of membrane-bound antigen [55].

Merkel cells, members of the paraneuronal cell system, are the third population of nonkeratinocytes present among basal cells in glabrous skin of the digital surfaces, lips, and gingiva and in the nail bed — all sites of high tactile sensitivity. They are found in lower frequency in hairy skin. Descriptive and experimental studies have led to the tentative conclusion that keratinocytes are the progenitors of Merkel cells [56,57]. Both cells are joined by desmosomes and contain keratins in their cytoplasm. K18 found in the Merkel cell is not present, however, in epidermal keratinocytes but rather is characteristic of simple epithelia [58]. Merkel cells are inconspicuous in histologic sections unless detected by an immunohistochemical reaction. At the ultrastructural level, they are easily distinguished by the presence of 100–140 nm-diameter, dense-core granules in the cytoplasm proximal to



a b

Figure 5. Electron micrograph of a Langerhans cell surrounded by keratinocytes within the epidermis (**a**) and an enlarged portion of the Langerhans cell showing the characteristic granules (small arrows) (**b**). (a) ×6, 270; (b) ×17, 325.

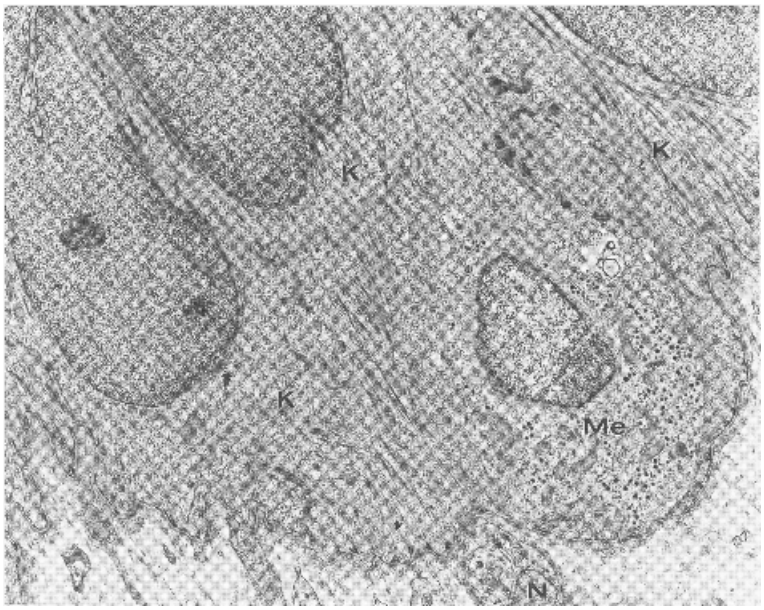


Figure 6. Electron micrograph of a Merkel cell (Me) among basal keratinocytes (K). The cell is identified easily by the presence of dense-core granules in the cytoplasm. A neurite (N) that is presumably associated with the Merkel cell is seen proximal to it, just below the basal lamina. ×8, 085.

the Golgi apparatus and adjacent to the site of apposition of a related intraepidermal neurite (Fig. 6). A junction with the structural features of a synapse may occur between the neurite and the Merkel cell, but there is no physiological evidence that transmitters are released. These sites may simply be specialized for adhesion.

The dense-core granules are similar in morphology to monoamine-storing granules in other cells; they contain the peptide transmitter substances met-enkephalin, vasoactive intestinal peptide (VIP), chromogranin A, and neuron-specific enolase [reviewed in 59,60]. Synaptophysin is also present in the Merkel cell on the surface of membrane-bound vesicles of variable size (hence not likely to be the dense-core granules) [61]; this last compound, along with neuron-specific enolase, suggests that the Merkel cell belongs to the diffuse neuroendocrine tissue of the body, and hence counters

evidence for a keratinocyte origin and suggests that instead Merkel cells may be derived from neural crest. The nature of the granule contents provides a basis for hypothesizing functions of the cell. Merkel cells are currently thought to be slow adapting type I mechanoreceptors that receive information when the keratinocytes they are in contact with are deformed. It is not clear, however, whether signal transduction is initiated at the keratinocyte or the neurite [62].

Epidermal organization. An interesting organization of epidermal cells is noted when epidermis is treated with strong alkali or hypotonic buffer solutions that cause the cells to swell. Under these conditions, one sees the cells aligned in columnar patterns, referred to as cell stacking [63,64]. Stacking is best seen in rodent epidermis, where there is evidence that the columns of cells are derived from the progeny of individual stem cells [11]. Cell stacking is also somewhat evident in normal adult human epidermis that is of typical body thickness, although the pattern is most apparent within the stratum corneum. Thick skin of the palms and soles and hyperplastic epidermis of healing wounds show no evidence of stacking. Higher rates of epidermal proliferation are thought to interfere with this organization. The stacking morphology may be important in barrier properties of the epidermis, because regions or conditions under which the stacking pattern is absent (normal or pathologic causes) permit greater epidermal water loss [64].

Epidermal appendages. The pilosebaceous structures, eccrine and apocrine sweat glands, nails, and teeth develop as appendages of the epidermis. Each of them begins to form during the fetal period soon after the embryonic–fetal transition (2 months' estimated gestational age), and requires the collaboration of epidermal and dermal cells. They are recognized in their earliest stages as clusters, cords or folds of epidermal cells that push downward into the dermis [65]. With few exceptions, the appendages form only during embryogenesis; regions of skin which lose follicles and sweat glands (in a burn wound, for example) will not spontaneously regenerate these structures with healing, although they can be reformed experimentally, indicating the potential still exists, assuming the right set of circumstances.

The Dermal–Epidermal Junction (DEJ)

Basement membranes or basement membrane zones are boundaries or interfaces between epithelia and connective tissue compartments assembled by collaboration of the two involved tissues, each contributing different structural, molecular, and antigenic components. The structural elaboration of the basement membrane zone appears to correlate with the mechanical stresses on the tissues joined and thus the presumed attachment strength required to maintain integrity at the interface. An epithelium, like the epidermis, that is subjected to significant physical stress typically develops mechanisms of attachment at the basement membrane zone ranging from modifications in tissue organization (rete pegs, rete ridges, microprojections of the basal surfaces of keratinocytes) to the formation of subcellular junctions (hemidesmosomes and anchoring fibrils).

The basement membrane between the epidermis and dermis is known as the dermal–epidermal junction or DEJ (Figs. 4,6,7). The DEJ separates these two distinct compartments of the skin and provides for adhesion between them, thus effecting the overall integrity of the skin. The DEJ excludes the transit of molecules based on size and charge, but it permits passage of migrating and invading cells under normal (e.g., melanocytes, Langerhans cells) or pathologic (e.g., lymphocytes, polymorphonuclear leukocytes, and tumor cells) conditions. All the nutrients needed by the epidermis, conveyed via the circulation, must cross the DEJ. The DEJ also supports the epidermis and influences the behavior of keratinocytes by modulating cell polarity, proliferation, spreading, migration, and differentiation. The last functions are particularly important during ontogeny, wound repair, and remodeling of the tissue.

Morphology

The ultrastructural morphology of the DEJ can be described in four planes (1) the basalmost borders of the basal keratinocytes; (2) the lamina lucida, an electron-lucent plane that lies beneath the epidermis and superficial to the lamina densa; (3) the lamina densa, an electron-dense plane also known as the basal lamina; and (4) the reticular lamina or subepidermal zone consisting of the connective tissue immediately subjacent to the epidermis (Fig. 7).

Hemidesmosomes join the epidermis to the dermis (Fig. 7). They span the keratinocyte plasma membrane, linking to other structures and molecules of the DEJ that promote attachment. The attachment plaque of the hemidesmosome is located along the basal cell plasma membrane. A few of the plaque proteins are common to the hemidesmosome and the desmosome [3,4]; others are unique to each structure. The bullous pemphigoid antigen (BPA) is associated with the attachment plaque of the hemidesmosomes where it appears to colocalize with the $\alpha 6\beta 4$ integrin receptor [2]. This combination of antigen and matrix receptor is believed

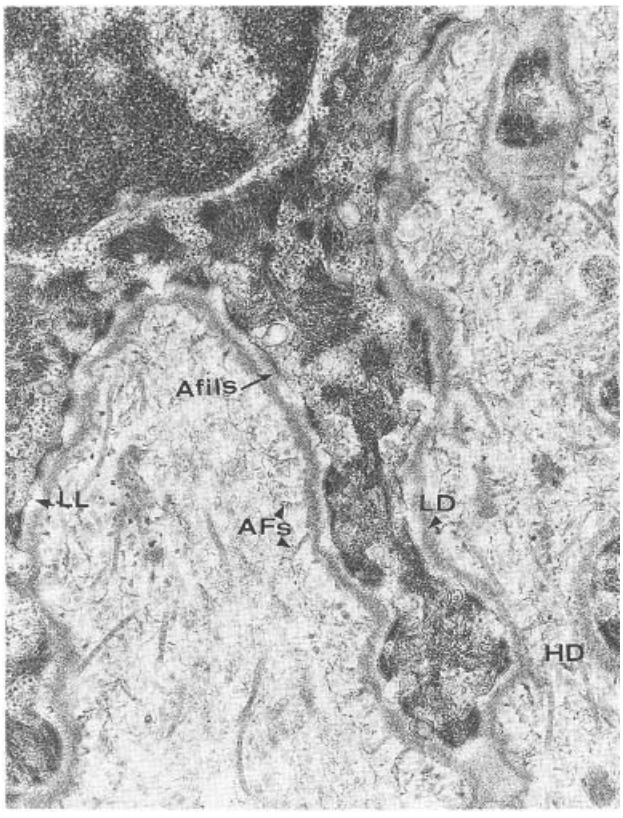


Figure 7. Electron micrograph of the dermal epidermal junction showing hemidesmosomes (HD), lamina lucida (LL), anchoring filaments (Afils), and anchoring fibrils (AFs). ×28, 350.

to provide a stable contact between epidermal cells and a yet-uncharacterized ligand in the basement membrane. There is some evidence that BPA may also be present in the lamina lucida adjacent to the hemidesmosome [66–68]. Keratin filaments of the basal keratinocyte insert into the attachment plaques, thus connecting the cytoskeleton with the cell surface and, ultimately, with the matrix of the papillary dermis. Juxtaposed to the attachment plaque, in the plane of the lamina lucida, is the subbasal dense plate (SBDP). Anchoring filaments that span the lamina lucida are usually increased in density beneath the hemidesmosomes (Fig. 7). From the dermal side of the lamina densa, banded, anchoring fibrils project into and loop through the reticular lamina network of loose fibrillar collagen (Fig. 7). Anchoring fibrils assemble from aligned dimers of type VII collagen. In most regions of the skin, both ends are embedded in the lamina densa [69–71]. In other regions, particularly notable in newborn foreskin, only one end of the type VII collagen dimer is embedded in the lamina densa, the other extending into the dermis, where it terminates in a small aggregation of type IV collagen and laminin termed an anchoring plaque [72].

The DEJ in human skin has the same general structure in all parts of the body, although there are certain age- and sex-related differences, and regional variation in the density and distribution of structures [73]. It is plastic and readily remodeled during wound healing and at sites of morphogenetic events (e.g., follicle formation).

Composition

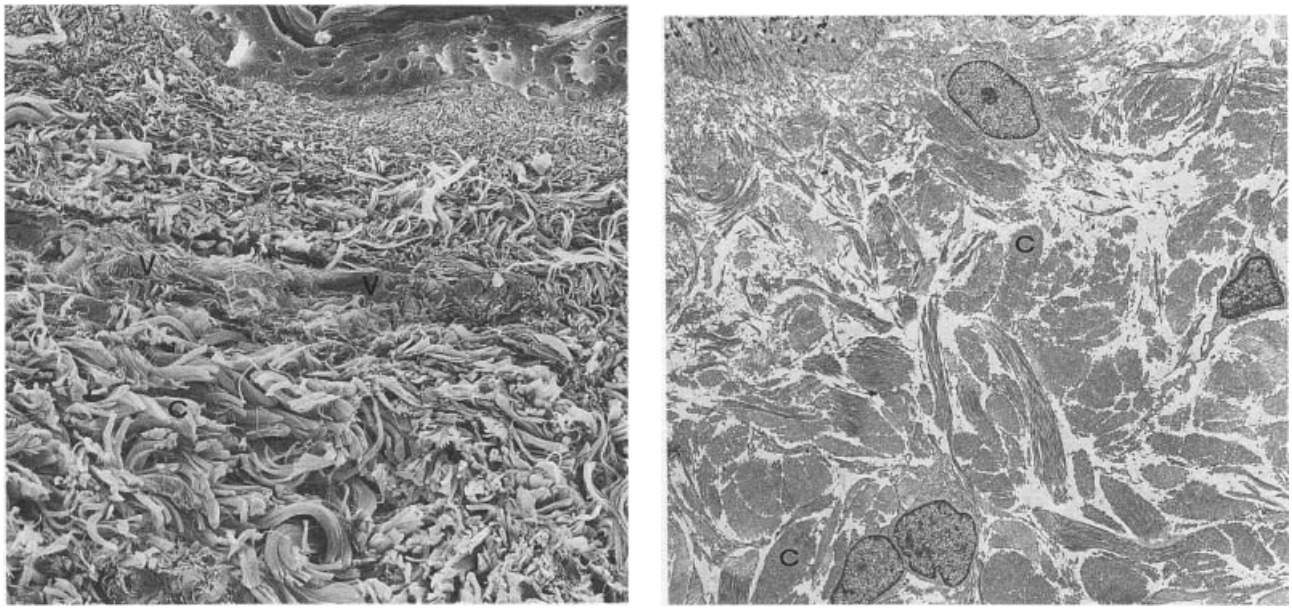
Several of the molecules of basement membranes (type IV collagen, laminin, and heparan sulfate proteoglycan) are common to all basement membranes, regardless of their position in the body or of species. Others are unique and may be tissue specific. The ubiquitous components may be the minimum essential requirements for adhesion between an epithelium and a connective tissue. The unique components may enhance the integrity required by the specific tissue, or they may provide additional functions. The molecular composition of many structures of the DEJ is understood, although the complexity of the region probably has only barely begun to be appreciated. Keratinocytes and fibroblasts both contribute molecules to the DEJ. Plaque proteins of the hemidesmosome, BPA, type IV collagen (lamina densa), type V collagen (lamina densa and reticular lamina), laminin (lamina lucida and lamina densa), heparan sulfate proteoglycan and chondroitin 6-sulfate proteoglycan (lamina densa), entactin/nidogen (lamina lucida [74,75]), and type VII collagen (anchoring fibril) are all products of the basal keratinocyte.

Fibroblasts of the papillary dermis contribute type V collagen [76] and fibronectin to the lamina densa, and types I, III, and V collagen to the reticular lamina. Oxytalan fibers, bundles of microfibrils of the elastin network, are also products of fibroblasts. These fibers originate in the lamina densa and project into the papillary dermis, where they join a horizontal network of elaunin-type elastic fibers (see below). Collagenase, a molecule which may be important in remodeling the DEJ and other matrix components of the dermis (see also Chapter 15, this volume), is synthesized by both keratinocytes and fibroblasts.

Dermis

Organization of the Dermis

The dermis is organized into regions on the basis of differences in connective tissue density and arrangement. The papillary dermis underlies the epidermis and is approximately two times its thickness (Fig. 8a,b). Small, collagen fiber



a b

Figure 8. Scanning (**a**) and transmission (**b**) electron micrographs of a portion of the epidermis (a and b), papillary dermis (a and b), and intermediate dermis (a). The vessel (V) in the scanning micrograph demonstrates the approximate boundary between the papillary and upper reticular (intermediate) dermis. In both micrographs, note the very fine bundles of collagen fibrils immediately beneath the epidermis and the increasingly larger collagen bundles (C) deeper in the dermis. (a) ×500; (b) ×2, 500.

bundles provide compliance to the tissue, accommodating deformation of the skin at its surface. Zones of the papillary dermis are recognized variously among individuals. In some, a subepidermal reticular lamina of densely organized connective tissue is seen prominently in stained sections of skin; in others, this region may be poorly developed and therefore cannot be delineated from the remainder of the papillary dermis. Projections of the dermis (termed dermal papillae) reciprocal to the epidermal rete ridges and pegs contain capillary loops and free nerve endings that terminate in close apposition to the DEJ. In some regions of specialized skin, such as the thick skin of palms and soles, encapsulated nerve endings are also present in the papillary dermis.

Fibroblastic cells are more abundant in the papillary dermis than in the reticular dermis and have unique properties: a higher rate of metabolic activity, an enhanced capacity for proliferation, and a longer replicative life span [77,78]; they also demonstrate increased plating efficiency, a higher cell density *in vitro*, and reach confluence more rapidly than fibroblasts isolated from the reticular dermis [79,80].

Matrix with the same consistency and organization as the papillary dermis surrounds the pilosebaceous structures as they extend deeply into the dermis. This connective tissue, known as the periadnexal dermis, is clearly different from the papillary dermis during embryonic development both in terms of cell types and matrix components, but it becomes more similar once the appendage attains adult characteristics. The deep boundary of the papillary dermis appears to correlate with the position of a vascular plane, the subpapillary plexus (Fig. 8a).

The reticular dermis provides the bulk of the dermis and of the skin as a whole. It is characterized by large, interwoven collagen fiber bundles and mature elastic fibers. Although differences in matrix organization are recognized abruptly at the transition between papillary and reticular regions, there is an increasing gradation in size of the collagen fiber bundles toward the hypodermis (Fig. 8a,b). As in the papillary dermis, these differences may be exaggerated, allowing different regions of the reticular dermis to be recognized. In some individuals an intermediate zone of the dermis is distinct from the deeper reticular dermis. Differences in the organization, mechanical properties, state of maturation of matrix components, and selective loss of elastic fibers from this region in certain disease states [81] provide additional evidence for the uniqueness of the region. Collagen fibrils are often heterogeneous in cross-sectional diameter in the intermediate dermis but homogeneous in the deep reticular dermis; collagen fibers and fiber bundles in this area are intermediate in size and density between the small bundles of the papillary dermis and the larger bundles of the deep reticular dermis. Elaunin elastic fibers (see below) are characteristic of the intermediate dermis, while mature elastic fibers are found deeper in the tissue, and, because of its relationship to the subpapillary vascular plexus, the intermediate dermis also has an abundance of cells belonging to varied populations. Compared with the papillary dermis, cells and vessels are relatively sparse in the deeper reticular dermis.

It is not clear what factors account for the structural and compositional differences between the papillary and the reticular dermis. It is well known, however, that there is communication between the epidermis and the dermis via soluble molecules and cells; thus it is possible that the structure and composition of the papillary dermis are regulated by these interactions. If this is correct, the papillary dermis may represent a zone of influence of the epidermis that corresponds to a minimum effective concentration of diffusible substances from keratinocytes. Similar interactions also may exist between cells in the deeper dermis and the hypodermis, although such interactions would involve fewer cells and the nature of the interactions is less apparent.

Extracellular Matrix

There is a rich variety of matrix molecules in the dermis, including several types of collagen molecules, elastic fibers of variable structure and composition, proteoglycans, and glycoproteins. The composition, ratios, and structural organization of these molecules vary with the specific region. Interstitial fibrillar collagen throughout the dermis is composed of types I and III collagens which may assemble together, and with type V collagen, into the same periodically banded fibrils [82]. Molecules of type V collagen may regulate fibril diameter in instances where they are incorporated with type I [83]. Type I collagen molecules that retain their amino terminal propeptide extensions (pN-collagen) are present specifically in the upper papillary dermis, where they form small-diameter fibrils [84]. Retention of the propeptide extensions is suggested to be one of the mechanisms which regulate fibril diameter [85]. Type V collagen is enriched in the papillary dermis and in the loose connective tissue sheaths surrounding nerves and vessels [76]. Based on the amounts of different collagens extracted from isolated dermal preparations, the relative proportions of these collagens in adult skin are estimated to be 80–90% type I collagen, 8–12% type III collagen, and less than 5% type V collagen. The high proportion of type I collagen contributes to the great tensile strength of the dermis and its ability to withstand deformation. In fetal and neonatal skin, type I collagen represents a smaller proportion of the total collagen [86,87]. Type VI collagen is also abundant within the dermis, where it is organized into 3 nm beaded filaments that are interwoven among the banded collagen fibrils and are within the diffuse matrix milieu between fiber bundles [88,89]. The widespread distribution of type VI collagen suggests it may play a role in organizing other matrix components of the dermis [90]. Ontogenetic studies demonstrating the early expression of type VI collagen in embryonic and fetal skin lend support to this hypothesis [91].

The elastic connective tissue fibers in the dermis organize in a continuous network that can be isolated when the skin is treated with strong alkali and autoclaved to digest all structures other than elastic fibers [92] (Fig. 9). Elastic fibers are composed of varying proportions of microfibrillar elements and elastin, depending upon the region of the skin. Vitronectin [93], fibronectin [94], amyloid P [95,96], and decay-accelerating factor [97] may also be associated with elastic fibers. The microfibrils establish a template of the elastic fiber network in the skin, while elastin imparts the elastic qualities. Oxytalan fibers, consisting of bundles of 10–12 nm microfibrils, are the elastic connective tissue components of the papillary dermis [98]. They extend perpendicularly from the DEJ into the papillary dermis, where they merge with elaunin fibers in the deep papillary and intermediate dermis. The microfibrils throughout the elastic fiber system have been shown to contain fibrillin, a 350 kDa glycoprotein [99] and an immunologically distinct 31 kDa microfibrillar-associated glycoprotein (MAGP)[100]. Other glycoproteins have also been revealed in microfibrils using monoclonal antibodies.

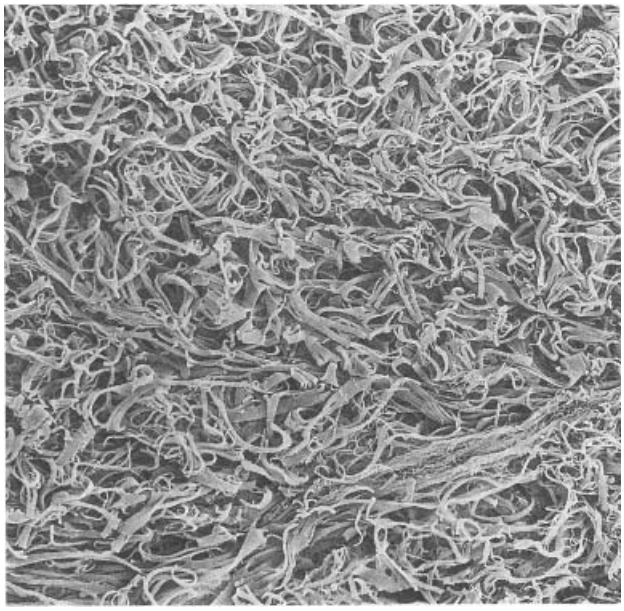


Figure 9. Scanning electron micrograph of the elastic network that remains after all of the other components of the dermis are removed by autoclaving. ×280.

Soluble elastin is deposited on the microfibrillar scaffolding of even the oxytalan fiber, as recognized by immunostaining with antielastin antibody [101]. Cross-linked, insoluble elastin is associated with elaunin fibers in the intermediate dermis [100]. Elaunin fibers, in turn, join with the "mature" fibers of the deeper reticular dermis. The latter fibers have the greatest proportion of elastin of all elements of the elastic fiber system (up to 90% [102]); microfibrils are evident only on the surface and embedded within the electron-lucent fiber, where they appear as densities. Mature elastic fibers are flat, branching structures that have the appearance of broad rubber bands (Fig. 9). They are responsible for the elastic recoil of stretched skin. Turnover of elastic fibers is relatively slow in normal dermis. They are susceptible to damage by ultraviolet radiation and particularly prone to changes with aging [103]; sun-exposed skin shows cumulative effects of radiation damage that result in matted, dysmorphic fibers that cease to function in maintaining skin elasticity [104]. There is great variability in the structural alterations of elastic fibers (fragmentation, granular degeneration, separate deposition of elastin and microfibrillar components, etc.) in the skin of patients with inherited disorders of connective tissue that involve both elastic (e.g., cutis laxa, the Buschke-Ollendorff syndrome, pseudoxanthoma elasticum) and collagenous (osteogenesis imperfecta, Ehlers-Danlos syndrome) connective tissue [105,106]. These changes appear to occur as both synthetic and degradative phenomena.

Proteoglycan, glycosaminoglycan (GAG), and glycoprotein molecules form a continuous matrix phase that surrounds and embeds the fibrillar components and cells of the dermis, and the structures of the DEJ. The proteoglycans are large molecules (100–2,500 kDa) that have several functional domains and attachment sites for from one to a large number of GAG molecules. All proteoglycans have a core protein which is characteristic for the specific molecule and determines, by its primary structure, the nature of the bound GAGs. The core protein may bind to hyaluronate. Proteoglycans bind cells to matrix molecules and in this capacity are involved in adhesion, regulation of proliferation, differentiation of cells, tissue repair (wound healing), and embryogenesis/morphogenesis (e.g., follicle formation); they bind growth factors (e.g., fibroblast and endothelial growth factors) and connect fibrillar (collagens and elastic fibers) and filamentous (e.g., fibronectin, laminin, vitronectin, and thrombospondin) matrix components via their GAG constituents, thus assisting in organizing the components of the extracellular matrix. Proteoglycans also bind water and thus are important space-filling molecules in the interstitium; the hydration of proteoglycans regulates the volume of the tissue and the compressibility of the skin. In addition to their position within the extracellular matrix, proteoglycans are also present on cell surfaces and in the basement membrane [107].

Proteoglycan molecules are visualized ultrastructurally as granules when the tissue is treated with cationic dyes (e.g., ruthenium red, alcian blue) that interact with anionic binding sites on the molecules (Fig. 10). They are recognized in routinely processed tissue only in certain connective tissue disorders (e.g., spondyloepiphyseal dysplasia) where there are abnormal proportions of the classes of proteoglycans in the dermis, or an excessive quantity of them. In these cases, flocculent material and filaments (presumably hyaluronate) are seen around collagen fibrils and in the usually "clear" interstitial spaces (Fig. 10). Immunohistochemical recognition of different proteoglycan species has been more difficult for other matrix molecules because the GAGs are not specific to any one proteoglycan and because the distinctive core proteins are well protected by the GAG side chains. Nonetheless, it has been achieved after "demasking" of the GAGs by enzymic digestion [108–110].

The major GAGs/proteoglycans in mammalian skin are hyaluronic acid (which exists free in the tissue as a GAG, heparin/heparan sulfate proteoglycan [111], and a chondroitin-6-sulfate proteoglycan [108] (restricted primarily to basement membranes), chondroitin sulfate/dermatan sulfate (assembled into large and small proteoglycans in the dermal matrix), and keratan sulfate (present in very low quantities). Dermatan sulfate represents 30–40% of dermal proteoglycans [109]. The small interstitial dermatan sulfate proteoglycan (PG II or decorin) associates primarily with type I collagen, where it binds, via its core protein, at regular periodicities along the length of collagen fibrils and with fibronectin [112]. These molecules are thought to influence the lateral growth of collagen fibrils, but they are not incorporated within the fibrils *per se* [113,114]. A second small proteoglycan, PG I or biglycan, seems to be absent from dermal matrix but is expressed by other cells in the skin such as keratinocytes and capillary endothelial cells. Fetal skin contains a higher content of hyaluronic acid [115] than adult skin, which correlates with a high rate of cell migration and inhibition of fibrillar matrix deposition. The nature of this tissue environment is thought to explain why embryonic and early fetal cutaneous wounds heal without scarring [116]. The transition from a hyaluronic acid-rich to a sulfated GAG-rich environment favors differentiation and stabilization of cells within the matrix and an increased accumulation of fibrous connective tissue.

Several glycoproteins in the dermis, laminin, fibronectin, thrombospondin, vitronectin, and epibolin, interact with the dermal proteoglycans, either by binding directly to their GAG moieties or as part of a complex involving both cells and proteoglycans. Specific matrix-binding and cell-binding

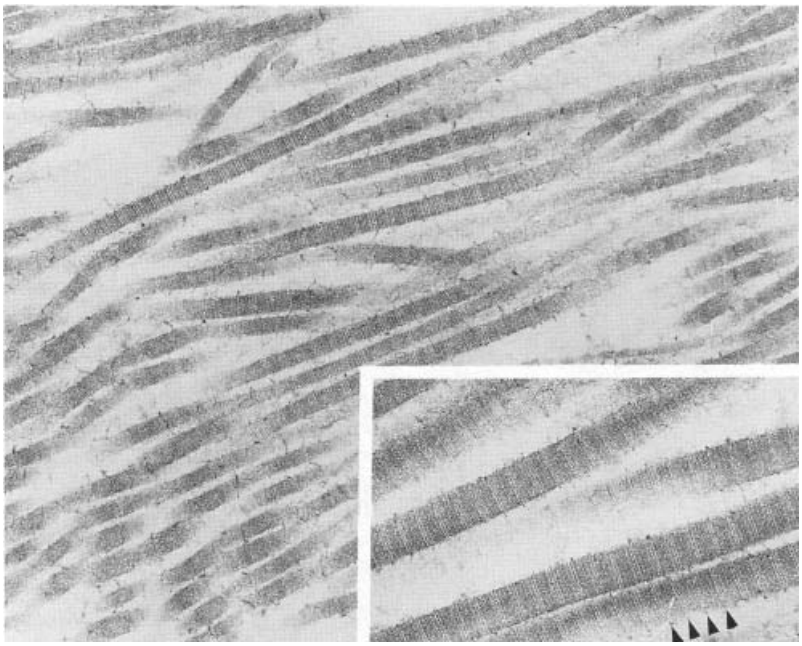


Figure 10. Transmission electron micrographs of collagen fibrils in the intermediate (**a**) and reticular (**b**) dermis fixed in the presence of ruthenium red. The glycosaminoglycan moieties (GAGs) of the proteoglycans associated with the fibrils are retained by binding with ruthenium red. GAGs decorate the surface of collagen fibrils (a) and can be seen in periodic association with regular banding patterns (b) (arrow-heads). (a) ×57, 000; (b) ×66, 700.

domains of the glycoprotein interact with integrin receptors on the cells (see below) to effect these interactions. Insoluble (cellular) fibronectin, for example, is abundant throughout the dermis, where it binds to collagen, fibrin, heparin, and cells. Cell–fibronectin complexes *in vivo* and *in vitro* are involved in tissue repair, wound healing, and embryogenesis. Fibronectin associated with cells also regulates cell migration, proliferation, and orientation.

It is apparent from the foregoing discussion that dermal fibroblasts are involved at various levels with the matrix. They are responsible for the synthesis and degradation of matrix molecules and they (and other cells of the skin, including keratinocytes and phagocytic and inflammatory cells) attach and interact with the matrix through a family of matrix receptors. The integrin matrix receptors are noncovalently-linked, heterodimeric, transmembrane proteins consisting of specific α and β subunits that, when assembled in various combinations, have specificities for matrix laminin, fibronectin, vitronectin, and collagens types I and IV [117,118]. Small intracellular domains of the integrin molecules are thought to interact with proteins of the cytoskeleton [118]. The binding of cells to matrix through integrin receptors thus establishes a continuous link with the cells' cytoskeletons, influencing their activity and function in the tissue.

Cutaneous Vasculature (Blood and Lymphatics)

Blood Vessels. Cutaneous vessels are important sites for the exchange of metabolites and cells in the skin. They play crucial roles in wound repair and various immunologic events. Like other regions and structures of the skin, a general pattern of the vasculature is characteristic for this organ (Fig. 11), but it is modified according to the region and

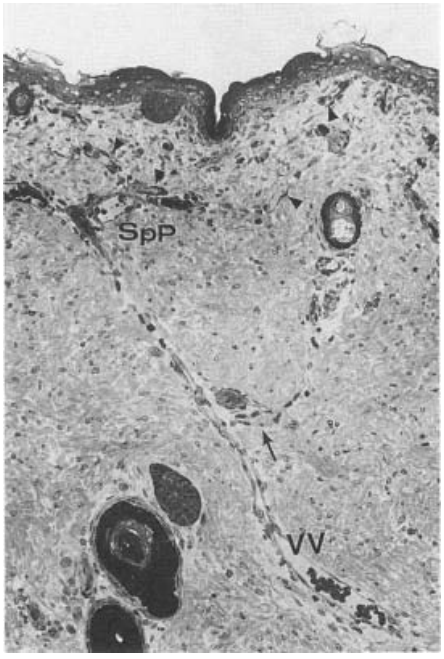
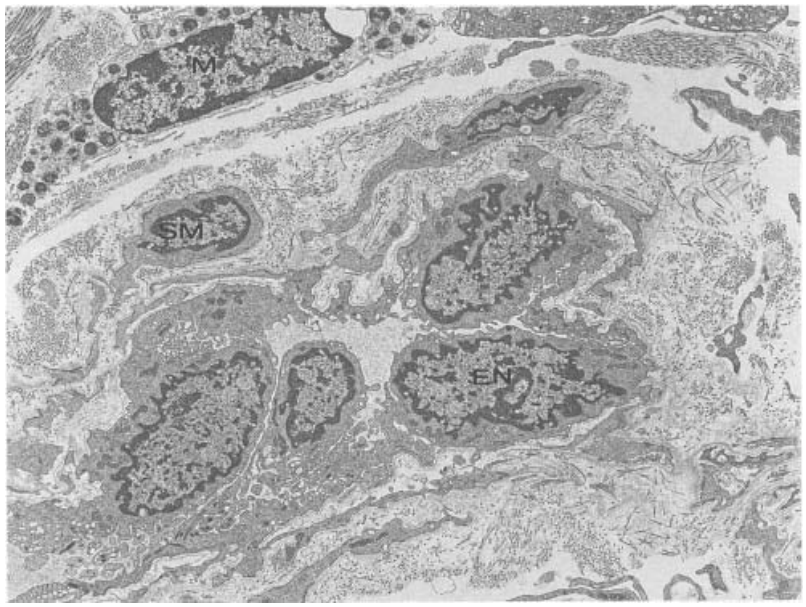


Figure 11. Histologic section of skin from a third-trimester human fetus showing the pattern of vasculature that is characteristic for late fetal and adult skin. Note the vertical vessel (VV) that either supplies or drains the subpapillary plexus (SpP) located at the boundary between the papillary and reticular dermis. Branches of the ascending vessels (arrow) supply the epidermal appendages and branches from the subpapillary plexus (capillaries) (arrowheads) supply the papillary dermis and epidermis. ×160.

age, environmental injury, and disease. The microvasculature (arterioles, venules, and capillaries) of the dermis originates from musculocutaneous vessels in the subcutaneous fat and muscle of the body wall. Vessels enter the skin and form a horizontal plexus at the junction between the hypodermis and deep reticular dermis. Arterioles extend vertically from the deep plexus toward the skin surface. They connect with one another by lateral branches, give rise to vessels which will supply the epidermal appendages, then terminate in the subpapillary plexus located at the approximate boundary between papillary and reticular dermis [119](Figs. 8a,12a). The structure of the vessel wall in this position permits easy migration of cells into the tissue, hence this plexus is commonly the site of an inflammatory reaction. Capillary loops extend from the subpapillary plexus toward the epidermis. Ascending and descending (arterial or venous) limbs of the loops differ in their wall structure [120]. Venous capillaries leave the dermal papilla to join postcapillary



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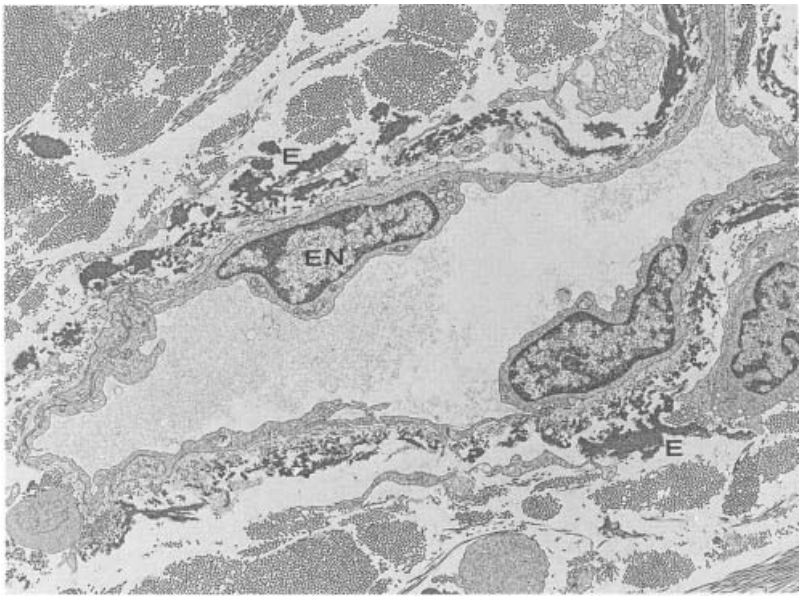


b

Figure 12. Electron micrographs of a postcapillary venule (**a**) and a lymphatic vessel (**b**) in the subpapillary plexus. A few smooth muscle cells (SM) surround the postcapillary venule and multiple layers of basal lamina are evident. Note the mast cell (M) adjacent to the vessel. The most conspicuous components in the wall of the lymphatic vessel are the elastic fibers (E). Note the flattened endothelial (EN) cells, in contrast to those of the venule, and the absence of other cells in the lymphatic wall. (a) ×5,200; (b) ×3,570.

venules in the horizontal plexus. The latter vessels are drained by venules, positioned in parallel with the ascending arterioles, that empty directly into the larger diameter venules of the dermal–subcutaneous horizontal plexus. Direct connections between arterial and venous segments of the microvasculature allow for bypass of congested capillary beds. In palms and soles, such connecting vessels have unusually well-developed smooth muscle layers and are referred to as glomus bodies. The most dense vasculature is found in the uppermost 1–2 μm of the skin [121,122].

Different vessels and regional segments of the microvasculature can be distinguished by size and position, external and internal vessel diameter, thickness of the vessel wall, and fine structure of the endothelial cells, basement membrane, and cells forming the vessel wall [121,123–125]. The papillary and intermediate dermis contain only vessels in the size range of the terminal arterioles, arterial and venous capillaries, and postcapillary venules. Larger diameter arterioles and venules with thicker walls are characteristic of the reticular dermis and subcutaneous fat [121]. Venules in the horizontal plexus at the dermal–subcutaneous interface have valves [126]. Venous capillaries associated with eccrine sweat glands and the hair bulb have bridged fenestrations of the endothelial wall which allow for rapid exchange of materials between the vessel and adjacent tissues. Such structures are not apparent in the capillaries of other regions of the skin in healthy individuals but are common in the skin of patients with psoriasis [127].

Lymphatic vessels. Lymphatic vessels, although less complex in organization and structure, are arranged in parallel with the venous network. Except for a few blind-ending lymphatic tubules (prelymphatics), lymph channels are absent from the normal papillary dermis. A prominent horizontal plexus of initial lymph vessels, so named because they are the origin of the lymphatic channels in the skin [128], is organized deep to the subpapillary venous plexus. In comparison with adjacent postcapillary venules, lymph vessels have a larger luminal diameter (not always apparent because of the normally collapsed state of the lymph capillaries), thinner walls lined by flattened, discontinuous endothelial cells, poorly developed structure of the basement membrane, and valves [129]. The endothelial cells do not show an immunopositive reaction with antibodies against factor VIII-related antigen (a prominent feature of endothelial cells of postcapillary venules) and only rarely contain Weibel-Palade bodies as ultrastructural markers in the cytoplasm. Lymph vessels are reinforced minimally with collagen fibers, but elastic fibers are conspicuous and characteristic as the major supporting elements of the wall (Fig. 12b). Bundles of filaments, called lymphatic anchoring fibrils, that have the morphology of elastin fiber microfilaments, appear to anchor the endothelial cells into the associated collagen matrix [130]. Continuity between the elastic fibers of the lymph vessel and the elastic network within the dermis may provide pathways along which fluid can track toward the lymph vessel; additionally, the elastic fiber systems may aid in movement of fluid in the lymphatics away from the skin [reviewed in 131]. Lymph vessels in the deep reticular dermis also have some smooth muscle cells as part of the vessel wall [131].

Lymph vessels in the superficial plexus are the primary sites for collection of extracellular fluid, soluble tissue debris, and proteins; they provide for the exit of macrophages and transient lymphocytes [129]. Fluid is passed quite rapidly through collecting lymphatics to lymph vessels in a deeper plexus at the junction between the deep reticular dermis and the subcutaneous tissue, then to another plexus of lymph vessels within the subcutaneous fat. Lymph appears to be cleared slowly from this site.

Cutaneous Innervation

Nerves that enter the skin are branches of large, myelinated, segmental, musculocutaneous branches of spinal nerves (cranial nerve V to the face) (Fig. 13a). Once within the skin, the fibers branch extensively, following pathways in parallel with the vasculature. Boundaries between segmental units of fibers are imprecise and each area receives overlapping innervation from more than one spinal nerve. The skin contains somatic sensory and autonomic nerve fibers that are distributed generally throughout the dermis and terminate as "bare" nerve endings (fibers surrounded by a Schwann cell sheath and basal lamina but lacking myelin) beneath and sometimes within the epidermis (Fig. 13b) (often associated with Merkel cells), within the dermal matrix and in specific relationships with sweat glands, pilosebaceous structures, and blood and lymphatic vessels. Other fibers end in encapsulated receptors, such as Meissner's corpuscles (touch receptors) of the papillary dermis and Pacinian corpuscles (pressure receptors) in the subcutaneous fat. These receptors, characteristic of ridged, nonhairy skin like the palm and sole, augment sensory reception by the bare nerve endings. Cutaneous sensory nerves receive mechanical, thermal, and chemical stimuli. Autonomic motor fibers influence secretion of sweat and sebaceous glands and supply the walls of vessels.

Nerves in the skin have traditionally been demonstrated by silver staining techniques or with routine dyes. More recently, immunohistochemical techniques and antibodies that recognize specific molecules in and on neuronal and non-neuronal cells have been used in conjunction with experimental procedures that delete specific populations of nerves according to their function. The results of these combined approaches have demonstrated that the innervation of the skin is remarkably complex and that the nerve fibers within this organ can be classified into *overlapping* categories on the basis of the following structural, compositional, and functional criteria:

1. General type of fiber (sensory or autonomic);
2. Size, degree of myelination, and density of the nerve fibers (large-diameter, myelinated, sensory A-α fibers are associated primarily with encapsulated receptors, whereas the smaller diameter, more sparsely myelinated, A-δ fibers and unmyelinated C-fibers are distributed generally and respond to heterogeneous sensory stimuli [132];
3. Pattern of distribution within the skin; i.e., within a specific portion of a structure of the skin (e.g., fiber or lamellar portion of a receptor, fiber or Merkel cell of the complex) or within a region of a neuronal or nonneuronal cell;
4. Enzymes and structural proteins expressed by neuronal cells;
5. Neurotransmitter and neuromodulatory substances released by neuronal cells;
6. Receptors expressed on neuronal and associated nonneuronal cells;
7. Functional activity of the nerve fiber.

Function is often predicted by the nature of the neuropeptides in the fibers, but the role of these substances in

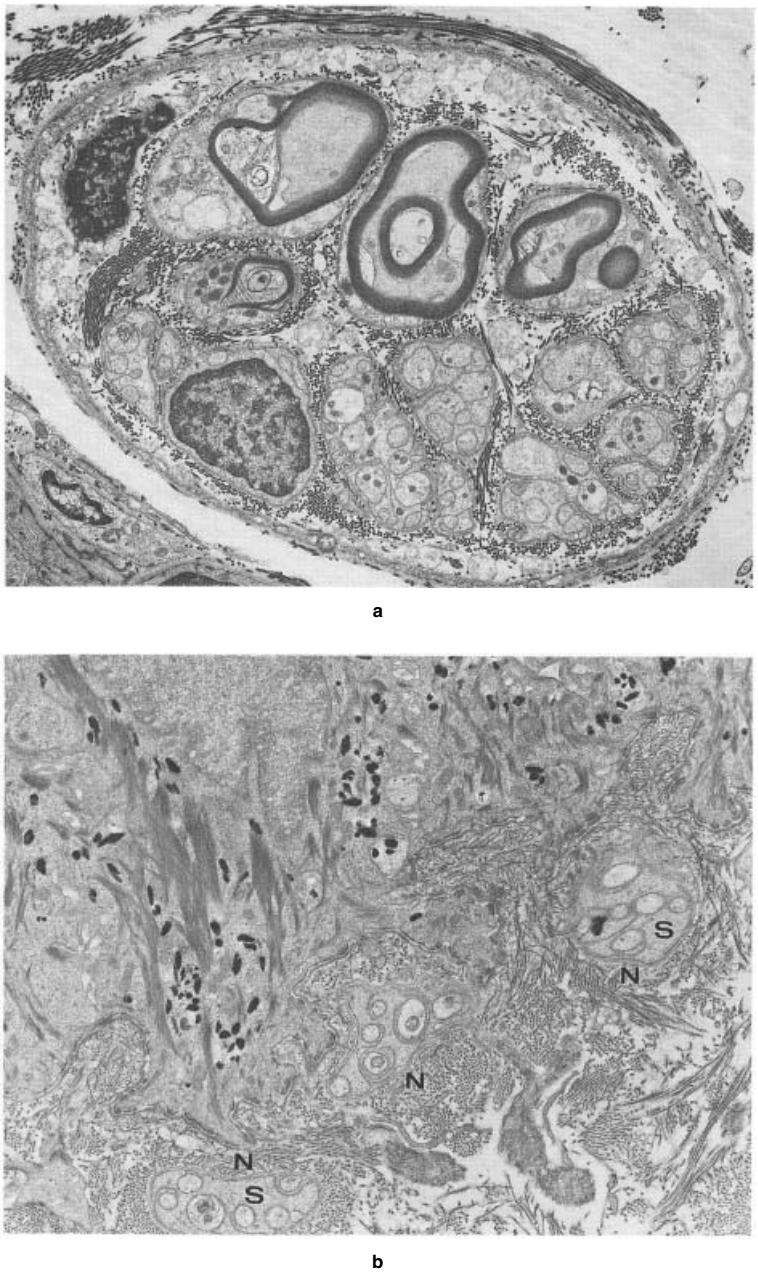


Figure 13. Electron micrographs of a large mixed nerve (**a**) and small unmyelinated nerves (**b**) in the reticular (a) and papillary (b) dermis. **a**: Nerve fibers contain myelinated and unmyelinated axons with investing Schwann cells. The endoneurium includes fibrillar collagen. The perineurium is composed of cells and basement membrane. **b**: The unmyelinated terminals (N) end immediately beneath the epidermis. Schwann cells (S) enclose the bare nerve fibers. (a) ×7, 220; (b) ×7, 670.

fibers of the skin is not always clear even though their roles may be well defined in other tissues. For example, acetyl cholinesterase is recognized immunohistochemically in areas innervated by sensory nerves, although how it acts in these sites is unknown [133]. Moreover, the effect evoked by the fiber may be secondary, as in the case of substance P, which stimulates the release of histamine from mast cells. Histamine in turn triggers the release of more substance P from the nerve fiber (axon reflex), causing itch, flare, and weal in the skin in a dose-dependent manner [134].

Any one structure of the skin may be innervated by different types of fibers and individual fibers may express more than one chemical substance. Small, unmyelinated sensory axons in the papillary dermis, for example, can contain both calcitonin gene-related protein (CGRP) and somatostatin, or CGRP and substance P [135], and may release them simultaneously. Substance P, neurokinin A, and CGRP all may be released from sensory fibers in response to physical or chemical irritation [134]. The description of fiber type and distribution and density for

TABLE 1. Neurotransmitter and Neuromodulatory Substances and Other Marker Compounds

Expressed by Sensory and Autonomic Fibers in the Skin			
Marker Compound	Sensory Fibers	Autonomic Fibers	Reference
Structural proteins			
NF (antineurofilament)	+	+	
PGP 9.5	+	+	133
NSE (neuron-specific enolase)	+	−	136
MBP (myelin basic protein)	+	+	136
S-100	+	+	136
Neurotransmitter/Neuromodulatory substances			
SP (substance P)	+	−	133,136,137
NKA (neurokinin A)	+	−	133,134,136
CGRP (calcitonin gene-related peptide)	+	−	133,135,137
VIP (vasoactive intestinal peptide)	+	+	59,133,136
NPY (neuropeptide Y)	−	+	133,136
TH (tyrosine hydroxylase)	−	+	133,136
SOM (somatostatin)	+	−	132,133
ANP (atrial natriuretic peptide)	−	+	133,137
AChE (acetyl cholinesterase)	+	+	133
Synaptophysin	+	−	133

one region may be inaccurate for another (e.g., hairy versus nonhairy skin).

Sensory fibers in the skin innervate the epidermis (sometimes via intraepidermal endings), the dermis, sweat glands, pilosebaceous structures, blood and lymphatic vessels, single cell receptors (Merkel cells), and encapsulated receptors (e.g., Meissner's corpuscles). They are immunoreactive for synaptophysin and somatostatin [132], substance P (transfer of nociceptive information), neurokinin A, vasoactive intestinal peptide, neuron-specific enolase, myelin basic protein, S-100, and an antineurofilament antibody [136] (Table 1). Blood vessels and lymph vessels, sweat glands, sebaceous glands, and the erector pili muscles also receive autonomic innervation [136]. Autonomic fibers to large blood vessels and to sweat glands are recognized in sections stained immunohistochemically with antibodies to neuropeptide Y (a vasoconstrictor), tyrosine hydroxylase (involved in the synthesis of catecholamines), vasoactive intestinal peptide (may increase sweat gland secretion and vasodilation, and thus play a role in thermoregulation), neuron-specific enolase, myelin basic protein, S-100 [136], and possibly somatostatin [132]. Nerves to sweat glands in particular are also immunoreactive for atrial natriuretic peptide, a substance which acts as a diuretic and vasodilatory substance in other tissues and which is therefore suggested to play a role in regulating water and electrolyte balance in the eccrine sweat glands of the skin [137]. Schwann cells, Meissner's corpuscles, and large nerve bundles are identified by antimyelin basic protein and anti-S-100 [136] (Table 1). Antinerve growth factor receptor antibody also stains Schwann cells of cutaneous nerves [138].

Hypodermis

The subcutaneous region, or hypodermis, is distinct from the dermis throughout life. Even in early embryonic skin, when there is little differentiation of body wall tissues into skin, subcutaneous connective tissue, and skeletal muscle, differences between the regions are recognized first by different densities and orientations of mesenchymal cells (embryo), then by differences in the orientation and composition of fibrous and diffuse connective tissues (fetus). Although the dermal and hypodermal regions are structurally distinct, they are well integrated through nerve, blood vascular, and lymphatic networks, and because the terminal portions of the anagen hair follicles and eccrine sweat glands are embedded deeply within the fat.

The distinguishing characteristic of the subcutaneous region is the presence of fat stores. These develop early in the second trimester within a finely constructed, fibrillar collagen stroma organized in a lobular pattern [65,139]. Assemblies of fat occur primarily at sites that are rich in blood vessels and lymphatics. Fibroblastic-appearing cells colonize the matrix and develop the differentiated adipocyte phenotype as they accumulate refractile lipid droplets within the cytoplasm. Individual lipid droplets coalesce into a large central lipid vacuole and the cells enlarge. A fine casing of connective tissue fibers surrounds individual fat cells, giving each cell the appearance of a ball of yarn.

In newborns, the fat lobules are well developed and measurements of skinfold thickness from a large population of infants between birth and the first year of life reveals a correlation between fat deposition, sex, and birth weight of the infant [140]. Subcutaneous fat deposition continues throughout life, increasing in mass either through the hyperproliferation of fat cells, recruitment of undifferentiated mesenchymal cells into adipocytes, and/or hypertrophy of existing adipocytes [141]. The size of fat cells increases with age and, accordingly, lipid synthesis increases in the larger cells. The latter is a property, however, primarily of growing individuals compared with mature individuals [142].

The thickness of subcutaneous fat can be measured as a function of skinfold thickness using skin calipers, computerized tomography (CT) [143], magnetic resonance imaging (MRI), radiography, and ultrasound [144,145]. The imaging modalities have been used, in particular, to determine whether correlations exist between the extent of

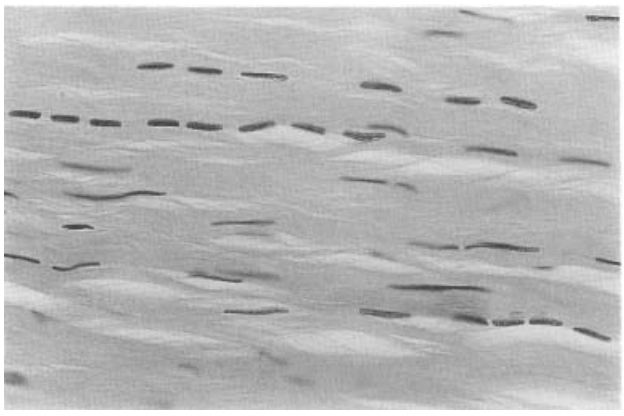
subcutaneous fat, body density, and visceral fat depots of males and females of different age groups. The purpose of these determinations is to assess whether the extent of subcutaneous fat correlates with the quantity of visceral fat and whether body fat distribution can be used to predict metabolic complications of obesity. The data at present are ambiguous and may depend upon the method used for data collection [143,144].

Fat in the skin is an important store of energy in the form of triglycerides. As a tissue, the adipose layer insulates the body, cushions the skin, and molds body contours. Absence of subcutaneous fat in certain disorders of the skin, such as Werner's syndrome, brings the dermis in direct contact with connective tissue of the bone. The absence of this richly vascularized tissue accounts for chronic ulcers that heal very slowly, very poorly, or not at all.

TENDON

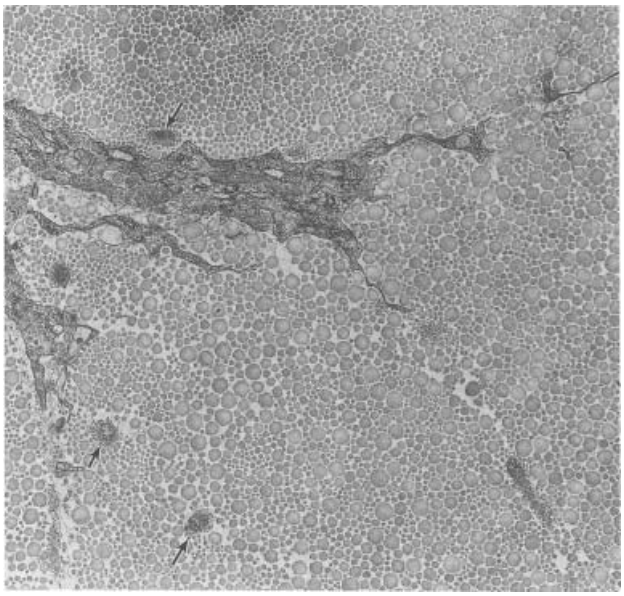
Tendons are connective tissue structures that link muscle to bone. They have great tensile strength, a degree of pliability, and a component of elasticity. Whereas muscle can withstand mechanical forces up to 77 lbs./sq. in. (531 kPa), the average tensile strength of human tendons ranges from 4,000 to 18,000 lbs./sq. in. (27,600–124,000 kPa) [146]. Tendons have a relatively sparse supply of blood and lymphatic vessels in contrast to muscle and bone with which they associate.

The characteristic structural units of tendon are densely packed parallel bundles of fibrous collagen that extend between the myotendinous and osteotendinous junctions. A tendon fascicle consists of groups of collagen fiber bundles and associated tendon cells, surrounded by a fine layer of loose connective tissue, referred to as the endotendon or endotenon. Within each fascicle, rows of elongated and flattened fibroblasts extend the length of the collagen fiber bundles (Fig. 14a) [147]. Groups of tendon fascicles are surrounded by a tendon sheath or epitenon. In certain specialized regions, tendon sheaths form synovia. Synovial sheaths surround the tendons and lubricate and nourish them, especially where they pass over joints.



a

Figure 14. Light (**a**) and electron (**b**) micrographs of adult human Achilles tendon. **a**: Artifactual separation of tendon fibers reveals the parallel organization of collagen matrix and linear alignment of tendon cells. **b**: Bimodal sizes of collagen fibrils are seen in cross section, along with small areas of elastin and microfibrils (arrows). (a) ×435; (b) ×15, 580.



b

Figure 14. (*Continued*)

Tendons may be very short relative to their width or they may be long and slender. Shorter, broader tendons include the attachments for the major muscles of the trunk, limbs, and head. The long, slender tendons that serve the digits of the hands and feet have special modifications. Vincula are shorter tendinous fibers that extend from the body of digital tendons to the associated bone, helping to keep the tendon aligned.

Collagen fibrils of the tendon fascicles are bimodal in diameter: Large fibrils are 150–200 nm in diameter and smaller fibrils are 40–80 nm in diameter (Fig. 14b). The relative proportions of the two sizes vary throughout a tendon. The collagen fibrils of tendon sheaths, however, show a narrower range of diameters and an average of 60 nm in diameter. They do not align in parallel, but are loosely interwoven in multiple orientations. Tendon sheaths contain primarily type I collagen, but they are relatively enriched with type III collagen compared to tendon fascicles. The organization of the tendon bundles ensheathed by loose connective tissue helps to keep the tendon matrix flexible and resistant to disruption by pulling. The loose connective tissue presents a potential path for cellular activity in maintenance and repair of tendon matrix. Small bundles of microfibrils also contribute to the tendon matrix, and these correspond to fine fibrillin-positive fibers observed by immunolabeling [99]. Tendon sheaths have more detectable amounts of elastic fibers (elastin and microfibrils) than the tendon matrix.

Scanning electron microscopy and polarization microscopy have demonstrated that tendon fibers have crimped, not helical, planar undulations, giving the appearance of wavy or zigzagged ribbons. This configuration appears to correspond to the nonlinear portion of the stress-strain curve when the contractile force of the muscle is applied to the tendon [148]. The crimped architecture is not observed in samples that have been treated with hyaluronidase [149], suggesting that hyaluronic acid and

perhaps other glycosaminoglycans may be important in maintaining this level of organization of tendon fibers. Dermatan sulfate, keratan sulfate, and chondroitin sulfate chains are found in varying proportions in different anatomical regions of tendons [150,151]. Decorin, dermatan sulfate proteoglycan II, is associated with the surface of tendon collagen fibrils and in periodic gaps along the fibril length, as it is in the skin [152].

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Chapter 1, Part II

Morphology and Chemical Composition of Connective Tissue: Cartilage

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INTRODUCTION

Cartilage is a highly specialized connective tissue that is distributed throughout the body. It performs many functions which, in general, help the body to withstand compressive forces and, to a lesser extent, resist shearing and torsional forces. It is essentially avascular and comprised primarily of extracellular matrix. Although various schemes have been used to classify different types of cartilage, i.e., embryonic versus permanent, calcified versus noncalcified, skeletal versus nonskeletal, it is most commonly divided into hyaline, elastic, and fibrocartilage based on its morphologic appearance and the composition of its extracellular matrix.

The cells that elaborate cartilage matrix have been called chondroblasts or chondrocytes, depending upon the amount of matrix surrounding the cells and the implied relative maturity of the cells [1]. Because this distinction is arbitrary, the term chondrocyte is employed in this chapter. It should be emphasized, however, that chondrocytes behave differently depending upon the particular circumstances. The sum total of this behavior, i.e., morphology, genes expressed, etc., is referred to as the phenotype [2]. Thus, chondrocytes have the capacity to express different phenotypes.

The macromolecules that comprise cartilage extracellular matrix are addressed in more detail in other chapters. This chapter focuses primarily on cartilage morphology and attempts to relate structure to development, since so many inherited disorders of cartilage are developmental in nature. Changes in molecular composition of the matrix and the identification of developmental control mechanisms are provided as examples and are not intended to be comprehensive.

DEVELOPMENTAL ASPECTS

Cartilage plays a central role in the formation and growth of the vertebrate skeleton because many bones form first as cartilaginous models that are only later replaced by bone tissue in a process called endochondral ossification. The current biological model of this process is derived from studies of limb development in lower vertebrates, especially the chick. Limb development begins early in embryogenesis with the formation of the limb buds comprised of mesenchymal tissue covered by a layer of ectoderm, the apical ectodermal ridge (AER) [3] (Fig. 1). Under the influence of the AER, the underlying mesenchymal cells form a progress zone where they remain undifferentiated, proliferate, and extend the limb bud distally [4]. The timing, location, and pattern of skeletal element formation are under the control of a variety of transcription factors, in particular the *Hox* genes and intercellular signaling or growth factor molecules such as sonic hedgehog, bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and Wnt7. In the limb, the latter originate from three signaling centers, the AER in the distal ectoderm, the zone of polarizing activity (ZPA) in the posterior mesenchyme, and the dorsal ectoderm. These maintain the proximo-distal, anterior-posterior, and dorsal-ventral axes of development, respectively [5].

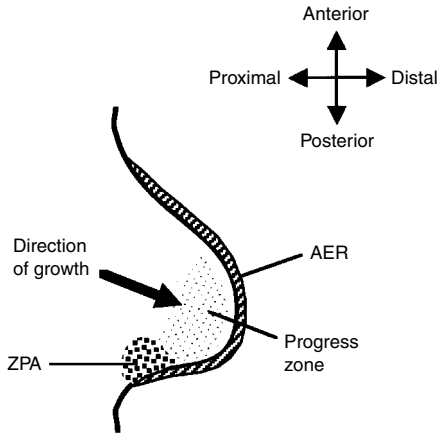

Figure 1. Regulation of early limb development. The proximal-distal axis is controlled by the apical ectodermal ridge (AER), which secretes fibroblast growth factors (FGFs) that maintain the outgrowth of proliferating mesenchymal cells in the progress zone. The anterior-posterior axis is determined by the zone of polarizing activity (ZPA), which is located in the posterior mesoderm and which utilizes signaling by Sonic hedgehog (Shh). The dorsal-ventral axis (perpendicular to the plane of the diagram) is determined by the dorsal ectoderm utilizing intercellular signaling by Wnt 7 molecules. These three signaling centers also require feedback from each other to be maintained.

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Beginning proximally, the mesenchymal cells in areas destined to become bone form condensations, a process that represents a key step in skeletal formation [6]. Chondrogenesis proceeds directly from these condensations as the cells differentiate into chondrocytes. Thus, the location, number and shape of the condensations initially define the endochondral skeleton. Aggregation of cells is mediated by the cell surface adhesion molecules, NCAM (neural cell adhesion molecule) [7] and N-cadherin [8], and includes the formation of gap junctions [9]. The size of the initial condensation appears to be determined more by the extent of recruitment of cells to the condensation than the proliferation of cells after condensation [6].

Modulation of the extracellular matrix is also required for condensation and chondrogenesis. The extracellular matrix produced by the poorly differentiated mesenchymal cells in the early limb bud contains collagen types I and III, fibronectin, and small amounts of noncartilaginous proteoglycans of small hydrodynamic size [3,10,11]. The expression of fibronectin, tenascin, versican, and syndecan 3, for example, increases during condensation, while the amount of hyaluronan decreases. The precise roles of these molecules are unclear but may be to enhance the migration of mesenchymal cells, as well as promote their response to growth factors or signaling molecules [6]. Some matrix changes associated with condensation involve modulation of matrix protein structure by alternative splicing of structural domains. Fibronectin contains multiple tandem type III structural modules, two of which are regulated by alternative splicing. Inclusion of one of these, extra domain A (EDA), appears to be essential for condensation, while its exclusion from the molecule appears to be necessary for subsequent differentiation to chondrocytes [12–15]. Coincident with condensation is the appearance of mRNAs for cartilage-specific proteins, including type II collagen and cartilage (large aggregating) proteoglycan (aggrecan) core protein [16,17]. An alternatively spliced form of type II collagen, type IIA, is characteristic of prechondrocytes or chondroblasts [18], and is expressed throughout the area of condensation [19–21]. Overt chondrogenesis, sustained by BMP signaling [22], begins at the middle of forming bones shortly after condensation with the synthesis and secretion of matrix rich in these macromolecules and others, including types IX and XI collagen, small proteoglycans, link protein, and cartilage matrix protein (CMP) [16,17,23–25]. Differentiation to the chondrocyte phenotype is accompanied by a switch to the collagen type IIB splice form as well as changes in the structure of type XI collagen brought about by alternative splicing [26–30]. Synthesis of noncartilaginous proteins, such as type I collagen, ceases. This process forms the *anlagen*, or models, of the future skeleton.

Cartilage formation (chondrogenesis), is followed by hypertrophy, degradation, and replacement of the cartilage by bone, a process called endochondral ossification. At the single cell level it can be viewed as the product of a differentiation pathway involving the sequential expression of three chondrocytic phenotypes: committed (prechondrocytic) mesenchymal cell, differentiated chondrocyte, and hypertrophic, or terminally differentiated chondrocyte. For the purpose of this chapter, the shaft of a long bone will be referred to as the diaphysis and its rounded ends, the epiphyses, at all stages of development.

To accomplish transformation from cartilage to bone, chondrocytes in the center of the diaphysis of the cartilage *anlage* enlarge and begin to synthesize molecules atypical of hyaline cartilage matrix, including type X collagen and fibronectin, and express activity of carbonic anhydrase and alkaline phosphatase [31–37]. These changes signal the expression of the hypertrophic chondrocyte phenotype, which is also characterized by reduced synthesis of type II collagen and possibly also that of cartilage proteoglycans, proteoglycan link protein, and protease inhibitors that prevent vascular invasion [31–39]. The net effect is that hypertrophic cartilage is degraded by invading vascular tissue, the hypertrophic chondrocytes undergo programmed cell death (apoptosis), and osteoblasts associated with the invasion deposit bone matrix in the space previously occupied by hypertrophic cartilage.

This transformation is accompanied by the formation of a layer of bone by osteoblasts in the perichondrium (periosteum) around the region of hypertrophic chondrocytes in the mid-diaphysis [40]. Because this bone forms from a layer of osteoblasts adjacent to the cartilage, but not by replacement of cartilage, it is called membranous or periosteal bone. This membranous bone may serve as a staging area and may be a prerequisite for vascular invasion of the underlying hypertrophic cartilage. In mice lacking the function of the transcription factor, Cbfa1, the overt expression of the osteoblastic phenotype is blocked, as is the formation of mineralized bone tissue [41,42]. Vascular invasion is also prevented, and the bone remains entirely cartilaginous, suggesting an interdependence of these two processes. Much of the cartilage is calcified and hypertrophic in appearance, showing that the hypertrophic chondrocyte can be a stable endpoint in chondrocyte differentiation and that apoptosis is not obligatory.

Once established in the center of a bone (mid-diaphysis), the ossification process spreads centrifugally as a front. Chondrocytes adjacent to the front terminally differentiate to hypertrophic chondrocytes, which, as above, facilitate the hypertrophy, degradation, and replacement of the cartilage. Much of the cartilage *anlage* is consumed by this process. However, as this front nears the ends of the bone (epiphyses), chondrocytes adjacent to the hypertrophic cells proliferate, producing new cells that synthesize cartilage matrix before they in turn hypertrophy. In other words, the source of cartilage that is replaced by bone changes from cartilage originally derived from mesenchyme to that produced by newly proliferated chondrocytes. This switch allows the epiphyseal cartilages at the ends of the bones to be pushed apart without being consumed. This proliferation-hypertrophy-replacement process becomes structurally organized into the so-called growth plate and occupies a narrow space between the epiphyseal cartilage and the expanding bone [43]. Once formed, the growth plate is responsible for linear skeletal growth. The mature growth plate can be viewed functionally as a dynamic structure with a leading edge where cells are born that synthesize cartilage template and a trailing edge where this template is replaced by bone. The coordination of these events is under precise and complex regulatory control, both autocrine and paracrine, involving signals from the perichondrium, from within the growth plate and from the newly formed bone.

Epiphyseal cartilage is not permanent. Specialization occurs at the surface to form articular cartilage, while secondary ossification centers develop in the center of the cartilage [44]. These centers correspond to the ''epiphyses'' seen on radiographs. They arise and grow through the same process that occurs in primary ossification, i.e., cartilage hypertrophy, vascular invasion, and replacement of cartilage

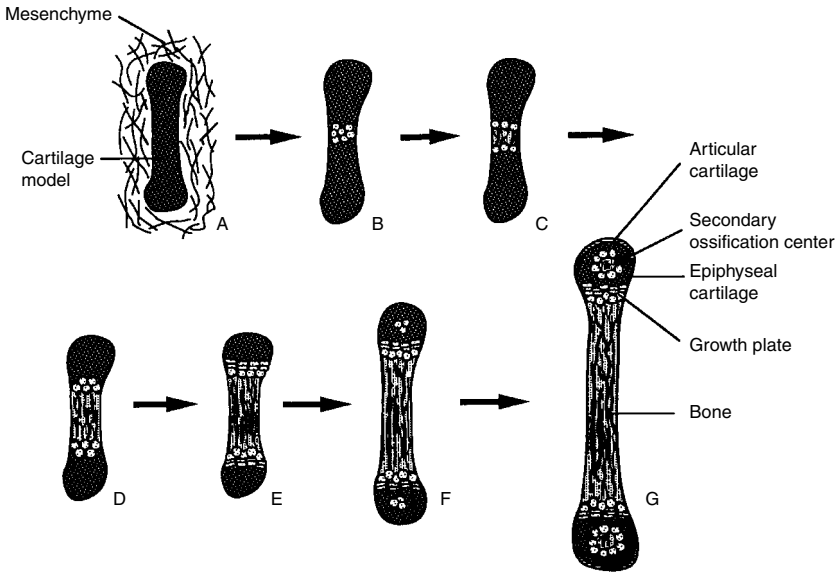


Figure 2. Schematic representation of bone formation and growth. **A:** The initial condensation of mesenchymal cells and their differentiation to chondrocytes defines the cartilage model. **B:** The differentiation of chondrocytes to prehypertrophic and then to hypertrophic chondrocytes (indicated as individual cells in the diagram) initiates in the diaphysis at the mid point of the long axis of the cartilage model. **C, D:** The differentiation to hypertrophic chondrocytes proceeds distally in a wave along the diaphysis. Differentiation of the loose mesenchyme surrounding the cartilage model into the perichondrium also begins at this midpoint and mirrors the differentiation of the underlying cartilage. As the wave of chondrocyte hypertrophy proceeds distally, a bony collar forms in the perichondrium adjacent to the cartilage, again beginning at the midpoint of the diaphysis. From here, blood vessels invade the cartilage model and cartilage is replaced by bone. **E:** The concerted wave of chondrocyte hypertrophy and bone formation proceeds along the diaphysis until the metaphyseal growth plates are formed. A steady state is reached where chondrocyte proliferation matches replacement by bone permitting regulated longitudinal bone growth while the epiphyses remain cartilage. **F, G:** As longitudinal bone growth proceeds, vascularization of the epiphyses by cartilage canals increases and chondrocytes in the center of the epiphyses become hypertrophic and are replaced by bone, forming the secondary ossification centers. In a process not yet understood, the articular cartilage is protected from ossification. Longitudinal growth ends when the metaphyseal growth plates are no long sustained and the primary and secondary ossification fronts fuse.

by bone [45,46]. Eventually, the entire epiphyseal cartilage is replaced by bone.

Skeletal formation occurs early in human development. For example, mesenchymal condensation is evident by 6.5 weeks of gestation, and the cartilage *anlage* of the tibia is formed by 8 weeks. Structural elements of the growth plate can be identified by 16 weeks, when approximately 60–70% of the cartilage *anlage* has been replaced by bone [47–49]. The growth plate brings about linear bone growth from this time through the end of puberty [43,48,49]. The formation and linear growth of a long bone is depicted schematically in Figure 2.

Permanent hyaline cartilage, other than articular cartilage, such as that in the nose, larynx, trachea, and bronchi, derives from local mesenchyme in a fashion similar to the formation of the cartilaginous models of the skeleton, but the chondrocytes do not progress to the hypertrophic stage [44]. The developmental history of other types of cartilage, such as the elastic cartilage of the ear, Eustachian tube, epiglottis, and certain laryngeal structures, and fibrocartilage, found in the intervertebral disks, symphysis pubis, and articular disks of many joints, is not as well defined as for hyaline cartilage [44,50].

STRUCTURE

Hyaline Cartilage

As the most abundant cartilage in the body, hyaline cartilage provides a good starting point to examine cartilage structure. At the molecular level, a great degree of complexity of the extracellular matrix is disguised by the simpler morphology. In addition to the many collagens, proteoglycans, and other proteins found abundantly or uniquely in cartilage, many gene products not typically associated with cartilage, such as basement membrane components [51–53] and complement proteins [54,55], are also expressed during the development of this tissue. Nevertheless, the defining feature of hyaline cartilage is an abundant extracellular matrix composed of an extensive system of collagen fibrils in which a network of aggregating proteoglycans is embedded. In combination, they impart the unique biomechanical properties typical of cartilage, rigidity without brittleness, and resistance to compression and shear. It must be pointed out that the structure, and especially the ultrastructure, of cartilage varies substantially according to the species, location, sublocation, and age of the cartilage, as well as to how it was prepared for study. A thorough description of all of these variations is beyond the scope of this chapter. Therefore, the general structure of hyaline cartilage is presented, with some emphasis on developmental cartilage, although a number of important deviations are addressed.

Hyaline cartilage appears glossy and almost translucent to the naked eye. At lower magnification, chondrocytes typically appear ovoid, but range in shape from round to flat (Fig. 3). Rounder cells tend to be found in the center of a cartilage structure, while flatter chondrocytes reside

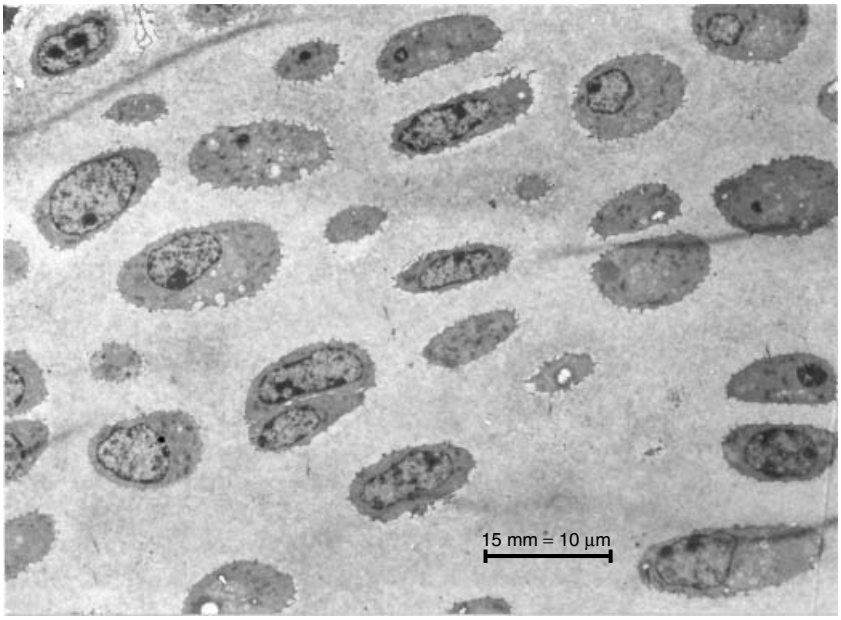


Figure 3. Transmission electron micrograph of epiphyseal cartilage from a newborn mouse. The cells have an even distribution, and the matrix stains relatively homogeneously, except for the region immediately surrounding the cells. Scale bar: 15 mm = 10 μm.

near borders, such as the perichondrium [56]. The nuclei are relatively large, usually centrally placed, and conform to the shape of the cell. Lipid droplets and poorly defined cytoplasmic structures may be seen. The distribution of cells in hyaline cartilage varies from relatively even to quite uneven. The space occupied by a chondrocyte and defined by the rim of extracellular matrix adjacent to the cell is often referred to as a lacuna. Two, or occasionally more, chondrocytes may occupy a single lacuna in hyaline cartilage, especially in young individuals, implying their recent division. The extracellular matrix appears relatively homogeneous by many stains. However, those that indicate the distribution of the cartilage proteoglycan, i.e., cationic dyes that form complexes with the glycosaminoglycan component of the macromolecules (toluidine blue, azure A), reveal a compartmental organization [56,57]. A densely staining pericellular compartment surrounds each cell or group of cells. This zone is circumscribed by a moderately dense-staining territorial matrix compartment, and a less intensely staining interterritorial matrix compartment occupies the intervening space. There may be a poorly staining ring called the border zone between the territorial and interterritorial compartments. An idealized chondrocyte and its matrix compartments are illustrated in Figure 4.

The fine structure of chondrocytes and matrix organization is much better defined by transmission electron microscopy (Fig. 5) [56,58,59]. The cytoplasm contains abundant rough endoplasmic reticulum, Golgi complexes, and secretory vesicles, which vary in relative amount according to the synthetic activity of the cell. Mitochondria are typically seen, as are fat droplets and glycogen deposits. Filamentous structures are extensive in some chondrocytes, and most cells contain a few microtubules. The plasma membrane is scalloped with many processes containing microfilaments, which may extend 1–2 μm into the matrix. Small invaginations called caveolae are prominent along the surface. A single small cilium may be detected in many chondrocytes, especially those from fetal cartilage.

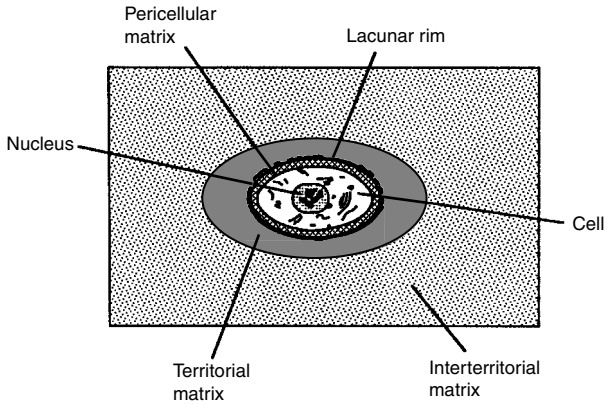


Figure 4. Idealized chondrocyte, depicting surrounding matrix compartments and other landmarks.

The fine structural organization of matrix compartments surrounding chondrocytes has been difficult to define for technical reasons, and the issue has been further clouded by the remarkable inconsistency in terminology that has been used. In some instances, the same structures have been given different names because they do not appear the same after different fixation procedures. In other instances, the same term has been applied to different structures. The nomenclature employed here is that given in Figure 4; it is consistent with that employed in reports of investigations in which the fine structure of the matrix has been well preserved [59–63].

Observations of tissues fixed by conventional chemical methods have implied the existence of a delicate lacunar

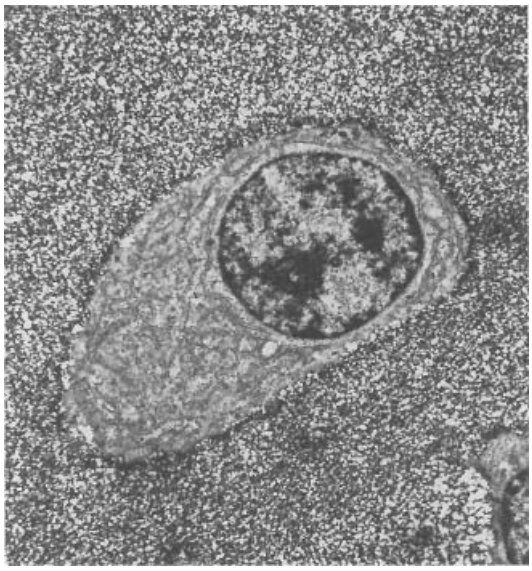


Figure 5. Transmission electron micrograph of an epiphyseal chondrocyte from a 9-day-old rabbit. Note the eccentrically placed nucleus and abundant rough endoplasmic reticulum. The section was stained with ruthenium red to demonstrate condensed proteoglycan granules, which obscure collagen fibrils. Note the slightly greater staining intensity of pericellular matrix. ×5600.

matrix occupying the space between the chondrocyte surface and the inner border of the lacuna [64]. The space has been called the lacuna, moat, halo, corona, and capsule [65–69]. However, fixation methods that preserve pericellular proteoglycans indicate that this space is an artifact of fixation, resulting from detachment and retraction of the cell membrane from the lacunar wall [65]. Fixation with substances that completely precipitate these proteoglycans, e.g., ruthenium hexamine trichloride, shows that the plasma membrane is contiguous with a dense proteoglycan coat that forms a pericellular envelope [60,62]. The collapsed proteoglycans are visualized as dense granules within this pericellular matrix compartment. The thickness of this compartment varies from cell to cell and from one region to another of the same cell and can be absent entirely [70]. Typically, few discernible collagen fibrils are seen. Fixation with ruthenium hexamine trichloride also indicates that the distribution of proteoglycans is relatively uniform in cartilage and thus the matrix compartments of cartilage are better defined and described by the organization and distribution of collagen fibrils [60].

Surrounding the pericellular envelope is a basket-like network of fine collagen fibrils aligned parallel to the surface of the chondrocyte which comprises the inner layer of the territorial matrix compartment [60,62], and is sometimes called the lacunar rim [56]. The fibrils are generally thin and some show faint 64-nm periodic banding, although others do not. Fine filamentous, as well as amorphous, structures can also be seen [71]. This capsular structure and its contents, referred to as a chondron, can be physically isolated from the tissue by mechanical shear or by enzymatic digestion. The structure of chondrons has been most extensively described in adult articular cartilage and they have been proposed to be the functional units of hyaline cartilage [57,72].

The remaining territorial matrix consists of randomly oriented bundles of collagen fibrils, which may exhibit faint periodic banding [73,74]. Specimens fixed in proteoglycan-precipitating agents show dense granules dispersed throughout the matrix [75] that, in many instances, are in close proximity to collagen fibrils. The interterritorial matrix is distinguished ultrastructurally by collagen fibrils, which may be oriented differently from those of the territorial matrix, depending on location [73,74], and which are, in general, thicker than those in the territorial matrix compartment. Proteoglycan granules are more abundant and are observed along and between collagen fibers. After cryotechnical preparation, an additional ultrastructural feature consisting of fine filaments, 10–15 nm in diameter and with crossbanding similar to collagen fibrils, has been observed in all matrix compartments [76]. However, the biochemical nature of these filaments is as yet undefined.

Fetal cartilage is characterized by a rather open network of collagen fibrils with diameters less than 25 nm and larger interfibrillar spaces that are filled with proteoglycan. In such a preparation fixed with ruthenium compounds, collagen fibrils are readily visible (Fig. 6A). Typically, the fibrils are individual rather than bundled as in adult cartilage and most other tissues and often show little preferred orientation. At times, fibrils of the territorial matrix near the chondrocyte run parallel to the plasma membrane in a concentric fashion, especially in the growth plate [60] and in developing articular cartilage [77]. After cryotechnical preservation, by which both the cell and the matrix are optimally preserved [78], no distinct pericellular envelope is visible and collagen fibrils are not excluded from the region adjacent to the plasma membrane (Fig. 6B). Fibrils are difficult to see embedded in the proteoglycan gel, but their distribution is not altered near the cell and the uniformity of appearance of the matrix is remarkable in light of previous studies using other methods of ultrastructural preservation.

The collagen fibrils are predominantly composed of the fibrillar collagen, type II, with lesser amounts of the related type XI collagen and the fibril-associated collagen (FACIT), type IX [79]. Polymerization of collagen molecules to form this structural unit of cartilage is complex and many factors may function in concert to maintain the small fibril diameter found in developing cartilage. Among these are the rate and order of processing of the propeptide domains [80], the presence of the fibril-associated proteins/proteoglycans decorin and biglycan [81] and fibromodulin [82], and the incorporation of the minor collagens, types IX and XI [83,84]. Mice with a perinatally lethal mutation in one of the genes for type XI collagen (COL11A1) fail to synthesize type XI collagen and produce a soft cartilage with compromised biomechanical integrity [85]. The cartilage extracellular matrix contains more sparsely distributed collagen fibrils with very large diameters suggesting an *in vivo* role for type XI collagen in maintaining (limiting) fibril diameter. The large amino-terminal noncollagenous domain of type XI collagen has been shown to be localized to the surface of thin cartilage fibrils [84], and it is likely that this domain sterically limits lateral growth of fibrils. The severe phenotype of this mutation demonstrates the fundamental importance of regulating such morphologic parameters.

Specific intermolecular interactions have been proposed to stabilize the proteoglycan-collagen fibril system beyond physical entanglement. Proteoglycan has been localized at regular intervals along collagen fibrils [86]. Recent evidence

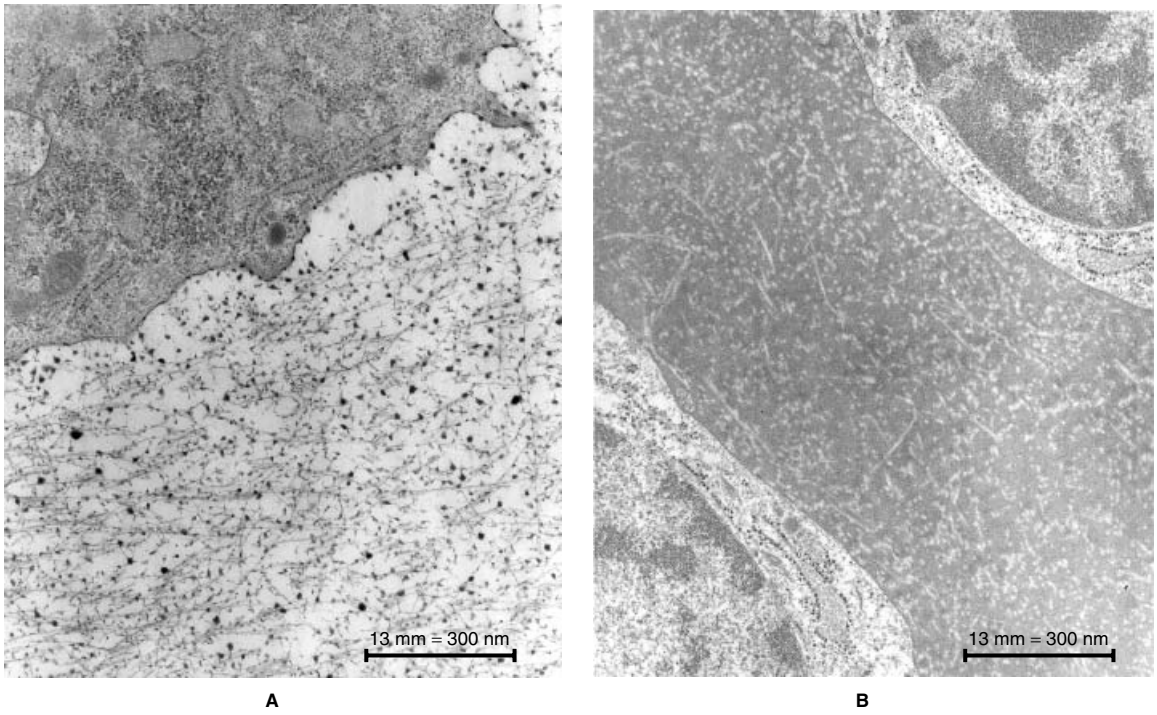


Figure 6. Transmission electron micrograph of 17-day fetal chick sternal cartilage, typical of nonpermanent fetal cartilage. **A:** The section was prepared by conventional aldehyde fixation and stained with ruthenium. It shows a somewhat higher magnification of the matrix than the previous figure. The collagen fibrils are thin and randomly oriented except very near the cell where they tend to run more parallel to the cell surface. Proteoglycans are collapsed into electron dense granules by this preparation revealing ''spaces'' between the fibrils. Combined with cell shrinkage, it suggests exclusion of fibrils from the immediate vicinity of the cell. The condensed proteoglycan granules appear associated with collagen fibrils in the matrix and perhaps slightly concentrated in the pericellular space created by cell shrinkage. **B:** The same tissue was prepared by high pressure cryotechnical fixation. The proteoglycans are not condensed so the area between fibrils appears electron dense, with no ''spaces.'' The matrix abuts directly on the plasma membrane of both cells because there is no cell shrinkage. Collagen fibrils (appearing light by this technique) are not excluded from the matrix near the cell surface and again tend to run parallel to it (seen mostly in cross section). Scale bar: 13 mm = 300 nm.

for an interaction between collagen molecules and the keratan sulfate domain of aggrecan occurring in the gap regions of the fibrils provides a putative mechanism for such a stabilizing connection [87]. Type IX collagen is located on the surface of the fibrils covalently associated with itself and type II collagen [88]. It contains a large globular domain, which can protrude from the fibril [89]. Type IX molecules interact with other type IX molecules via this domain [90] supporting the proposition that type IX collagen stabilizes inter-fibril interactions [91]. Mice lacking type IX collagen show normal skeletal development, but a progressive loss of structural integrity is evident from the early and severe osteoarthritis that develops [92]. There are many other proteins associated with the fibril surface; it is possible that one or more mediates similar interactions between the fibrils and the aggrecan complex. Finally, matrix-stabilizing interactions may be provided by the activity of transglutaminase which has been implicated in cross-linking cartilage extracellular matrix components, especially in the hypertrophic zone of the growth plate [93,94].

Despite the presence of matrix compartments and differing organization of collagen fibrils, histochemical and immunohistochemical staining of fetal cartilage suggest the composition of the matrix is consistent and homogeneous. Thus, the fibrillar components, collagen types II, IX, and XI, and the aggrecan network are found throughout fetal cartilage. Noncollagenous proteins such as cartilage oligomeric matrix protein (COMP) and CMP are rather evenly distributed as well [25]. However, there are exceptions and changes occur as the skeleton matures. For example, type XI collagen is found throughout the matrix of fetal cartilage, but the protein isoform containing the peptide encoded by exon 6B of *Col11a1* is found preferentially in diaphyseal cartilage but not in epiphyseal cartilage (Fig. 7). In fetal bovine cartilage, the FACIT collagens, types XII and XIV, are found more abundantly near the articular surface and around cartilage canals, and staining diminishes toward the growth plate [95]. The glycosaminoglycan component of cartilage proteoglycans appears to differ in the territorial and interterritorial compartments. The territorial compartment is relatively enriched in chondroitin sulfate compared with keratan sulfate side chains, whereas the opposite is observed in the interterritorial compartment [33,56]. CMP is present in fetal cartilage but is absent from developing and adult articular cartilage [25]. In a complementary fashion, fibromodulin is preferentially localized in developing articular cartilage [25,96]. Lumican, which is difficult to detect in early fetal cartilage, is present in neonatal/juvenile cartilage and is abundant in adult cartilage [97]. These and other modulations of matrix composition suggest that not all chondrocytes and their associated matrices are functionally equivalent.

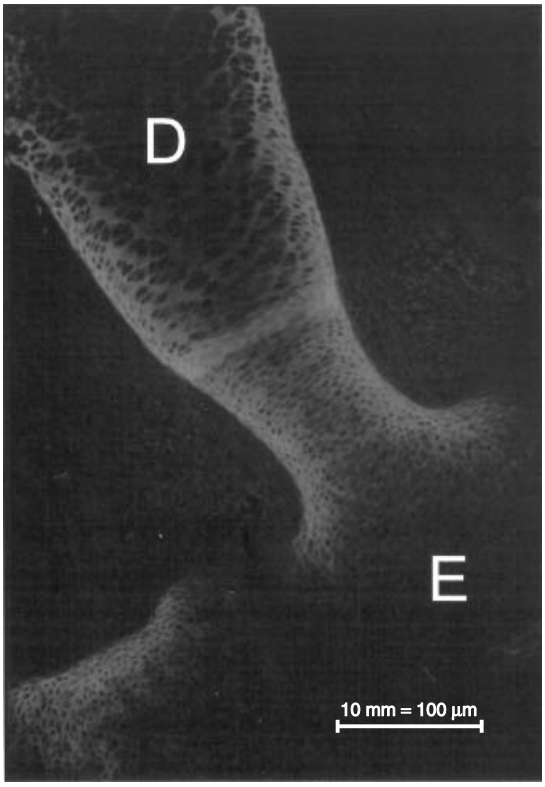


Figure 7. Indirect immunofluorescence staining of 18-day fetal rat elbow cartilage with antibodies to type XI collagen. Antiserum recognizing all isoforms of type XI collagen stains the cartilage uniformly. Staining with a monoclonal antibody specific to the isoform of type XI collagen containing the peptide encoded by exon 6B of the α1(XI) chain shows that in the humerus, this isoform is restricted to diaphyseal regions, D, adjacent to the perichondrium and is not found in the epiphysis, E, at this stage. Although the function of this isoform is unknown, this observation reveals complexity and nonuniformity of the cartilage matrix that are not readily apparent from morphology or ultrastructure alone. The ulna is at the lower left. Scale bar: 10 mm = 100 μm.

Perichondrium

The surface of most cartilage is covered by a fibrous covering known as the perichondrium. This is continuous with the periosteal cover of bone and blends into surrounding fibrous connective tissue [56]. The articular surface and cartilage-bone interfaces of articular cartilage and the growth plate lack a perichondrium. The structure is usually divided into an outer fibrous and an inner chondrogenic layer. The outer layer contains bundles of typical banded collagen fibrils and elastin. Types I, III, and V collagen have been localized by antibodies to this layer, whereas staining for proteoglycans is much less intense than in cartilage matrix [33]. Fibroblasts are detected between the fibrous bundles, and blood vessels are frequently seen. The inner layer of perichondrium blends into the subperichondrial cartilage. Chondrocytes in this region are flat and aligned tangentially with the cartilage margin. The cell density is higher here than deeper in the cartilage; the gradual change in density correlates with a change in cell shape from flat to ovoid (Fig. 8).

The development of the perichondrium begins at the middle of the embryonic bone as a concentration of polygonal cells that covers the cartilage *anlage* [98,99]. Indeed, each successive stage of development is first detected at this point; it then propagates distally in both directions correlating with the state of differentiation of the underlying chondrocytes. As the chondrocytes become hypertrophic, the perichondrial cells adjacent to them elongate in the direction of the long axis of the bone. These cells form overlapping layers and then develop cell-cell contacts or syncytia. Finally, the outer layers of cells form a fibrous extracellular matrix that separates the cells. The most differentiated perichondrium is associated with mature or hypertrophic chondrocytes. The more distal proliferative regions of developing cartilage have a less developed perichondrium. The epiphyseal regions do not have a true perichondrium as defined morphologically. The fact that the most organized perichondrium is associated with the narrowest parts of the developing bone suggests that the perichondrium imposes a physical constraint on the lateral growth of the rudiment. Such a constraint would promote longitudinal growth as chondrocytes in the center produced matrix or enlarged as a result of hypertrophy, as well as contribute to the difference between the width of the cartilage at the diaphysis and epiphysis [98]. It has also been suggested that the perichondrium provides an inhibitory signal for chondrocyte proliferation [100].

During development, the perichondrium immunohistochemically and by *in situ* hybridization stains intensely for a variety of extracellular matrix proteins, including types XII and XIV collagen [101,102], fibrillin [103], and tenascin and the cell surface proteoglycan, syndecan 3 [104]. Their specific roles are unknown, but it is likely that fibrillin and type XII collagen contribute to mechanical constraints and that syndecan 3, the expression of which is localized to the perichondrium of proliferating regions of cartilage, contributes to growth factor function [105]. It is now clear that the perichondrium is much more than a passive restraint and covering. Components of many molecular signaling systems such as BMPs [106,107], Indian hedgehog (Ihh) [108], transforming growth factor-β (TGF-β) [109], and retinoic acid [110,111], which have pronounced effects on chondrocyte differentiation, can be found in the perichondrium.

Vascular Canals

Some hyaline cartilages, including epiphyseal, costal, and laryngeal cartilages, contain vascular canals [56]. These enter from perichondrial vessels and penetrate the cartilage, occasionally forming anastomoses. A typical cartilage canal is comprised of capillaries surrounding a central arteriole and venule (Fig. 9) [56,112]. A variable amount of interstitial connective tissue is usually present, and banded collagen fibrils can be seen in bundles. Types I and III collagen and fibronectin localize immunohistochemically to the interstitial tissue and vessel walls [33]. The chondrocytes in the immediate vicinity of the vascular canals are often larger than typical chondrocytes, and some resemble hypertrophic chondrocytes. The cartilage matrix around vascular canals is enriched in type XII collagen [95]. Younger cartilage (i.e., fetal, newborn) tends to contain more canals than older cartilage. They are often located in the vicinity of the growth plate, and "communicating canals" between the epiphyseal and metaphyseal vessels may sometimes be detected in the growth plate [113].

Growth Plate

The growth plate, or physis, is a very specialized form of hyaline cartilage that is responsible for linear

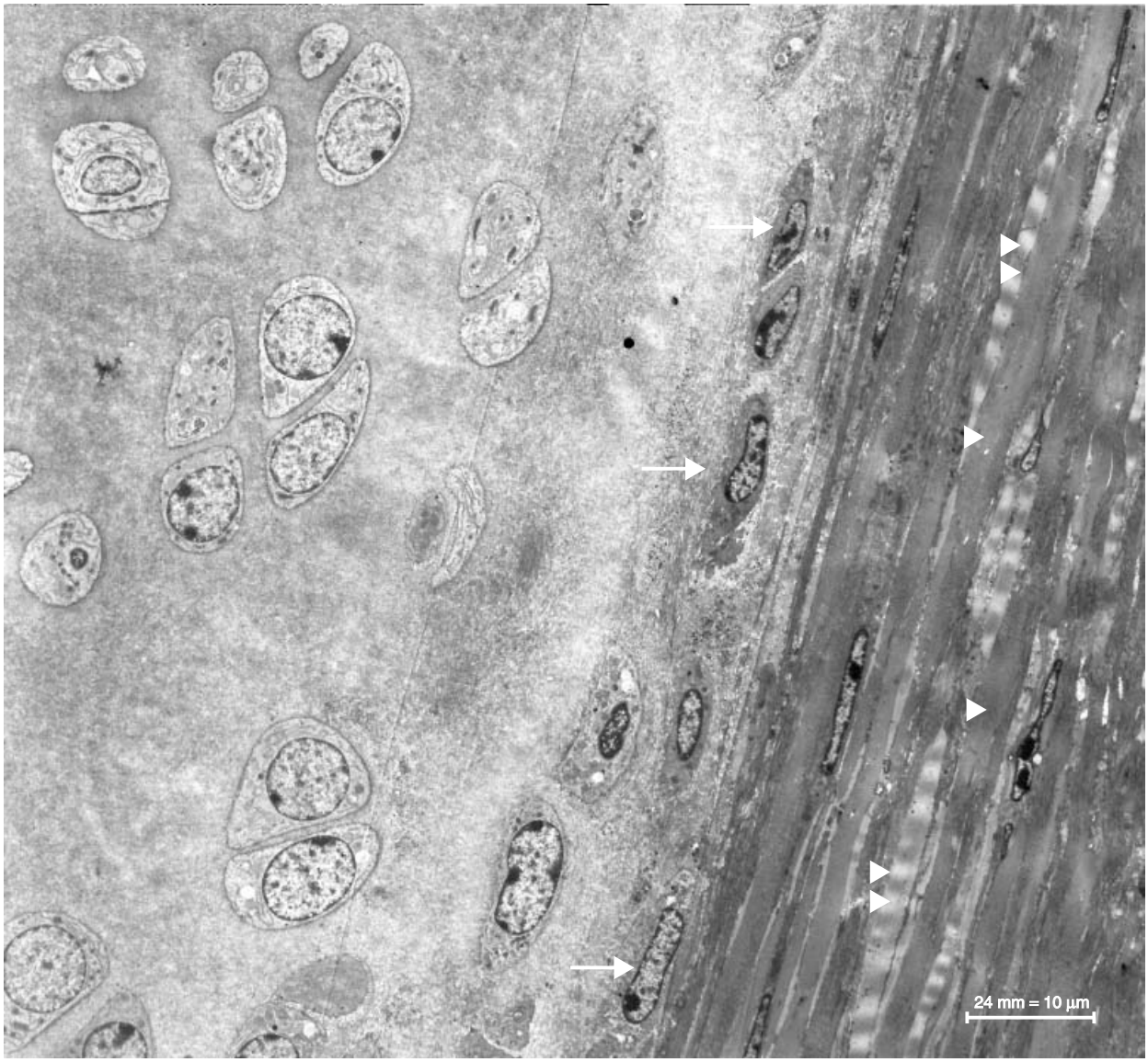


Figure 8. Transmission electron micrograph of newborn rat perichondrium. The perichondrium consists of flattened cells and very dense bundles of collagen fibrils running more in parallel to the section (arrowheads) or more in cross section (double arrowheads) (see also Fig. 14). The chondrocytes adjacent to the perichondrium (arrows) are more flattened than those farther into the cartilage. The matrix around them appears coarser due to larger, more densely arranged collagen fibrils indicating a transitional matrix containing cartilage and non-cartilage components. Scale bar: 24 mm = 10 μm.

skeletal growth. It has been the subject of several lengthy reviews [43,47–49,114]. Its structure is shown in Figure 10. The growth plate is a transition tissue that occupies only a few millimeters of space between the epiphyseal cartilage at the ends of growing bones and the bone proper. Chondrocytes in the growth plate play out the latter stages of the differentiation scheme outlined earlier. After proliferating in the direction of bone growth, chondrocytes enter a maturational or prehypertrophic stage where they actively secrete matrix, which pushes the cells apart from one another. As they complete terminal differentiation, the growth plate chondrocytes enlarge, taking on the form of hypertrophic chondrocytes. These cells alter the matrix, so that some areas become mineralized, while others become degraded. Finally, the hypertrophic chondrocytes die and are replaced by osteoblasts that deposit bone in place of cartilage. For any one cell, this sequence of events occurs at one site over time. However, because the bone is growing, these temporal events are represented spatially in the tissue. The chondrocytes at the earliest stages of the scheme are furthest from, and those at the latest stages closest to, the ossification front where bone is formed. Since neighboring chondrocytes are at approximately the same stage of the scheme, they tend to be the same distance from the front. This arrangement creates the appearance of columns or elongated clusters of cells aligned parallel to the direction of growth and zones of cells with similar characteristics perpendicular to this axis. The names of

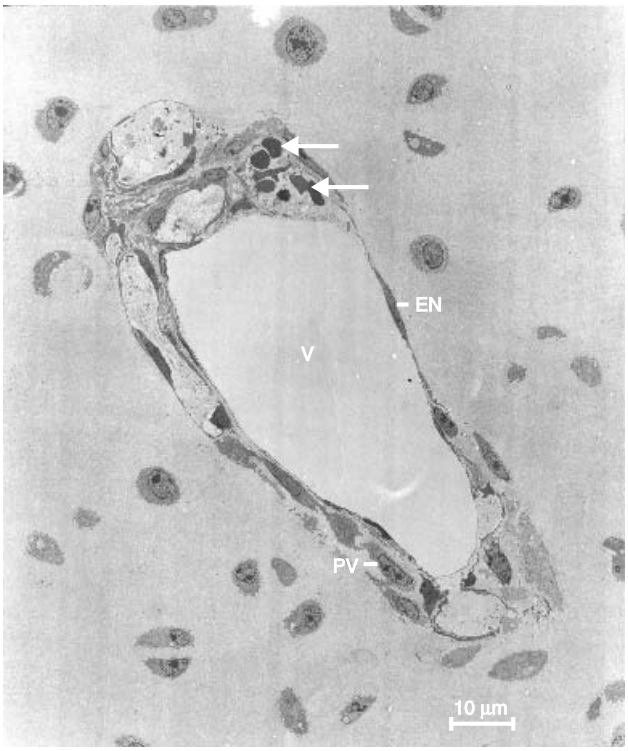


Figure 9. Transmission electron micrograph of a vascular canal from an early second trimester fetal calf. A cartilage canal contains an arteriole, one or more venules (V) and associated capillaries. Note the endothelial (EN) and perivascular (PV) cells. A few capillaries containing red blood cells are indicated by arrows. Scale bar: 15 mm = 10 µm.

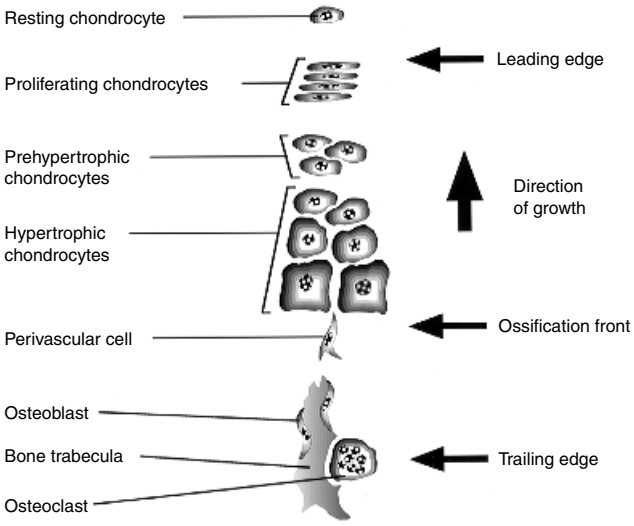


Figure 10. Schematic representation showing the different zones of the growth plate in relationship to bone growth and ossification. Also depicted are the changes in growth plate structure with time, and the life cycle of a single chondrocyte. The cell starts as a resting chondrocyte, differentiates to a hypertrophic chondrocyte, and finally dies as the ossification front approaches and passes through the site occupied by the cell.

the zones reflect their predominant activity, i.e., reserve or resting, proliferative, maturational or prehypertrophic, and hypertrophic, schematically represented in Figure 10 and shown in the photomicrograph in Figure 11. It must be emphasized, however, that the borders between

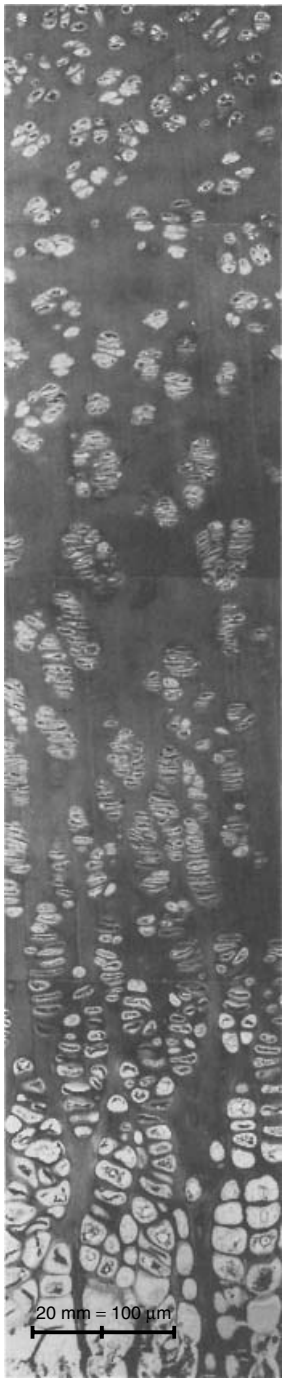
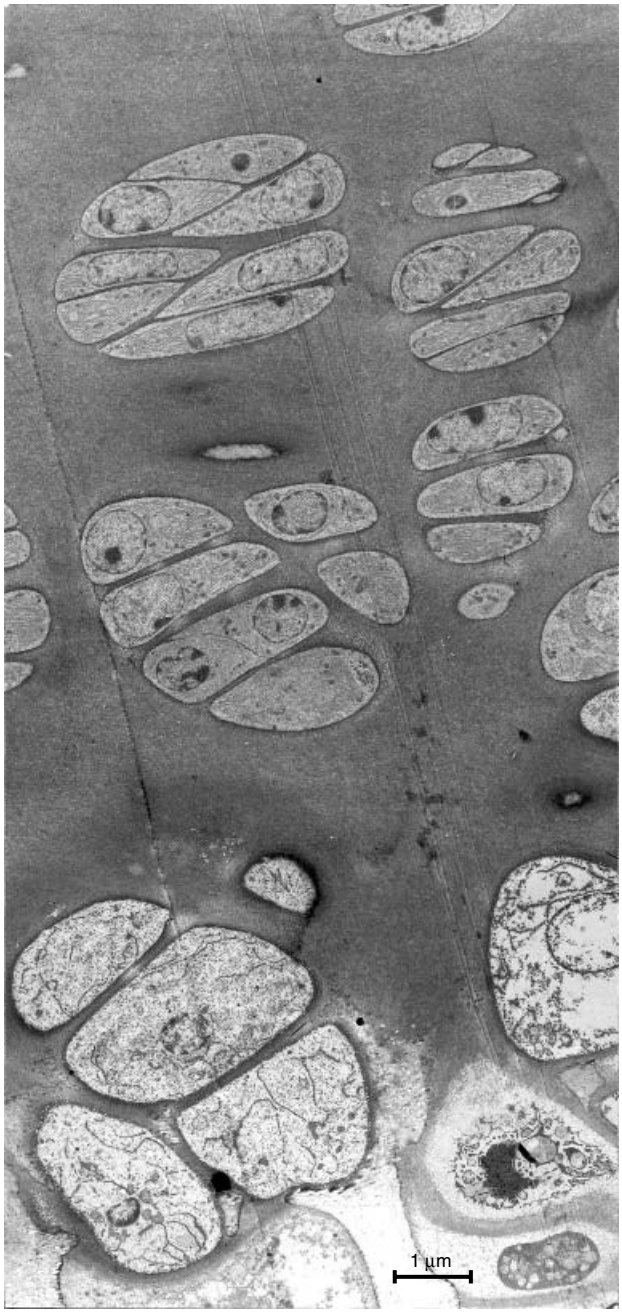


Figure 11. Light photomicrograph (composite) of a rib growth plate from a newborn infant. Note the gradual transition from the reserve zone at the top to the zone of vascular invasion at the bottom, where the invasion of several chondrocyte lacunae can be seen. Methylene blue/basic fuchsin. Scale bar: 20 mm = 100 µm.

these zones are not precise and that the cellular and matrix changes that occur in the growth plate happen gradually, rather than in distinct steps, as implied by zonal designations.

Although all growth plates share the same general structure, they differ slightly from site to site and from species to species, as documented in the literature and in the experience of the authors. Growth plates from fast-growing bones are wider than those from slow-growing bones [115]. Hence, a femoral growth plate from a fetus or infant will be wider than one from an older child, and a femoral growth plate will be wider than an iliac crest or vertebral body growth plate from the same child. Similarly, the growth plates of fast-growing bones, such as a tibia or femur, tend to exhibit the columnar arrangement of cells, whereas the elongated cluster arrangement is more typical of slowly growing bones, e.g., iliac crest. Fetal growth plates are not as well organized as those from newborn infants or older children; the younger the fetus, the less the organization. It is not clear whether this reflects the faster growth rate or the "immaturity" of the fetal bone.

A



B

Figure 12. Transmission electron micrographs of chondrocytes from the growth plate proliferative **(A)** and hypertrophic **(B)** zones. In the proliferative zone **(A)**, note the distinct, more amorphous-appearing territorial matrices that fuse together to create transverse septa, and the vertical alignment of collagen fibrils along the lateral borders of the micrograph, which correspond to longitudinal septa of the growth plate. The hypertrophic zone **(B)** shown in this micrograph illustrates that the proliferative cells, prehypertrophic cells, and hypertrophic cells can appear as asymmetric clusters rather than distinct columns. Scale bars: **(A)** 24 mm = 10 μm; **(B)** 15 mm = 1 μm.

Figure 12. (*Continued*)

The zone farthest from the center of the growing bone is the zone of reserve or resting chondrocytes. It merges with the epiphyseal cartilage, which it closely resembles except for the orientation of the ovoid-shaped chondrocytes. Their long axes are oriented perpendicularly to the axis of bone growth.

As shown in Figure 12A, the proliferative zone contains flattened chondrocytes in columns or clusters parallel to the axis of growth [33,49]. The cells are close together, implying

recent division; however, mitotic cells are difficult to detect. Uptake of ^{3}H-thymidine or the deoxynucleotide analogue, bromodeoxyuridine (BrdU), reflecting DNA synthesis and cell replication, is restricted mainly to this zone of the growth plate [116,117]. The cells are rarely separated by only their plasma membranes; more commonly, each cell is surrounded by its own territorial matrix compartment, which may merge with that of an adjacent cell [60]. Chondrocytes in the lower proliferative zone contain relatively more rough endoplasmic reticulum, Golgi complexes, and secretory vesicles than cells in the upper part of the zone [65]. Single cells and surrounding territorial matrices in the lower proliferative zone are separated by interterritorial matrix with the ultrastructural characteristics described earlier; however, all the chondrocytes within a cluster share a common territorial matrix [33,60]. Thus, a cluster of cells in this region with its shared territorial matrix compartment is the chondron, or functional unit, of the growth plate. It is comprised of the daughter cells of a single differentiated cell in the reserve or upper proliferative zone, i.e., a clone.

Although the histochemically defined differences between the territorial and interterritorial matrix compartments of typical hyaline cartilage (including epiphyseal cartilage and the growth plate reserve zone) are the same in the proliferative zone, the orientation of collagen fibers changes from random to the direction of growth (longitudinal or vertical by convention) in the interterritorial matrix. This presumably results from stretching of this compartment by the directional cell division and compression from the accompanying secretion of new matrix. The net result is the formation of septa between the chondrons, which exhibit a longitudinal or sometimes diagonal orientation. This interterritorial matrix is continuous with that of the reserve and epiphyseal cartilage. Small membrane-bound structures called matrix vesicles are found in these septa [118,119]. They measure approximately 100–200 nm in diameter and occasionally contain ribosomes. They are thought to be derived from the cell processes of nearby cells and participate in mineralization of the matrix in the lower growth plate. Matrix vesicles are not normally found in the transverse septa that separate individual cells, which is composed of territorial matrix [60].

As one moves to the hypertrophic zone, there is a gradual increase in cell size that corresponds to the expression of the hypertrophic chondrocyte phenotype (Fig. 12B). Earlier studies, using conventional chemical fixation, suggested that cells in the lower hypertrophic zone degenerated. However, studies employing cryofixation combined with methods to preserve matrix proteoglycans indicate that they remain intact throughout the hypertrophic zone to the point of metaphyseal vascular invasion [60,61,63,120]. Using this approach and stereologic procedures, Hunziker et al. were able to quantify and compare a number of parameters of chondrocytes of the proliferative and lower hypertrophic zones of the rat tibia [61]. Hypertrophy was associated with increases in cell volume and surface area of approximately ten- and fourfold, respectively. The increases were due mainly to changes in vertical diameter. The absolute amounts of rough endoplasmic reticulum, Golgi complexes, and mitochondria increased in the hypertrophic chondrocytes two-, four-, and threefold, respectively. However, the relative amounts of these organelles per cell volume decreased, suggesting a large influx of fluid during the process. Although the absolute numbers differed, Buckwalter et al. reached the same conclusions using a somewhat different methodology to examine the mouse tibial growth plate [63]. These observations illustrate the dramatic changes in cell morphology that occur during chondrocyte hypertrophy.

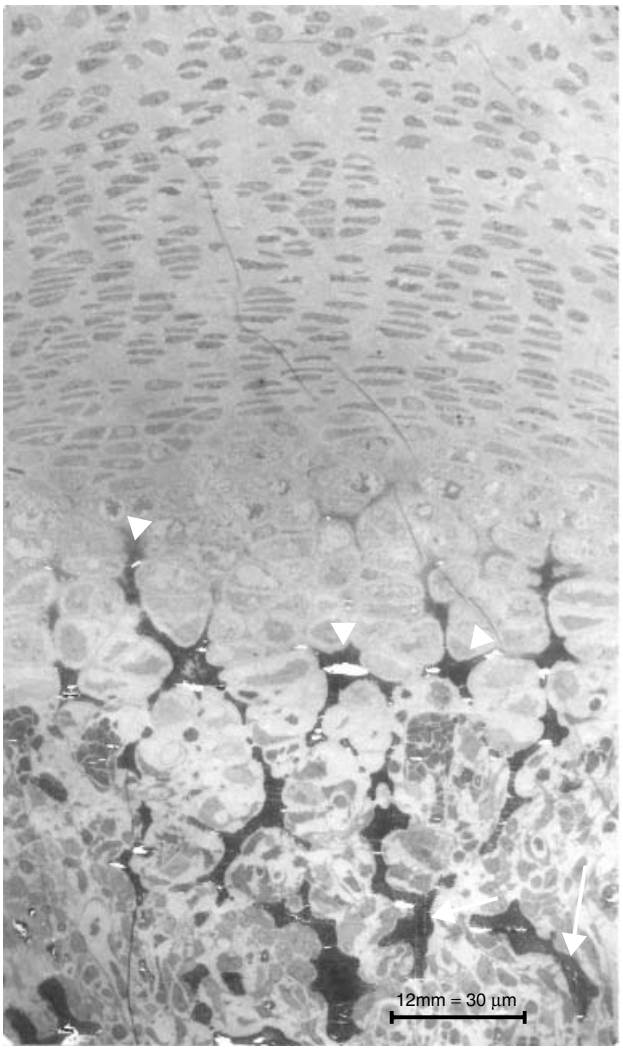
More extensive morphometric analysis of the hypertrophic zone indicates that extracellular matrix changes also contribute to longitudinal growth, albeit to a lesser extent than changes in cellular volume. The collagen content and the volume of both the territorial and interterritorial matrix compartments are expanded relative to the proliferative zone, with the territorial matrix making the larger contribution [121]. These matrix changes are accompanied by extensive remodeling, especially in the longitudinal septa, as evidenced by the turnover of type II collagen predominantly in this region [122].

The structural organization of matrix compartments in the hypertrophic zone is similar to that in the proliferative zone. By light microscopy, the septa appear more compressed by the enlarging cells, but electron microscopy shows that the collagen fibrils of the territorial matrix are arranged parallel to the cell surface and are more tightly packed than the longitudinally aligned fibrils of the interterritorial matrix [60]. Mineralization can be detected by electron microscopy as crystalline needles of apatite inside matrix vesicles in the septa of the upper hypertrophic zone [119]. In the middle of the zone, the crystals protrude from the vesicles and clusters of crystal needles extend outside the matrix vesicles. Coalescence of the clusters is detected in the lower hypertrophic zone, where the mineralization is visible by light microscopy [33]. Mineralization occurs normally only in the interterritorial matrix of the longitudinal septa (Fig. 13A). The territorial matrix and thus the transverse septa, which are composed of common or adjacent territorial matrices, are not mineralized (Fig.13B) [60,118]. The restricted pattern of mineralization is likely to be due in part to the production by lower hypertrophic chondrocytes of matrix Gla protein, a calcium-binding protein that inhibits mineralization [123]. The mineralization of cartilage matrix is often called provisional calcification.

Histochemical methods have shown changes in the matrix of the hypertrophic zone that are not evident morphologically. These changes are mainly in the territorial matrix of the hypertrophic cells. Most notable is the presence of type X collagen, a collagen that appears to be synthesized only by hypertrophic chondrocytes in the growth plate [31]. There is also evidence that this collagen is also expressed in articular cartilage in mouse, pig, and human late in development or in mature tissue [124,125]. Nevertheless, type X collagen is typically used as a marker for hypertrophic chondrocytes and the hypertrophic zone. The organization of type X collagen in the matrix is not entirely clear. Type X collagen forms a filamentous network in association with collagen fibrils near cells [126], and it contains typical lysine-derived collagen cross-links [127]. Type X collagen can also form a more elaborate hexagonal network *in vitro* [128] and so it can both self-associate and presumably interact with other collagens. It has also been shown to interact with proteoglycans [129,130]. The function of type X collagen is unclear, but changes in the organization of proteoglycans, matrix vesicles, and subchondral trabecular bone have been observed in type X-null mice [131].

Type I collagen and fibronectin have been localized intracellularly and in the pericellular region of cells in the lower hypertrophic zone [33]. Also, several other proteins more often associated with bone matrix are produced

by hypertrophic chondrocytes. These include osteocalcin, osteopontin, bone sialoprotein, and osteonectin [40,132], and alkaline phosphatase activity is associated with cell membranes and matrix vesicles in the same region [118,133]. It is possible that expression of these proteins by both hypertrophic chondrocytes and osteoblasts reflects a convergent requirement for the mineralization of their respective matrices [132]. However, it is also possible that some hypertrophic chondrocytes in appropriate circumstances escape apoptosis and functionally become osteoblasts [40,134,135].



A

B

Figure 13. Transmission electron micrograph showing the zone of provisional calcification. **(A)** The calcified areas appear black (arrowhead) and are found primarily in the longitudinal septa composed of interterritorial matrix between columns/clusters of lower hypertrophic chondrocytes. As the ossification front advances, this calcified cartilage forms the trabeculae (arrow) in the subchondral bone. The territorial matrix that lies adjacent to the longitudinal septa and the fused territorial matrices that form the tranverse septa are not mineralized. This is readily seen at higher magnification **(B)**, where the hydroxyapatite crystals, dark area (left), are excluded from the territorial matrix (center) of the hypertrophic chondrocyte (right). Scale bars: **(A)** 12 mm = 30 μm; **(B)** 13 mm = 300 nm.

Figure 13. (*Continued*)

Compared to the interterritorial matrix in the septa of the lower hypertrophic zone, the adjacent territorial compartment appears to stain less intensely with antibodies to type II collagen, proteoglycan, and proteoglycan link protein; however, this may be an artifact of compression [33].

Metaphyseal blood vessels penetrate the transverse septa and lacunae of the last (terminal) hypertrophic chondrocytes in the zone of vascular invasion. This can be seen at the bottom of Figure 11. Capillary sprouts and perivascular cells resembling macrophages are seen in direct contact with the transverse septa [136]. Partially degraded transverse septa can be identified in some instances, while dissolution of the septa with protrusion of the capillary sprouts into the lacunae can be seen in others. Extravasated red blood cells and platelets may be found in perforated lacunae. Degeneration of the terminal chondrocytes, i.e., contraction and rupture of the plasma membrane, may be observed in some lacunae in which the transverse septa are partially intact. Degradation of the territorial matrix of the terminal hypertrophic chondrocytes and neighboring longitudinal septa in some cases is also seen.

The normal and timely advance of blood vessels into the cartilage matrix is dependent on the expression of the matrix metalloproteinase, MMP-9 (gelatinase B). Analysis of MMP-9-null mice has provided insight into the regulation of cartilage degradation and bone formation in the growth plate [137]. Observations suggest that MMP-9 is produced by a cartilage-degrading cell, or chondroclast, originating in the bone marrow. The activity of MMP-9 leads to the release of angiogenic factors and promotes apoptosis

of hypertrophic chondrocytes. In the model derived from the MMP-9-null mouse, chondroclasts from the vascular bed of newly forming bone arrive at this last layer of hypertrophic chondrocytes initiating a degradative cascade by the secretion of MMP-9. This degradation provides both access and stimulus for further vascularization. The destruction of cartilage and/or the approach of capillaries induce the apoptosis of nearby hypertrophic chondrocytes that is observed near invading capillaries [120,138]. Finally, the capillaries introduce osteoblasts that deposit osteoid (bone matrix) in the remodeling matrix and lacunae vacated by the terminal chondrocytes. Not surprisingly, the highest levels of expression of interstitial collagenase (MMP-1) [139,140] and collagenase-3 (MMP-13) [141] during embryonic development occur at this interface of hypertrophic cartilage and forming bone.

Proximal (metaphyseal) to vascular invasion is the so-called primary spongiosa or zone of primary ossification [114]. Trabeculae oriented in the direction of growth contain an inner core of cartilage matrix that is frequently contiguous with the longitudinal septa of the growth plate, and which corresponds to the interterritorial matrix that was not degraded by vascular invasion [39]. Osteoblasts deposit bone matrix, osteoid, on these cartilaginous cores immediately beneath the sites of vascular invasion. The cartilage cores are gradually replaced by bone matrix during remodeling of metaphyseal bone [114]. Thus, the extensions of the longitudinal septa into the metaphyseal bone provide scaffolding for the deposition of bone matrix in subchondral bone, physically anchor the growth plate to the bone, and, in essence, provide continuity between cartilage and bone elements of the growing skeleton.

Much of the growth plate is bordered peripherally by a structure called the perichondrial ossification groove (of Ranvier) [48,142]. It encircles the lower growth plate and extends to the proliferative zone, where the groove is deepest (Fig. 14). The outer fibrous layer is continuous with the periosteum and, to a large extent, with the perichondrium. The innermost cells are densely packed and are thought to be mesenchymal precursor cells. It has been proposed that in the lower growth plate they become osteoblasts, which give rise to the sheaths of so-called "bone bark" that surround the zone of vascular invasion and part of the hypertrophic zone. In the upper growth plate, they may differentiate along chondrocytic lines and contribute to appositional growth of epiphyseal cartilage [142].

There has been much recent progress in understanding how the growth plate is regulated. This has focused on the control of chondrocyte proliferation and terminal differentiation because these events ultimately determine the rate at which terminally differentiated hypertrophic chondrocytes are presented to the ossification front. At least three mechanisms have been identified. The best defined is a regulatory circuit in the form of a negative feedback loop, involving FGFs, Ihh, PTH-related peptide

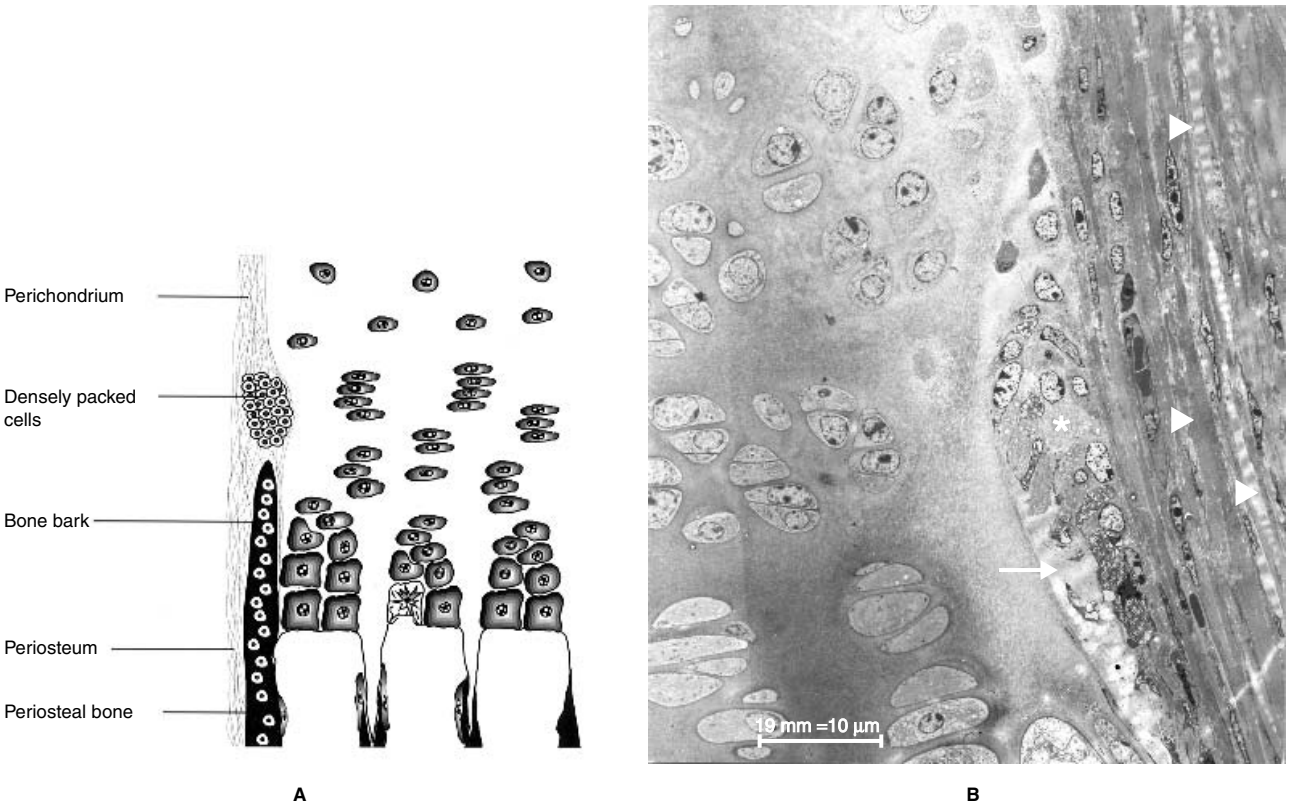


Figure 14. Schematic representation of the lateral border of a growth plate, showing the ossification groove (of Ranvier) and its components (bone bark and zone of densely packed cells). A transmission electron micrograph of the ossification groove is on the right. In this micrograph, the densely packed cells (asterisk) lacking extracellular matrix can be seen adjacent to the perichondrium. The mineralized bone bark is light in color (arrow). Note the thick bundles of collagen fibrils in the outer perichondrium (arrowheads). Scale bar: 19 mm = 10 μm.

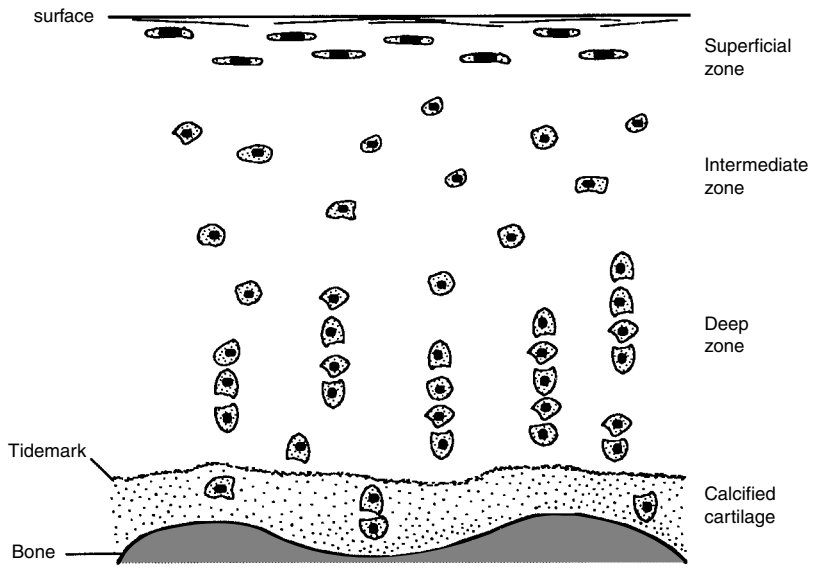


Figure 15. Schematic representation of adult articular cartilage, showing the zones and organization of articular chondrocytes.

(PTHrP) and, possibly, BMPs that controls the rate of formation of prehypertrophic cells [108]. FGFs acting through FGF receptors, especially FGFR3, constitute the second mechanism. FGFR3 activation inhibits bone growth, most likely through blocking both proliferation and terminal differentiation of growth plate chondrocytes [143]. The third mechanism involves extracellular matrix proteins that appear to affect chondrocyte proliferation and terminal differentiation by influencing the diffusion and presentation of growth factors to cells and by directly interacting with receptors on cell surfaces [144].

Articular Cartilage

Articular cartilage is usually divided into four zones: superficial (tangential), intermediate (transitional), deep (radial), and calcified (Fig. 15). The alternative designations reflect the alignment of collagen fibers as visualized by light microscopy [59]. The zones are sometimes referred to as zones I–IV, respectively. As with other cartilages, there are differences in cellular and matrix morphology depending on joint location, degree of weight bearing, and age and species of the cartilage. For example, it is difficult to distinguish the intermediate from the deep zone in young cartilage compared with articular cartilage from an adult [74]. Similarly, the articular cartilage of a growing bone does not have a calcified zone; rather, it blends into epiphyseal cartilage that resides between the articular surface and the growth plate (Fig. 16). This is more evident in species with large bones (and large epiphyseal cartilages), such as man, and becomes less apparent as secondary ossification centers in the epiphyseal cartilage encroach upon the articular region. Despite these differences, articular cartilages share many features with each other as well as with other cartilages. With the variations noted below, the ultrastructure of the chondrocytes and matrix, the organization of matrix into compartments, and cell-matrix relationships are similar to what has already been described.

The superficial zone is thinnest along the articular surface and widest at the articular margin, where it merges with the perichondrium. The articular surface is comprised of a thin layer of densely packed, fine collagen fibrils running parallel to the surface [73]. The fibrils have characteristic periodic banding, and many form bundles [71,74]. There are no cells in this fibrous layer. However, the lower part of the superficial zone contains flattened cells aligned parallel to the surface. These chondrocytes exhibit a cell-surface polarity [59,73]. The cell membrane facing the surface tends to be smooth and to contain prominent invaginations, or caveolae, while many cytoplasmic processes are found on the other (basal) side of the cell. Rough endoplasmic reticulum and Golgi complexes are sparse [73]. The typical basket-like pericellular capsule and territorial matrix compartment are not prominent in this zone. Cationic dyes reveal less proteoglycan than in deeper zones; however, proteoglycans and proteoglycan link protein can be detected by immunostaining [59,86]. The chondrocytes of the intermediate zone are more spherical, and the surface polarity seen in the superficial zone is much less apparent [59,73]. The cells have more prominent perinuclear filaments, and rough endoplasmic reticulum and Golgi complexes are more abundant. The organization of matrix compartments is more typical, and chondrons with concentric capsular layers are evident [62,71]. Collagen fibrils are thinner and more widely spaced, and may exhibit faint periodic banding. Their orientation tends to be random, and bundles are rarely observed [74]. The matrix of the intermediate zone stains intensely with cationic dyes for glycosaminoglycans [56].

The deep zone, when present, is distinguished by the tendency of the cells to align themselves perpendicularly to the joint surface and of the collagen fibrils of the interterritorial compartment, which are thicker than in the intermediate zone, to exhibit the same orientation [59,86]. Rough endoplasmic reticulum and Golgi complexes are prominent in the chondrocytes [73,75]. Cationic stains and immunostaining show abundant proteoglycan in this zone [86]. Schenk and colleagues have noted that the cells of the upper deep zone (closest to the articular surface) are rich in intermediate filaments and glycogen deposits compared with the cells of the lower deep zone near the border of this zone and the calcified cartilage zone, the so-called tidemark [59]. Matrix vesicles can be identified in the

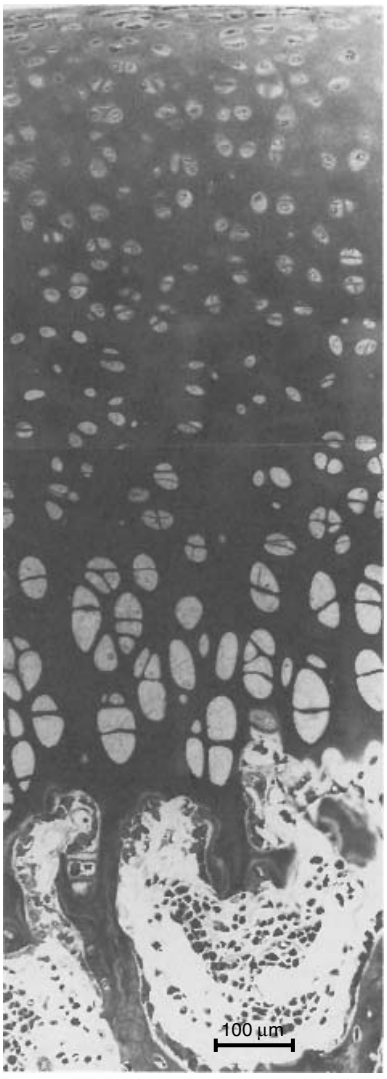


Figure 16. Light photomicrograph of articular cartilage from the femur of a 9-day-old rabbit. The articular surface, with flattened chondrocytes, is seen at the top. Since the rabbit is still growing, the articular cartilage merges into the growth plate at the bottom. Toluidine blue. Scale bar 20 mm = 100 μm.

interterritorial matrix of the deep, as well as the superficial and intermediate zones, but they are not mineralized [71].

The tidemark, which demarcates the deep from the calcified zone in adult articular cartilage, is a narrow band of fine vertical striations that corresponds to aggregates of mineral associated with matrix vesicles that protrude a short distance into the interfibrillar spaces of the lower deep zone [59]. They resemble cement lines of bone. The appearance of mineral in the calcified cartilage zone of articular cartilage closely resembles that in the lower growth plate, although matrix vesicles appear to be less numerous. As in the growth plate, mineralization occurs only in the interterritorial matrix compartment. There is less intense cationic dye staining of proteoglycan in the calcified cartilage zone than in the intermediate and deep zones [56].

Molecular heterogeneity contributes to the complexity of the articular cartilage matrix. The extracellular matrix of the superficial zone contains specialized proteins, superficial zone protein (SZP) [145,146] and lubricin [147], not found in the deeper zones. SZP is also made by synovial cells and both proteins are found in synovial fluid, suggesting that they may contribute a lubricating function. A novel cartilage glycoprotein, cartilage intermediate layer protein (CILP), is found in the territorial matrix only in the intermediate zone [148,149], suggesting a distinction between chondrocytes in this zone and those in the superficial and deeper layers. The changes in fibril diameters associated with different regions of articular cartilage are accompanied by a heterogeneous composition of the fibrils. The small proteoglycan, decorin, is preferentially associated with the population of fibrils with larger diameters, while type IX collagen is associated with the thinner fibrils [150].

Articular cartilage develops after the initial condensation defines the bone. Mesenchymal cells in the interzone, the space between the adjacent ends of developing bones that will eventually become the joint space, progressively differentiate into chondrocytes and thus form a subset of cells distinct from the rest of the epiphyseal cartilage [151]. In rabbit, the articular cartilage can be distinguished from the underlying epiphyseal cartilage at birth by the vertical orientation of collagen fibrils and at 2 weeks by the vertical and linear columns of chondrocytes or chondrons [77]. Formation of articular cartilage is independent of the secondary ossification center in the epiphysis, and growth of the articular cartilage to keep pace with the rest of the bone occurs by cell division in the vertical columns of cells and by the production of matrix.

Elastic Cartilage

Elastic cartilage has many of the characteristics of hyaline cartilage [50]. The cells are round to ovoid and may occasionally contain two nuclei. Fat droplets and glycogen aggregates may be extensive and, in such cases, the chondrocytes come to resemble typical adipose cells [152]. Otherwise, the cellular ultrastructure is similar to that of other chondrocytes [153,154]. Extracellular matrix compartments have not been as well defined as in hyaline cartilage. Collagen fibrils are relatively thin and do not show periodic banding. The most distinctive feature is the presence of a dense network of elastin, which can be selectively stained by orcein for light microscopy. By electron microscopy, elastin appears as irregular strands of amorphous material associated with poorly defined peripheral microfibrils [56,153] (Fig. 17). The cells near the perichondrium have a flattened shape, and faintly banded collagen fibrils can be detected. The elastic fiber component of elastic cartilage is produced by the chondrocytes. This appears to be a general capability of chondrocytes in as much as chondrocytes isolated from hyaline cartilage, which lacks elastic fibers, synthesize elastin and fibrillin within 24 hours of cell culture [155]. However, the elastic component is poorly regenerated during repair [152]. Elastic cartilage develops from a condensation of mesenchyme. The production of elastin, like chondrogenesis, is dependent on this condensation phase and coincides with the differentiation of mesenchymal cells to chondrocytes and the expression of the typical cartilage components, aggrecan and type II collagen [156].

Fibrocartilage

Fibrocartilage has been considered an intermediate tissue between hyaline cartilage and dense fibrous tissue and morphologically can vary between these two extremes [56,58]. It

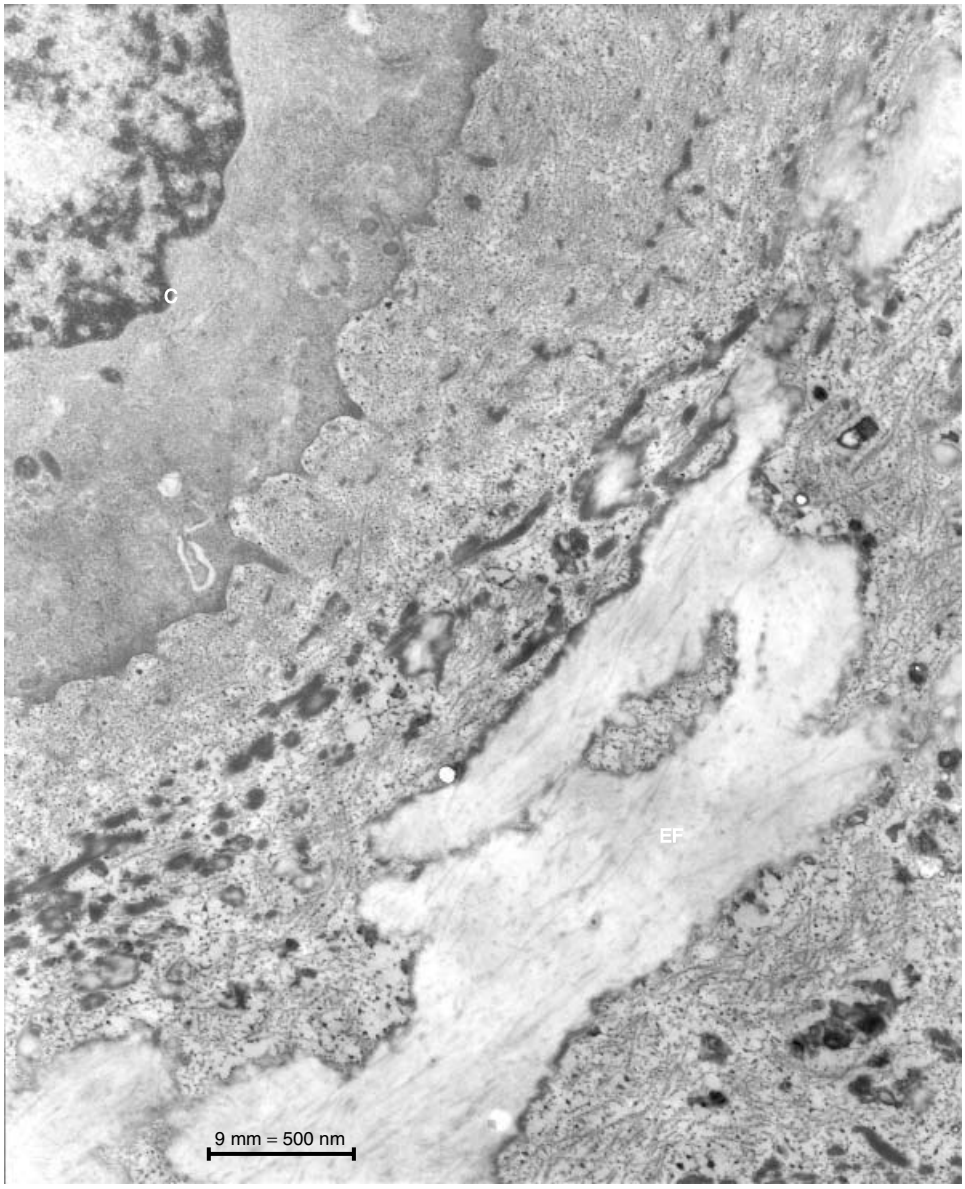


Figure 17. Transmission electron micrograph of elastic cartilage of the human ear. The extracellular matrix closest to the chondrocyte, C, has the more typical appearance of cartilage while the matrix more distant from the cell is a little coarser. The large elastic fibers, EF, occupy a considerable portion of the extracellular matrix. Scale bar: 9 mm = 500 nm.

is never found alone, but merges gradually with neighboring dense fibrous tissue or hyaline cartilage [50]. Fibrocartilage combines the properties of resistance to compression like cartilage with resistance to shear as in tendon [157]. Not surprisingly, both of these properties of fibrocartilage are intermediate in magnitude between cartilage and tendon. Fibrocartilage is typically found in areas where both of these properties are required, such as the intervertebral discs, menisci of the knees, and at the articular surfaces in the coracoclavicular and temporomandibular joints. Fibrocartilage is also found at the sites of tendon attachment to long bones but only at the epiphyses. Here the fibrocartilage is calcified to provide additional stabilization to this connection. The chondrocytes are interspersed, sometimes in rows, between dense fibrous bundles containing type I collagen. The territorial matrix around the cells is cartilage-like, containing collagen types II, IX, and XI [158] and proteoglycan, while the remaining extracellular space is occupied by thick interwoven bundles of collagen fibrils with the characteristic banding pattern (Fig. 18). The cells are round to elongated and resemble other chondrocytes ultrastructurally. Some fibrochondrocytes at the ligament- or tendon-bone interfaces express type X collagen [159,160] even though they do not appear hypertrophic. Fibrocartilage contains no definite perichondrium. Like hyaline cartilage, fibrocartilage is mostly avascular. Where they occur, blood vessels are limited to the periphery of the fibrocartilage. The nutritional state of the cells is maintained by diffusion aided by compression of

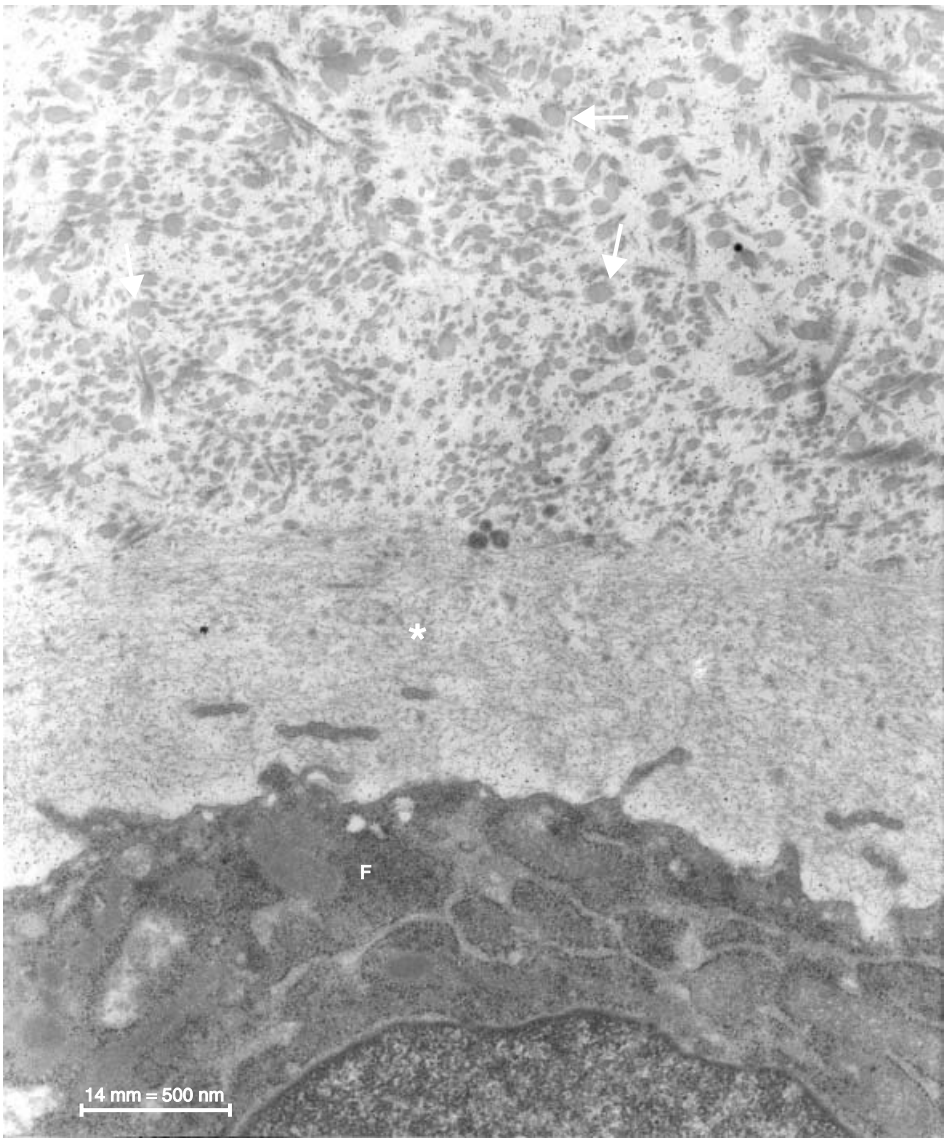


Figure 18. Transmission electron micrograph of fibrous cartilage of the human annulus fibrosus. The fibrochondrocyte, F, is surrounded by a cartilaginous matrix (*) and together they are embedded in a typical noncartilaginous fibrous connective tissue matrix containing large diameter banded collagen fibers, seen here mostly in cross section (arrows). Scale bar: 14 mm = 500 nm.

the tissue. Fibrocartilage usually develops from fibrous tissue in response to a loss of vascularity and/or to compressive forces [157]. The chondrocytes arise by metaplasia of existing fibroblasts and produce a cartilaginous matrix within the context of the preexisting fibrous matrix. However, fibrocartilage can also develop from hyaline cartilage or from condensing mesenchyme, where a fibrous tissue forms first and is followed by the appearance of chondrocytes [161].

RECENT DEVELOPMENTS

Matrilins

Cartilage matrix protein (CMP) is the first of a new family of proteins called matrilins (for review, see [162,163]). CMP is designated matrilin-1, and three other members have been identified, matrilin-2 [164], matrilin-3 [165], and matrilin 4 [166]. Only matrilin-4 has not been found in developing or mature endochondral skeleton. Starting at the amino terminus, matrilins contain a von Willebrand factor A domain, one or more EGF motifs, a second von Willebrand factor A domain (lacking in matrilin-3), and, finally, a coiled coil sequence responsible for oligomerization that results in either trimers or tetramers.

In the cartilage of developing bones, matrilin-3 and matrilin-4 generally co-distribute [167]. They are absent from the hypertrophic zone of the growth plate and from developing and mature articular cartilage [168]. Analysis of their molecular organization as recombinantly expressed proteins [169], or in tissue [167], indicates that they assemble into homotrimers of matrilin-1, homotetramers of matrilin-3, and heterotetramers comprising different combinations of

both. Matrilin-1 and matrilin-3 have been shown to assemble into collagen fibril-dependent and collagen fibril-independent filamentous networks [168,170]. Within cartilage, matrilin-2 is found in the hypertrophic zone [171,172], appears to form homotrimers [173], and can form filamentous networks [172].

At its simplest level, the matrilin system is an example of the complexity and regional specialization of cartilage extracellular matrix composition. However, because matrilin-1 has been shown to associate with type II collagen [174] and proteoglycans [175], and to show integrin-mediated effects on chondrocyte attachment and spreading [176], it is likely that matrilins contribute to the structural integrity of developing cartilage. Two different knockouts of matrilin-1 in mice showed no developmental effects [177,178], although one did show an alteration of collagen fibril formation and organization in the maturation zone of the growth plate [178]. It is possible that functional redundancy between matrilin-1 and matrilin-3 may mitigate the effects of gene inactivation. However, two different missense mutations within the von Willebrand factor A coding region of matrilin-3 have been found to be associated with autosomal dominant multiple epiphyseal dysplasia [179] (see Chapter 23, Part II, this volume). This suggests a dominant negative effect of mutant matrilin-3 molecules on the function of the matrilin-1/matrilin-3 network and an important role for matrilins in the structure of cartilage. Autoimmune reactivity to matrilin-1 is associated with relapsing polychondritis [180], and has been used to generate the disease in a mouse model [181].

Perlecan

An additional example of the functional significance of matrix macromolecules in endochondral bone formation is the heparan sulfate proteoglycan, perlecan. Perlecan is a multidomain, heparan sulfate proteoglycan, usually associated with basement membranes and other tissues, and can function as both a cell surface-attached and -unattached molecule. In addition, it has many binding activities, including the potentiation of fibroblast growth factor activity [182,183]. Perlecan protein is widely distributed in fetal cartilage templates that will be replaced by bone [184–186], in which it is expressed primarily after overt chondrocyte differentiation and appears in greater amounts in hypertrophic cartilage.

Evidence that perlecan plays an essential role in endochondral ossification has been provided by two studies of targeted perlecan gene inactivation [187,188]. In homozygous −/− mice in both cases, skeletal development was substantially perturbed, features including dwarfism and pronounced bowing of long bones. Pattern formation, mesenchymal condensation, joint formation, and chondrogenesis appeared normal, consistent with the expression of perlecan later in chondrocyte differentiation. However, the cartilage was soft, endochondral ossification was delayed, and the organization of primary and secondary ossification centers was disturbed. It is likely that these effects accounted for much of the skeletal phenotype observed. Collagen fibrils were fewer in number, and shorter, and disorganization of the growth plate was acute in that columns of cells and distinct zones were lacking and hypertrophic chondrocytes were fewer in number. Mineralization of the hypertrophic cartilage was also disorganized and was often horizontally oriented rather than being restricted to the longitudinal septa as in normal cartilage. Finally, the expression of Indian hedgehog, a regulator of chondrocyte hypertrophy, could not be detected in the growth plate.

Two ideas have been advanced for the function of perlecan that would lead to the effects of gene inactivation mentioned [182]. Perlecan may be required for the orderly process of matrix degradation during the complex and extensive tissue remodeling associated with endochondral ossification. Indeed, the expression of vertebrate collagenase is higher in the growth plate than in any other location in the developing vertebrate embryo. Evidence suggests that perlecan may protect matrix from inappropriate degradation and that its absence may lead to pervasive matrix degradation [187]. Alternatively, or in addition, perlecan may participate in growth factor function and developmental signaling, particularly with regard to FGFs and Indian hedgehog, which are central to the regulation of chondrocyte differentiation in the growth plate [188].

Mutations in the human perlecan gene, *HSPG2*, have now implicated this molecule in the function of human cartilage (see Chapter 23, Part II, this volume). Functionally null mutations have been detected in patients with the lethal autosomal recessive disorder, dyssegmental dysplasia, Silverman-Handmaker type (MIM 224410) [189]. Affected individuals have a skeletal phenotype very similar to that observed in perlecan knockout mice. Missense mutations and small truncations of perlecan result in the milder Schwartz-Jampel syndrome (MIM 255800), in which mutant perlecan is nevertheless secreted into the matrix and likely retains partial function [190].

Joint Formation

Recent evidence has shed light on the mechanism of joint formation. While some joints form at the interface of separately formed skeletal elements, more frequently they do so as a result of the subdivision of the prechondrogenic mesenchymal condensation. This is especially the case in the appendicular skeleton. Joint formation begins with the de-differentiation of a band of chondroblastic cells to form a joint interzone. Apoptosis then leads to cavitation and the physical separation of skeletal elements. A few growth and differentiation factors and developmental signaling molecules are expressed at the sites of developing joints, and their identities are now becoming apparent [191]. The first factor shown to be involved in regulating joint formation is the growth and differentiation factor, GDF5 [192,193]. Mutations in the corresponding gene result in the absence of certain joints in both mice and humans [194-196]. Paradoxically, ectopic expression of GDF5 results in chondrogenesis, not de-differentiation, apoptosis or other manifestation of joint formation. It is now apparent that the intercellular signaling molecule, Wnt-14, plays a key role in initiating joint formation [197]. Ectopic expression of Wnt-14 results in cellular changes associated with joint interzones, as well as the activation of genes normally expressed in the interzone and the suppression of genes not normally expressed there. Misexpression of Wnt-14 in developing cartilage also suppresses the formation of the nearest joints, suggesting that it may also be involved in regulating the spacing of joints.

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Chapter 1, Part III

Morphology and Chemical Composition of Connective Tissue: Bone

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Morphology of Bone

R. Schenk

INTRODUCTION

Bone is considered the last and most perfect achievement in the evolution of supporting tissues. As far as mechanical properties are concerned, connective tissue made the first decisive step with the elaboration of collagen fibrils, which offer a superb tensile strength but no resistance to other sorts of deformation. In cartilage, proteoglycans were extruded into the interfibrillar space. Trapped within the collagen network, their osmotic or electrostatic swelling capacity provides an intercellular matrix that can withstand compression and shear elastically. The high water content of this material allows the metabolic supply of the enclosed chondrocytes by diffusion over distances of several millimeters. The tissue could also be shaped as a skeleton and provides a unique capacity for interstitial growth.

The final step toward a solid building material was mineralization. Calcified cartilage is the first step in this direction. Calcium phosphate and hydroxyapatite are precipitated in the interfibrillar space formerly occupied by proteoglycans. This filler stiffens the cartilaginous mold, but also hampers the transport of nutrients to the cells. This was a severe limitation on the construction of massive components in the skeleton. In bone, the elaboration of cytoplasmic connections between osteocytes and the surface lining cells was the prerequisite for a further increase in mineral density, and the direct association between collagen and the inorganic phase improved the mechanical properties. In addition, the cytoplasmic net within the canaliculo-lacunar system gave access to the mineral phase, which has become the ultimate source of calcium ions in land-dwelling organisms.

STRUCTURE AND FUNCTION OF BONE CELLS

Osteoblasts and Bone Formation

Bone is formed by osteoblasts. These cells not only deposit osteoid, the organic matrix of bone, but also initiate and control its subsequent mineralization. Osteoblasts cover all active bone formation sites. At regular intervals, some osteoblasts are predetermined to become osteocytes, stop matrix extrusion on their mineral-facing side, and become buried within the calcifying matrix by adjacent cells [1]. They maintain communication with overlying osteoblasts and neighboring osteocytes via cytoplasmic processes.

Structural Organization of Osteoblasts

Fully developed osteoblasts form a monolayer upon bone-forming surfaces (Fig. 1A–C). In contrast to true epithelia, the intercellular gaps are not sealed by tight junctions. This indicates that osteoblasts do not have full control over the ion exchange between the extracellular fluid compartment and bone. They exhibit, however, a functional polarity: matrix is only extruded along the osteoblast-bone interface. Accordingly, the shape of an osteoblast determines the size of its secretory territory and reflects to some extent the local intensity of bone formation; columnar, cylindrical, or cuboidal osteoblasts achieve a higher linear rate of matrix apposition than flattened cells.

The cytoplasm of osteoblasts is highly basophilic, except for a clear juxtanuclear area representing the Golgi-negative. Electron microscopy reveals an abundant rough endoplasmic reticulum loaded with ribosomes, and a prominent Golgi complex (Fig. 1C). The secretory activity is further reflected by a great number of energy-producing mitochondria and a pronounced alkaline phosphatase reaction. Along the lateral portion of the cell membranes, gap junctions transfer information to and from neighboring osteoblasts. Numerous processes originate from the osteoid-facing surface, contained within canaliculi and connected to the lacunae of adjacent osteocytes (Fig. 2A). Again, gap junctions are described at the contact sites of the cytoplasmic processes [2]. This opens the way for the intercellular transport of small molecules, in addition to passive diffusion via the extracellular bone fluid. The transport capacity of the canaliculo-lacunar system is limited to a distance of approximately 100 μm, a critical

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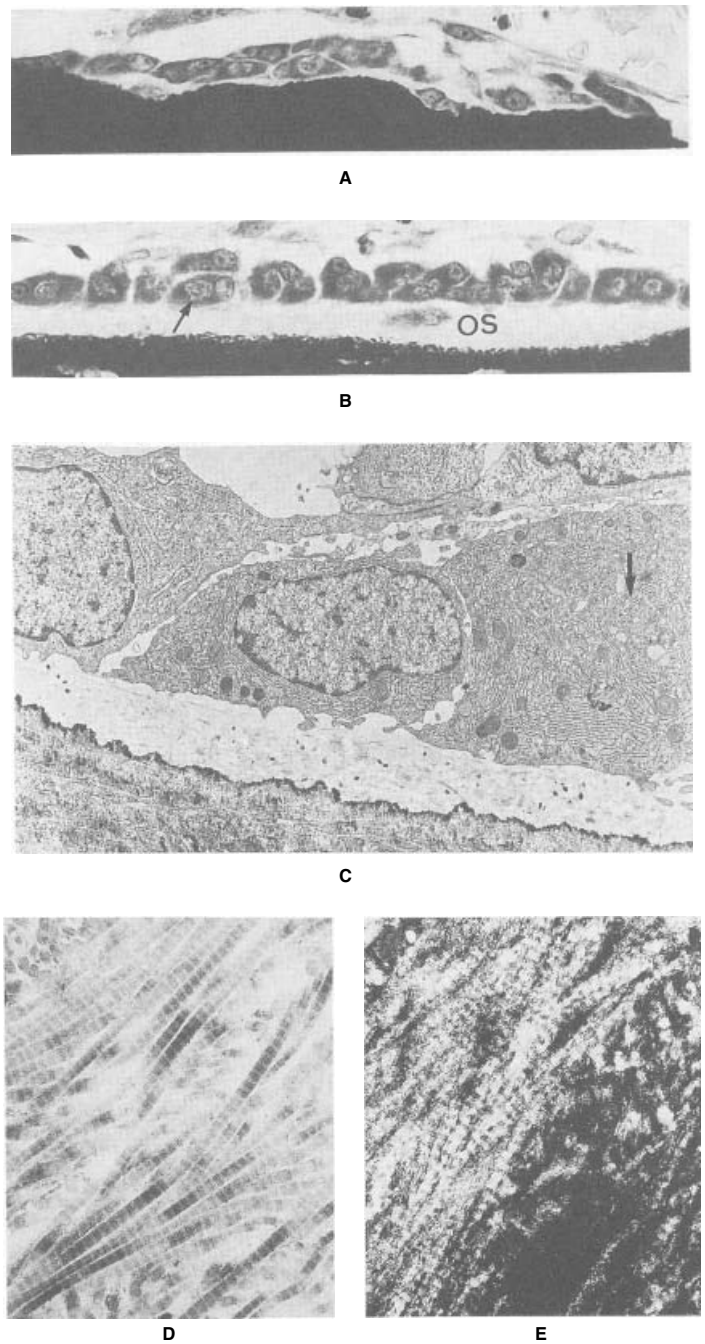

Figure 1. Osteoblasts and bone formation. **A:** Woven bone formation in the primary metaphyseal spongiosa of a rat's tibia. The osteoid seam is extremely thin. Von Kossa-McNeal, × 400. **B:** Lamellar bone formation on the endosteal cortical surface of the same section. Golgi-negative (arrow), osteoid seam with osteoid osteocyte (OS), × 400. **C:** Electron micrograph of a similar location to show the epithelial-like arrangement of osteoblasts in the primary spongiosa. There is abundant rough endoplasmic reticulum, and an extensive Golgi zone (arrow), × 3,700. **D:** Electron micrograph of human lamellar osteoid, × 22,000. **E:** Electron micrograph of mineralized human lamellar bone matrix. × 22,000. (C,D, and E reprinted with permission from [388].)

value for the wall thickness of osteons in compact bone, as well as for the diameter of plates or trabeculae in cancellous bone [3]. Gap junctions may also facilitate a concerted response of the interconnected cells to stimuli regulating calcium metabolism.

Structural Organization of Bone Matrix

In the current literature, only two types of bone tissue are commonly distinguished: woven bone and lamellar bone. Based upon Weidenreich's classification of 1928 [4], Pritchard [5] listed five different bone types, some of which

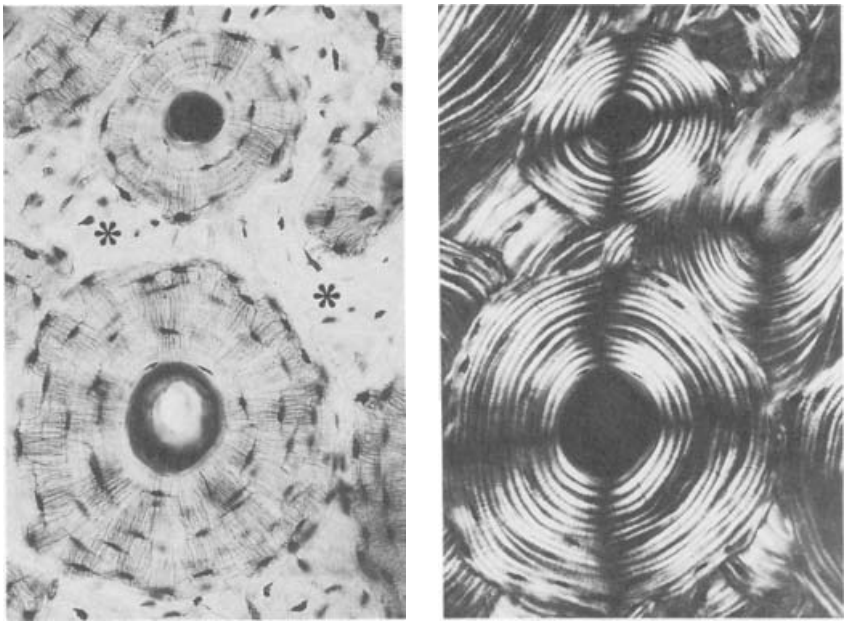

Figure 2. Transverse section through secondary osteons in adult human compact bone. **A:** A fresh, undecalcified ground section, stained with basic fuchsin, demonstrates osteocytes and their cytoplasmic processes. Most of the interstitial lamellae (*) are devitalized (or micropetrotic). × 150. **B:** Same section to show the lamellae under polarized light.

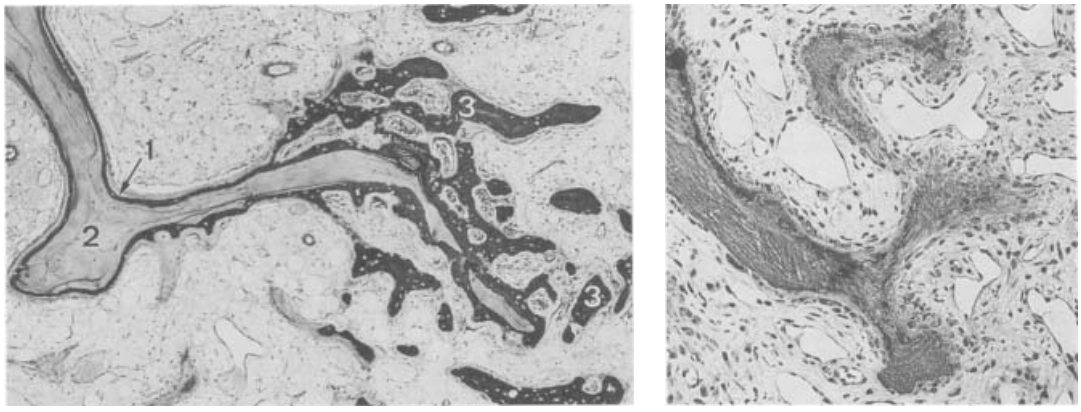

Figure 3. Activation of lamellar and woven bone formation in a 3-week-old fracture of a human femoral neck. **A:** Newly formed bone is identified by the more intense surface staining with toluidine blue. Lamellar bone (1) is strictly deposited along the surface of preformed trabeculae (2). Woven bone (3) is able to construct a scaffold of beams protruding into the intertrabecular space and possibly across the fracture gap. × 60. **B:** A microtome section stained with Goldner's trichrome clearly shows the association of osteoblasts and blood vessels in the interfragmentary granulation tissue. × 120.

occur only in lower vertebrates. For the mammalian skeleton, Pritchard restricted this number to three, namely woven bone, lamellar bone, and the mixed type. Our view, in accordance with that of the group of Marotti [1], is that the category "parallel-fibered, finely bundled bone" or simply "parallel-fibered bone," should be revived on the basis of observations made in recent experimental studies [6,7].

Woven bone. This form of bone is structurally characterized by the random orientation of its collagen fibrils, the great number of large, often irregularly shaped osteocytes, its short mineralization lag time, a diffuse uptake of fluorochromes, and often by its high mineral density (see Fig. 10C). This is due to the fact that apatite is not only deposited upon and within the collagen fibrils but equally in the interfibrillar spaces. Under polarized light, the matrix is not birefringent. The most important features, however, are its pattern and dynamics of growth: it forms rapidly growing, branching struts and plates, and is able to occupy a relatively large territory within a short space of time. Woven bone is formed predominantly in embryos and growing children, and is later

substituted by lamellar bone. In the adult, it reappears when accelerated bone formation is demanded, as in the bony callus during fracture repair (Fig. 3) and in pathological conditions with accelerated osteogenesis such as Paget disease, renal osteodystrophy, hyperparathyroidism, and fluorosis.

Parallel-fibered bone. In parallel-fibered bone, the collagen fibrils are oriented parallel to the surface, but otherwise do not follow a preferential course. Formation of parallel-fibered bone requires a preformed solid base, consisting of either woven bone, calcified cartilage, or, in the case of bone repair, preexisting lamellar bone. Parallel-fibered bone is not, or is only faintly, birefringent. Fluorochrome labels appear as relatively wide bands with poorly defined boundaries. The apposition rate is higher than in lamellar bone and amounts to 3–5 μm per day. Parallel-fibered bone enables accelerated bone deposition in both intramembranous and endochondral ossification in the growing skeleton, as well as in bone healing.

Lamellar bone. Lamellar bone is characterized by 3–5 μm-wide layers of parallel collagen fibrils. The course of the fibers changes from lamella to lamella. This pattern was first analyzed by polarized light (Fig. 2B) and later confirmed by electron microscopy. The exact delineation of the lamellae and the detailed arrangement of the fibrils, however, is still under debate [8]. Lamellar bone requires a preformed solid scaffold for its deposition, and the newly formed lamellae run strictly parallel to the underlying surface. Under such conditions, it is not surprising that the linear apposition rate for lamellar bone amounts to only about 1–2 μm per day. Fluorochrome labels appear as thin, clearly delineated bands, and the birefringence under polarized light strongly emphasizes the lamellar pattern resulting from the parallel arrangement of the collagen fibrils (Fig. 2B).

Formation and Mineralization of Bone Tissue

Bone formation always starts with the deposition of osteoid, which subsequently mineralizes. Woven bone is formed more rapidly than lamellar bone, and the interval between osteoid deposition and mineralization is short (1–3 days). The osteoid seams are small and sometimes barely visible. The mineralization of woven bone is initiated by matrix vesicles, spherical, membrane-bound bodies with a diameter of 100–200 nm. They are pinched off from cytoplasmic osteoblast processes. The role of matrix vesicles was originally described and extensively discussed with regard to cartilage calcification in the growth plate. Later on, they were also demonstrated in woven bone (for a review, see [9]). Matrix vesicles are also described in parallel-fibered bone [1]. In both instances, the first needle-like calcium phosphate deposits appear within the matrix vesicles in close contact with the inner leaflet of the membrane. This localization has been confirmed by anhydrous embedding techniques (high-pressure freezing and freeze substitution) [10,11]. After rupture of the membrane, the crystals are set free and serve as secondary nucleation centers. These morphological observations strongly support the assumption that osteoblasts directly participate in woven bone mineralization.

Lamellar bone formation is slower. The orderly deposition of the collagen fibrils restricts the linear rate of osteoid production to about 1–2 μm per day. The cause and control of the alternating fiber orientation in successive lamellae are still poorly understood. Ascenzi and Benvenuti [12] postulate a periodical rotation of osteoblasts in order to change the direction of the collagen fibrils. Others [13] assume that electrical forces accumulate with increasing thickness of a lamella and rotate incoming fibrils and attached osteoblasts when a critical level is reached. In any case, the final pattern is already set during osteoid deposition.

Lamellar bone formation requires a relatively flat surface upon which collagen fibrils can be deposited in parallel and/or concentric layers. Unlike woven bone, lamellar bone is not able to construct ridges or beams. Mineralization occurs along a clearly delineated calcification or mineralization front. It is separated from the osteoblast-bone interface by an osteoid seam (Fig. 4A). An active mineralization front exhibits some peculiar staining properties and, above all, quite a number of substances are adsorbed and permanently bound to the rapidly growing apatite crystals. This mechanism forms the basis of labeling techniques, especially with fluorochromes, allowing accurate measurements of the mineral apposition rate (Fig. 4B). The daily linear advance of 1–2 μm mentioned above was, in fact, determined at the level of the mineralization front. As long as the osteoid seams maintain a constant width, it is also true for osteoid deposition. The mean thickness of osteoid seams is about 10 μm. From this value one can also derive a mineralization lag time of roughly 10 days in between osteoid production and mineralization. The nature of the postulated maturation process, however, is not clear.

In contrast to woven bone, matrix vesicles are not, or are only rarely, seen in mineralizing lamellar osteoid, and up to now no structural element besides collagen has been reported that could take over their role. There is general agreement on some basic requirements for undisturbed mineralization [14], such as an adequate concentration of calcium and phosphate ions, the presence of a calcifiable matrix and of nucleating agents, and finally the control by regulators, i.e., promoters

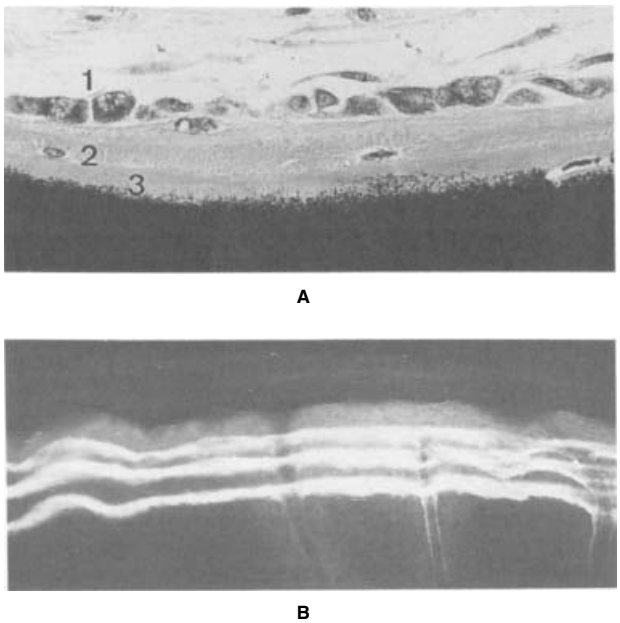

Figure 4. Lamellar bone formation. **A:** Osteoblasts (1), osteoid seam (2) including osteoid osteocytes, and calcification front (3). Human iliac crest biopsy, von Kossa-McNeal. × 400. **B:** Formation of circumferential lamellae by periosteal lamellar bone apposition in a foal. Sequential polychrome labeling at intervals of 3 and 4 weeks, corresponding to a daily mineralization rate of 2.1 μm.

and inhibitors. Collagen is a strong candidate for being a nucleator among other contenders. In lamellar bone there is, in fact, a much closer association between collagen and apatite crystals than there is in woven bone (Fig. 1D,E). The mineral deposits accentuate the typical 64-nm banding pattern of the collagen. It has been suggested that the crystals are located within the fibrils, preferentially in the hole regions resulting from the quarter-stagger arrangement of the tropocollagen molecules. Numerous *in vitro* tests have shown that native collagen with a 64-nm periodicity is capable of precipitating apatite in metastable solutions of calcium and phosphate ions [15,16]. It has been postulated that the nucleating capacity of collagen depends on its combination with osteonectin [17] or phosphoproteins [18].

A number of promoters and an even greater number of inhibitors of calcification are described in the literature (for a review, see [18]). Some inhibitors are of particular interest for the prevention of ectopic calcification (e.g., pyrophosphate, bisphosphonates) [19]. Highly aggregated proteoglycans inhibit mineralization *in vitro* and possibly do so also in cartilage [20]. There is much controversy, however, about the definition of a "calcifiable matrix" and the physiological role of nucleators such as collagen or possible candidates for such among the noncollagenous proteins, phosphoproteins and proteolipids.

The Fate of Osteoblasts — Bone Lining Cells

When bone formation ceases, osteoblasts flatten out and transform into bone lining cells (see Fig. 15C). These cells definitely belong to the osteoblastic family, but other names like inactive osteoblasts, resting osteoblasts, and surface osteocytes create unnecessary confusion. Lining cells still maintain cytoplasmic connections with osteocytes. Whether they act as a barrier for the ion exchange between bone and extracellular fluid is doubtful, as is also their potential to revert directly into osteoblasts. Lining cells may participate in the initiation of bone resorption through an active contraction that is thought to expose the bone for the attachment of osteoclasts [21]. Lining cells may also produce activating factors for the generation of osteoclasts. The exact composition of the intercellular substance underneath the lining cells is not clear, and it is not certain whether it is mineralized up to the surface [22] or coated by a layer of nonmineralized matrix [23,24]. If the latter were the case, the nonmineralized matrix would have to be removed before the osteoclasts could get access to the calcified bone [25]. For a discussion, see Vaes [26,27].

Osteocytes

Differentiation

The origin of osteocytes and the formation of the canaliculo-lacunar system has been described above. After being fully encompassed by mineralized matrix, osteocytes first go through a short osteoblastic or formative phase, and continue to deposit a thin layer of matrix upon the walls of their lacunae. At this time, the cytoplasm still contains a considerable amount of rough endoplasmic reticulum and a well-developed Golgi apparatus. Later on, these organelles disappear and mitochondria, as well as vesicles and lysosomes, prevail. These changes may indicate a transition into a resorptive phase, or, as Bélanger and Jande named it, an osteocytic osteolysis [28,29]. This concept assumed that osteocytes were capable of forming larger resorption cavities, but now it is generally agreed that osteocytes only remove minute layers of the lacunar and canalicular walls, too thin to be detected by light microscopy. Electron microscopy, however, indeed gives evidence of such alternating formative and resorptive activity [30]. This mechanism is thought to contribute to the calcium exchange between bone mineral and extracellular body fluids, and may be regulated by parathyroid hormone and calcitonin.

Fate of Osteocytes

The canalicular system and the cytoplasmic connections of the osteocytes are essential for their metabolic activity. Ultimately, this metabolism depends upon the blood vessels within the cortical canals and along the periosteal and endosteal surfaces. With undisturbed vascularization, osteocytes stay alive for years or decades. With increasing age, however, some vessels become obliterated, the corresponding domains (osteons in cortical bone) become deprived of nutrients and oxygen, and the osteocytes die and disintegrate. Such devitalized or necrotic areas are recognized by empty lacunae and, after some time, by a higher mineral density of the matrix (see Fig. 10C). Finally, the canaliculi and lacunae become obstructed and filled with mineral (micropetrosis) [31]. An accumulation of micropetrotic areas makes bone more brittle and also reduces the surface available for calcium exchange. A reasonable proportion of vital, vascularized bone tissue in the aging skeleton, however, is maintained by internal remodeling. On the other hand, such remodeling creates cement lines that may interrupt the canalicular pathway and thus produce another physiological type of micropetrotic compartment (see below, Cortical Bone Remodeling).

Osteoclasts and Bone Resorption

Structure and Function of Osteoclasts

Osteoclasts belong to a family of giant cells specialized in the breakdown of calcified matrices (bone, dentin, enamel, calcified cartilage) or of the mineral phase alone (calcium-phosphate-ceramics). They differ from other giant cells, especially foreign body giant cells, and are conventionally identified by their location in resorption foci, their light- and electron-microscopic structure, and a positive tartrate-resistant acid phosphatase (TRAP) reaction [32,33].

Osteoclasts had already been accurately described in 1873 by Kölliker [34]. Actively resorbing osteoclasts adhere to the bone surface and produce localized pits called Howship's lacunae (see Fig. 16A). They are mobile and possibly form grooves in the bone surface resembling the tracks of bark beetles. The cell diameter varies from 30 to 100 μm and the number of nuclei from 3 to 30. The cytoplasm is acidophilic and often contains vacuoles. A perpendicular striation, sometimes mistaken for a brush border, is frequently seen on the bone-facing side.

Electron microscopy has provided relevant information regarding the cytoplasmic organization of osteoclasts (Fig. 5). A detailed description of the osteoclast-bone interface has been given by Holtrop and King [35]. The marginal area of the osteoclast (clear zone, sealing part) consists of cytoplasm rich in actin-like filaments but devoid of organelles. There, the membrane adheres to the mineralized surface and seems to seal off an actual resorption chamber. In this central part, the cell surface is enlarged by numerous cytoplasmic folds forming the ruffled border. The increase in surface membrane may be related to the release of hydrogen ions for acid production. After dissolution of the mineral, the exposed collagen fibrils are digested by lysosomal and nonlysosomal enzymes (for reviews see [26,36], and Chapter 7, this volume).

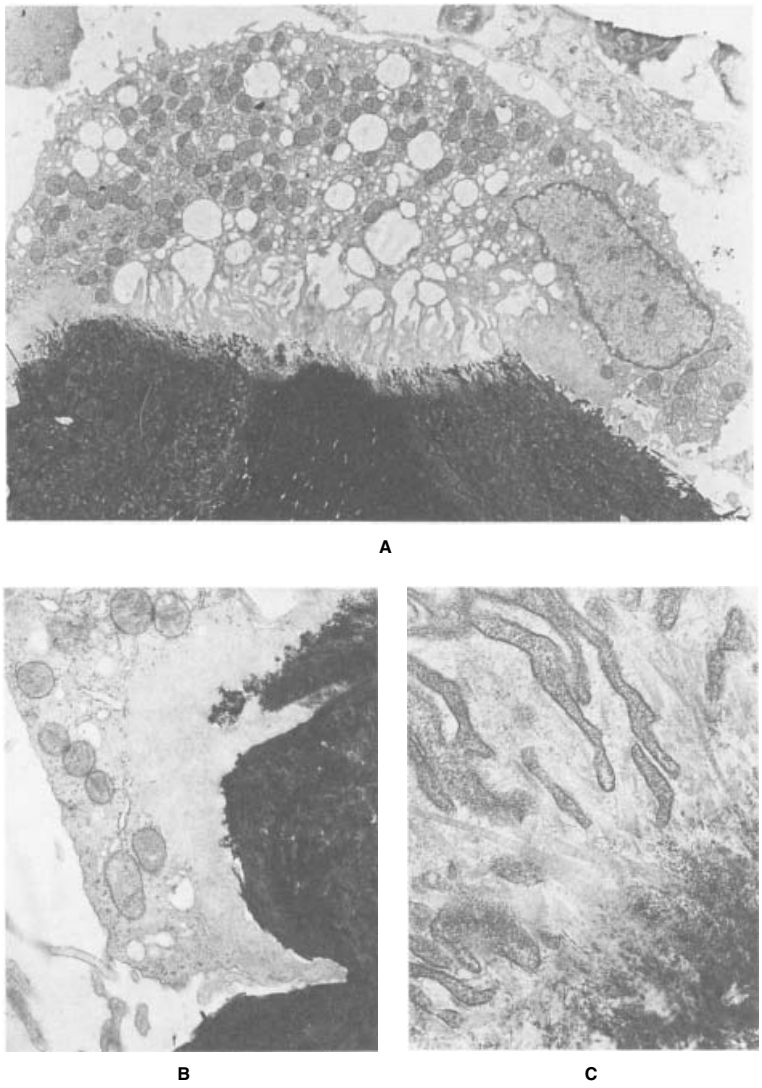

Figure 5. Osteoclastic bone resorption (reprinted with permission from [388]). **A:** Low-power electron micrograph of an osteoclast in a rat's tibial metaphysis. This section exhibits only a single nuclear profile. The cytoplasm shows numerous mitochondria, vesicles, and vacuoles. A marginal clear zone is visible surrounding the ruffled border along the osteoclast-bone interface. × 3, 050. **B:** The cell membrane of the clear zone (sealing part) is in intimate contact with the mineralized matrix. × 7, 000. **C:** A higher magnification of the ruffled border suggests that the mineral is dissolved before the collagen fibers are enzymatically digested. × 14, 000.

The cytoplasm of osteoclasts is extremely rich in mitochondria. These are the ultimate source of H^+ ions and supply the energy necessary for all other activities that enable osteoclasts to resorb a 50-μm or more thick layer of calcified bone per day. A well-developed rough endoplasmic reticulum, free ribosomes, multiple Golgi fields, and many vesicles and vacuoles give further proof of the equipment needed to fulfill this task.

Fate of Osteoclasts

The average life span and the ultimate fate of osteoclasts is still debated. They cannot be compared with regular mononuclear cells. They undergo continuous renewal by fusion with additional mononuclear precursors, and obviously can also dispose of exhausted organelles and nuclei ("nuclear shedding"). After the injection of tritiated thymidine into adult beagle dogs, the average life span of labeled nuclei in the osteoclasts was estimated to be 11 days [37]. Inactivated osteoclasts (for example, those following inhibition by bisphosphonates) may persist in the tissue for weeks or months and reach giant dimensions with high numbers of nuclei. This suggests that fusion with new precursors might continue, whereas nuclear shedding from such paralyzed cells is slowed down or arrested.

MACROSCOPIC STRUCTURE OF BONE

Long bones are subdivided into epiphysis, metaphysis, and diaphysis (Fig. 6). The epiphysis is confined by an articular surface covered with hyaline cartilage. The body of the epiphysis is formed by cancellous or spongy bone consisting of plates and trabeculae. During growth, the metaphysis and epiphysis are separated by the cartilaginous growth plate. In the adult, a discontinuous transverse bone plate

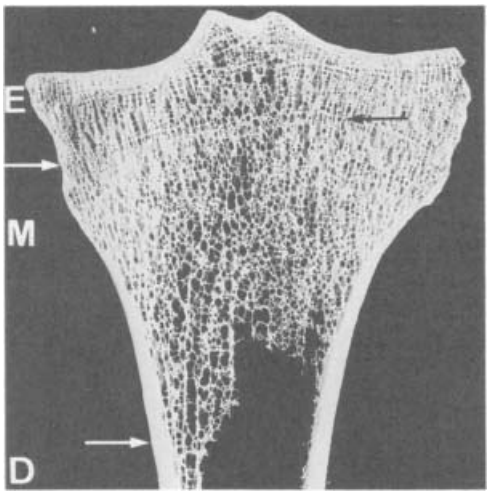

Figure 6. Frontal section through the proximal end of a human tibia to show the location and borderline of the epiphysis (E), epiphyseal line (black arrow), metaphysis (M), and diaphysis (D). White arrows mark the borderlines between the metaphysis and diaphysis, and between the metaphysis and epiphysis.

remains to indicate its original position, and may be identified radiographically as an epiphyseal line. The metaphysis is composed of cancellous bone encompassed by a compact cortical layer. The boundary between metaphysis and diaphysis is not so clearly defined. Anatomically, it corresponds to the level where the spongiosa ends and the marrow cavity begins. Age-related rarefaction of the spongiosa and corresponding expansion of the marrow cavity, however, will shorten this segment, with corresponding expansion of the diaphysis. The diaphysis, finally, has a compact wall surrounding a uniform marrow cavity.

Small bones, such as the vertebral bodies and the carpal and tarsal bones, lack the diaphysis, and the metaphyses have merged. Ribs also consist of cancellous bone surrounded by a cortical layer, grow only from the anterior costo-chondral junction, and can be considered as extremely lengthened metaphyses.

Bone is separated from adjacent tissues by three bone envelopes: the periosteum, the endosteum, and lining cells within the intracortical (endocortical) canals (the Haversian envelope). These three envelopes share two important features: osteogenic potential and abundant vascularization. This enables the envelopes to participate in modeling and remodeling activities, as well as in bone repair. The periosteum covers the outer surface of the bones, with the exception of the articular surfaces and tendon and ligament insertions. Its structure varies from delicate, loose connective tissue sheets to dense fibrous membranes. Osteoprogenitor cells are commonly located in the vicinity of blood vessels near the bone surface. The cells of the endosteum belong to the stroma of the bone marrow or are derived from resting osteoblasts, which most probably constitute the main source of the lining cells in the cortical canals.

DEVELOPMENT AND GROWTH OF BONE

Types of Ossification

Bone formation depends, among other things, on two important prerequisites: an ample blood supply and mechanical support. Osteoblasts function only in the proximity of blood vessels. Lack of oxygen seems to change the gene expression of osteoblasts toward the production of cartilage, fibrocartilage, or fibrous tissue. Bone matrix production and mineralization requires a mechanical rest, and, except in early embryonic life, bone is only deposited upon a solid base as provided by preexisting bone or calcified cartilage. These principles serve as a key for the understanding of the two modes by which bone develops. In intramembranous ossification, connective tissue serves as a mold into which bone is deposited. Endochondral ossification, the much more prevalent type, uses mineralizing cartilage as a model and solid base which is first covered with, and then substituted by, bone.

Intramembranous or Direct Ossification

In the early embryonic stage, bony trabeculae appear as condensations in the mesenchymal tissue, always in the vicinity of blood vessels. Intramembranous ossification mainly occurs in the head and results in the formation of the following bones in the neurocranium: frontal, parietal, squamous temporal, interparietal portion of the occipital bone; and in the viscerocranium: premaxilla, maxilla, palatine, nasal, lacrimal, zygomatic, tympanic bone, and the vomer.

The mandible also forms directly in the mesenchyme lateral to Meckel's cartilage. In the 10th fetal week, a secondary cartilage appears upon the future capitulum of the condylar process. This cartilage contributes to the growth of the collum by endochondral ossification and finally differentiates into articular cartilage.

The clavicle also ossifies directly in a mesenchymal condensation. Later on, secondary cartilages appear upon its medial and lateral ends. They function as endochondral ossification centers and finally transform into the articular cartilages of the sterno-clavicular and acromio-clavicular joints.

In comparison with endochondral ossification, intramembranous ossification plays only a minor role in embryonic osteogenesis. It gains, however, in importance in postnatal bone repair, in both defect filling and direct fracture healing. In these conditions, the granulation tissue that has previously organized the blood clot serves as a matrix for bony ingrowth, and, again, bone formation appears to be tightly coupled to angiogenesis

Perichondral and Endochondral Ossification

Most of the bones in the skeleton, and especially all long bones, have a cartilaginous precursor. With the onset of chondral ossification, the cartilage undergoes mineralization and is thus converted into a firm material that serves as a solid base for bone deposition (Fig. 7). Bone is formed either by apposition upon the outer surface of the calcifying model (perichondral ossification), or by substitution of the calcified cartilage in the primary or secondary ossification centers (endochondral ossification).

Primary ossification centers arise in short bones like the vertebrae, and in the midshafts of long bones. Secondary ossification centers originate in the epiphyses of long bones and expand by endochondral ossification until the cartilage is restricted to the articular surface and the growth plate. Articular cartilage and growth plate remain connected by a cartilaginous bridge that is still covered by perichondrium (groove of Ranvier). This is the only location where appositional cartilage growth endures, thereby enlarging the transverse diameter of the epiphyseal plate. During growth,

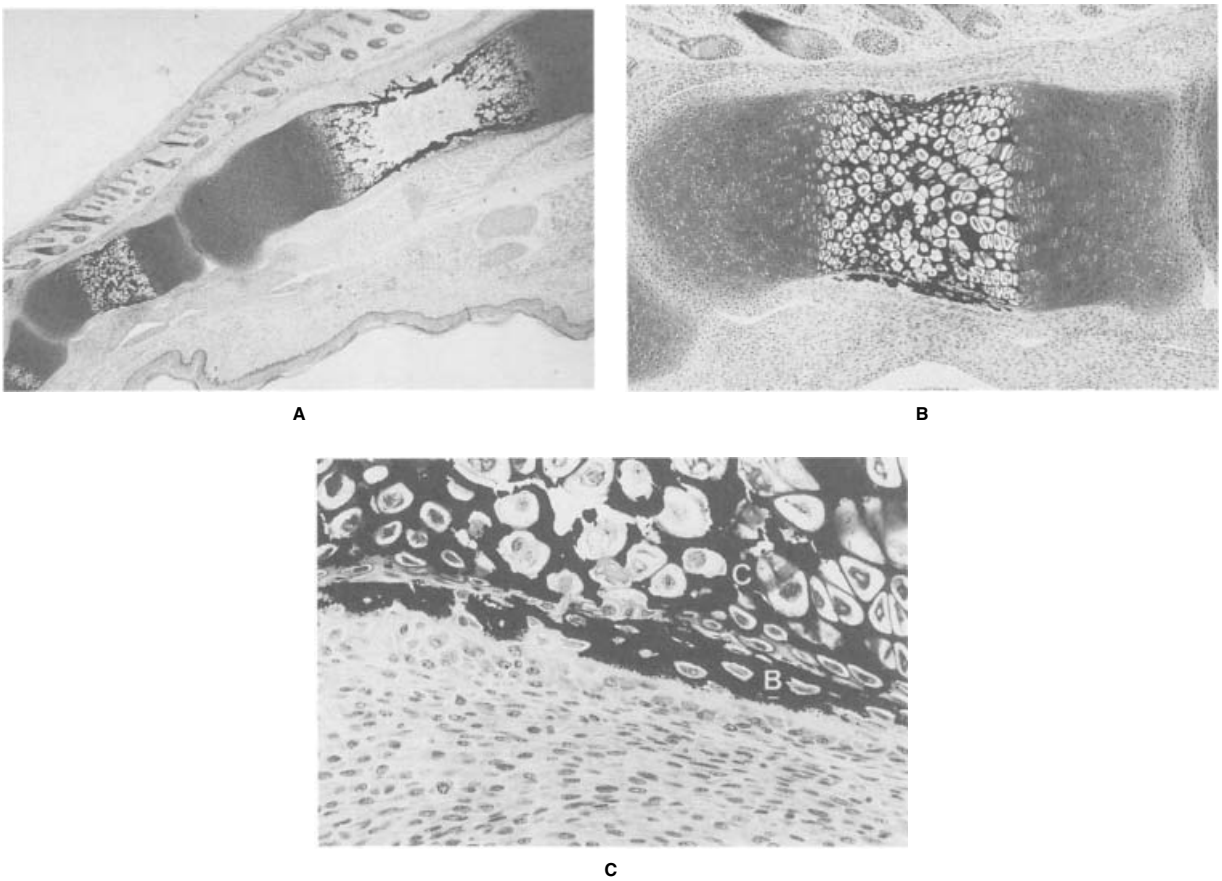

Figure 7. Perichondral ossification in the foot of a 2-day-old rat. Undecalcified microtome section, von Kossa-McNeal. **A:** Three stages of the developing primary ossification center in the end phalanx, ground phalanx, and first metatarsal bone. × 20. **B:** Cartilage calcification in the diaphyseal part of the ground phalanx. × 60. **C:** Perichondral ossification. Note the difference between newly deposited woven bone (**B**) and calcified cartilage (**C**). × 250.

the articular cartilage is responsible for the enlargement and precise shaping of the articular surface, whereas the growth plates function as a center for longitudinal growth.

Histological pattern of endochondral ossification. Although all sites of endochondral ossification exhibit the same basic pattern, the growth plates present the histological events in their most elaborate form. In fully developed growth plates, the chondrocytes are organized in columns that represent functional growth units. Quantitative data given in this chapter are always related to this unit [38]. In the longitudinal direction, we subdivide the growth plate into three zones, according to the prevailing function of each: 1) interstitial growth, 2) preparation for ossification, and 3) formation of the primary spongiosa (Fig. 8).

The nonmineralized part, except for the reserve zone, participates in interstitial growth, which is based on three cellular activities: proliferation, matrix production, and cell hypertrophy.

The proliferative zone comprises pools of relatively flat chondrocytes arranged in columns. Mitotic figures are found over the whole length of this part of the column. In rats, the mitotic frequency is about 1 in 48 hours [38]. All cells go through a finite number of cell divisions before they stop DNA replication at the borderline with the hypertrophic zone [39]. During the interphase the cells take part in matrix production. The matrix volume produced within 24 hours by each proliferating cell is quite constant and roughly equals the cell volume.

Cell hypertrophy is probably the most significant contribution of the chondrocytes to longitudinal growth. In rats, chondrocytes increase their volume up to 10 times in becoming hypertrophic. The formerly flat cells become spherical or ovoid, thereby increasing their longitudinal diameter by a factor of four. The elongation of the column during the process of becoming hypertrophic calls for supplementary matrix production. If hypertrophic chondrocytes are adequately preserved for electron microscopy, they appear well equipped with cytoplasmic organelles required for matrix production (rough endoplasmic reticulum, Golgi membranes), and mitochondria for production of the energy related to osmotic work. The fate of the "last chondrocyte" in the column is not clear. It disintegrates rapidly, either by apoptosis or under the attack of macrophages accompanying the invading capillaries.

Preparation for ossification essentially means the elaboration of a solid scaffold for bone deposition in the metaphysis. This is achieved by cartilage mineralization. Mineralization is confined to the longitudinal, intercolumnar septa. The

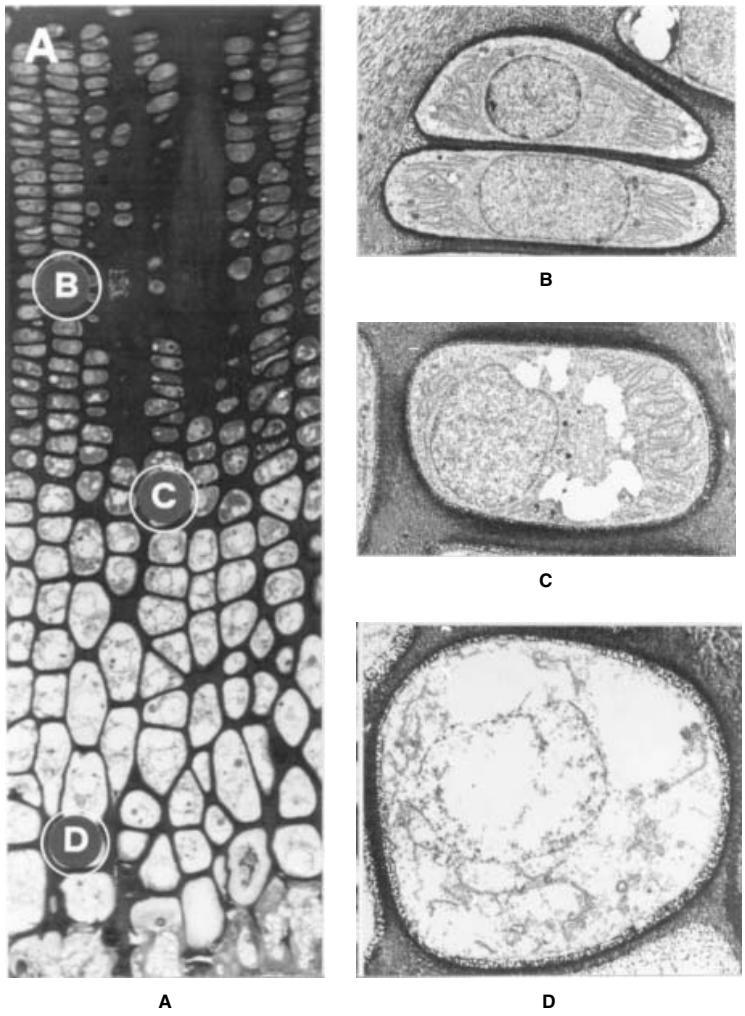

Figure 8. Light and electron microscopy of the growth plate in the proximal tibia of a 120 g Wistar rat. **A:** Thick section, toluidine blue, × 275. The encircled letters indicate the locations of the cells within the electron micrographs; B, C × 3720; D × 2100. **B:** Cells in the proliferative zone. **C:** Chondrocyte in the upper hypertrophic zone. **D:** Chondrocyte in the lower hypertrophic zone. Fixation with ruthenium hexamine trichloride [389] preserves the proteoglycans *in situ*. The membrane remains attached to the pericellular matrix, but shrinkage causes vacuoles in the cytosol.

transverse septa separating the chondrocytes within a column remain unmineralized. Resorption of the unmineralized transverse septa by macrophages opens the way for vascular invasion. Resorption of calcified longitudinal septa by multinucleated chondroclasts reduces the number of calcified cartilage septa and widens the intertrabecular spaces [40]. In rats, about half or two thirds of the calcified cartilage septa are removed to make room for the ingrowing bone forming elements, i.e., osteoprogenitor cells and osteoblasts. The formation of the primary spongiosa results from the deposition of parallel-fibered bone upon the persisting calcified cartilage septa. The primary spongiosa in the metaphysis differs from that formed by intramembranous ossification in two respects: firstly, it is anisotropic, i.e., its trabeculae are oriented parallel to the long axis of the bone. Secondly, the trabeculae are built around a central core of calcified cartilage, instead of a core consisting of woven bone (see Fig. 9A,B).

The primary spongiosa in the metaphyses subsequently undergoes extensive remodeling and is transformed into the secondary spongiosa, which consists solely of bone, mostly of the lamellar type (see Fig. 9C). At the junction with the diaphysis, its trabeculae are finally resorbed and replaced by the marrow cavity.

In conclusion, the primary function of the growth cartilage is the continuous and well-controlled elaboration of a solid scaffold for the deposition of trabecular bone of the primary spongiosa. Growth rates vary considerably, and so do the turnover of the cells and matrix in different locations and with age. A detailed histomorphometric analysis of these relationships has been carried out in the proximal tibia of rats [85].

Development and Growth of Cortical Bone

In long bones, the final shape and structure of cortical bone is the result of four different processes: 1) perichondral ossification, 2) periosteal and endosteal bone apposition, 3) corticalization of metaphyseal cancellous bone, and 4) modeling of the external shape by the envelopes.

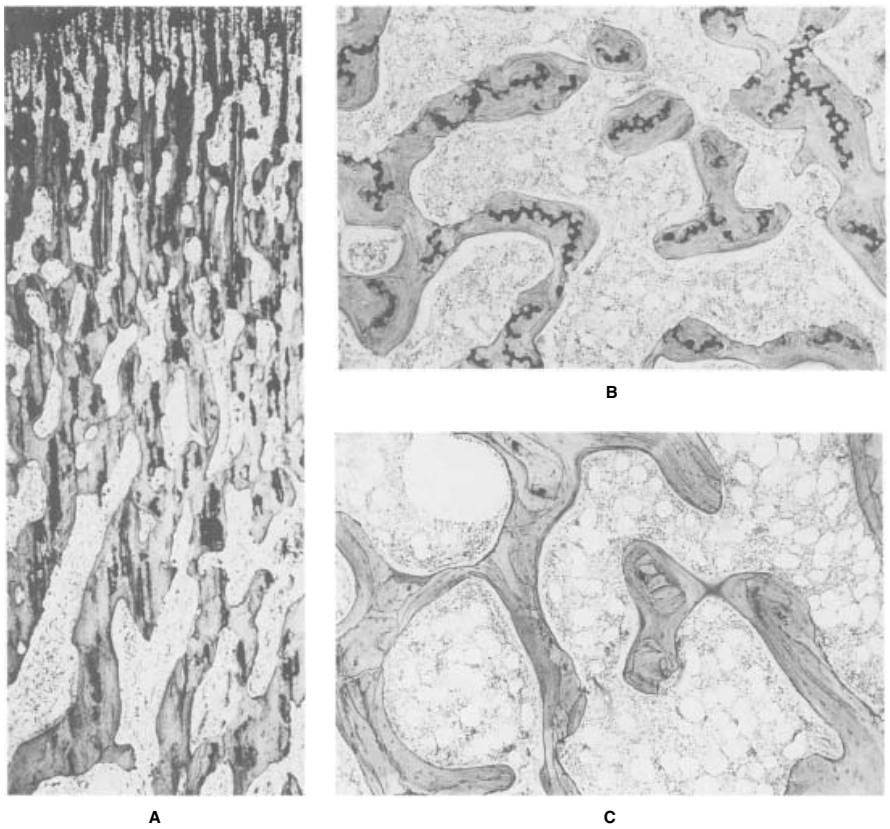

Figure 9. Cancellous bone remodeling during growth. Surface-stained ground sections reveal calcified cartilage inclusions by the heavy uptake of toluidine blue. **A:** Frontal section through the proximal metaphysis of a rabbit's tibia, × 20. There is a gradual disappearance of the calcified cartilage inclusions towards the marrow cavity. **B,C:** Transverse sections of a tibial metaphysis in a mini-pig at the level of the primary (**B**) and secondary (**C**) spongiosa. Note the numerous cement lines in the secondary trabecular profiles which indicate an intense remodeling. × 50.

Perichondral Ossification

The onset of perichondral ossification coincides with the formation of the primary ossification center in the midshaft of the cartilaginous precursor. The original perichondrium converts into a periosteum and deposits a bony cuff around the calcified cartilage that is resorbed or replaced by endochondral ossification. The initially formed woven bone is broken up by longitudinal vascular spaces, which then become narrowed by an accumulation of parallel-fibered bone. Thus, perichondral ossification strictly represents appositional bone formation, and not the substitution of cartilage by bone.

Periosteal and Endosteal Bone Apposition

Both the periosteal and endosteal envelopes contribute to the growth and shaping of bones by appositional bone formation. Lamellar bone formation results in the deposition of outer or inner circumferential lamellae. It is handicapped by the small apposition rate of 1–2 μm/day. In sites where a more rapid apposition is required, a composite of woven bone, parallel-fibered bone, and lamellar bone is deposited, resulting in the formation of primary osteons (Fig. 10). Essentially, the capacity of woven bone to form crests and ridges is activated in order to form grooves and, later on, tunnels around longitudinally oriented blood vessels. Subsequently, the tunnels are narrowed by the deposition of concentric bone layers until the primary osteons are completed. Primary osteons resemble secondary osteons, but are not confined by cement lines (see below). They are embedded within the initially formed woven bone, which can be identified in microradiographs by its higher mineral density (Fig. 10C).

Corticalization of Metaphyseal Cancellous Bone

The metaphyses of most long bones exhibit a conical shape. This shape reverses the pattern of apposition and resorption during longitudinal growth: formation prevails along the endosteal aspect of the cortex, whereas the periosteal surface is subjected to resorption (see Fig. 11A). When the metaphyseal cone advances toward the epiphysis, some peripheral trabeculae are engulfed in the cortical layer. Corticalization is accomplished by filling the intertrabecular space with parallel-fibered or lamellar bone, similar to primary osteons, and finally the compact composite is reconstructed by cortical remodeling (see Fig. 11B).

Modeling of the External Shape by the Envelopes

The midshaft of long bones grows in diameter by periosteal apposition and endosteal resorption. The cortex increases in thickness as long as apposition surpasses resorption. In the adult, both activities cease and a constant cortical diameter is preserved. In the elderly, they resume, at least to a certain extent, and endosteal resorption may exceed periosteal apposition, resulting in an increase in diameter of the marrow cavity at the expense of cortical width. This basic pattern is valid for bones with a straight diaphysis, but is subjected to manifold alterations in bones with a curved

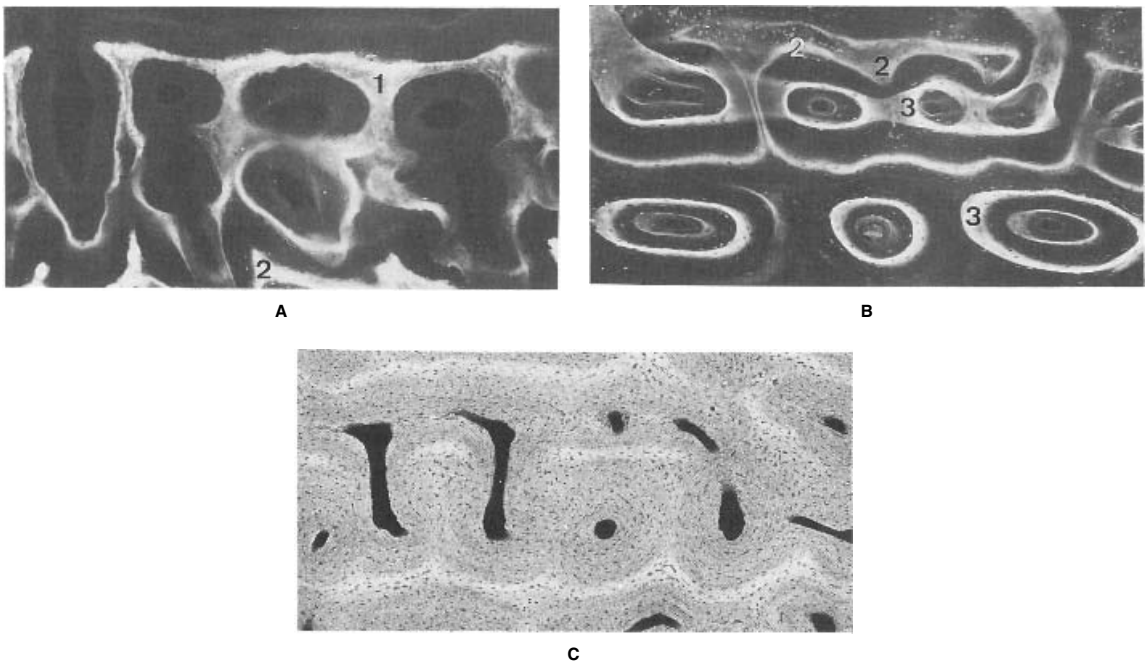

Figure 10. Formation of primary osteons during appositional growth in the periosteal envelope of an equine metacarpal bone. Three sequential polychrome labels at intervals of 3 and 4 weeks. The daily apposition amounts to 25.8 μm (compared with 2.1 μm in Fig. 4B). × 60. **A:** The woven bone initially deposited by the periosteum shows a diffuse uptake of the fluorochome label (1) given 2 weeks before sacrifice. This bone confines the walls of the future vascular canals. **B:** In a deeper layer, labels 2 and 3, given 4 and 7 weeks before sacrifice, demonstrate the concentric lamellar filling giving rise to the wall of the primary osteons. **C:** In a microradiograph, the initially formed woven bone compartment is identified by its brighter appearance, due to a higher mineral density. Its structure corresponds to the diffusely labeled woven bone in A above.

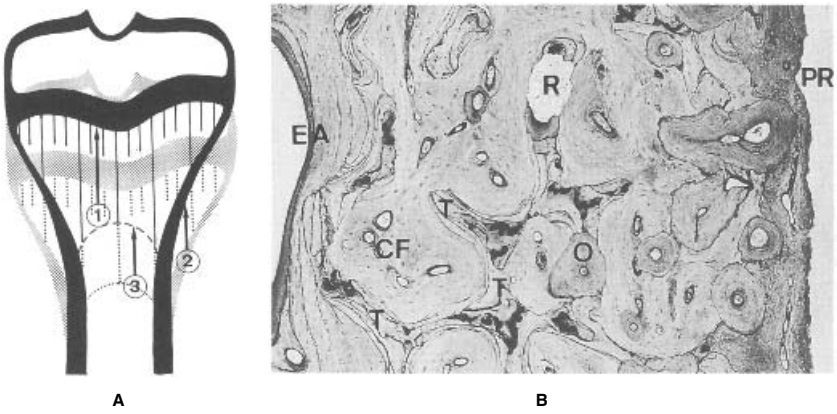

Figure 11. Conical modeling and formation of the cortex in the metaphysis of a long bone. **A:** Schematic superposition of two outlines of the tibial head, corresponding to 1 week of growth in a 35-day-old rat. Reprinted with permission from [390]. The numbers indicate the main sites of osteoclastic resorption: (1) calcified cartilage resorption, (2) subperiosteal cortical bone resorption, (3) enlargement of the marrow cavity by resorption of the metaphyseal spongiosa. **B:** Transverse section of the metaphyseal cortex of the proximal tibia of a growing mini-pig. Surface-stained ground section, × 40. Endosteal apposition of circumferential lamellae (EA), subperiosteal resorption (PR), incorporated trabeculae of primary spongiosa (T), concentric lamellar filling of the former intertrabecular space (CF), resorption canal (R), and completed secondary osteons (O).

or more complex anatomical shape. Enlow [41] and others have carefully analyzed the principles of shape-deforming modeling in a variety of bones. Instructive examples are the conical shaping of the metaphyses and the cortical drift in the shaft of curved long bones like the femur. Lengthening at the epiphyseal ends steadily increases the bend. This must be compensated for by a shift of the cross-section in the anterior direction, as illustrated in Figure 12. To this end, the anterior cortex undergoes periosteal apposition and endosteal resorption, whereas the opposite pattern occurs in the posterior cortex.

The growing ribs also have to displace in space to reach the outer circumference of the thorax (Fig. 13). Thereby, the outer layer undergoes periosteal apposition and endosteal

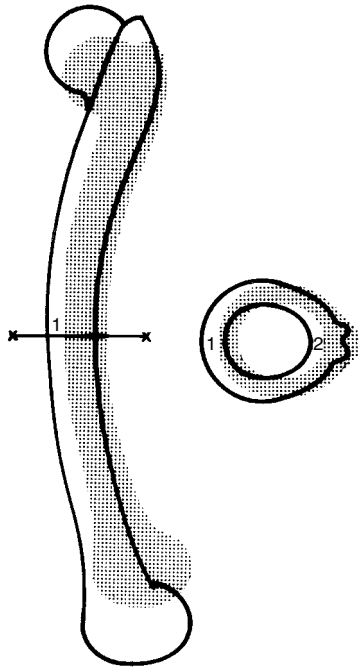

Figure 12. Cortical drift during growth and modeling of a curved long bone, e.g., the femur. Dotted area = growth stage, black outline = final stage. The scale of the cross section is three times that of the profile. (1) Zone of periosteal apposition. (2) Zone of endosteal apposition Reprinted with permission from [390].

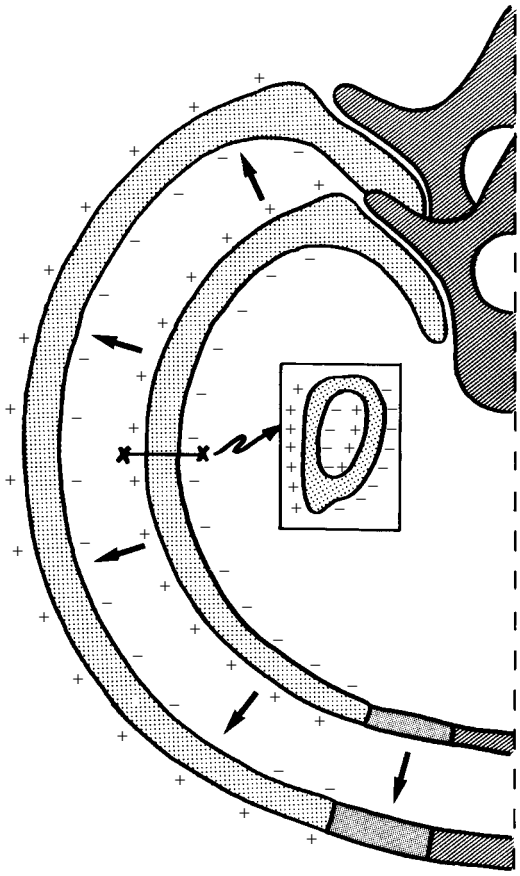

Figure 13. Schematic drawing of the cortical drift in a growing rib, indicating the reversal of periosteal and endosteal apposition, as well as resorption by the outer and inner cortical envelopes.

resorption, whereas the inner cortex is subject to endosteal apposition combined with periosteal resorption.

REMODELING OF BONE

It is legitimate to make a distinction between modeling and remodeling activities in the skeleton. Modeling is a shape-modifying process, carried out by the formative and resorptive activities of the envelopes. Remodeling results in the substitution or replacement of bone moieties and thus means the renewal of material without structural change. Modeling is limited to the growing skeleton, and becomes only exceptionally operative in the adult, i.e., after the malalignment of fractures. Remodeling occurs lifelong; during growth it contributes to the maturation of bone, while in the adult it provides metabolically active bone tissue for calcium homeostasis, eliminates avascular, necrotic bone compartments, and prevents fatigue by local repair of microcracks and microfractures.

Cortical Bone Remodeling

Bone apposition by the cortical envelopes results in the formation of circumferential lamellae and primary (or appositional) osteons. Both are at least partially replaced by secondary (or replacement) osteons (Fig. 14A–E). Replacement means substitution, and this is initiated by resorption followed by formation. First, a resorption canal is formed by osteoclasts. Shortly thereafter, osteoblasts appear and start refilling this canal with concentric lamellae, until it is narrowed to the final diameter of the vascular canal of the newly formed secondary osteon or Haversian system, the bone structural unit (BSU) of the cortex. In human bone, secondary osteons reach an outer diameter of about 200–250 μm and an inner diameter of 50–80 μm. The wall thickness amounts to 70–100 μm [42] and varies somewhat with age [43]. Secondary osteons always run parallel to the long axis of the bones, but measurements of their length are impeded by frequent branching or ramifications. As a uniform cylindrical structure, they are rarely more than 2–3 mm long. In addition, they are interconnected by transverse vascular channels (Volkmann's canals) at intervals of 0.5–1 mm.

In contrast to primary osteons, secondary osteons are always delineated from the surrounding bone matrix by a cement line (Fig. 14C). Although cement lines are easily identified by their refractive properties and by various staining methods, their exact composition is not clear. Cement lines appear at sites where the bone surface stays quiescent (undergoing neither resorption nor formation) for some time. Microscopically, two types are distinguished: Resting (or arresting) lines appear when bone formation is temporarily arrested and then resumes again. Resting lines are smooth and run strictly parallel to the lamellae (Fig. 15B). Reversal lines have a crenated appearance, which indicates that bone formation was preceded by osteoclastic resorption. Cement lines surrounding secondary osteons belong to this latter type. They form the boundary between adjacent osteons or interstitial lamellae, the latter representing remnants of former osteon generations or circumferential

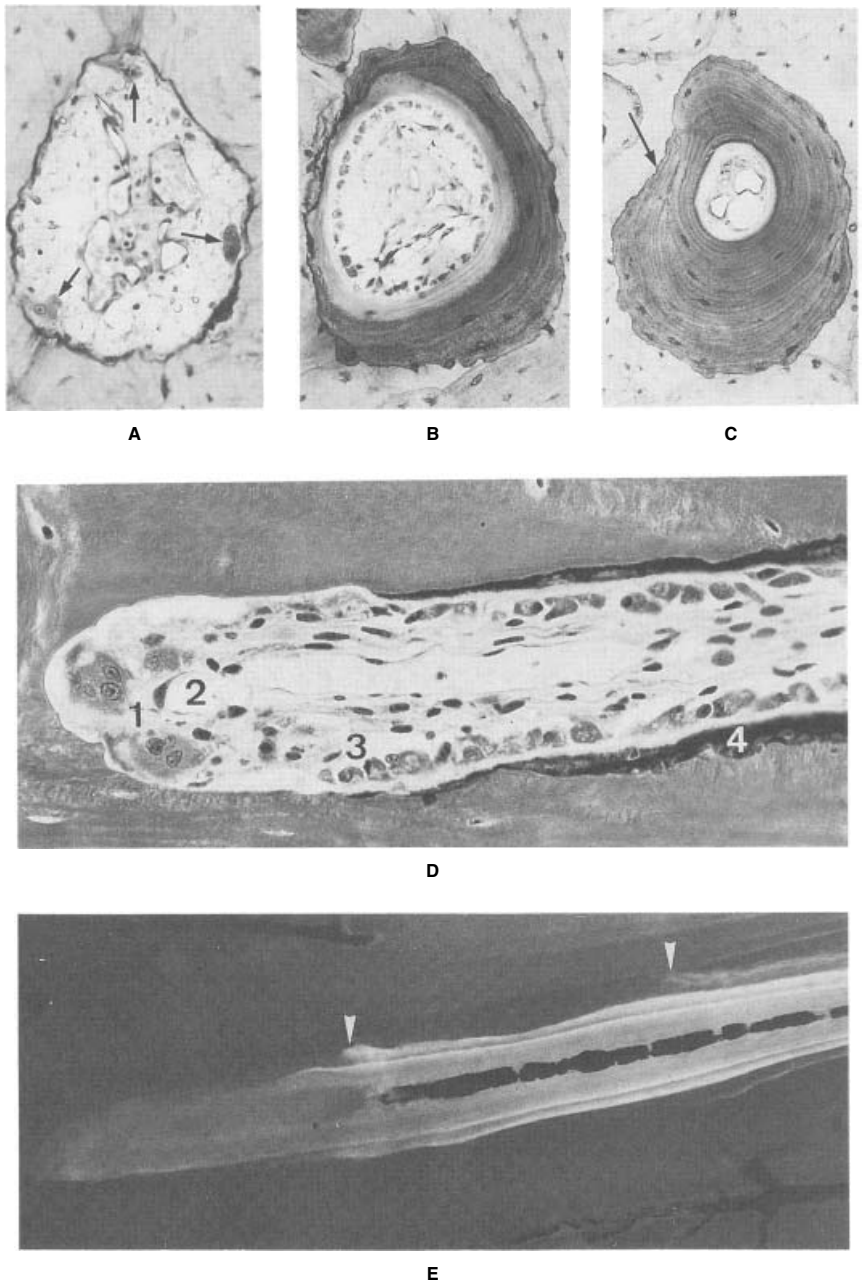

Figure 14. Cortical bone remodeling. **A**–**C:** Diaphysis of an adult human humerus, surface-stained ground sections, × 220. **A:** Resorption canal (arrows point to osteoclasts). **B:** Deposition of concentric lamellae. **C:** Completion of a secondary osteon (arrow points to the cement line). **D:** Longitudinal section through the tip of an evolving secondary osteon during fracture repair in a canine radius. Reprinted with permission from [391]. Goldner-stained, 5 μm-thick microtome section, × 320. Osteoclastic cutter cone (1), vascular loop (2), osteoblasts (3), and osteoid seam (4). **E:** Sequential fluorochrome labeling of an evolving osteon in a canine tibia. The daily osteoclastic resorption rate is calculated by measuring the distance between the tips of the labels (arrowheads) given at weekly intervals, × 120.

lamellae (Fig. 2). It is generally assumed that reversal lines are not crossed by canaliculi, and thus prevent communication between osteocytes. As a result, interstitial lamellae are often devitalized and become micropetrotic. In other locations, however, osteocytes may re-establish cytoplasmic connections with newly generated osteoblasts after resorption has ended. These connections assure the further nutrition of adjacent compartments via canaliculi across a cement line.

An important advance in our understanding of bone remodeling was the observation that bone resorption and bone formation are coupled in space and time, and occur in discrete remodeling sites or "bone multicellular units," also known as "bone metabolizing units" (BMUs) [44,45]. The sequence of resorption and formation has already been described in transverse sections. Longitudinal sections through cortical BMUs confirm this coupling. Within the tip

of a resorption canal, a group of osteoclasts is assembled in a cutter cone (Fig. 14D). This lengthens the resorption canal and, at the same time, widens it to the final diameter of the future osteon. The resorption canal is well vascularized. The vessels are accompanied by perivascular cells, possibly including osteoclast and osteoblast precursors. After osteoclasts have passed by, the wall is lined by ill-defined mononuclear cells. This portion, however, is only 100–200 μm long and represents a reversal phase of 1 or 2 days. During this phase, the cement line may be formed. Then osteoblasts appear and start depositing lamellar osteoid that will undergo mineralization 8–10 days later. Because the system advances longitudinally, the canal will assume a conical shape (closing cone). Lamellar bone formation continues until, several weeks or months later, the evolving osteon is completed.

The dynamics of the remodeling units at the cellular level have been analyzed by sequential fluorochrome labeling (Fig. 14E). A linear osteoclastic resorption rate can be measured in longitudinal sections [46,47]. In the radius and tibia of dogs, the daily longitudinal advance of the cutter cone is 50–60 μm and the average daily osteoblastic apposition rate is 1.5–2 μm [48]. In dogs, the mean outer diameter of completed new osteons is 140–170 μm and the wall thickness about 70 μm. Accordingly, the completion of a new osteon (the life span of a BMU) in a dog will take 5–6 weeks, corresponding to a length of the closing cone of 2–3 mm. In human osteons with a wall thickness of 90–100 μm and an apposition rate of only 1 μm/day, the life span of a BMU is estimated to be at least 3–4 months [49].

Cancellous Bone Remodeling

The remodeling of cancellous bone has attracted much attention in recent years because it can be studied quantitatively in biopsies. In human iliac crest cancellous bone, about 5% of the trabecular surface is covered by osteoblasts and 0.5–1% by osteoclasts. But cancellous bone exposes an enormous surface to the endosteal envelope of the bone marrow, and unbalanced resorption and formation has a profound influence on its overall density. With a diameter of roughly 200 μm, the trabeculae stay within the limits of the canaliculo-lacunar transport distance, and therefore are seldom penetrated by vascular canals. The constant remodeling results in a surprisingly complicated structure (Fig. 15B). Staining of the cement lines reveals a complex assembly of lamellar bone compartments or lamellar packets. These bone structural units must have been deposited at different times, either during growth or in a later modeling or remodeling phase.

Whereas the modeling of cancellous bone results for the most part in the functional adaptation of the framework to the mechanical load, remodeling is thought to replace discrete portions of the scaffold with new bone. This improves the quality of the tissue, with regard to both its mechanical

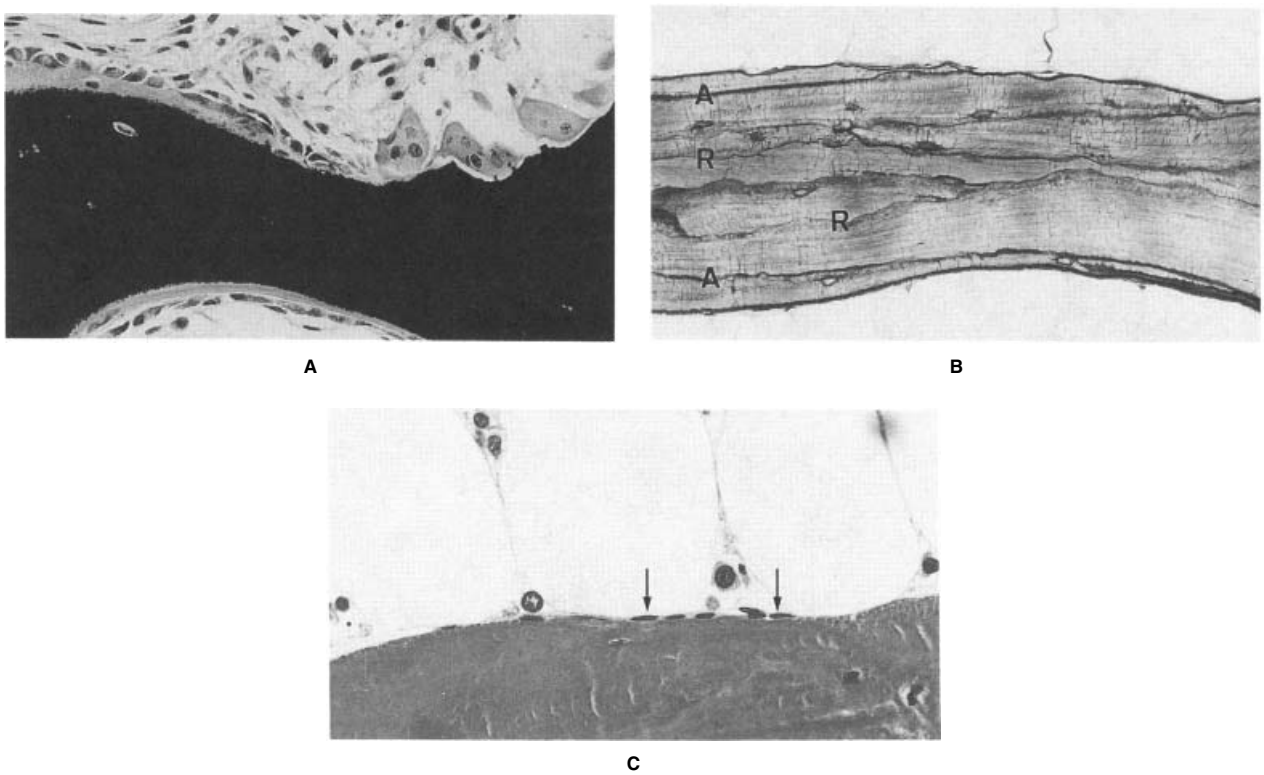

Figure 15. Surface characteristics and composition of adult human cancellous bone trabeculae. **A:** Resorption and formation on the trabecular surface in an iliac crest biopsy of a patient with increased bone turnover (renal osteodystrophy). Osteoclastic and osteoblastic activities appear to be coupled as in cortical BMUs. Von Kossa-McNeal, × 250. **B:** Surface staining of polished ground sections reveals a surprisingly complex assembly of packets, separated by cement lines. Note two smooth arresting lines (A) and multiple reversal lines (R), × 200. **C:** Inconspicuous, extremely flattened lining cells (arrows) cover the quiescent bone surface, which in this instance seems to be mineralized up to the bone-marrow interface. Goldner's trichrome stain, × 500.

properties and the availability of surface for ion exchange. Microscopically, the formation of a new packet also begins with the recruitment of osteoclasts (Fig. 16). These "dig" a cavity in the trabecular surface with a diameter of 0.5–1 mm and an average depth of 50 μm, seldom more than 70 μm. When resorption is completed, the osteoclasts are replaced by inconspicuous mononuclear cells. After a short reversal phase, osteoblasts appear, deposit new lamellae into the excavation, and possibly fill it up completely. The resulting lamellar packets represent the BSUs of the trabeculae and are analogous to secondary osteons (Fig. 16D). It is assumed that this type of cancellous bone remodeling also exhibits similar dynamics to the BMUs in cortical bone [50,51]. It is possible that resorption, followed by formation, also moves along the endosteal surface like one-half of the cutter cone and closing cone in a cortical BMU, although this is, for geometrical reasons, difficult to demonstrate in microscopic sections [52] (Fig. 15A).

Regulation of Bone Remodeling

The systemic activation of bone remodeling is attributed to the actions of growth hormone and thyroid and parathyroid hormones, whereas calcitonin and cortisone have a systemic inhibitory effect. Bisphosphonates, which are known as potent inhibitors of osteoclastic bone resorption, also reduce the overall bone remodeling rate.

Local activation is observed after mechanical lesions of bone, such as fractures, surgical manipulations, or the insertion of implants. A tremendous activation follows any temporary interruption of the blood supply. It accompanies revascularization and results in the substitution of necrotic zones by vital bone tissue.

The coupling of resorption and formation, which is so obvious from morphological considerations, poses many difficult questions as far as its regulation is concerned. The term "coupling" was first used to describe the local collaboration of osteoclasts and osteoblasts, explained by their derivation from a common osteoprogenitor cell [53]. It has to be recalled that Frost [44] defined the BMU as a basic multicellular unit and considered it as a typical example of intercellular communication. This concept initiated the search for coupling factors [54]. At present, the idea that a rather complex interaction of multiple factors is involved in the initiation and synchronization of resorptive and formative activities is favored. This sequence includes, 1) osteoclast activation, possibly mediated by lining cells, 2) factors released from the bone matrix by the resorbing osteoclasts to induce osteoblast proliferation and differentiation, and 3) factors produced by other local cells apt to stimulate all of these processes [55].

In a more comprehensive version, the term "coupling" is now not only applied to the recruitment and activation of the various cells involved but it is also suggested that their performances are balanced with respect to each other. In other words, the resorption of a certain amount of bone must be matched by the replacement of the same volume by new bone formation [50,51].

As long as resorption and formation remain coupled and balanced, cancellous bone remodeling results in substitution of bone tissue without any change in trabecular dimension

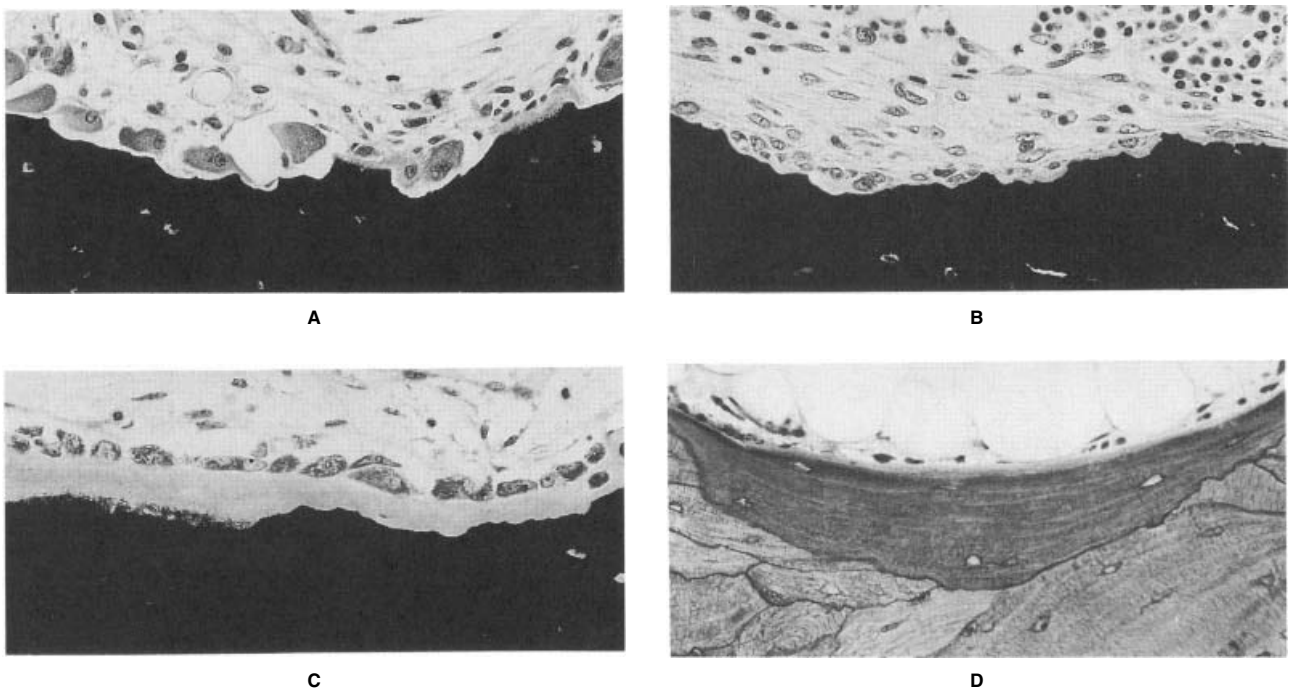

Figure 16. Stages of cancellous bone remodeling in the adult. Human iliac crest biopsies, **A**–**C**, von Kossa-McNeal, × 250; **D**, surface staining with toluidine blue, × 200. **A:** Resorptive phase: osteoclasts erode the trabecular surface. **B:** Reversal phase: Howship's lacunae, now filled with mononuclear cells of unknown origin, disclose the former osteoclastic activity. **C:** Early formative phase: Osteoblasts start filling the resorption cavity. To the right, only osteoid has yet been deposited upon the lacunar surface; to the left, a calcification front is already established. **D:** Newly formed lamellar packet, or bone structural unit (BSU), is clearly delineated by a reversal line. Note again the complexity of the adjacent trabecular structure revealed by the staining of the cement lines.

and shape. Generalized and persisting negative balance at the BMU level is, according to the current view of bone histologists, a plausible mechanism for the pathogenesis of bone loss and osteoporosis. Localized imbalance or uncoupling, however, will not only change the dimension but also the shape of the trabeculae and thus is able to modify the architecture of the spongiosa, but not the external shape of the bone. This might be an adaptive response to changes in load and physical activity, as postulated by Julius Wolff in 1892 [56] or more recently by Frost [57,58] in his concept of a "mechanostat." The functional adaptation of bone to cementless orthopedic prostheses and dental implants is another area where these structure-function relationships are convincingly confirmed.

Bone Formation

W. Hofstetter

Osteoblasts are the cells responsible for bone formation. They differentiate from mesenchymal stem cells and thus share a common origin with other mesenchymal cell lineages such as chondrocytes, adipocytes, fibroblasts, and myoblasts. Below are discussed some of the mechanisms essential for the development of the skeleton and the differentiation of cells of osteoblast lineage. Emphasis is placed on the exciting findings of the past few years, while reference is made mainly to review articles for earlier data.

BONES ARE ORGANS, BONE IS A TISSUE

The development of organs constitutes a sequence of complex processes that are all tightly regulated by a network of interacting signals. Although the results of these developmental processes are vastly different, it has become clear over time that organogenesis is controlled by members of a few gene families that have been highly conserved during evolution.

In its adult state, the skeleton comprises two main tissues, bone and cartilage, containing various cell populations, namely chondrocytes, osteoblasts, osteocytes and lining cells, and chondroclasts and osteoclasts. Among these cell populations, it is the osteoblasts that are responsible for bone formation. This task encompasses the elaboration of the components of the extracellular bone matrix and the regulation of its mineralization. The osteoblasts, however, not only produce the bone material, but are also instrumental in regulating the recruitment and/or activation of the bone resorbing cells, the osteoclasts, resulting in their having a central position in bone metabolism. The aim of this section is 1) to summarize the fundamental processes regulating the development and patterning of the skeleton; 2) to describe the development of osteoblasts from mesenchymal stem cells to the final differentiation states of either active osteoblasts, osteocytes, or resting lining cells; and 3) to discuss the role and function of various components of the bone matrix.

MORPHOGENESIS OF BONE

In recent years, many of the genes involved in the formation of bone have been identified by elucidating the molecular mechanisms underlying heritable skeletal disorders. Furthermore, as knowledge has increased, it has become evident that molecular cascades guide the formation of different organs and that the components of these cascades have been highly conserved during evolution.

Among the earliest events in the formation of the limb buds of the appendicular skeleton is the induction of structurally undifferentiated mesenchyme to transform into condensed mesenchyme. Such regions of high cell density already represent the outlines of the future skeletal elements. Subsequently, the cells in the centers of the condensations change their phenotype, differentiating into prechondrocytes. This step is accompanied by a switch from the production of a mesenchymal matrix to that of a cartilaginous matrix, as is described later in more detail. The cartilage anlage of the developing bone grows rapidly to resemble the future bone, in both size and shape. The chondrocytes in the center of the future diaphysis start to hypertrophy and the extracellular matrix becomes mineralized, while on the perichondrial surface osteogenic cells develop into osteoblasts and lay down a bone collar around the hypertrophying cartilage. Concomitantly, osteoclasts develop, which, together with blood vessels, penetrate the mineralized cartilage of the forming bone rudiment and dissolve the mineral [59,60]. Osteoblasts, following the invading osteoclasts, start to lay down bone material onto persisting residues of the mineralized cartilage septa forming the cores of the primary trabeculae, which are gradually replaced during the remodeling phase of osteogenesis (Fig. 17). By this process, which is called endochondral ossification, the major part of the skeleton is formed. In intramembranous bone formation, however, which gives rise to the bones of the skull and the clavicles, the cells of the mesenchymal condensations differentiate directly into osteoblasts that synthesize the extracellular matrix of bone. For further discussion of these processes, see the preceding part of this chapter (Chapter 1, Part II).

Some time ago, it became increasingly evident that early skeletal patterning is governed by the *Hox* genes, a family of homeobox transcription factors that was originally identified in *Drosophila melanogaster* and has been highly conserved during evolution. The gene products act as molecular switches that regulate the initiation of complete developmental programs. The roles of the individual Hox proteins have been elucidated either by investigating the molecular defects in heritable genetic disorders or by producing and analyzing gene knockout mice [61]. Using these approaches, *Hox* genes were found to act on both

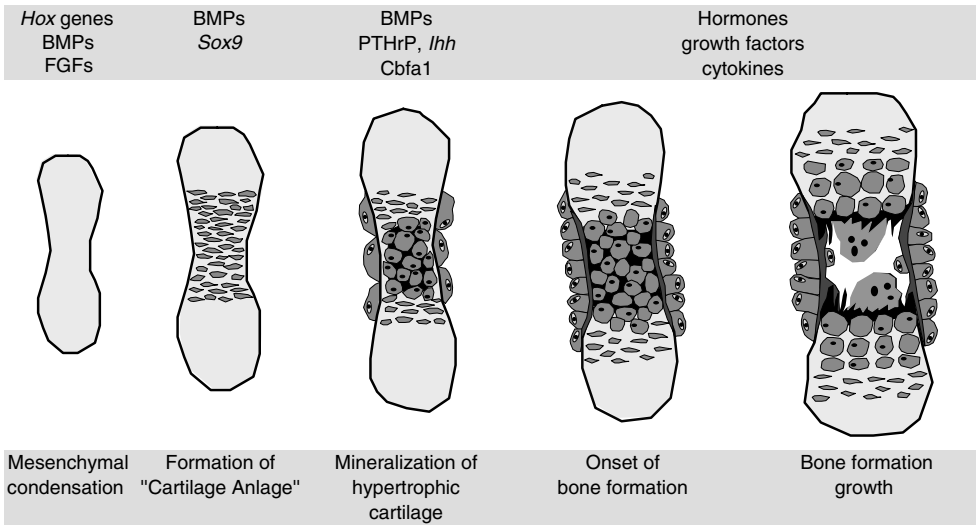

Figure 17. Schematic representation of long bone development. Specific gene products regulate each step from the mesenchymal condensation through commitment to the chondrocytic lineage, induction of the osteoblastic phenotype and the growth and development of osteoclasts.

initial mesenchymal condensation and differentiation and the later processes of proliferation and maturation of chondrocytes [62,63]. As a consequence, the inactivation or ectopic expression of *Hox* genes results in the deletion or addition of skeletal elements or in the transformation of certain elements into shapes resembling other elements [64]. For example, the misexpression of *HoxD-11* in the chicken limb bud leads to an additional phalanx in digit I with a morphology similar to that of digit II [62]. Similarly, expansions of a polyalanine stretch within the amino-terminal non-DNA binding region of *Hox-D13* have been shown to cause synpolydactyly in heterozygous individuals, characterized by the insertion of an extra digit between digits III and IV in association with variable syndactyly [64]. Such experiments have further revealed that, in mammals, *Hox* genes do not act as individual entities, but, rather, that multiple *Hox* genes function together to mediate a program by regulating common target genes. Thus, for appropriate development, the temporal, spatial, and stoichiometric expression of *Hox* genes operating within the developing tissue is critical [65].

A second class of proteins instrumental in the early events of morphogenesis of the skeleton are the bone morphogenetic proteins (BMPs), which are members of the transforming growth factor-β (TGF-β) superfamily [66]. The first BMPs were originally characterized by their capacity to induce ectopic cartilage and bone formation when implanted into experimental animals. The name, bone morphogenetic protein, however, is somewhat misleading since there is strong genetic and experimental evidence that these molecules regulate diverse biological processes such as cell proliferation, differentiation, apoptosis, and morphogenesis [67]. Several BMPs are expressed in the early mesenchymal condensations in a manner that suggests a role for these proteins in patterning of the developing bone. BMP2, BMP4, and BMP7 are all expressed in the apical ectodermal ridge of the limb buds, where they may function in regulating cell proliferation, differentiation, and/or activation [68]. In the mesenchyme of the developing limb, however, the expression patterns of these genes differ significantly from each other locally as well as temporally. The essential role of BMPs in the development of the skeletal elements was first demonstrated when the genetic defect in the murine *short ear* mutation was found to be located in the gene for BMP5 [69,70]. The phenotype of homozygous mutant mice is characterized by an alteration in the number of ribs and in additional more subtle changes in shape and size of various bone and cartilage elements. BMPs, however, are not only required for differentiation but also for the induction of apoptosis in the supernumerary cells of the interdigital web. Experimental application of BMP2 or BMP4 to developing limbs induces an acceleration of programmed cell death in mesenchymal cells [71,72], while TGF-β diverts the cells from the death program and induces the formation of ectopic cartilage or extra digits [72].

Another family of growth factors and receptors that is involved in these early processes of morphogenesis are the fibroblast growth factors (FGFs) and their receptors [73,74]. In concert with Sonic hedgehog, a member of the hedgehog family of signaling proteins [74,75], FGFs are thought to act upstream of Hox proteins and BMPs, regulating their respective expressions. Hedgehog proteins constitute a conserved family of secreted molecules that provide key signals in embryonic patterning of many organisms (for a review, see [76]).

DEVELOPMENT OF CARTILAGE

After the formation of the condensed mesenchyme, the constituent cells become committed to developing along the chondrocyte lineage. The cells in the centers of the condensations change their phenotype and hypertrophy, becoming large and round, while at the periphery a sheath of spindle-shaped cells forms the perichondrium. The central cells cease to proliferate and switch the composition of their extracellular matrix from a mesenchymal matrix that contains mainly collagens I and III to a cartilaginous matrix containing collagens II, IX, and XI and noncollagenous extracellular matrix proteins and proteoglycans such as aggrecan [77].

The chondrocytes undergo further differentiation and hypertrophy, producing a matrix that is abundant in collagen X and partially calcifies.

The molecular mechanism causing the commitment of the mesenchymal cells to the chondrocytic lineage is largely unknown as yet. Sox [Sry (testis determining factor) - type HMG (high mobility group) Box] proteins, which constitute a subfamily of DNA-binding proteins with an HMG domain, exert critical functions in a number of developmental processes, including sex determination, neurogenesis, and skeletal formation [78]. In humans, mutations in *Sox9* are associated with campomelic dysplasia (see Chapter 23, Part V, this volume), a disease characterized by skeletal malformation and XY sex reversal [79]. During morphogenesis of bone, *Sox9* is expressed in the cells of the mesenchymal condensations that develop into chondrocytes [80]. The protein is coexpressed with collagen II, binds to a specific sequence within the collagen II promoter, and induces the ectopic expression of collagen II in transgenic mice [81–83], further strengthening the notion that Sox9 contributes in a critical manner to the commitment of pluripotent mesenchymal precursors to the chondrocytic lineage. Sox9, however, does not act by itself in the regulation of the expression of chondrocyte products, but acts with other members of the gene family in the regulation of collagen II, and possibly other genes [84].

While *Sox9* contributes to the commitment of mesenchymal cells to the chondrocyte lineage, the proliferation, differentiation, and activation of chondrocyte precursors and mature chondrocytes is regulated by a diverse set of growth factors and cytokines, the nature of which is gradually being unravelled. Longitudinal growth is determined by chondrocyte proliferation, differentiation, and activity within the growth plate. Growth acceleration is achieved by cell-shape modeling, specifically by an increase in cell height, whereas cell volume, proliferation rate, and net matrix production remain largely unchanged [85]. A physiological reduction of the growth rate, on the other hand, is achieved by simultaneous reduction of cell height, volume, and proliferation rate. In growth cartilage, growth hormone has been shown to stimulate the proliferation of resting chondrocytes [86], while the mitogenic effect on proliferating chondrocytes is mediated through insulin-like growth factor 1 (IGF-1) [87]. Mutations in either growth hormone-releasing hormone [88] or IGF-1 [89] lead to reduced growth, suggesting that deficient chondrocyte proliferation leads to reduced bone size. The family of fibroblast growth factors and their receptors has also been implicated in the regulation of chondrocyte proliferation. Achondroplasia, a frequent form of osteochondrodysplasia, has been shown to be the result of a mutation in a gene of the FGF receptor family (*FGFR3*) [90] (see also Chapter 23, part IV, this volume).

Since the molecular characterization of parathyroid hormone-related peptide (PTHrP) [91], major progress has been made in defining the role of this polypeptide in development (for review see [92]). Originally, PTHrP was characterized as a tumor product causing humoral hypercalcemia of malignancy, a major contributor to morbidity and a complication in clinical treatment [93]. In contrast to IGF-1 and FGF, PTHrP does not act primarily to stimulate the proliferation of chondrocytes but rather to promote cellular development. Mice in which the gene for PTHrP has been artificially disrupted ("knockout" or "null-mutation") die shortly after birth and exhibit widespread abnormalities of endochondral bone development. These animals are characterized by the premature maturation of chondrocytes and accelerated bone formation [94]. Because the peptide and its receptor are expressed in close spatial proximity during development, it has been suggested that, in cartilage, PTHrP acts mainly as a paracrine/autocrine factor, its main effect being to delay chondrocyte differentiation during endochondral bone development [95]. The essential role of PTHrP in endochondral bone formation has been further demonstrated in $PTHrP^{-/-}$ mice carrying a PTHrP transgene targeted to chondrocytes. Targeting was achieved by placing the PTHrP transgene under the control of the collagen II promoter. The mice displayed a normal bone phenotype, while the development of other tissues, such as the mammary gland, was still impaired [96]. On the other hand, overexpression of PTHrP [97], or the expression of a constitutively active receptor [98,99], leads to chondrodysplasia due to a delay in chondrocyte differentiation and delayed mineralization. These findings indicate that PTHrP exerts a profound control on endochondral bone formation by regulating the development of chondrocytes into hypertrophic chondrocytes, and emphasize the necessity of its tightly regulated expression for normal cartilage development.

The expression of PTHrP in growth cartilage is regulated through a feedback mechanism that involves Indian hedgehog (Ihh) and its direct targets Patched (Ptc) [100] and Gli [101]. During their differentiation from proliferating to prehypertrophic to hypertrophic cells, chondrocytes transiently express PTHrP/PTH receptors, Ihh, and collagen X [102]. Simultaneously, Ptc and Gli are expressed in the perichondrium. This leads to the conclusion that PTHrP is under the control of Ihh. In this regulatory pathway, prehypertrophic chondrocytes signal through Ihh to the perichondrium. The response to Ihh in the perichondrium induces, either directly or indirectly, the synthesis of PTHrP in the articular perichondrium, blocking proliferating chondrocytes from hypertrophying [103]. This block in differentiation subsequently leads to a drop in Ihh levels, causing an interruption of the signaling cascade from the perichondrium to the periarticular cartilage, which, in turn, ceases to produce PTHrP, thus releasing the block of PTHrP on cartilage differentiation. Although proliferating chondrocytes express only low levels of PTHrP/PTH receptors, the regulation of chondrocytic differentiation is dependent on this pathway [104]. Thus, Ihh regulates the expression of PTHrP in a common feedback loop that regulates the rate of chondrocyte differentiation and thereby balances growth and ossification of long bones.

OSTEOBLASTS

Determination of the Osteoblast Lineage

Osteoblasts are the skeletal cells responsible for bone formation. They synthesize and regulate the deposition, maturation, and mineralization of the extracellular matrix. But, as described elsewhere in this chapter, osteoblasts also function in the modulation of bone resorption through both the secretion of soluble factors and the expression of surface molecules that act via cell–cell contact.

Cells of the osteoblast lineage are of mesenchymal origin and derived from mesenchymal stem cells (for a review see [105]). They include committed osteoprogenitor

cells, osteoblasts, osteocytes, and lining cells, and share a common precursor with myocytes, fibroblasts, adipocytes, and chondrocytes. A goal of recent research has been to identify molecules that are essential for the development of pluripotent precursor cells. The search has been successful, since it has identified the MyoD group of basic helix-loop-helix muscle regulatory factors as determinants of a muscle phenotype (for a review, see [106]) and the peroxisome proliferator-activated receptor γ2 (PPAR γ2) [107] as responsible for determination of an adipocyte phenotype.

The identification of transcription factors regulating the differentiation of osteoblasts has proven to be more elusive. Recently, however, evidence has been obtained demonstrating that the transcription factor Cbfa1 is essential for the development of the osteoblast phenotype. *Cbfa1* belongs to the *Runt* domain gene family and is homologous to the *D. melanogaster* pair-rule gene *runt* that plays a role in the formation of the segmented body pattern as well as in sex determination and the development of the nervous system [108]. In humans, mutations in *Cbfa1* have been shown to cause cleidocranial dysplasia, an autosomal dominant condition characterized by hypoplasia/aplasia of clavicles, patent fontanelles, supernumerary teeth, short stature, and other changes in skeletal patterning and growth [109] (see Chapter 23, Part V, this volume). Disruption of the *Cbfa1* gene in knockout mice has revealed the necessity of the transcription factor in determination of the osteoblast phenotype [110,111]. *Cbfa1*$^{-/-}$ animals died shortly after birth due to an inability to breathe. The homozygous animals had short limbs, but all organs were normal. The most striking effect caused by the lack of Cbfa1 was a total deficiency of bone. The skull was a thin layer of fibrous connective tissue, while the tibiae contained only calcified cartilage, this at an age at which bone has normally formed [112]. Mice in which only one of the *Cbfa1* alleles had been mutated showed defects that again were typical of cleidocranial dysplasia [111].

The molecular identification of Cbfa1 as a transcription factor inducing the commitment of a precursor cell to the osteoblast lineage was undertaken on the basis of previous studies that had identified putative transcription factors stimulating the expression of osteoblastic gene products [113]. Low stringency cloning and *in situ* hybridization experiments revealed that, during development, Cbfa1 is expressed in an osteoblast-specific manner. Its forced expression in nonosteoblastic cells induces the expression of osteoblast-specific genes [114], among them osteocalcin (bone Gla protein, BGP), presently the only protein known to be expressed specifically by osteoblasts [115]. Cbfa1 binds to *cis*-acting elements within the osteocalcin promoter (osteoblast-specific element, OSE) that have previously been shown to be essential for transcription of the osteocalcin gene [116,117].

Development of Osteoblasts

The development of osteoblasts, together with osteoclast formation and activation, determines the mass and shape of the bone formed. The regulation of osteoblasts takes place at several levels, namely, the proliferation of precursors, the differentiation of precursors to mature osteoblasts, the activity of mature osteoblasts, and the further development of osteoblasts to osteocytes or to inactive lining cells (Fig. 18). While many growth factors and cytokines are known to affect the proliferation and

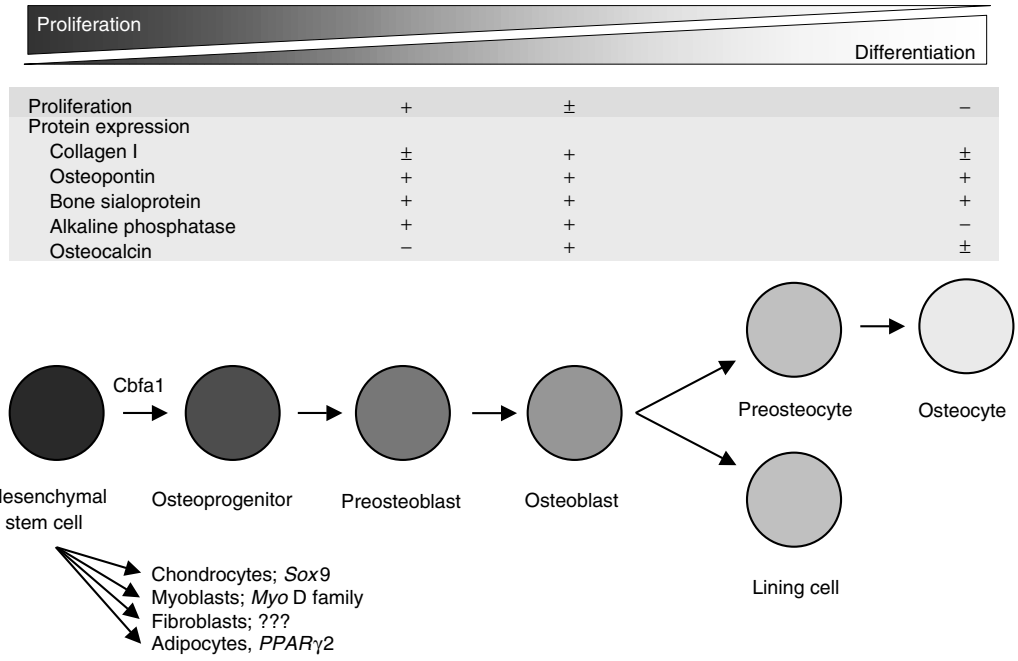

Figure 18. Development of cells of the osteoblast lineage. Osteoblasts are derived from mesenchymal stem cells. Expression of the transcription factor CbfaI commits these cells to become osteoprogenitor cells. During their development, the cells of the osteoblast lineage express specific genes, some of which are expressed during the whole differentiation pathway (like osteopontin and bone sialoprotein), whereas some of them are expressed predominantly by mature, matrix-producing osteoblasts (collagen I, osteocalcin). The ingrowth of vessels is not shown in this drawing for the sake of clarity (see also Fig. 7). Figure drawn after [118].

differentiation of the cells of the osteoblast lineage, little to nothing is known about the regulation of the further development of the osteoblasts to inactive lining cells or to osteocytes.

As noted, osteoblasts are derived from mesenchymal stem cells. During the process of differentiation, these cells pass through a number of distinct stages that can be characterized by the pattern of expression of osteoblastic marker proteins (for a review, see [118]). In the first phase, osteoblast progenitors are highly proliferative, expressing no markers specific for the lineage. With increasing maturation, the cells produce matrix proteins such as collagen I, osteopontin, and bone sialoprotein. This stage of matrix maturation is accompanied by a decrease in the potential to proliferate. Mature osteoblasts express osteocalcin and have virtually ceased proliferating. Subsequently, some of the active, matrix-synthesizing osteoblasts are induced by as yet unknown signals to arrest matrix production and are entombed as osteocytes in the bone matrix by neighboring cells. Lastly, the osteoblasts cease producing extracellular matrix and become "silent" lining cells.

The different steps in the development of osteoblasts are regulated by growth factors and cytokines. Depending on the state of the target cells, the respective factors exert differing effects. Thus, platelet-derived growth factor (PDGF), TGF-β, and the IGFs act chemotactically on osteoblast precursors. Additionally, TGF-β, PDGF, and FGFs stimulate the proliferation of their target cells [119,120]. The bone-forming capacity of TGF-β has been demonstrated in animal studies in which subperiosteal injections of the growth factor into murine calvaria induced an increase of bone apposition and a thickening of the bone layer [121,122]. The effect of TGF-β on bone, however, is even more complex because bone resorption is also modulated by this growth factor [123]. Lastly, BMP2 supports the differentiation and activation of osteoblastic cells. Animal studies have revealed that BMP2 has the capacity to induce bone in skeletal defects. When defects are filled with a collagenous carrier matrix impregnated with BMP2, osteoblasts invade the matrix and form bone within the collagen mesh, indicating the clinical potential of this growth factor [124–127]. Elucidating the actions of these cytokines and growth factors is complicated by the fact that, to a large extent, they are produced by osteoblasts themselves, enabling both autocrine and paracrine actions, and are integrated into the extracellular matrix during bone formation. As a consequence, the liberation and activation of these factors during bone resorption may be a possible mechanism for coupling the processes of bone formation and resorption.

The Functions of Osteoblastic Proteins

The composition of the extracellular matrix of bone defines its unique properties among the various extracellular matrices in the organism. Although mineral is required for resistance to pressure, it is the organic fraction, mainly the collagen fibers, that are responsible for the tensile strength and elasticity of bone. Correspondingly, mineral constitutes approximately two thirds of the dry weight of bone, while the organic portion is predominantly (approximately 90%) collagenous, the remaining 10% consisting of various noncollagenous proteins. While collagen is considered extensively elsewhere in this book (see Chapter 2), the major noncollagenous bone matrix proteins are briefly described below. Because it is not within the scope of this review to discuss the components of bone matrix in detail, and because this topic is reviewed elsewhere in this book, the present focus is on those proteins, the function of which has been studied recently using transgenic or knockout mouse models.

The noncollagenous matrix proteins, which define the specific functions of the bone matrix, can be divided roughly into four groups: proteoglycans such as decorin and biglycan [128], the RGD (arginine-glycine-aspartate)-containing proteins osteopontin (OP) [129] and bone sialoprotein (BSP) [130], glycoproteins such as osteonectin (ON) [17] and the γ-carboxyglutamic acid (Gla)-containing proteins, osteocalcin (BGP) [131], and matrix-Gla-protein [132]. Although the functions of these proteins have remained largely unknown until recently, with the establishment of knockout strains, some aspects of their biological roles in the formation and maintenance of bone have been elucidated.

Biglycan is an extracellular matrix proteoglycan that is enriched in bone. Mice deficient in biglycan appear normal at birth, but during growth they display a phenotype characterized by a reduced growth rate and decreased bone mass [133].

Osteopontin is a widely expressed protein that is also found in bone matrix. The expression of osteopontin is increased in transformed cells [134] or in response to stimuli such as myristate acetate [135]. Due to the presence of the cell attachment RGD-sequence, osteopontin has been proposed to be involved in cell adhesion as well [136]. Mice homozygous for a null mutation in the osteopontin gene develop normally during embryogenesis and reach fertility. They do, however, show alterations in wound healing, suggesting a role for osteopontin in tissue remodeling [137]. Double knockout mice for osteopontin and the RGD-binding vitronectin receptor are also viable, suggesting that other ligand/receptor pairs compensate for the two proteins [137]. Preliminary data suggest that osteopontin deficiency may affect osteoclast activity [138], because $OP^{-/-}$ mice are characterized by an increase in the number of osteoclasts that resorb bone inefficiently.

Only preliminary data have been presented relating to the phenotype of mice deficient in BSP, which suggest a decrease in the number of osteoprogenitor cells in $BSP^{-/-}$ animals, and subtle disturbances in the regulation of bone formation [139].

Osteonectin is thought to play a role in the mineralization of bone as a result of its affinity for hydroxyapatite and type I collagen [17]. Furthermore, it has been postulated to modulate the proliferation of cells of osteoblast lineage. Mice deficient in osteonectin develop normally up to the age of 6 months, at which time they develop severe eye pathology [140]. At birth, no skeletal abnormalities can be observed, but within 4 months the animals become severely osteopenic, $ON^{-/-}$ mice having 50% less bone mass than normal littermates. The numbers of osteoblasts and osteoclasts are reduced, causing a decrease in bone remodeling with a negative bone balance [141].

The role of the only bone-specific protein, osteocalcin (BGP), also proved elusive in the past. The protein is characterized by its high content of γ-carboxyglutamic acid, an amino acid that derives posttranslationally from glutamic acid by addition of a carboxyl group. The carboxylation reaction is catalyzed by the enzyme γ-carboxylase, which uses vitamin K as a cofactor. Rats treated with warfarin, an antagonist of vitamin K, show excessive mineralization at the growth plate [142], suggesting a putative role of osteocalcin in the regulation of mineralization. Somewhat contradictory data were also presented, suggesting a role for osteocalcin as a chemoattractant for osteoclasts. The protein has been described both as being required [143] and as being dispensible [144] for the dissolution of subcutaneously implanted bone particles. Since osteocalcin is not expressed during initial bone formation, it has been suggested that it may play a role in the remodeling and maturation of bone [145,146]. This hypothesis is supported by the finding that $BGP^{-/-}$ mice develop normally but show an increase in bone mass. Bone resorption, on the other hand, is not affected by the mutation. Therefore, it has been proposed, in accordance with the earlier warfarin experiments, that the main role of osteocalcin in bone metabolism consists in limiting bone growth [147].

As noted, osteoblasts pass through a series of stages before reaching maturity and laying down the bone matrix. A critical step in this complex process is the recruitment of osteoprogenitor cells to the sites of future bone formation. Extracellular matrix proteins that are known to serve in other tissues as substrates for cell attachment and to act as guides for cell migration are, therefore, of special interest. One of these proteins is heparin-binding growth-associated protein (HB-GAM), which is required for the development of neuronal tissues [148,149]. Transgenic mice that specifically overexpress HB-GAM in osteoblasts show an increase in bone mass resulting from an increase in bone formation [150]. Furthermore, HB-GAM is upregulated at the surface of damaged bone, where the recruitment and activation of new osteoblasts occurs. Potential sources of the protein are cells of osteoblast lineage, including osteocytes. The data suggest that growth factors, cytokines promoting bone formation, and even mechanical strain, might act through the production of HB-GAM to attract and activate osteoprogenitor cells and osteoblasts [150].

MINERALIZATION IN BONE AND CARTILAGE

Besides producing the organic components of the extracellular matrices of bone and cartilage, osteoblastic and chondrocytic cells control the mineralization of these matrices. For this purpose, chondrocytes and osteoblasts release matrix vesicles that induce, and subsequently serve as nuclei for, mineralization (for a review, see [151]).

Matrix vesicles are extracellular membrane vesicles that are released by budding from the surfaces of chondrocytes and osteoblasts [152]. Two pieces of evidence suggest that only specific compartments of the cell perimeter can give rise to matrix vesicles. Firstly, the protein composition of the matrix vesicles is far less complex than that of cell membranes [153]. Secondly, the release of matrix vesicles from the cells is a highly polarized process. The deposition of vesicles within the calcifying matrix is restricted to those areas in which mineralization will begin. In cartilage, matrix vesicles are localized to the calcifying longitudinal septal matrix [154], while in developing bone they are deposited within the newly formed osteoid beneath the osteoblasts [155].

At the time of their release, matrix vesicles are rich in phosphatases (among them alkaline phosphatase) and calcium-binding proteins. They do not, however, contain any mineral. The mineralization process, which is regulated and initiated by matrix vesicles, can be divided into two phases, phase one being the initiation of mineralization, which is enzymatically regulated, and phase two being the propagation of mineralization, which is a self-perpetuating process.

In both bone and cartilage, matrix vesicles are the first sites of mineralization. The first mineral crystals appear within the vesicles, often in close alignment with the membrane. This first phase of mineralization begins with the attraction of calcium into the vesicles by concentrating calcium-binding lipids and proteins [156,157] and by the action of calcium channels in the matrix vesicles [158]. The accumulation of calcium is followed by the accumulation of phosphate, which may result from the action of phosphatases within the vesicles or from membrane-localized phosphate transporters [159]. When sufficient calcium and phosphate have been accumulated within the vesicles, calcium phosphate precipitates [160]. The first precipitate consists of amorphous, noncrystalline calcium phosphate, which is subsequently converted to the less soluble hydroxyapatite [160,161].

While growing, the hydroxyapatite crystals penetrate the vesicle membrane, thereby initiating the second phase of the mineralization process. After penetration of the membrane by the growing crystals, a process that may be facilitated by the hydrolytic action of phospholipases [162,163], the hydroxyapatite becomes exposed to the extracellular fluid, in which calcium and phosphate concentrations are sufficiently high to cause spontaneous mineral proliferation. During this second phase of mineralization, the hydroxyapatite crystals, which originally formed within the matrix vesicles, serve as nuclei for crystal formation. Because crystal growth is dependent only on the concentrations of calcium and phosphate in the extracellular fluid, mechanisms that contain the progress are of major importance to avoid excessive mineralization. Cellular control of mineralization is achieved by regulating the budding of new matrix vesicles and by balancing mineralization and resorption. Molecular control, as mentioned above, may involve osteocalcin, which, in animal models, has been shown to regulate bone growth, eventually by interfering with the mineralization process [147].

THE OSTEOBLAST–OSTEOCLAST CONNECTION

As noted above, the central role in bone metabolism is assigned to osteoblasts, because cells of osteoblast lineage are thought to synthesize the components of the extracellular matrix and also to regulate, at least in part, the recruitment and activation of osteoclasts [164]. Indeed, it has been known for some time that hormones or cytokines like parathyroid hormone (PTH), interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α) indirectly exert their stimulatory effect on bone resorption through osteoblasts [165–167].

The molecules, however, that function as mediators between osteoblasts and osteoclasts have not yet been characterized [168–171]. Only recently, two molecules have been identified that are products of cells of osteoblast lineage and which, together, are sufficient to support the formation of functional osteoclasts from hematopoietic precursors. Colony-stimulating factor-1 (CSF-1), also called macrophage colony-stimulating factor (M-CSF), was originally described as essential for the proliferation, differentiation, activation, and survival of the cells of the mononuclear phagocytic system. This growth factor is expressed constitutively by mesenchymal cells *in vitro* and by activated macrophages [172]. It binds to a single class of high-affinity receptors encoded by the proto-oncogene *c-fms*. Interest in the role of CSF-1 in bone resorption was initiated by the finding that the osteopetrotic murine mutant strain *op* was characterized by a decreased number of macrophages and impaired bone resorption as a result of a virtual absence of osteoclasts. On the basis of these findings, it was suggested that osteopetrotic *op* mice were deficient in a growth factor for macrophages [173]. Subsequently, *op* mice were demonstrated to lack biologically active CSF-1 [174,175], the deficiency being the result of a point mutation within the coding region of the gene encoding the cytokine [176]. That the deficiency of CSF-1 was indeed responsible for the observed osteopetrosis was shown when injections of CSF-1 into *op/op* animals reversed the osteopetrotic phenotype [174,177]. Supporting a local effect of CSF-1 during bone development and osteoclast recruitment were the findings that osteoblasts, both *in vivo* and *in vitro*, produced the cytokine [178,179]. Furthermore, in bone, transcripts encoding c-fms can be detected by *in situ* hybridization only in osteoclasts and their precursors [180,181].

Previously, CSF-1 had been shown to be synthesized as either a rapidly secreted molecule or as a membrane-bound molecule expressed on the cell surface and released slowly through proteolytic cleavage (for a review, see [182]). The major portion of the secreted molecule is post-translationally modified by the attachment of a glycosaminoglycan side chain that may serve as an anchor for integration into the extracellular matrix [183,184]. Cells of osteoblastic lineage synthesize both the secreted and the membrane-bound molecules [179,185], suggesting that the cytokine may act either chemotactically by attracting osteoclast precursors or via cell-cell contact to induce final differentiation, support survival, and modulate the activity of osteoclasts.

Although it has been shown unequivocally that CSF-1 is synthesized by osteoblastic cells in close local and temporal relationship with bone resorption, little is known about the regulation of its expression and the respective contributions of the different molecular forms to the formation of osteoclasts. In murine osteoblastic MC3T3-E1 cells, CSF-1 expression is constitutive and can be further increased by PTH, IL-1, TNF-α, and dexamethasone [181,186–188]. *In vivo*, however, the expression of the cytokine is tightly regulated. Thus, it is dramatically induced during pregnancy by 17β-estradiol and progesterone in the uterus [189]. In bone, expression of the cytokine is affected by estrogen deficiency in stromal cells [190–192], and by this mechanism may contribute to the onset of postmenopausal osteoporosis. Even less clear than the *in vivo* regulation of CSF-1 are the biological functions of its different molecular forms. It has previously been hypothesized that the membrane-bound form is required for full biological activity [193]. Only recently, however, has evidence been obtained that the expression of this form is specifically modulated in osteoblasts and that it is highly efficient in promoting the formation of osteoclasts [194].

While CSF-1 acts on a common precursor of macrophages and osteoclasts, a second factor synthesized by osteoblasts and essential for osteoclast formation has been found to act at a later stage of osteoclast differentiation. Osteoclast differentiation factor (ODF/RANKL/TRANCE)[1] is a member of the membrane-bound tumor necrosis factor ligand family [195,196]. The recombinant soluble form of ODF, together with CSF-1, supports the formation of osteoclasts *in vitro* demonstrating that the two factors are sufficient to induce the respective developmental programs in hematopoietic precursor cells [197]. The action of ODF on osteoclast formation is counteracted by another osteoblastic product, known as osteoprotegerin (OPG) [198] or osteoclast inhibitory factor (OCIF) [199]. OPG is a member of the tumor necrosis factor receptor superfamily, is synthesized by cells of osteoblastic origin, and is expressed as a soluble protein. It binds to ODF and, in this manner, blocks its osteoclastogenic effect [199]. Knockout mice carrying a null mutation in the OPG gene are characterized by severe trabecular and cortical bone porosity and a high incidence of fractures, demonstrating the critical role of OPG in the regulation of postnatal bone mass [200]. In consequence of the important role of OPG/ODF in the regulation of the formation of osteoclasts by cells of osteoblast lineage, several studies have been performed on the regulation of the expression of these genes. OPG mRNA is upregulated in response to TNF-α and TNF-β, indicating that the factor may help counteract the bone-destroying activity of these cytokines [201]. Other data, however, indicate that the ratio of ODF:OPG is increased by osteoclastogenic hormones and cytokines such as $1,25(OH)_2D_3$, PTH, and IL-11 [202]. Thus, OPG may be synthesized by osteoblastic cells to counteract the stimulatory effects of these hormones and cytokines on bone resorption, and, by this mechanism, the extent of loss of bone mass may be reduced.

Taking all these results together, it would appear that CSF-1 may be a factor required for the formation of osteoclasts and that it constitutes a critical part of the microenvironment of hematopoietic precursor cells, while the ODF/OPG pair provides local control for the fine tuning of the resorptive response.

To elucidate further the interactions between osteoblasts and osteoclasts, and, in particular, the coupling of bone formation and bone resorption, a mouse model that can be depleted of differentiated, active osteoblasts has been established [203]. When osteoblasts are destroyed in these animals at the age of 6 weeks, the rate of bone resorption is not affected, suggesting that this process does not require the presence of mature osteoblasts. Although great care has to be taken in the interpretation of the model [204], it shows that by combining physiology, morphology, and molecular biology, many exciting discoveries may be made, leading step by step to a better understanding of the mechanisms that allow bone to work in the magnificent way it does.

[1] Abbreviations: ODF, osteoclast differentiation factor; TRANCE, tumor necrosis factor-related activation-induced cytokine; RANKL, receptor activator of c-jun N-terminal kinase and NF-κB ligand

Bone Resorption

R. Felix

Bone, like other tissues, is continuously turned over by cellular processes. The osteoblast forms the bone matrix, and the osteoclast, a multinucleated cell, resorbs it. Thus, bone mass depends on the balance between these two processes. In childhood, bone formation is more rapid than resorption, and bone mass increases; between the ages of 20 and 40 years, the two processes are equal and it remains constant; in later years, resorption is more rapid than formation, and bone mass decreases. The rate of bone resorption is determined by both the number of osteoclasts and their activity. Osteoclast numbers are consequent upon their rates of formation and disappearance, the latter occurring through apoptosis. Whereas osteoclasts have a single function, namely to resorb bone, osteoblasts have two functions, not only forming bone matrix, but also regulating its resorption. This regulatory function is performed by stimulating osteoclasts in response to hormonal and cytokine signals. Further, osteoblasts support the development of osteoclasts through the synthesis of matrix proteins and growth factors. Osteocytes, also, probably have an influence on osteoclasts, but no information about this is available as yet.

The aim of this section of the present chapter part is to provide an overview of present knowledge concerning the development of osteoclasts and their function as bone matrix resorbing cells, but it does not cover every detail. Nor is there any information relating to the signal transduction involved in the generation and activation of osteoclasts. Additional information may be obtained from reviews elsewhere [205–208].

THE DEVELOPMENT OF OSTEOCLASTS

The Hemopoietic Origin of the Osteoclast

Thirty years ago, it was thought that osteoblasts and osteoclasts originated from the same precursor cell. In elegant work, several groups have shown this to be a misconception, and demonstrated that the hemopoietic stem cell is the precursor of the osteoclast.

In a parabiotic procedure, two rats, in one of which all hematopoietic tissue and local precursors had been destroyed by irradiation, were joined via a common circulation [209]. This demonstrated that the cells that fused to form multinucleated osteoclasts in a healing fracture in the irradiated rat must have been derived from the nonirradiated rat through the circulation. Another useful model for studying the origin of osteoclasts is osteopetrosis, an inherited disorder characterized by bone sclerosis resulting from deficient osteoclastic bone resorption. In this model, both the parabiotic union of osteopetrotic mice with normal littermates and the transplantation of hemopoietic stem cell suspensions from normal to affected siblings have been shown to cure some forms of the disorder [210,211]. These experiments indicated that precursors of osteoclasts are of hemopoietic origin and present in the circulation.

In a third approach, quail-chick chimera were investigated [212,213]. The embryonic long bones of one species were grafted onto the chorioallantoic membrane of the other, and it was possible to exploit the known morphological difference between chick and quail cell nuclei to determine the origin of different types of cells present in the developing bone. It was shown that osteoclasts formed through the fusion of host mononuclear cells derived from the vasculature of the chorioallantoic membrane, whereas the origin of osteoblasts was the local donor graft.

More recently, the hemopoietic origin of osteoclasts was also demonstrated in co-culture systems *in vitro*, in which hemopoietic precursor cells were grown either with fetal bone rudiments devoid of osteoclast progenitor cells, or with stromal cells [214,215]. Through these experiments, it also became evident that the stromal/osteoblastic cells play an important role in supporting the generation of osteoclasts. Scheven succeeded in generating osteoclasts in culture from hemopoietic stem cell populations [216]. Recently, macrophage cell lines that can be induced to form osteoclast-like cells [217,218] and immortalized mouse osteoclastogenic cell lines [219,220] have been generated.

The Pathway of Osteoclast Differentiation

Osteoclast markers are important for the identification of osteoclast-like cells grown in culture and osteoclast precursors observed *in vivo* or *in vitro*. Such markers have been identified when hemopoietic precursor cells develop into osteoclast-like cells in co-cultures of bone marrow cells and osteoblasts in the presence of 1,25-$(OH)_2D_3$ [221]. Precursor cells at the state before fusion (prefusion osteoclasts) have been isolated [222,223]. Postmitotic osteoclast precursor cells are positive for nonspecific esterase (NSE) and antigens Mac-1 and Mac-2, but negative for the macrophage-specific antigen F4/80. Mac-1 and Mac-2 are rat monoclonal antibodies against mouse macrophage-associated antigens. Mac-1 recognizes C3bl. C3bl is expressed preferentially on monocyte-macrophages, granulocytes, blood monocytes, and neutral killer cells, but not thymocytes. Mac-2 reacts with

Abbreviations: BMP: bone morphogenetic protein; CSF-1: colony-stimulating factor-1; GM-CSF: granulocyte-macrophage colony-stimulating factor; IGF-1: insulin-like growth factor-1; IL-1: interleukin-1; LIF: leukemia inhibitory factor; NFκB: nuclear factor κB (a transcription factor); *oc*: osteosclerotic; ODF: osteoclast-differentiation factor (also named: OPGL: OPG ligand; RANKL: RANK ligand; TRANCE: TNF-related activation-induced cytokine); *op*: osteopetrotic; OPG: osteoprotegerin (also named: OICF: osteoclast inhibitory factor); OSM: oncostatin M; PGE_2: prostaglandin E_2; PTH: parathyroid hormone; PTHRP: PTH-related protein; RANK: receptor activator NFκB; TNF-α: tumor necrosis factor-α; TRAP: tartrate-resistant acid phosphatase.

a 32-kDa glycoprotein that is expressed in a subpopulation of mouse macrophages including peritoneal macrophages provoked by thioglycolate. Neither resident macrophages, splenic macrophages, granulocytes, thymocytes, nor most marrow cells express this antigen. The rat monoclonal antibody to F4/80 recognizes a 160-kDa glycoprotein, which is specifically expressed in mouse macrophages. During further differentiation to committed osteoclast precursors, these cells become TRAP-positive and express receptors for calcitonin. An almost simultaneous expression of TRAP and calcitonin receptors has also been observed in differentiating osteoclasts in cultured embryonic murine metatarsals [224]. During subsequent differentiation to mononuclear preosteoclasts, NSE and the Mac-1 antigen disappear, while antigen Mac-2 remains. The expression of Mac-2 antigen as a marker in preosteoclasts has also been verified *in vivo* in *op/op* mice (osteopetrotic mice deficient in CSF-1) injected with CSF-1 [225]. Mature multinucleated osteoclasts express carbonic anhydrase II, c-Src, the vacuolar proton ($V\text{-}H^+$) ATPase, the 92-kDa type IV collagenase (matrix metalloproteinase 9), the osteoclast-specific cysteine proteinase cathepsin K, integrin $\alpha_v\beta_3$, and a high number of receptors for calcitonin [223,226]. $1,25\text{-}(OH)_2D_3$ has been shown to affect positively the expression of carbonic anhydrase II and integrin $\alpha_v\beta_3$ [227-229]. These marker proteins are characteristic for osteoclasts, but are produced also by other cell types, such as macrophages. Markers highly specific to osteoclasts are the binding of calcitonin and the production of cAMP on stimulation with calcitonin. Osteoclasts are also the only cells with the capability to resorb mineralized matrix (when cultured on mineralized matrix, they excavate lacunae). Mononuclear osteoclasts which have not yet fused to multinucleated cells also resorb bone [230].

The Function of Stromal and Osteoblastic Cells in Osteoclast Generation

When Suda and colleagues succeeded in growing osteoclast-like cells expressing osteoclastic markers in bone marrow cell culture in the presence of $1,25\text{-}(OH)_2D_3$, they observed that these cells appeared to be in contact with cells positive for alkaline phosphatase [231]. This suggested that the alkaline phosphatase positive cells may support the generation of osteoclasts. Indeed, it was demonstrated that it was necessary to co-culture marrow-derived stromal cells with hemopoietic precursor cells from spleen to support the development of osteoclasts [215], and that cell-cell contact between the two cell types was required [232,233]. Several stromal and osteoblastic cell lines supporting the formation of osteoclast-like cells in co-culture have now been characterized: MC3T3-G2/PA6 [215], KS-4 [234], ts8 [235], TM8 [236], MR1.8 [223], BMS2 [237], and MS1 [238]. If such cell lines are not available, osteoblastic cells isolated from murine calvaria can be used for co-culture with hemopoietic cells [239]. The co-culture system allows the production of a high number of osteoclast-like cells that can be used for biochemical investigations [240,241].

It has proved possible to grow osteoclasts from human bone marrow cells aspirated from healthy volunteers [242]. In an initial phase of culture, the bone marrow cells are grown on plastic. This is then followed by a second phase in which the cells are plated on bone slices where they resorb bone and form pits (lacunae). In contrast to murine marrow cultures, CSF-1 has to be added during the first phase to support the generation of osteoclasts. However, just as with the murine culture, high concentrations of CSF-1 suppress the formation of osteoclasts [243]. Human osteoclasts have also been generated by culturing precursor cells obtained from peripheral blood samples in both the presence and the absence of murine stromal cells [244–246]. The mesenchymal cells support the generation of osteoclasts by producing matrix proteins (see also "Bone Formation") and growth factors.

The Role of Matrix Proteins in Osteoclast Generation

Matrix proteins such as osteopontin, vitronectin, and collagen bind to their corresponding receptors on the cell surface, the integrins. Osteoclasts express the vitronectin receptor $\alpha_v\beta_3$ [247], the collagen receptor $\alpha_2\beta_1$ [248] and the fibronectin receptor $\alpha_v\beta_1$ [249]. On binding of the respective ligand, the integrins transduce signals into the cell in the same way that hormone and growth factor receptors do, inducing proliferation and differentiation of the cell. That matrix proteins support the development of osteoclasts is demonstrated by the observation that, in fetal long bone rudiments, the tripeptide arginine-glycine-aspartic acid (RGD), which competes with matrix proteins for the vitronectin receptor, inhibits the fusion of mononuclear precursors to form multinucleated osteoclasts [250]. Osteocalcin, the most abundant noncollagenous protein of the bone matrix, has also been suggested to support the differentiation of osteoclasts [251]. However, recent data obtained from transgenic osteocalcin-deficient mice demonstrate that the absence of osteocalcin does not impair bone resorption. The extent of bone surface covered with osteoclasts and their numbers are even greater in the mutant mouse, and ovariectomy causes a rapid increase in bone resorption that ultimately leads to osteoporosis [147].

Factors Required for the Development of Osteoclasts

Colony-Stimulating Factor-1 and Osteoclast Differentiation Factor

The factors, CSF-1 and ODF, are essential for the generation of osteoclasts. Since they are synthesized by osteoblasts, their role in bone resorption is described in the previous section "Bone Formation." ODF, a new member of the TNF receptor-ligand family, has only recently been identified, and knowledge about this membrane-integrated protein is still accumulating. An important point to note here is that the hormones and cytokines that stimulate the formation of osteoclasts act indirectly through osteoblasts and induce the synthesis of ODF by them. The receptor for ODF, named RANK, is expressed on both osteoclast precursor cells and mature osteoclasts. Thus, ODF binds to RANK and induces differentiation of the precursor cells to mature osteoclasts (Fig. 19) [252,253]. Osteoprotegerin, a novel member of the TNF receptor superfamily, binds as a soluble decoy receptor to ODF and inhibits its action.

Osteopetrosis Indicates Essential Factors Required for the Generation of Osteoclasts

Mutations in genes required for the generation of osteoclasts result in low numbers of osteoclasts, or even in their absence. Consequently, bone resorption is impaired, leading to osteopetrosis. Various types of heritable defects in osteoclast generation are known in the mouse (Fig. 20). In some types, genes encoding transcription factors are mutated, in others genes encoding growth and differentiation factors.

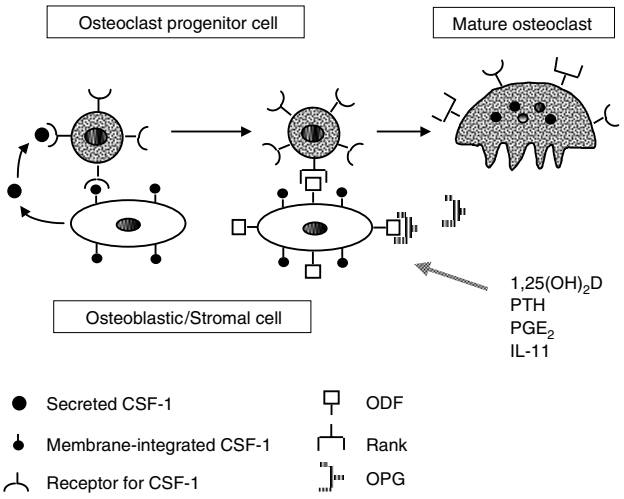

Figure 19. The roles of colony-stimulating factor-1 (CSF-1), osteoclast differentiation factor (ODF) and osteoprotegerin (OPG) in the generation of osteoclasts. CSF-1, either secreted or integrated within the membrane, and ODF, also integrated within the membrane, are the two proteins synthesized by osteoblastic/stromal cells. CSF-1 is essential for the generation of osteoclasts and ODF for their generation and activation. Secreted CSF-1 also binds partially to the extracellular matrix (not shown). Whether CSF-1 and ODF are expressed by a single osteoblastic/stromal cell or by different cells is not known. CSF-1 is constitutively synthesized, and its production is probably not much influenced by hormones and cytokines stimulating bone resorption (an exception is estrogen deficiency). In contrast, the expression of ODF seems to be strongly regulated by these hormones and cytokines. The receptors for CSF-1 and ODF are expressed on osteoclasts and their precursor cells. OPG binds ODF and prevents binding to RANK. This results in inhibition of osteoclast formation and activation. OPG is a critical regulator of postnatal bone mass. Other names for ODF are RANKL (RANK ligand), OPGL (osteoprotegerin ligand), and TRANCE (TNF-related activation-induced cytokine). Abbreviations: 1,25-dihydroxycholecalciferol ($1,25(OH)_2D$); parathyroid hormone (PTH); prostaglandin E_2 (PGE_2); interleukin-11 (IL-11).

Regarding the very early steps in the ontogeny of the osteoclast, one might expect an absence of transcription factors governing myeloid differentiation to result in osteopetrosis. Indeed, transgenic mice lacking the myeloid and B lymphoid transcription factor PU.1 (also called Spi-1 or Sfpi-1) fail to generate macrophages and osteoclasts, consequently developing sclerotic bone disease [254].

Further along the pathway of differentiation, the *op* mouse presents a defect in the osteoblastic/stromal cells, specifically, a point mutation in the coding region of the CSF-1 gene that leads to a lack of biologically active CSF-1 [176]. Injection of recombinant CSF-1 reverses the osteopetrotic phenotype, demonstrating the essential function of this cytokine in the generation of osteoclasts [174,177].

Resident macrophages and osteoclasts, both of which require CSF-1 for their early development, derive from the same precursor cell. The question arises as to which factor determines the branching of the pathway toward either macrophage or osteoclast, and the osteopetrotic *c-fos* knockout mouse provides the answer. This mouse generates macrophages but no osteoclasts [255]. This indicates that *c-fos* expressed in the hemopoietic lineage is involved in the commitment to differentiation of postmitotic osteoclast precursors towards osteoclasts.

Another type of osteopetrotic mouse unable to generate osteoclasts is the transgenic mouse lacking the transcription factors $NF\kappa B_1$ and $NF\kappa B_2$ [256,257]. The defect appears to be located within cells of the hemopoietic lineage. These mice fail to generate osteoclasts and B cells, and the function of macrophages is impaired. The role of NFκB in the formation of osteoclasts can be explained by the finding that these transcription factors are activated by ODF, resulting finally in the differentiation of osteoclasts [196]. ODF binds to its receptor RANK (receptor activator of NFκB), which is expressed by osteoclast precursor cells and induces the activation of NFκB [258,259]. NFκB is also involved in cell survival. Inhibitors of NF-κB induce apoptosis of rabbit mature osteoclasts [260]. Both ODF and IL-1 promote the survival of murine osteoclast-like cells [261,262]. Thus, the NFκB transcription factors, activated by ODF or IL-1, are probably required for the differentiation of osteoclast precursor cells and for the survival of osteoclasts. Consequently, their absence leads to osteopetrosis.

In the naturally occurring osteopetrotic microphthalmia (*mi*) mouse, the number of osteoclasts is normal, but they do not form ruffled borders. The *mi* locus encodes a member of the basic-helix-loop-helix-leucine zipper (bHLH-Zip) protein family of transcription factors [263]. Investigating osteoclast formation in cultured metatarsals/metacarpals, and in cell cultures prepared from neonatal calvaria from these mice,

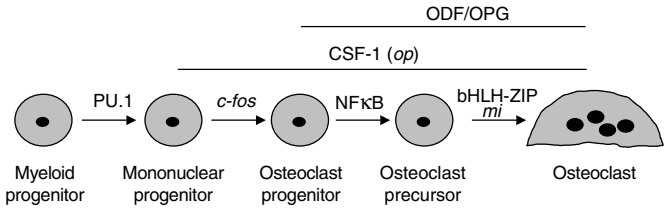

Figure 20. Osteopetrosis indicates essential factors required for the generation of osteoclasts. The expression of various genes is required for the generation of osteoclasts and, consequently, mutations of these genes result in osteopetrosis. Investigations of these inheritable defects helped to elucidate the pathway of osteoclast differentiation. PU.1 (also called Spi-1 or Sfpi-1) is a transcription factor required for the early differentiation of the myeloid precursor to the mononuclear progenitor cell. The transcription factors c-Fos, NFκB, and bHLH-Zip (basic helix-helix-loop-leucine zipper) act later in the differentiation pathway. CSF-1 (colony-stimulating factor-1) is a growth factor required for the generation of osteoclasts. An inactivating mutation in the gene encoding CSF-1 results in an osteopetrotic phenotype, as is the case in the osteopetrotic mouse strain *op*. The other factor required for the differentiation of osteoclasts is ODF (osteoclast differentiation factor). Consequently, transgenic mice deficient in ODF are osteopetrotic. Furthermore, transgenic mice overexpressing OPG (osteoprotegerin), which binds ODF and inhibits its action, are osteopetrotic.

indicated a defect in the fusion of osteoclast precursor cells [264,265].

The function of OPG as an inhibitor of bone resorption has been demonstrated in mice that overexpress OPG in the liver [198]. The high amount of OPG released into the circulation binds ODF on the surface of osteoblasts and inhibits the interaction of this ligand with its receptor, RANK, on osteoclasts and their precursor cells, resulting in impaired formation and activation of osteoclasts, and subsequent osteopetrosis.

Instead of overexpressing OPG, the ODF gene has been disrupted in mice. This results also in osteopetrosis [252].

Nonessential Factors Supporting the Formation of Osteoclasts

To generate osteoclast-like cells in bone marrow culture or in co-cultures of hemopoietic cells and osteoblastic/stromal cells, one of the following hormones has to be added: 1,25-$(OH)_2D_3$, PTH, PTHRP, or PGE_2 [231,266,267]. The action of these hormones is mediated by osteoblasts/stromal cells, since they, and not osteoclastic precursor cells, express receptors for these hormones [268,269].

PTH and PTHRP induce osteoblasts to synthesize cytokines that stimulate the generation of osteoclasts. They stimulate IL-6 production by osteogenic cells *in vitro* [270] and the expression of IL-6 and LIF *in vivo* [271]. PTH has also been shown to stimulate the expression of CSF-1 in a human osteosarcoma cell line [194]. For the formation of human osteoclasts, prostaglandin synthesis seems to be essential as indomethacin, an inhibitor of cyclooxygenase, blocks this process [272].

1,25-$(OH)_2D_3$ may also have several direct effects on osteoclast precursor cells. As already noted, it induces the expression of carbonic anhydrase in myeloid cells and of $\alpha_v\beta_3$ integrins in avian osteoclast precursors. Furthermore, it has been shown to induce fusion in macrophages [273].

PGE_2 may also act directly on osteoclast precursor cells. It antagonizes the inhibitory effect of both IL-4 and interferon γ (IFN-γ) on the osteoclastic cell-forming potential of bone marrow macrophages [274]. This may be related to the positive effect PGE_2 has on the generation of osteoclasts in co-cultures of bone marrow cells and osteoblasts. Addition of 10^{-6} M PGE_2 in combination with 1,25$(OH)_2D_3$ completely blocks the formation of any macrophages [240] (and personal observation).

Interleukin-1 and Tumor Necrosis Factor-α

TNF-α has been shown to support osteoclast formation in cultured long bone rudiments [275]. IL-1 stimulates the formation of osteoclast-like cells by a mechanism involving PGE_2 production both in mouse bone marrow cell culture and in co-cultures of osteoblasts and spleen cells since indomethacin completely blocks the effect [276]. In human cell culture, IL-1 and TNF-α also enhance osteoclast formation and bone resorption by increasing the synthesis of PGE_2 [272].

Cytokines Acting Through gp130

IL-6 is one of the cytokines that acts through the signal-transducing protein, gp130. It exerts its activity via a cell surface receptor that consists of two components, an α subunit, gp80, a ligand-binding protein (IL-6 receptor, IL-6R), and a non-ligand-binding β subunit, gp130, that is shared by other cytokines such as OSM, LIF, and IL-11 (Fig. 21) [277]. Besides the IL-6 binding α subunit associated with the membrane, a recombinant human and mouse soluble IL-6 receptor (α subunit) lacking transmembrane and cytoplasmic domains is also able to mediate the IL-6 signal through gp130 [278]. Native soluble IL-6 receptors have been detected in urine and serum from healthy subjects [279,280]. Their levels are increased in patients with multiple myeloma [281].

IL-6 stimulates the formation of osteoclasts. Thus, in cultured embryonic metatarsals/metacarpals, IL-6 has been shown to increase bone resorption by stimulating the generation of osteoclasts [270]. When IL-6 was added to co-cultures of mouse osteoblasts and bone marrow cells, it had no effect on the formation of osteoclasts in this culture system. However, simultaneous treatment with IL-6 and the soluble IL-6 receptor strikingly induced osteoclastogenesis [282], indicating that IL-6 supports the generation of osteoclasts, provided the soluble receptor is

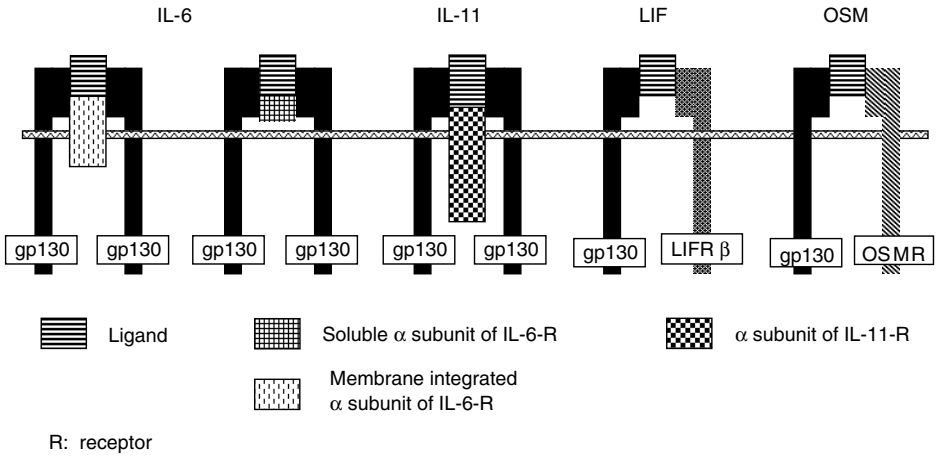

Figure 21. The receptors for interleukin-6 (IL-6), interleukin-11 (IL-11), leukemia inhibitory factor (LIF), and oncostatin M (OSM). Members of the subfamily of cytokines that include IL-6, IL-11, LIF, OSM, and ciliary neurotrophic factor (not shown) induce the assembly of related multicomponent receptors. The α subunit of the IL-6 receptor appears also in a soluble form that can bind IL-6 and assemble with, and activate, gp130. The β subunits, but not the α subunits, transduce the signal into the cell.

available. Related cytokines such as IL-11, OSM, and LIF, which also exert their function through gp130, also induced the formation of osteoclast-like cells.

The experiments above did not show whether IL-6 exerted its effect directly by binding to osteoclast precursors or indirectly by binding to osteoblasts. However, dexamethasone was subsequently found to increase the expression of IL-6 receptor mRNA in osteoblasts and, in parallel, their sensitivity toward IL-6 [283]. Finally, in co-cultures of osteoblasts and spleen cells derived from transgenic mice constitutively expressing the human membrane-anchored IL-6 receptor and from normal mice, osteoblasts were demonstrated to be the target cells for IL-6 action. Thus, IL-6 stimulated the formation of osteoclasts if the osteoblasts were taken from the transgenic mice and cultured with normal spleen cells. In contrast, it had no effect if the spleen cells were from the transgenic mice and the osteoblasts were normal [283]. These results indicate that the ability of IL-6 to induce osteoclast formation is indirect and depends on signal transduction mediated by the IL-6 receptor expressed on osteoblastic cells but not on osteoclast progenitors. The preceding data also suggest that the action of IL-6 is dependent on the concentration of the soluble IL-6 receptor and that this concentration may be affected by drugs and in certain diseases.

Whereas IL-6, as described below, may participate in the stimulation of the osteoclastogenesis that occurs after estrogen loss (see below) and in other diseases (e.g., rheumatoid arthritis, myeloma), IL-11 seems to regulate osteoclast formation under normal physiological conditions. IL-1, TNF-α, PGE_2, PTH, and 1,25-$(OH)_2D_3$ all induce the production of IL-11 in osteoblasts [284]. In accordance with these results, an anti-IL-11 neutralizing antibody was found to suppress osteoclast development in bone marrow induced by 1,25-$(OH)_2D_3$, PTH, IL-1, and TNF-α [285]. The effects of IL-11 on osteoclast development were also blocked by indomethacin, an inhibitor of prostaglandin synthesis, but did not depend on the estrogen status of the bone marrow donors.

Three different signal transduction pathways are activated by the various nonessential factors that support the generation of osteoclasts [206]. One mechanism, for PTH, IL-1, and PGE_2, is mediated by signaling involving cAMP. Whereas PTH and PGE_2 stimulate the production of cAMP directly, IL-1 acts through the induction of prostaglandin synthesis. The second mechanism, for 1,25-$(OH)_2D_3$–induced osteoclast formation, is mediated by the nuclear vitamin D receptor independently of cAMP. It is not known whether the recently described membrane-integrated vitamin D–binding receptor also plays a role in the generation of osteoclasts [286]. The third mechanism, for gp130 signaling, activated by cytokines such as IL-11, IL-6/sIL6 receptor, LIF, and OSM, is an additional important pathway for the formation of osteoclasts. However, as described above, some hormones and cytokines act through more than one signal pathway. Thus, 1,25-$(OH)_2D_3$, PTH, IL-1, and TNF-α partially stimulate the generation of osteoclasts via the activation of IL-11 production [285]. In contrast to IL-11, IL-6 is not involved in osteoclast formation induced by IL-1 and 1,25-$(OH)_2D_3$ [282], but it does partially mediate the effect of PTH.

The unifying mechanism by which the above-mentioned hormones and cytokines modify osteoblasts to support the generation of osteoclasts can now be explained [253]. As described above, most of them, if not all, induce the osteoblastic/stromal cells to synthesize ODF which is, in addition to CSF-1, essential for the formation of osteoclasts. Additionally, some of them also decrease the synthesis of the inhibitor of osteoclast formation, OPG. Extensive studies into ODF, RANK, and OPG are in progress, which will demonstrate the exact function and regulation of these three proteins.

Cytokines Inhibiting the Generation of Osteoclasts

The cytokines IL-4, IL-10, and GM-CSF suppress the generation of osteoclasts [274,287]. They act directly on the osteoclast precursor cells. The production of GM-CSF has been demonstrated in mouse bone marrow culture, and its inhibition by dexamethasone increases the generation of osteoclasts [288]. IL-10 increases the expression of CSF-1 mRNA. Since an excess of CSF-1 has been shown to inhibit osteoclast generation in favor of macrophage formation [289], it may be, at least in part, that an increased CSF-1 production is the mechanism by which IL-10 suppresses the formation of osteoclasts. IL-18 inhibits osteoclast formation by stimulating T cells to release GM-CSF, suggesting one possible means by which T cells may modulate bone resorption [290].

APOPTOSIS OF OSTEOCLASTS

The number of osteoclasts depends on the rates of both formation and apoptosis. Glucocorticoids and estrogen are known to induce apoptosis in mature osteoclasts. Glucocorticoids cause a dose-dependent decrease in bone resorption by rat osteoclasts incubated on bone slices [291]. This decrease is accompanied by a parallel loss in the number of osteoclasts due to apoptosis. Glucocorticoids seem to act directly on osteoclasts, since immunocytochemistry reveals the presence of glucocorticoid receptors in osteoclasts, and glucocorticoid-induced apoptosis can be prevented by a glucocorticoid antagonist [291]. Estrogen has also been shown to inhibit bone resorption by isolated rabbit osteoclasts by apoptosis. Its effect has been blocked completely by a pure antagonist and partially by a partial one, which suggests that the effect is mediated by the estrogen receptor [292]. The presence of mRNA for this receptor was also demonstrated in the rabbit osteoclasts. The results suggest a direct action of estrogen on osteoclasts, inducing apoptosis and consequently a decrease in bone resorption. The induction of apoptosis by estrogen has also been observed in murine osteoclasts, both *in vitro* and *in vivo* [293]. In contrast to the results obtained with rabbit osteoclasts, the effect of estrogen is mediated by transforming growth factor-β (TGF-β) in these cells. Estrogen induces the production of TGF-β by osteoblasts, and a neutralizing antibody against TGF-β abolishes apoptosis, demonstrating the role of TGF-β as a mediator in this process [293].

Apoptosis can also be induced by cytokines that stimulate the production of nitric oxide. Nitric oxide inhibits bone resorption by inducing apoptosis of osteoclast progenitors and suppressing osteoclast activity [294]. This may be a mechanism for protecting bone against aggressive bone resorption in inflammation induced by IL-1 and TNF-α. IFN-γ, synthesized by inflammatory cells, stimulates the formation of nitric oxide and inhibits bone resorption, at least partially [295].

Cytokines that prevent apoptosis are CSF-1, IL-1, and IGF-1 [296–299]. Furthermore, the recently described

ODF also supports the survival of osteoclasts [300]. One group also reported calcitonin to be a survival factor for osteoclasts [301], but this finding has not been confirmed by others, who have observed it to have only a minor effect on the survival of osteoclasts [208].

RESORPTION OF THE BONE MATRIX BY OSTEOCLASTS

The Role of the Osteoblast in Bone Resorption

Osteoblasts are the cells that regulate the bone-resorbing activity of osteoclasts. It is not osteoclasts, but osteoblasts that possess receptors for the hormones and cytokines that stimulate bone resorption, and these do so by inducing the expression of ODF by osteoblasts. ODF is a type II transmembrane protein that belongs to the new class of members of the TNF receptor family. It not only has the capacity to support the differentiation of osteoclast precursor cells (see above), but is also the factor that mediates the signal for bone resorption from osteoblasts to mature osteoclasts. Osteoclasts express the receptor RANK, which binds ODF [252,253], (see Fig. 19 and also the section "Bone Formation").

In addition to expressing ODF, osteoblasts also support osteoclastic bone resorption by removing the organic material that covers the bone surface and prevents osteoclasts from attaching to the mineral. For this purpose, osteoblasts secrete metalloproteinases and plasminogen activator and digest proteins covering the mineral surface [302–305]. Subsequently, osteoclasts attach to the now exposed mineral surface and start resorbing the bone [306]. It has also been suggested that collagen fragments released during digestion by osteoblast interstitial collagenase activate osteoclasts to resorb bone [307]. In recent years, several metalloproteinases have also been identified in osteoclasts (see below). Therefore, it seems likely that the organic material covering the bone surface is not digested by osteoblasts alone, but may be degraded by metalloproteinases of both osteoblasts and osteoclasts acting in concert [305].

Interaction of Osteoclasts with Matrix Proteins

When osteoclasts attach to bone matrix, they do so through integrin adhesion receptors. Osteoclasts express on their surface the classical vitronectin receptor $\alpha_v\beta_3$ [136], the collagen receptor $\alpha_2\beta_1$ [248], and integrin $\alpha_v\beta_1$ [249]. When osteoclasts are exposed to matrix proteins, such as osteopontin [308], sialoprotein [309], vitronectin, fibronectin, and collagen type I, but not collagen type IV or laminin [310], they become stimulated to resorb bone. Accordingly, antibodies against vitronectin receptors inhibit bone resorption *in vitro* [311]. Similarly, RGD peptides, which compete with RGD-containing matrix proteins in binding to vitronectin receptors, diminish bone resorption both *in vitro* and *in vivo* [312–314]. However, the mechanism by which molecules interact with the vitronectin receptor to inhibit bone resorption is not clear. Echistatin, an RGD-containing peptide and a strong inhibitor of bone resorption, inhibits bone resorption in mice with secondary hyperparathyroidism but, surprisingly, no histologic evidence for inactivated osteoclasts has been observed [315]. Echistatin treatment increased the area of osteoclast-covered bone surface; the osteoclasts appeared normal and the proportion of cells containing a ruffled border and a sealing zone was similar to that in controls. The peptide was localized in the basolateral membrane and the intracellular vesicles of actively resorbing osteoclasts, but not in the sealing zone. The number of osteoclasts was increased in the treated mice, which may be explained by a reduced osteoclast efficiency leading to a feedback-driven osteoclast recruitment. Antibodies against $\alpha_v\beta_3$ or RGD-containing peptides cause osteoclast retraction and detachment *in vitro*. However, much higher concentrations of echistatin are required to block osteoclast attachment than to reduce osteoclast activity. This suggests that echistatin inhibits bone resorption by a mechanism other than osteoclast detachment [312].

Searching for a drug that binds to $\alpha_v\beta_3$ integrin-binding sites and inhibits bone resorption, a synthetic chemical peptide mimetic, β-[2-[[5-[(aminoiminomethyl)amino]-1-oxopentyl]amino]-1-oxoethyl]amino-3-pyridinepropanoic acid, bistrifluoroacetate (SC56631) was synthesized. When continuously infused intravenously to ovariectomized rats, it prevented the massive bone loss that normally occurs within 6 weeks after ovariectomy [316].

Integrins play an important role not only in adhesion of osteoclasts to the bone surface, but they are also necessary for the movement of these cells upon it [317]. Since these receptors are also present on the serosal side, solubilized fragments of matrix proteins can bind at this location and activate osteoclasts.

It was demonstrated some years ago that mammalian osteoclasts can resorb mineral containing little or no matrix protein, such as that in egg and oyster shell [306,318]. This shows that matrix proteins are not required for the activation of osteoclasts; however, attachment to a solid surface is an essential step in such activation. This was demonstrated when factors inducing osteoclast polarization were examined [319]. An indication for the polarization of an osteoclast attached to a solid phase is the formation of an actin ring, which consists of closely assembled filamentous actin dots. The actin ring is similar to the sealing zone, which is formed when an osteoclast attaches to the bone surface and resorbs the matrix (see below). When osteoclasts grown in co-cultures of mouse osteoblastic cells and bone marrow cells were placed on plastic, calcified dentin, or calcium phosphate in the presence of fetal calf serum, they formed actin rings. However, when they were placed on demineralized matrix or type I collagen gels, no actin rings were formed. These results indicate that the physical properties of bone, such as its rigidity and hardness, are required to induce the polarization of osteoclasts. The mineral components may also be important in maintaining the polarized state.

The physical properties of bone may also play an important role in the interaction of the sealing zone membrane with the mineralized bone surface. The sealing zone is an organelle-free area of the osteoclast, rich in actin filaments, where the plasma membrane is closely apposed to the bone surface (Fig. 5) [320]. It is essential for bone resorption; it surrounds the ruffled border that defines the area beneath the osteoclast to be resorbed (see below). The membrane proteins of the sealing zone that interact with the bone surface have not been identified to date. $\alpha_v\beta_3$ integrins seem not to be present in the sealing zone, and some other as yet uncharacterized proteins must mediate the interaction of actin with the extracellular portion of the sealing zone. The tight seal may be formed between the plasma membrane and bone mineral and/or mineral-associated proteins that may be liberated from bone during decalcification and, therefore, may not be identified easily [321].

The Process of Bone Resorption

During the process of bone resorption, the osteoclast adheres to the bone surface at the sealing (clear) zone, the special structure that seals off the area to be resorbed. There is a very tight interaction of the plasma membrane with the mineralized matrix, the gap being less than 10 nm. This distance is narrower than any other interaction between cells and matrix [321]. The sealing zone surrounds the so-called ruffled border, an area of intense membrane folding where the process of bone resorption occurs.

Bone is composed of organic and inorganic material. About 90% of the organic material is collagen type I, the remaining 10% being noncollagenous proteins, while the inorganic material is hydroxyapatite, a calcium phosphate salt. During the resorptive process, osteoclasts fulfill the unique task of dissolving the mineral phase of bone with acid. To accomplish this highly specialized role, osteoclasts express an array of enzymes and transport systems (Fig. 22). Carbonic anhydrase II (EC 4.2.1.1) catalyzes the formation of carbonic acid (H_2CO_3) from water and carbon dioxide, which is the end product of the oxidative metabolism that occurs at a high rate in osteoclasts. Carbonic acid spontaneously dissociates to bicarbonate ions (HCO_3^-) and protons (H^+). The protons are subsequently transported through the cell membrane to the bone surface by a proton pump, a vacuolar H^+-ATPase, located in the ruffled border [322–326]. The mineral is dissolved in the acidic environment thereby created in the lacuna beneath the osteoclast. While protons are actively transported through the membrane of the ruffled border, chloride ions are translocated through a Cl^- channel, resulting in the net transport of hydrochloric acid. The bicarbonate ion produced by the dissociation of carbonic acid is eliminated from the cytoplasm via a HCO_3^-/Cl^- exchanger, which replaces the Cl^- secreted into the resorption lacuna [327]. Each of these steps is essential to the overall bone resorption process. This has been demonstrated in experiments in which bone resorption is impaired if the enzyme reactions or transport systems are blocked [326,328].

After dissolution of the mineral, the organic material is exposed and can now be digested by lysosomal enzymes and metalloproteinases secreted by the osteoclasts. Cathepsins (see Chapter 7, this volume) have been demonstrated to be present in osteoclasts and to play an important role in digesting matrix proteins [329,330]. That most abundantly expressed is cathepsin K, originally called cathepsin O [331,332], which is essential for bone resorption to occur. This has been demonstrated in experiments in which cathepsin K antisense oligodeoxynucleotides were found to inhibit the bone resorbing activity of rabbit osteoclasts cultured on dentin slices [333]. In addition, several metalloproteinases have been shown to be present in osteoclasts [334–336]. While the acid pH in the lacuna below the resorbing osteoclast favors the activity of lysosomal enzymes, it has been more difficult to find an explanation as to how the neutral metalloproteinases can participate in the digestion process in the acid environment of the lacuna. Recent data suggest that after dissolution of the mineral, the lysosomal cysteine proteinases first digest part of the organic bone matrix and finally, when the pH has increased, metalloproteinases exert their activity [330].

After having formed a lacuna, the osteoclast probably stops resorbing and migrates to a new site where it starts the process again. Metalloproteinases integrated in the plasma membrane seem to be involved in these migratory and attachment activities [336]. In developing bone, TRAP-positive mononuclear cells migrate from the periosteum into the bone, fuse to form multinucleated osteoclasts, resorb the mineralized matrix, and form the bone marrow cavity. Inhibitors of metalloproteinases prevent the migration of osteoclast precursor cells from the periosteum to the developing marrow cavity of mouse fetal metatarsals in culture, indicating the important role of these enzymes in the migration of osteoclasts and their precursors [337].

Hydrolytic digestion seems not to be the sole mechanism for breaking down bone matrix. Superoxide and other

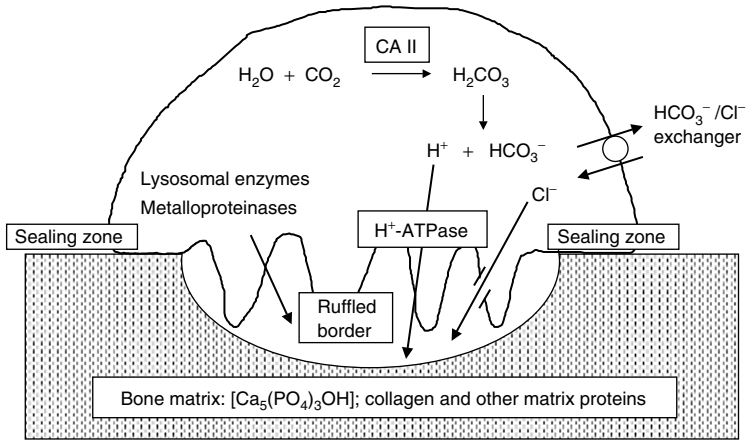

Figure 22. Enzyme reactions and transport systems involved in the bone resorption process. The area of bone below the ruffled border and surrounded by the sealing zone is resorbed and an excavation, a lacuna, is formed. Carbonic anhydrase II (CAII) catalyzes the formation of carbonic acid (H_2CO_3) from water and carbon dioxide, which dissociates into a proton (H^+) and a bicarbonate ion (HCO_3^-). The vacuolar H^+-ATPase transports the proton through the membrane of the ruffled border. Together with the proton, a Cl^--ion migrates through a chloride-channel into the lacuna, resulting in the net transport of hydrochloric acid. In the lacuna, the acid environment generated by the H^+-ATPase dissolves the mineral (hydroxyapatite: $Ca_5(PO_4)_3OH$). The bicarbonate ion produced by the dissociation of carbonic acid is eliminated from the cytoplasm via a HCO_3^-/Cl^-exchanger, replacing the chloride ion secreted into the lacuna.

reactive oxygen molecules produced by activated osteoclasts seem also to participate in the complex process of bone resorption [338]. The enzyme catalyzing the production of superoxide, NADPH-oxidase, has been demonstrated to be expressed in resorbing osteoclasts [339]. Reactive oxygen molecules and ions seem also to stimulate the generation of new osteoclasts [340]. The exact mechanism of these processes is, however, not known.

The lacuna and the ruffled border under the resorbing osteoclast are sealed off by the sealing zone. Consequently, a major question is how is the material released during resorption eliminated. It was earlier suggested that an osteoclast might move on when a certain amount of solubilized material had accumulated in the lacuna. Newer data, however, demonstrate that degraded proteins and inorganic matrix components are transcytosed in vesicles to the membrane of the osteoclast opposite the ruffled border [341,342]. This transcytosis may be more than just a disposal pathway. Bone formation stimulating factors, such as TGF-β, IGF, and BMP, which are incorporated in the matrix and released during resorption, could be translocated by this transport system to neighboring osteoblasts and, as coupling factors, induce bone formation. This may be part of the mechanism coupling bone resorption and bone formation.

Osteopetrosis Due to Mutations in Genes Required for Bone Resorption

Five types of osteopetrosis are known, three in humans and two in the mouse, in which a gene encoding a protein required for bone resorption is mutated, and results in osteopetrosis (Table 1).

The human gene for carbonic anhydrase II (EC 4.2.1.1) was identified as the site of the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification (MIM 259730) [343] (see also Chapter 19, this volume).

Nonsense, missense, and stop codon mutations in the gene encoding cathepsin K (EC 3.4.22.38) are the cause of pycnodysostosis (MIM 265800), a rare autosomal recessive osteochondrodysplasia characterized by osteosclerosis and short stature [344]. It seems that due to these mutations, cathepsin K is not synthesized as a stable protein, or that its activity is impaired.

Craniometaphyseal dysplasia is a rare craniotubular bone dysplasia transmitted in both autosomal dominant (MIM 123000) and autosomal recessive (MIM 218400) forms. It is characterized by hyperostosis of the cranial bones and deformities of the metaphyses of the long bones. Investigation of osteoclast-like cells cultured from the bone marrow of a 3-year-old patient suggested that osteoclast dysfunction due to impaired expression of the vacuolar proton pump leads to this disease [345].

A defect in the proton pump is also seen in the osteosclerotic (*oc/oc*) mouse. Osteoclasts from this mouse fail to form ruffled border and resorption pits (lacunae) on dentin [346]. Further studies have indicated that the vacuolar proton pump is present throughout the cytoplasm but not on the apical membranes as in phenotypically normal littermates [347].

Finally, there is the *src*-deficient mouse. Targeted disruption of the *c-src* proto-oncogene has been shown to induce an osteopetrotic disorder [348]. The gene product c-Src is a tyrosine kinase that, in addition to many other functions, participates in the reorganization of the cytoskeleton. It has been identified in the ruffled border of osteoclasts of normal mice [349,350]. Osteoclasts in the *src*-deficient mouse develop normally, but fail to form a ruffled border and do not resorb bone [351]. The c-Src protein seems to play a unique role in the formation of ruffled borders and the bone resorption process that cannot be replaced by other members of the Src-kinase family.

Hormones and Cytokines Which Stimulate Bone Resorption by Acting Directly on Osteoclasts

Interleukin-1, Interleukin-6, and Interleukin-11

Osteoclasts express receptors for IL-1, IL-6, and IL-11. This has been demonstrated by identifying their mRNAs, and for IL-6 also by immunostaining the receptor protein [284,352,353]. The direct effect of IL-11 on mature osteoclasts is not known. As mentioned earlier, IL-1 is a survival factor for osteoclasts. It can also directly activate the bone resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells [261]. IL-6, which is also produced by both osteoblasts and osteoclasts, has been shown to reverse the calcium-induced inhibition of the bone resorbing activity of osteoclasts and to attenuate their calcium sensing. Furthermore, high calcium concentrations enhance IL-6 and IL-6 receptor gene expression and IL-6 secretion. These results suggest that IL-6 production increases when osteoclasts become exposed to an inhibitory calcium level during resorption and that IL-6 sustains resorption, at least in part, by attenuating extracellular calcium sensing [353].

TABLE 1. Osteopetrosis Due to Mutations in Genes Involved in the Bone Resorption Process

Enzyme Reaction Affected[1]	Disease
$CO_2 + H_2O \xrightarrow[\text{anhydrase}]{\text{Carbonic}} H_2CO_3$	Human osteopetrosis with renal tubular acidosis and cerebral calcification
Hydrolysis of proteins by Cathepsin K	Pycnodysostosis: osteochondrodysplasia characterized by osteosclerosis and short stature
H^+-ATPase	Craniometaphyseal dysplasia in humans
H^+-ATPase not associated with apical membrane	Osteosclerotic mouse (*oc*)
No c-Src ⟶ no ruffled borders	Osteopetrotic *c-src* knockout mouse

[1]Mutations in genes encoding enzymes involved in the bone resorption process resulting in osteopetrosis

Insulin-like Growth Factor-1

The expression of IGF-1 receptors on osteoclasts has been demonstrated [299]. In contrast to insulin, IGF-1 stimulates the bone resorbing activity of mature osteoclasts. It also prevents apoptosis of these cells. The stimulatory effect of growth hormone seems to be partly mediated by IGF-1 [354].

Inhibition of Bone Resorption

When bone resorption is greater than bone formation, bone loss and finally bone fracture occurs. Thus, to protect bone against such damage, it seems necessary that inhibitors of bone resorption exist that counteract hormones and cytokines stimulating bone resorption. Native inhibitors (e.g., calcitonin, estrogen) are also applied as drugs in disorders with high bone resorption (e.g., postmenopausal osteoporosis). In addition to native inhibitors, there are synthetically produced drugs like the bisphosphonates, which are potent inhibitors of bone resorption.

Osteoprotegerin

OPG inhibits bone resorption by binding ODF and preventing its association with its receptor, RANK (see Fig. 19 and also previous section). Adolescent and adult mice deficient in OPG exhibit a decrease in total bone density characterized by severe trabecular and cortical bone porosity, marked thinning of the parietal bone of the skull, and a high incidence of fractures [200]. These findings demonstrate that OPG is a critical regulator of postnatal bone mass. Unexpectedly, OPG-deficient mice also exhibit medial calcification of the aorta and renal arteries, suggesting that regulation of OPG, its signaling pathway, or its ligand(s) may play a role in the long observed association between osteoporosis and vascular calcification.

Estrogen

The decrease in estrogen levels that occurs after the menopause causes bone loss through increased bone resorption and may finally result in osteoporosis. Postmenopausal osteoporosis and the effect of estrogens on bone is a major field of research [355] that will be touched on only briefly in this chapter. The main function of estrogens in bone is to regulate the number of osteoclasts and, to a lesser extent, their activity. They stimulate the cell death of osteoclasts as previously mentioned, but, probably more importantly, suppress their formation by inhibiting the synthesis of cytokines IL-1, TNF, and IL-6. The relative importance of these cytokines in mediating the effect of estrogen loss is still unclear, some proposing TNF and IL-6 as crucial factors [355,356], others, IL-1 and TNF [357,358].

Experiments with transgenic mice have demonstrated that IL-1 and TNF play an essential role in mediating the bone loss induced by estrogen deficiency. Transgenic mice unresponsive to IL-1 because of deficient expression of IL-1 type I receptor were shown to be protected against bone loss after ovariectomy (the type II receptor is a "decoy" receptor that has similar extracellular and transmembrane domains to IL-1 type I receptor but lacks critical intracellular elements that are necessary for postreceptor signaling) [359]. The results, demonstrating that IL-1 is an essential mediator of the effect of estrogen deficiency on bone, are particularly convincing because the mice displayed both normal bone mass and normal growth rates. By a similar approach, the essential role of TNF in bone loss due to estrogen deficiency was also demonstrated. Transgenic mice unresponsive to TNF due to soluble TNF-receptor overexpression, are also protected against bone loss induced by ovariectomy [360]. Although the finding that the functional blocking of either IL-1 or TNF is sufficient to prevent estrogen deficiency-induced bone loss may appear difficult to reconcile, it should be emphasized that in most biological systems IL-1 and TNF have synergistic effects.

As far as IL-6 is concerned in mediating the effect of estrogen deficiency on bone loss, it has been reported that transgenic mice deficient in IL-6 are protected from bone loss caused by estrogen depletion [361]. However, whereas these results support the essential role of IL-6, other data do not. Thus, in mice overexpressing IL-6, bone turnover was suppressed and bone resorption and the number of osteoclasts were reduced [362]. Furthermore, in contrast to the situation with TNF-binding protein, neutralizing antibody against IL-6 failed to prevent osteoclastogenesis, an increase in bone resorption, and bone loss induced by ovariectomy in mice [363].

Accordingly, IL-1 and TNF seem to be the important factors in estrogen deficiency. They stimulate the stromal cells in the bone marrow to synthesize CSF-1, IL-6, and IL-11, each of which supports the generation of osteoclasts [190]. IL-1 and TNF are thus regarded as "upstream" cytokines necessary to induce the secretion of "downstream" factors that stimulate hemopoietic osteoclast precursors.

Calcitonin

Osteoclasts express a high number of calcitonin receptors on their surface [226]. In contrast to the indirect effects of hormones that stimulate bone resorption, calcitonin binding to osteoclasts leads to a rapid cessation of osteoclast mobility and bone resorbing activity [364]. The growth of osteoclasts *in vitro* is also reduced by calcitonin given during the whole culture period [231]. When calcitonin is used therapeutically, resistance to the hormone develops rapidly. This phenomenon was originally noted in calcitonin-treated organ cultures and described as "escape" [365]. With the aim of explaining this phenomenon, T.J. Martin and colleagues in Australia have investigated extensively the regulation of osteoclast calcitonin receptor levels. Pretreatment of osteoclasts with calcitonin *in vitro* leads to down-regulation of the receptor and a reduced bone-resorbing capacity [366]. Importantly, treated osteoclasts are 100 times less sensitive to rechallenge with calcitonin. In contrast, dexamethasone increases the number of calcitonin receptors, and pretreatment with this drug lessens somewhat calcitonin-induced down-regulation of the calcitonin receptor [366]. Dexamethasone treatment results in a higher level of calcitonin receptor mRNA as a result of increased transcriptional activity [367]. Calcitonin seems to increase the rate of decay of calcitonin receptor mRNA. It was noted some years ago that calcitonin in combination with glucocorticoids protects against the development of calcitonin resistance in clinical therapy [368]. The above results found in *in vitro* studies seem to provide an explanation for this observation. Finally, when calcitonin is added to bone marrow cultures, even for short periods, developing osteoclast-like cells possess a normal capacity to resorb bone, but the number of calcitonin receptors is reduced and remains low [369,370]. The data suggest that the "escape" phenomenon may result from a prolonged calcitonin-induced loss of calcitonin responsiveness, at least in part due to reduced synthesis of calcitonin receptors and the development of osteoclasts deficient in calcitonin receptors.

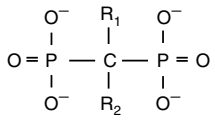

Figure 23. Structure of bisphosphonates: The structure "phosphonate–C–phosphonate" binds with high affinity to mineral. R_1 is generally H or OH and influences binding only slightly. R_2 together with the bisphosphonate structure determines the biological activity of a bisphosphonate, i.e., its potency to inhibit bone resorption.

Bisphosphonates

Bisphosphonates, synthetic compounds with a high affinity for mineral, are used in the management of patients with various disorders affecting the skeleton, including osteoporosis, metastatic bone disease, and Paget's disease of bone [371]. Their specific pharmacological properties include selective uptake at active bone sites, suppression of osteoclast-mediated bone resorption, and long-time skeletal retention.

The structure of a bisphosphonate molecule consists of a C-atom, to which two phosphonate groups and R_1 and R_2 groups are bound (Fig. 23). The structure "phosphonate-C-phosphonate" is the portion of the molecule that binds with high affinity to the mineral [372]. Because of this property, bisphosphonates accumulate in bone and mineralized cartilage, but not in other tissues. The nature of R_2, together with the bisphosphonate group, determines the potency with which bone resorption can be inhibited. When various bisphosphonates have been synthesized, R_1 has not been varied much. It is normally H or OH, and this variation does not much influence the inhibitory activity.

During bone resorption, osteoclasts take up bisphosphonates bound to the bone surface and become inactive [373]. Over the years, the mechanism of inhibition has been elucidated. It was found that bisphosphonates with R_2 containing an amino group inhibit steroid synthesis from mevalonate [374]. However, the significance of this observation was not realized until M.J. Rogers and R.G.G. Russell and their colleagues demonstrated that bisphosphonates containing nitrogen inhibit the mevalonate pathway and prevent post-transcriptional prenylation of GTP-binding proteins [375–377]. Various intracellular proteins involved in signal transduction require prenylation for attachment to the cell membrane and for activation. Inhibition of prenylation disturbs the intracellular regulation of cell function, which finally results in apoptosis [378]. Other bisphosphonates, not containing nitrogen in R_2, are incorporated into adenine nucleotides by aminoacyl-tRNA synthetase enzymes. These products cause both necrotic and apoptotic cell death [379].

Although bisphosphonates, when taken up, disturb the function of every cell, only osteoclasts are the targets of this drug. The reason for this is, as mentioned above, that bisphosphonates accumulate only in bone because of their high affinity for mineral. Any excess is rapidly secreted through the kidney. Furthermore, osteoclasts are the only cells that resorb bone and, therefore, take up bisphosphonates.

While in the mechanism described above, the osteoclast is the target of bisphosphonates, there have been *in vitro* experiments that suggest that bisphosphonates induce osteoblasts to release inhibitory activities that reduce the bone resorbing activity and the formation of osteoclasts [380–384]. The mechanism of this bisphosphonate action on osteoblasts is not known, and up to now there is no indication that bisphosphonate action is mediated *in vivo* by osteoblasts.

Extracellular Calcium

It has been suggested that an increase in local calcium concentrations in the resorption lacunae suppresses osteoclastic bone resorbing activity through an increase in intracellular calcium mediated through a specific receptor. A calcium-sensing receptor has been identified in mature osteoclasts and an increase in extracellular calcium reduces the bone resorbing activity of osteoclasts cultured on bone slices [385,386]. As mentioned above, some data also indicate that high calcium concentrations stimulate the secretion of IL-6, which reduces the calcium-induced inhibition of bone resorption by attenuation of osteoclast calcium-sensing [353]. Such an autocrine-paracrine loop may sustain osteoclastic activity in the face of an inhibitory calcium level generated during resorption.

Insulin

Insulin is known to stimulate bone formation through its effect on osteoblasts. Recently, it was demonstrated that both murine osteoclast-like cells generated *in vitro* and primary osteoclasts isolated from mouse and rat bone express the insulin receptor [387]. Insulin added to osteoclast-like cells cultured on dentin slices inhibited bone resorption. This is consistent with the well-known effect of insulin on bone formation, and together they result in net bone growth.

RECENT DEVELOPMENTS

Bone Formation

W. Hofstetter

Coupling of Bone Formation and Bone Resorption

The paradigm for the maintenance of bone mass states that bone formation and resorption are tightly coupled. In transgenic mouse strains which overexpress the Fos family members Fra-1 [392,393] and ΔFosB [394], respectively, however, an increase in bone formation occurs without a concomitant increase in bone resorption. In both cases, the mice are normal at birth, but over time an increase in bone

formation becomes evident throughout the skeleton and an osteosclerotic phenotype develops.

ΔFosB is a naturally occurring splice variant of FosB. In osteoblasts, ΔFosB is further truncated to the Δ2ΔFosB isoform, which seems to be responsible for the observed bone phenotype [394]. Thus, alternative splicing of *FosB* mRNA during the differentiation stage of osteoblasts may regulate the development and function of these cells. Proteins of the Fos family associate with members of the Jun family of proteins to form the heterodimeric transcription factor AP-1. Most interestingly, FosB and Fra-1 lack a C-terminal transactivation domain [395], suggesting that their transcriptional potential depends on their partners in the heterodimer or on transactivation factors.

In mice overexpressing ΔFosB, the osteosclerotic phenotype is accompanied by a decrease in adipogenesis [394]. Since osteoblasts and adipocytes both derive from the mesenchymal stem cell [396], it was suggested that ΔFosB supports osteogenesis at the expense of adipogenesis [394]. In *Fra1* transgenic mice, however, adipogenesis is not affected, indicating that in these animals the development of cells of the osteogenic lineage is independent of adipogenesis. This suggests a sequential action of ΔFosB and Fra-1 during osteoblast development, rather than an inverse relationship between the two cell lineages with a common bi- or multipotential precursor.

Since the phenotype in the development of adipocytes is seen only in the Δ*FosB*, but not the *Fra1* transgenic mice, a role for adipocyte-derived products in the regulation of bone mass in these animals can be excluded. Leptin is a polypeptide hormone secreted primarily by adipocytes and acts through specific receptors in the hypothalamus. Mice deficient in leptin or its receptor are obese and have a high bone mass phenotype, which is resolved by the infusion of leptin into normal or receptor-deficient mice, supporting the concept of a central regulation of bone mass through the activation of a hypothalamic relay [397].

Effect of Estrogen on Bone Cells

Estrogen deficiency exerts profound effects on the hematopoietic microenvironment, stimulating lymphocytes, amongst other cells, to express osteoclastogenic cytokines [398]. It has now been shown that osteoblastic cells require estrogen for survival, demonstrating that it also has a direct effect on bone formation [399]. This effect is not mediated at the genetic level, but involves the sequential activation of a signal transduction cascade involving Src/Shc/ERK [400,401]. It is noteworthy that estrogen as well as androgens exerts its effects through ERα and ERβ, as well as through the androgen receptor, demonstrating a striking promiscuity of the receptors in their choice of ligands [399].

Chondrocytes Regulate the Migration of Osteoclasts into Developing Bone Rudiments

Recent findings suggest that hypertrophic chondrocytes in developing long bones induce osteoclasts to invade mineralized cartilage by producing the chemotactically active vascular endothelial cell growth factor (VEGF) [402]. Blocking VEGF activity with chimeric anti-VEGF antibodies caused a suppression of blood vessel invasion, concomitant with impaired trabecular bone formation and an expansion of the zone of hypertrophic chondrocytes in experimental animals [403]. VEGF is liberated from cartilage matrix by the action of gelatinase B/matrix metalloproteinase-9 (MMP-9) [404]. Consequently, MMP-9 deficiency delays osteoclast invasion, possibly due to impaired function of VEGF [405].

The Role of Cbfa1 in the Development and Function of Skeletal Cells

Chung et al. have demonstrated that Ihh not only regulates chondrocyte development, but also defines the sites of initial bone formation. Using chimeric mice containing both wild type and $Ihh^{-/-}$ cells, induction of a bone collar was observed in the perichondrium of wild type prehypertrophic and hypertrophic chondrocytes, but not in the vicinity of $Ihh^{-/-}$ prehypertrophic and hypertrophic chondrocytes in developing long bones [406]. This data supports previous results suggesting that cartilage development and bone formation are coupled through the action of Ihh. Analysis of $Ihh^{-/-}$ mice demonstrated a failure in the development of osteoblasts in endochondral bones due to a deficiency in Cbfa1 induction in the perichondrium of bone rudiments [407]. The fact that vascular invasion is blocked in $Ihh^{-/-}$ mice provides additional evidence that this process is induced by cartilage cells producing specific growth factors. The aforementioned production of VEGF by hypertrophic chondrocytes is critical for triggering vascularization and invasion of osteoclasts into the mineralized cartilage rudiment [402]. In the absence of chondrocyte differentiation, the cells will not synthesize VEGF and, as a consequence, the mineralized cartilage will not be vascularized and resorbed.

Further study of *Cbfa1* knockout mice has revealed that not only is bone tissue not formed, but that the development of chondrocytes is impaired as well. Calcification of cartilage occurs in $Cbfa1^{-/-}$ mice in the distal limbs, where Ihh is expressed in the hypertrophic chondrocytes, but in femur and humerus, however, chondrocyte differentiation is blocked before the onset of hypertrophy and Ihh is not expressed [408]. In animal models in which Cbfa1 is constitutively overexpressed in cartilaginous tissues, accelerated endochondral calcification due to precocious chondrocyte maturation is observed [409,410]. In $Cbfa1^{-/-}$ mice, targeting Cbfa1 expression to chondrocytes restored hypertrophy and vascularization of cartilage rudiments. Although no osteoblasts develop in such animals, vessels and multinucleated, tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts are found within mineralized cartilage [409], further confirming the role of chondrocytes in regulating vascularization and invasion by osteoclasts. An additional piece of evidence for the role of Cbfa transcription factors in chondrocyte development has been provided by the demonstration that expression of the metalloproteinase MMP-13, which solubilizes unmineralized cartilage upon stimulation by MMP-9, is directly affected by a cooperative function of Cbfa/Runt proteins with the transcription factor complex AP-1 [411].

Bone Resorption

R. Felix

RANK, RANKL and OPG

A standard nomenclature for new tumor necrosis factor family members involved in the regulation of bone resorption has been recommended [412]. The following names were selected from the various acronyms: RANK for the receptor, making obsolete ODAR and TNFRSF-11; RANKL for the ligand, making obsolete ODF, OPGL, TRANCE, SOFA and TNFSF-11; and OPG for the decoy receptor, making obsolete OCIF, TR-1, FDCR-1 and TNFRSF-11B.

A number of reports have described the effects of hormones and cytokines on the mRNA and protein levels of RANKL and OPG *in vitro* [413, 414], and indicate that the number of osteoclasts is regulated by the ratio of RANKL to OPG. This has been confirmed *in vitro* by the correlation that exists between bone remodelling indices in normal human cancellous bone and this ratio, although this is not the case in osteoarthritis [415].

CSF-1 has been found to stimulate the expression of RANK in c-Fms$^+$ early-stage precursor cells; IL-3 and GM-CSF are less effective [421]. The timing of RANK expression and the subsequent binding of RANKL are critical for osteoclastogenesis. Fully differentiated macrophages have lost the potential to form osteoclasts. TNF, as well as RANKL, have been shown to upregulate RANK expression in precursor cells [417].

Using reverse transcriptase–polymerase chain reaction (RT-PCR), three RANKL isoforms have been identified [418], each of which when transfected into NIH 3T3 cells is translated. Bone marrow stromal cells and the osteoblastic cell line ST2 express the originally described membrane-bound RANKL (RANKL1) and the soluble RANKL3. This would suggest that cell-cell contact is not required for the stimulation of osteoclasts and their precursor cells by osteoblasts. Lymphocytes express all three isoforms, the pattern being dependent on the type of lymphocyte. Membrane-bound RANKL seems also to be released by proteolytic cleavage, and two shedding activities distinct from the TNFα convertase have been characterized [419].

TNFα Acts on Osteoclast Formation Similarly to RANKL

TNFα can replace RANKL as a factor necessary for the differentiation of osteoclasts [420, 421], supporting their formation in two ways: in combination with RANKL, it synergistically up-regulates RANK [417] thereby stimulating osteoclastogenesis at permissive levels of RANKL [422], and it also seems to induce differentiation to mature osteoclasts in the absence of RANKL. Since the signaling pathways of RANKL and TNFα overlap, it is not surprising that both factors support osteoclastogenesis [417, 423]. However, in contrast to RANKL [424], TNF-α does not directly activate the bone resorbing activity of mature osteoclasts [420, 421]. Although osteoclast precursor cells express TNF receptors 1 (p55r) and 2 (p75r), TNFα-induced osteoclastogenesis seems mainly dependent on p55r and soluble TNFα [425]. An important role of TNF in bone resorption induced by inflammation and the possibility that it may be the main mediator of periprosthetic osteolysis is suggested [426].

RANKL-Induced Intracellular Signalling

RANKL induces the survival, formation and activation of osteoclasts by activating the transcription factor NF-κB, JNK (cJun N-terminal kinase) and p38 mitogen activated (MAP) kinase. TNFα, IL-1 and LPS (lipopolysaccharide) seem to affect osteoclasts and their precursors also via these signaling proteins [417,423,427–429]. Activation of the p38 MAP kinase, JNK and finally the transcription factor AP-1 play an important role in RANKL and TNFα-induced osteoclast differentiation from precursor cells [423,429,430]. NF-κB seems to be important for the induction of the bone resorbing activity of osteoclasts [431], but is also activated during osteoclast formation [432]. Finally, RANKL activates protein kinase B (Akt/PKB) via c-Src and PI 3-K (phosphoinositide 3-kinase) and thus supports the survival of osteoclasts, and may also activate NF-κB and affect cytoskeletal reorganization by this pathway [433,434]. In contrast to RANKL, CSF-1 required for the formation and survival of osteoclasts activates the MAP kinase p42/44-ERKs (extracellular signal-regulated kinase) [429,431].

Effect of Immune Cells

Recent data have drawn attention to the importance of lymphocytes in regulating the formation and activity of osteoclasts and their precursors. Immune response often results in tissue destruction, especially in enhanced osteoclastic bone resorption. In a T-cell dependent model of rat adjuvant arthritis characterized by severe joint inflammation, bone and cartilage destruction is induced by T-cell derived RANKL [435]. Additionally, IL-1 and TNF-α, cytokines typically produced in inflammatory conditions, increase bone marrow stromal cell production of IL-7 [436]. This factor upregulates the production of osteoclastogenic cytokines by T cells that are different from CSF-1 and RANKL, as antibodies against these latter factors do not or only partially inhibit the formation of osteoclasts [437]. Immune cells have also been demonstrated to inhibit the formation of osteoclasts. Depletion of CD4 and CD8 T lymphocytes (nonactivated cells) in mice *in vivo* enhanced the formation of osteoclasts in cultured bone marrow treated with $1,25(OH)_2D_3$ [438]. This stimulation was dependent on prostaglandin synthesis, suggesting that nonactivated lymphocytes produce factors that inhibit prostaglandin synthesis or that lymphocyte depletion affects the type of stromal cell that populates the bone marrow. Furthermore, mature B lymphocytes inhibit osteoclast formation in peripheral blood stem cell cultures, a system that generates

functional osteoclasts in the absence of stromal cells, through the production of TGF-β [439].

TGF-β Supports Osteoclast Formation

The stimulation of osteoclast formation by TGF-β in the presence of CSF-1 and RANKL has been reported [440–442]. The cytokine seems to prevent the final differentiation of macrophages to the mature cell type and hence prolongs the period during which osteoclast development is possible. Since TGF-β is incorporated in the bone matrix, its release during bone resorption may enhance the osteoclast-forming potential of precursors as they migrate towards sites of cell-bound RANKL. This migration is probably controlled by CSF-1 and TGF-β both acting chemotactically on osteoclasts [443–445]. TGF-β may stimulate osteoclasts directly, but inhibit them indirectly by inducing the production of OPG by bone marrow stromal cells [446]. The generation of osteoclasts from human peripheral blood mononuclear cells in the presence of CSF-1 and RANKL suggests an even more complicated action of TGF-β. It abrogates the suppressive effect of non-adherent lymphocytes, and its effect on osteoclastogenesis seems to be determined by the cytokine milieu of the microenvironment and the state of activation by TGF-β of the target cell [447].

Stimulators of Bone Resorption

Vascular endothelial growth factor (VEGF). In aged *op/op* mice, the numbers of osteoclasts and macrophages increase and the bone marrow cavity expands, resulting in resolution of the osteopetrosis. The mechanism of this recovery has been investigated. A single injection of recombinant human VEGF into *op/op* mice induced osteoclast recruitment similar to that induced by CSF-1, demonstrating that VEGF and CSF-1 have an overlapping function in the support of osteoclastogenesis [448]. Endogenously produced VEGF may be responsible for the age-related resolution of osteopetrosis in *op/op* mice. VEGF is also essential for osteoclast recruitment into developing long bones [449]. The receptor for VEGF (Flt-1) has been identified as a surface marker as well as a functional molecule for the monocyte-macrophage lineage in humans [450].

GM-CSF and IL-3. IL-3 and GM-CSF are also suggested to induce osteoclast formation in aged *op/op* mice. Serum concentrations of each are elevated in these animals, and their injection into both young and aged *op/op* mice expands the bone marrow cavity [451].

Fibroblast growth factor. Fibroblast growth factor (FGF) receptors 1 and 4 have been detected in isolated mouse osteoclasts. FGF-2 stimulates directly, and indirectly mediated by osteoblasts, the bone resorbing activity of rabbit and mouse osteoclasts *in vitro* [452].

Chemokines. Macrophage inflammatory protein 1-α (MIP 1-α) induces osteoclast formation from human bone marrow cells cultured in medium containing 20% horse serum [453]. This chemokine enhances PTHrP-, IL-6- and RANKL-induced osteoclast formation and appears to act directly on human osteoclast precursors and to stimulate independently of RANKL in the later stages of osteoclast differentiation [454].

ADAM8. ADAM8, a member of the ADAM (a disintegrin and metalloproteinase) family of proteinases, has been identified in a murine osteoclast precursor cell line, and found to increase both the formation of osteoclasts when present in the second half of the culture period, and the activity of osteoclasts cultured on dentine slices [455].

Prostaglandins. Prostaglandins stimulate bone resorption indirectly, activating the synthesis of RANKL by osteoblasts via a cyclic AMP-dependent mechanism. Recent data suggest that prostaglandin receptors E4 and, to a certain extent, E2, respond to prostaglandin E_2 and induce bone resorption [456–458].

Extracellular matrix proteins. Investigations into the differentiation of osteoclast precursor cells in nonadherent and adherent systems point to a two stage process, in which precursor cells are initially bipotential and capable of anchorage-independent growth, the further proliferation and differentiation of osteoclast-committed precursor cells subsequently becoming anchorage-dependent, differentiation being enforced by the activation of $\alpha_v\beta_3$ integrins [459]. Matrix proteins, including vitronectin, osteopontin and bone sialoprotein, bind to $\alpha_v\beta_3$ integrins resulting in the activation of signaling pathways, including PI 3-K, c-Src, protein tyrosine kinase 2 (PYK2), a major adhesion-dependent tyrosine kinase in osteoclasts, and Crk-associated substrate (Cas). Various results suggest that activation of these pathways plays a role in linking the adhesion of osteoclasts to the bone matrix with their cytoskeletal organization and polarization and activation for bone resorption [460]. CSF-1 induces the spreading and migration of osteoclasts, and the signals induced by either adhesion or CSF-1 have been investigated and compared [461]. While c-Src is involved in the activation of prefusion osteoclasts by matrix proteins, CSF-1-initiated signaling modulates the $\alpha_v\beta_3$ integrin-mediated cytoskeletal reorganization in these cells in the absence of c-Src. This is different from the situation in mature osteoclasts grown in culture where CSF-1-induced spreading has been found to be c-Src dependent [443,462].

Osteopontin. Osteopontin exists both as an immobilized extracellular matrix protein and as a cytokine in body fluids regulating inflammation, tissue remodeling and cell survival [463]. Studies with osteopontin-deficient animals indicate that it is required for bone resorption. In organ culture, bone resorption was impaired in osteopontin-deficient bones stimulated by PTH or RANKL, and the development of osteoclasts was also reduced [464]. Ectopic bone implantation experiments have demonstrated that osteopontin facilitates angiogenesis, the accumulation of osteoclasts, and bone resorption [465]. Further, the enhancement of osteoclastic bone resorption and the suppression of osteoblastic bone formation in response to reduced mechanical stress does not occur in bone of osteopontin-negative mice [466]. Mechanical stress itself seems to increase the expression of osteopontin since oscillatory fluid flow increased osteopontin mRNA levels in the MC3T3-E1 osteoblastic cell line [467]. Finally, osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption [468].

Collagenase induced in mesenchymal cells is important in bone resorption. PTH-induced bone resorption in collagenase-resistant mutant mice is diminished, proving the importance of osteoblastic collagenase in bone resorption [469].

Microvascular endothelial cells regulate osteoclastogenesis induced by inflammatory cytokines. Vascular endothelial cells, known to be intimately associated with osteoclasts both during their formation and during the resorption of bone, have now been demonstrated to express RANKL and OPG,

and the inflammatory cytokines TNFα and IL-1, although not PTH and $1,25(OH)_2D_3$, to regulate this expression [470]. Therefore, cytokine-activated vascular endothelial cells may contribute to inflammatory bone loss via the regulated production of RANKL and OPG. The latter may also serve as an autocrine signal to inhibit blood vessel calcification. It has also been reported that osteoclast precursor cells adhere to endothelial cells that have been treated with TNFα and IL-1 [471]. This is the first step before precursor cells leave the blood, cross the endothelial cell layer and migrate to the bone surface.

Inhibitors of Bone Resorption

Osteoprotegerin. A potential therapeutic role for OPG has been suggested in diseases with enhanced bone resorption. A single adenoviral gene transfer to sham and ovariectomized mice produced a persistently high-level of OPG expression and protected ovariectomized mice from bone loss [472]. In a murine model of humoral hypercalcemia of malignancy, osteoprotegerin prevented and reversed hypercalcemia and reduced osteoclast activity [473]. Most interestingly, OPG decreased the skeletal tumor burden in a mouse model of experimental bone metastasis [474], and in a prostate tumor model, prevented the establishment of mixed osteolytic/osteoclastic tibial tumors [475]. In neither model did it have any effect on the metastatic tumor burden in soft tissue organs. Inhibitors of bone resorption thus appear promising as tools for the management of skeletal metastases, especially if they can be introduced early in cancer therapy [476]. In postmenopausal women, a single, well tolerated, subcutaneous dose of 3 mg OPG per kg body weight decreased urinary levels of collagen I N-telopeptide, a marker for bone resorption (see Appendix II, this volume), by 80% and 14%, respectively, 4 days and 6 weeks after dosing [477]. Bone-specific alkaline phosphatase, a marker for bone formation, did not change for 3 weeks after dosing. The results indicate that OPG acts primarily on osteoclasts, and suggests that OPG may be effective in the treatment of diseases with increased bone resorption such as osteoporosis.

Estrogen. Using mouse models, progress in understanding the mechanism of estrogen action has been made [478]. While it was previously assumed that the inhibition of bone resorption by estrogen was mediated by bone marrow stromal cells and macrophages, new findings indicate that it acts also on immune cells. Thus, estrogen inhibits the proliferation of TNF-producing T-cells [479]. Estrogen deficiency results in increased numbers of pre-B cells in bone marrow, as well as in the production of prostaglandin E_2 which induces the expression of RANKL on pre-B cells, leading to accelerated osteoclastogenesis [480]. It also acts directly on osteoclast precursors by down-regulating RANKL-induced JNK activation [481-483]. Estrogen seems also to exert bone-protective effects by selective modulation of IL-1 receptor levels in osteoclasts, thereby reducing their IL-1 responses and cell survival [484]. Estrogen apears to be the dominant sex steroid regulating bone resorption in normal elderly men, while both it and testosterone are important in maintaining bone formation [485].

Interferon-γ. Interferon has long been known to inhibit bone resorption, and it has now been found to interfere with the RANKL-RANK signaling pathway by inducing rapid degradation of the RANK adaptor protein, TRAF (TNF receptor-associated factor) 6, thereby inhibiting activation of the transcription factor NF-κB and JNK [486,487].

Peroxisome proliferator-activated receptor-γ and IL-4. Activation of the nuclear receptor and transcription factor PPAR-γ (peroxisome proliferator-activated receptor-γ) pathway inhibits osteoclast differentiation [488,489]. The suppression of osteoclast formation by IL-4 seems, at least partially, to occur via activation of PPAR-γ1. In addition, IL-4 abrogates osteoclastogenesis via STAT 6 (signal transducer and activator of transcription 6)-dependent inhibiton of NF-κB. In $STAT^{-/-}$ mice, IL-4 failed to block osteoclastogenesis [490].

Osteoclast inhibitory lectin. A 207 amino acid residue type II transmembrane C-type lectin expressed in osteoblasts, chondrocytes, and a variety of extracellular tissues, has been cloned, and demonstrated to inhibit the formation of osteoclasts *in vitro* [491].

Osteopetrosis

Infantile Malignant Osteopetrosis

In several patients with a diagnosis of infantile malignant autosomal recessive osteopetrosis (MIM 259700) (see also Chapter 19, this volume), mutations have been found in *TCRG1*, which encodes the osteoclast-specific 116 kDa subunit of the vacuolar proton pump [492]. Mice lacking *Atp6i*, which encodes a putative osteoclast-specific proton pump subunit of 116 kDa, are also osteopetrotic [493], and osteoclasts within these mice have lost the function of extracellular acidification, although intracellular lysosomal proton pump activity is not impaired. In the osteosclerotic (*oc/oc*) mouse, a deletion in the gene encoding the 116 kDa subunit of the vacuolar proton pump has also been found [494]. All of this demonstrates that this particular gene is necessary for osteoclast-mediated extracellular acidification, and that its mutation is a cause of malignant osteopetrosis in man.

Pycnodysostosis, Microphthalmic $Mitf^{mi/mi}$ Mutant Mice and Cathepsin K Null Mice

A phenotypic similarity between microphthalmic $Mitf^{mi/mi}$ mutant mice, cathepsin K null mice, and the human disease pycnodysostosis has been observed. Mitf is a member of a helix-loop-helix transcription factor subfamily which contains the potential dimerization partners TFE3, TEFB, and TFEC. Cathepsin K has now been identified as a transcription factor target of Mitf and TFE3 via three consensus elements in the cathepsin K promoter [495], explaining the similarities between the phenotypes caused by cathepsin K and Mitf mutations.

Glanzmann Mutation in β_3 Integrin Specifically Impairs Osteoclast Function

Mice lacking β_3 integrin are osteosclerotic because of dysfunctional osteoclasts [496]. To identify the components of the β_3 cytoplasmic domain regulating osteoclast function, six point mutations known to affect integrin β_3 signaling, have been generated. Only one, a Ser752Pro substitution, which also characterizes a form of the human bleeding disorder Glanzmann thrombasthenia (MIM 273800), failed to rescue $\beta_3^{-/-}$osteoclasts or restore ligand-activated signaling [497]. This suggests that the bone mass in patients with Glanzmann thrombasthenia may be increased.

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Chapter 1, Part IV

Morphology and Chemical Composition of Connective Tissue: The Cardiovascular System

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INTRODUCTION

The cardiovascular system allows the transport of blood and lymph through the tissues. The central motor of the entire system is represented by the heart, which functions to pump blood through the vascular system to the most distal parts of the body, ensuring its return to the heart, and indirectly regulating the correct distribution of lymph from the peripheral tissues back to the vascular network [1]. After being distributed through the tissues, blood returns to the right atrium of the heart through the superior and inferior venae cavae and passes into the right ventricle from where, via the pulmonary arteries, it is pumped to the lungs (Fig. 1). Here blood is oxygenated and becomes ready to reenter the heart in the left atrium being transported by the pulmonary veins. Finally, it passes into the left ventricle, enters the aorta, and is distributed in the systemic circulation [2].

The continuous activity of the heart is assured by the presence of specialized myocardial fibers that are responsible for the maintenance of the cardiac rhythm and for the contraction of atria and ventricles without any direct role for the nervous system. In fact, innervation by the autonomic nervous system does not initiate cardiac muscle contraction, but exerts a regulatory role according to body needs. Thus, impulses are generated in the sinoatrial node and transmitted through the atrial wall to the atrioventricular node, the atrioventricular bundle, and the Purkinje fibers. The nodes and the atrioventricular bundles are small cardiac muscle cells, whereas Purkinje fibers are large modified myocardial cells allowing a faster conduction of impulses.

Cells, nutrients, growth factors, and oxygen are transported through the vascular system to all tissues in response to local and/or systemic factors; reduced oxygen level, for

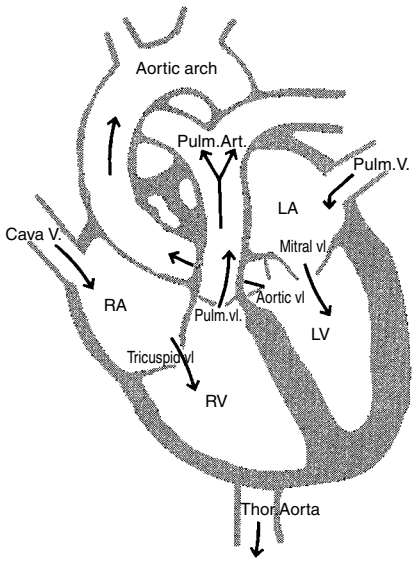

Figure 1. Drawing of the heart chambers (RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle) and valves (tricuspid vl., mitral vl., aortic vl., and pulmonary vl.) with main vessel insertions (aortic arch, thoracic aorta, vena cava, pulmonary artery and veins). Arrows indicate the direction of blood flow in and out of the heart.

Abbreviations used: β-MHC, β-myosin heavy chain; ACE, angiotensin I-converting enzyme; ANF, atrial natriuretic factor; ANP, atrial natriuretic peptide; bFGF, basic fibroblast growth factor, CAM, cell adhesion molecule; C-CAM, cell-cell adhesion molecule; CEA, carcinoembryonic antigen; CRP, complement regulatory proteins; CTGF, connective tissue growth factor; EGF, epidermal growth factor; ELAM-1, endothelial leukocyte adhesion molecule-1; FBN-1, fibrillin-1; GAGs, glycosaminoglycans; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-γ; IGF-1, insulin-like growth factor-1; IL-1, interleukin-1; LDL, low density lipoproteins; LPS, lipopolysaccharide; MAG, myelin-associated glycoprotein; MAGP, microfibril-associated glycoprotein; MAP-kinase, mitogen activated protein kinase; MEF2, myocyte enhancer factor-2; MMP-2, matrix metalloproteinase-2; NADPH, nicotinamide adenine dinucleotide phosphate; N-CAM, neural-cell adhesion molecule; NO, nitric oxide; NOS, nitric oxide synthase; PDGF, platelet-derived growth factor; PDGF-rβ, platelet derived growth factor-receptorβ; PECAM, platelet-endothelial cell adhesion molecule; PF-4, platelet factor-4; RGD, arginine-glycine-aspartate; SkA, skeletal α-actin; TGF-β, transforming growth factor-β; TIMP, tissue inhibitor of metalloproteinases; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; VE-cadherin, vascular endothelial-cadherin; VEGF, vascular endothelial growth factor.

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instance, causes a relaxation of arterial smooth muscle cells and increased blood flow, whereas the secretion of norepinephrine exerts the opposite effect. Although there are many systems regulating traffic between blood vessels and the extracellular milieu, capillaries, due to their permeability, are mostly responsible for exchanges of cells and molecules to and from tissues.

STRUCTURAL ORGANIZATION

Heart

In a normal adult, the heart is about 98 mm wide, 107 mm long, and 52 mm thick; however, dimensions differ depending on thoracic conformation and exercise. Similarly, its weight depends greatly on height and development of the muscle mass and varies from about 21 g at birth to 300–350 g in adulthood, with a decrease in the ratio of heart weight to body weight from 6×10^{-3} at birth to 4.5×10^{-3} in adulthood.

The heart is covered by a thin layer of dense connective tissue called pericardium that is in close continuity with the external layer of the major vessels arising from the heart. It is a muscular organ divided into four cavities (Figs. 1 and 2): two atria and two ventricles that are organized into the following three layers:

- The epicardium represents the outer surface of the organ and is mainly comprised of mesenchymal cells and scarce collagen fibrils.
- The myocardium is the major constituent of the organ, and is composed of myocytes that are organized into a thicker layer in the ventricles compared with the atria.
- The endocardium, the inner surface of the heart, is 50–500 μm thick depending on the area, being more pronounced in the atria than the ventricula; its inner surface is covered by an endothelium that lies on the tunica propria, which is rich in elastic fibers, and it consists of smooth muscle cells, collagen fibrils, and proteoglycans. The impulse-conducting system of the heart is located in the deep region of the endocardium.

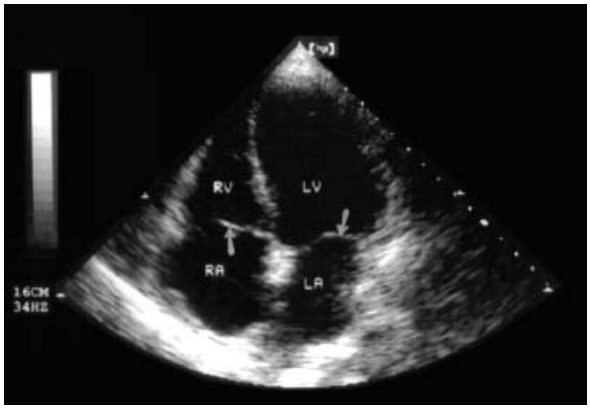
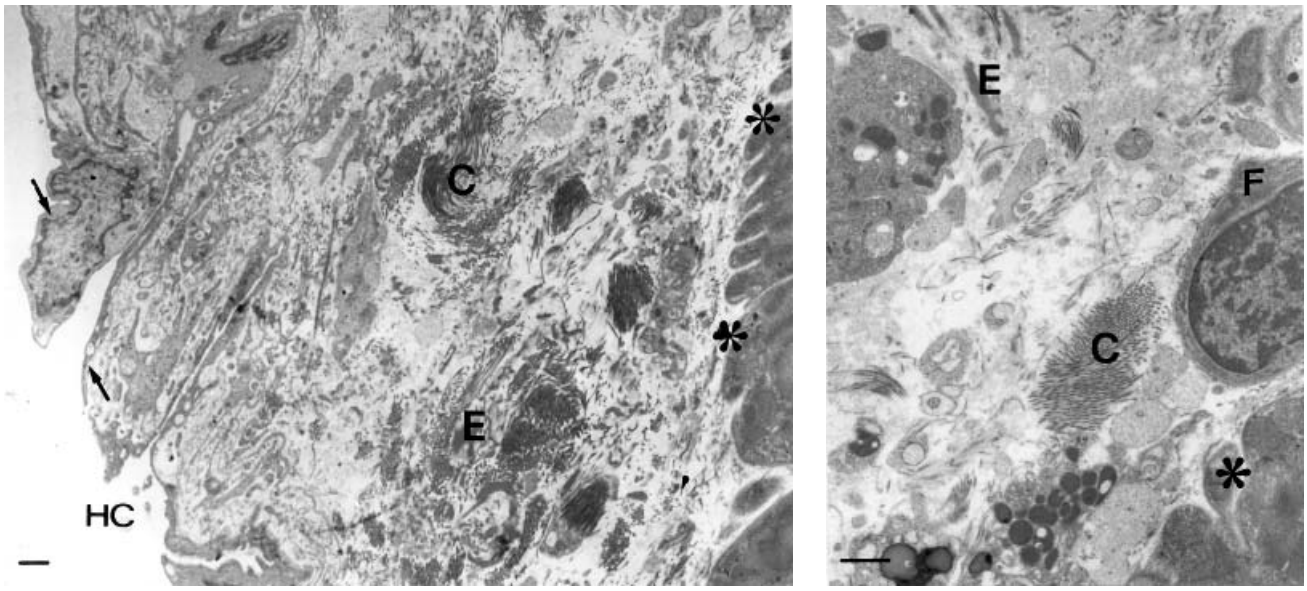


Figure 2. An apical ''4-chamber'' view from a normal adult by two-dimensional echocardiography. Leaflets of the tricuspid and mitral valves (arrows) are seen between the right atrium and ventricle (RA and RV) and between the left atrium and ventricle (LA and LV), respectively. (Figure courtesy of L.Targa, MD, Division of Cardiology, Este Hospital, Padova, Italy.)

Although extracellular matrix molecules are present in the endocardial layer and in the interstitial spaces between myocytes (Fig. 3), the major sites of deposition of cardiac



a b

Figure 3. Electron micrographs showing the organization of the extracellular matrix in the endocardium **a** and myocardium **b** of an adult heart. The endocardium consists of a layer of loose connective tissue, 50–500 μm thick, separated from the heart cavity (HC) by endocardial cells (arrows) and extending to the contractile myocytes (asterisks). It is formed by collagen bundles (C) and small elastic fibers (E) scattered among hydrated proteoglycans and glycoproteins. The connective tissue within the myocardium (b) (asterisks) contains very small collagen bundles (C) and elastic fibers (E) together with scarce fibroblasts (F). Bars: 1 μm

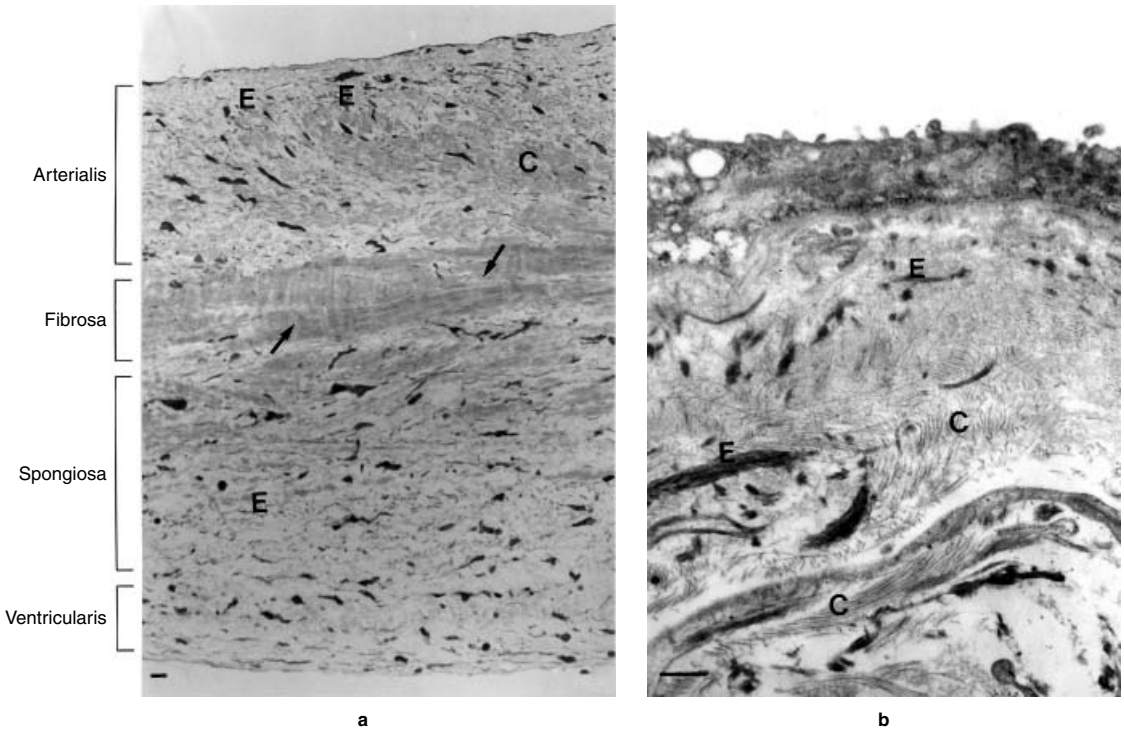


Figure 4. Light **a** and electron **b** micrographs showing a portion of the aortic valve. The different organization of the extracellular matrix can be appreciated in the different areas (arterialis, fibrosa, spongiosa, and ventricularis) of the valve wall. In particular, the free surfaces of the valve are covered by endothelial cells (a, top and bottom). The arterialis consists of loose connective tissue containing scarce cells, collagen fibrils (C) and elastic fibers (E) immersed in an hydrated matrix, as can be appreciated by electron microscopy (b). The fibrosa is characterized by compact collagen bundles (arrows), whereas the spongiosa is highly hydrated with few cells, small collagen bundles and some elastic fibers (E) that are present in the thin ventricularis. Bars: a) 10 μm; b) 1 μm.

connective tissue are at the insertions of the aorta and pulmonary artery, and continue in the atrioventricular rings, forming a network for the attachment of the valves. The cuspid valves located between the atria and the ventricles and the semilunar valves of the aorta and pulmonary artery assure the unidirectionality of blood flow. These valves are covered on both surfaces with endothelial cells, beneath which a few layers of connective tissue are present (Fig. 4). The aortic valve, for instance, is characterized by areas of different composition and function, called arterialis, fibrosa, spongiosa, and ventricularis [3]. The arterialis is comprised of small and spread collagen bundles and elastic fibers similar to those of the arterial intima. The fibrosa has a dense extracellular matrix, whereas the spongiosa has few cells immersed in a loose extracellular matrix, mainly comprised of glycosaminoglycans, especially dermatan and chondroitin sulfates. The ventricularis is a thin layer made of collagen bundles and contains more elastic fibers than other areas. The adult human aortic valve cusps comprise about 10–15% of elastin and 45–55% of collagen (dry weight ratio), mainly collagen type I, even though small amounts of collagen type III can also be measured [3].

Blood Vessels

The blood vessels represent a complex network that can be divided into several categories according to function (pulmonary and systemic circulation), structure (arteries, veins, and capillaries), or size (small, medium, and large). Generally speaking, the walls of arteries and veins are comprised of a tunica intima, tunica media, and adventitia [4] (Fig. 5 and Table 1).

The tunica intima consists of a single layer of flat and elongated endothelial cells underlined by a thin space made of loose connective tissue and limited, in human arteries, by the internal elastic lamina. Endothelial cells exhibit their long axis parallel to blood flow and are connected one to the other by tight and gap junctions in order to strictly regulate the exit of blood cells and diffusion of nutrients from the blood stream. The most efficient system allowing the transport of nutrients through endothelial cells is via pinocytotic vesicles. Endothelial cells may present Weibel-Palade bodies, rod-like cytoplasmic inclusions exhibiting an electron dense structure and containing coagulation factor VIII.

The tunica media is characterized by layers of smooth muscle cells, with their long axes concentric to the lumen, which are responsible for the production of the extracellular molecules in which they are situated. Smooth muscle cells are surrounded by a basal lamina, except where they are connected to one another by gap junctions and where they make close contacts with elastic fibers (Fig. 6). Among the cells, collagen bundles and proteoglycans can be seen; concentric elastic lamellae alternating with smooth muscle cells are present in the arteries and their number is highly variable according to the functional role of the vessel, being more numerous in the ascending aorta and decreasing proportionally to the reduction of blood pressure in the abdominal aorta. The number of lamellae in the aortic arch varies from 10 to 13 in the rat and from 40 to 70 in

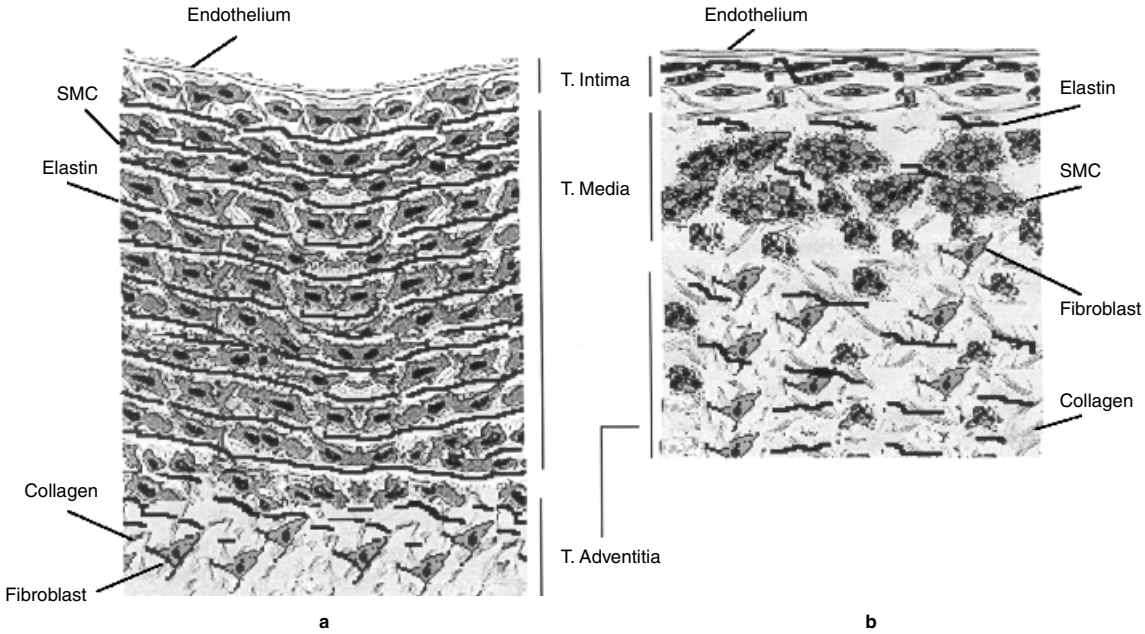


Figure 5. Schematic views of the organization of arteries **a** and veins **b**. The lumen of all vessels is covered by endothelial cells that lie on a basement membrane. The tunica intima is very thin at birth and tends to become thicker with age in arteries. Arteries and veins have different organization of the tunica media as far as thickness and composition are concerned, this being thick and mostly formed by elastin and smooth muscle cells (SMC) in arteries, and rather thin and mainly made of smooth muscle cells in veins; the external layer (tunica adventitia), on the contrary, is thicker in veins than in arteries of comparable size, and consists of collagen, fibroblasts, and smooth muscle cells.

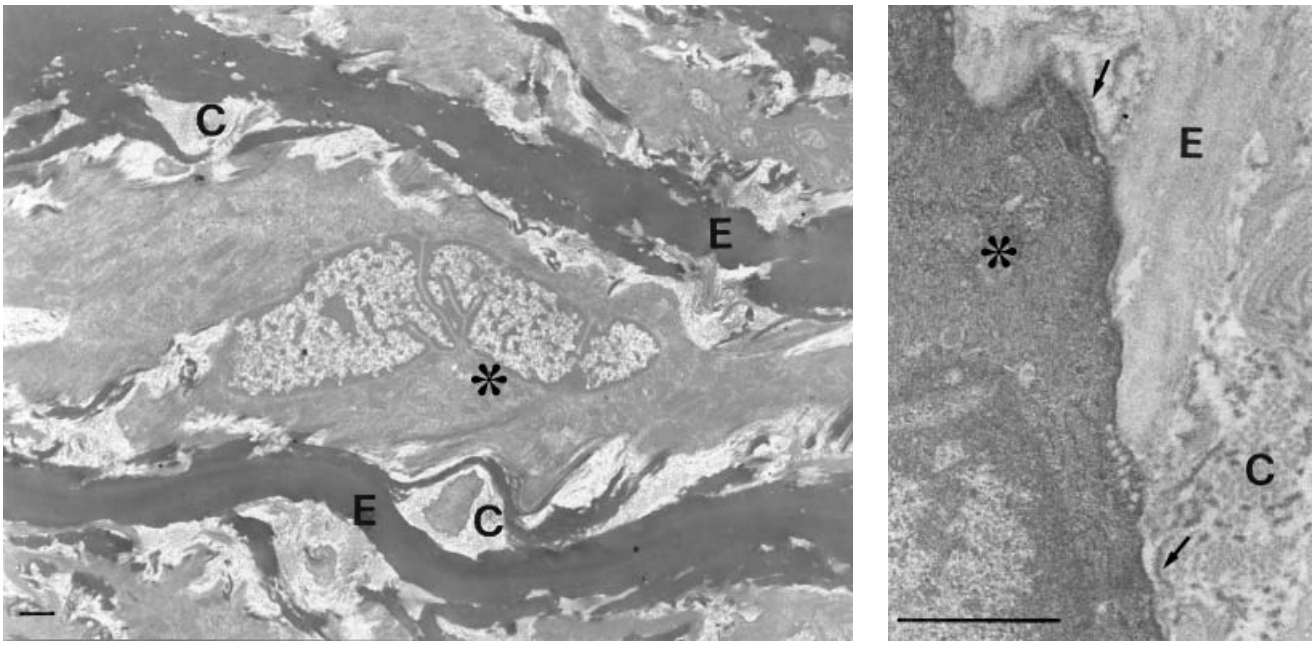


Figure 6. Electron micrographs showing a typical aortic smooth muscle cell (asterisk) in the space between elastic laminae (E) **a**. Collagen bundles can also be appreciated (C). At higher magnification **b**, it appears that the smooth muscle cell basal lamina (arrows) is interrupted in areas where the cell is in contact with the elastic fiber (E). Bars: 1 μm.

TABLE 1. Vessel Parameters in Humans

	Type	Diameter	Tunica Intima	Tunica Media	Tunica Adventitia
Arteries					
	Elastic	>1 cm	50–100 μm thick (increases with age), endothelium, basement membrane, proteoglycans, a few collagen fibrils, internal elastic lamina	1–3 mm thick, layers of smooth muscle cells, layers of elastic lamellae, small collagen bundles, proteoglycans	300–400 μm thick, fibroblasts, scarce smooth muscle cells and elastic fibers, thick collagen bundles, vasa vasorum
	Muscular	0.1–10 mm	50–100 μm thick (increases with age), endothelium, basement membrane, proteoglycans, a few collagen fibrils and smooth muscle cells, prominent internal elastic lamina	0.1–1 mm thick, layers of smooth muscle cells, scarce elastic lamellae, small collagen bundles, proteoglycans	200–400 μm thick, fibroblasts, scarce smooth muscle cells and elastic fibers, thick collagen bundles, vasa vasorum
	Arterioles	<100 μm	Thin endothelium, basement membrane, little collagen, thin elastic lamina	one to two layers of smooth muscle cells, scarce elastin, collagen, and proteoglycans	Thin, ill-defined sheets of connective tissue
	Capillaries	<10 μm	Endothelium, basement membrane	None	None
Veins					
	Postcapillaries	10–30 μm	5–20 μm thick (increases with age), endothelium, basement membrane, pericytes	None	None
	Small	0.1–1 mm	20–50 μm thick, endothelium, basement membrane, pericytes, rare smooth muscle cells, scarce collagen fibrils and proteoglycans	50–100 μm thick, smooth muscle cells (one to three layers), scarce collagen, and proteoglycans	100–200 μm thick, fibroblasts, collagen bundles, some elastic fibers
	Medium	1–10 mm	20–50 μm thick, endothelium, basement membrane, rare smooth muscle cells, scarce collagen fibrils, and proteoglycans	100–500 μm thick, smooth muscle cells (up to eight layers), scarce collagen bundles, elastic fibers and proteoglycans	200–500 μm thick, fibroblasts, scarce smooth muscle cells, collagen bundles, some elastic fibers
	Large	>1 cm	20–50 μm thick, endothelium, basement membrane, rare smooth muscle cells, scarce collagen fibrils, and proteoglycans	>0.5 mm thick, few fibroblasts, smooth muscle cells (up to 15 layers), collagen bundles, elastic fibers, proteoglycans	>0.5 mm thick, fibroblasts, smooth muscle cells, collagen bundles, some elastic fibers

man. Fenestrations occasionally present in the elastic laminae facilitate movements of cells and nutrients through the vessel wall [5].

The tunica adventitia is composed of dense connective tissue mainly comprised of collagen bundles and a few spread elastic aggregates. Fibroblasts are the most abundant cell type in this area of the vessel wall, but a few macrophages can also be seen.

Arteries

Arteries, on the basis of the major constituent of the tunica media, are classified into elastic arteries, muscular arteries, and arterioles. Their diameters and structural organization progressively change from heart to peripheral tissues (Table 1). The aorta and the pulmonary arteries are the most important elastic arteries, and are characterized by numerous elastic laminae intercalated by layers of smooth

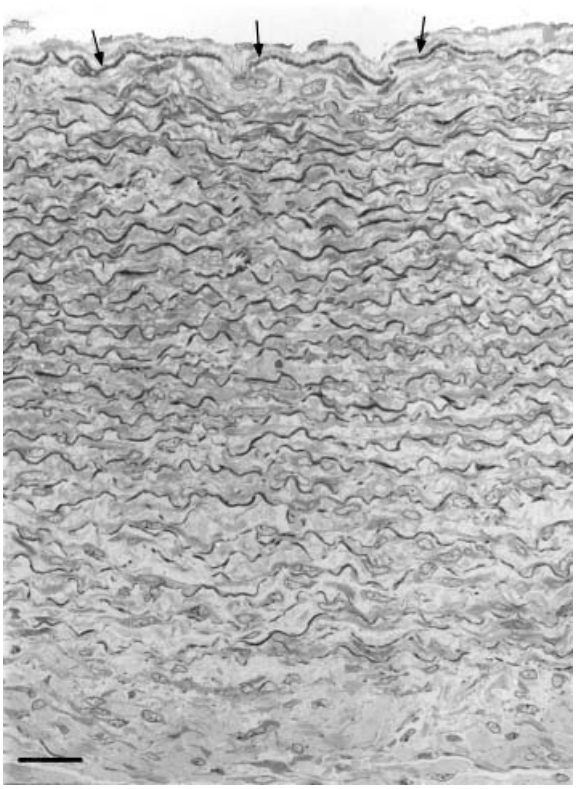


Figure 7. Light microscopy of the aorta from a newborn individual. Endothelial cells (**top**) cover a thin tunica intima that is separated from the tunica media by a still incomplete internal elastic lamina (arrows). In the tunica media, layers of elastic lamellae (waved dark lines) alternate with smooth muscle cells and collagen bundles. The organization of the adventitia (**bottom**) is less ordered and is characterized by scanty elastic fibers, fibroblasts, and collagen bundles. Semithin section stained with toluidine blue. Bar: 100 μm.

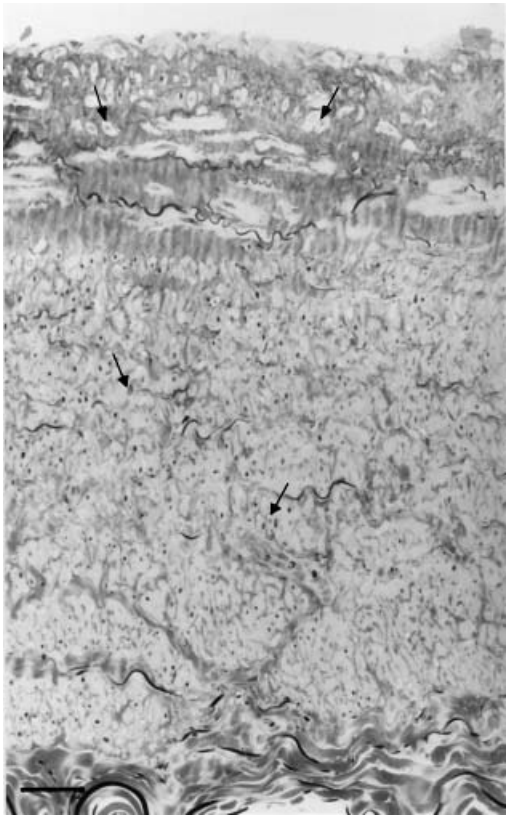


Figure 8. Light microscopy of a portion of the saphenous vein from an adult subject. Underneath the endothelium (**top**), scanty and irregular elastic fibers (waved dark lines) are immersed in a matrix containing numerous smooth muscle cells in different orientations that occupy the greatest part of the vessel wall (arrows). Part of the adventitia of the vein is shown at the bottom and is mainly formed by collagen bundles. Semithin section stained with toluidine blue. Bar: 20 μm.

muscle cells (Figs. 5 and 7). Muscular arteries are sometimes difficult to distinguish from elastic arteries, although they have more smooth muscle cells and less elastin compared with the elastic ones. In small arteries and arterioles, the number of smooth muscle layers in the tunica media is reduced to seven or eight and one or two, respectively. Moreover, the internal elastic lamina is frequently absent from arterioles.

Veins

Veins, as arteries, present a tunica intima, a tunica media, and a tunica adventitia, although these areas are less well defined and thinner than in arteries (Figs. 5 and 8); moreover, their lumens are generally wider than those of comparable arteries. In contrast to arteries, the tunica media of large veins contains also a few fibroblasts in addition to smooth muscle cells (Table 1). Veins also contain valves to allow them to convey blood against gravity. Postcapillary venules are characterized by an endothelial lining and are surrounded by pericytes enclosed by basal lamina material that is continuous with that of the endothelium.

Capillaries

Capillaries are the smallest blood vessels and are characterized by a single layer of endothelial cells resting on a basal lamina and connected to each other by specialized junctions (Fig. 9). Depending on their structure, capillaries may be distinguished into continuous, fenestrated, and discontinuous [6]. In particular, continuous capillaries, which are typical of muscle, lung, and central nervous system, are delimitated by thin and elongated endothelial cells exhibiting a cytoplasm enriched with pinocytotic vesicles, about 70nm wide, that allow transport of substances across their plasma membranes. Sometimes, associated with endothelial cells, pericytes, enclosed by a basal lamina, can be recognized (Fig. 10). Pericytes, also called Rouget cells or mural cells, exhibit some structural differences according to tissue; moreover, they seem to contribute to the regulation of vessel growth and blood flow. Interactions and exchanges between endothelial cells and pericytes occur at different levels, and a number of endothelium-related growth factors (PDGF, bFGF, EGF, CTGF) stimulate pericyte proliferation, migration and also differentiation into osteoblasts, phagocytes, or adipocytes. Moreover, gap junctions, cell adhesion molecules, and integrins can mediate transmembrane signaling within pericytes and play a role in the activation of growth factors such as TGF-β [7]. In fenestrated capillaries, which are mainly present in the endocrine glands, gallbladder and intestine, endothelial cells have a cytoplasmic membrane with fenestrations of 80–100 nm that contribute, together with a few pinocytotic

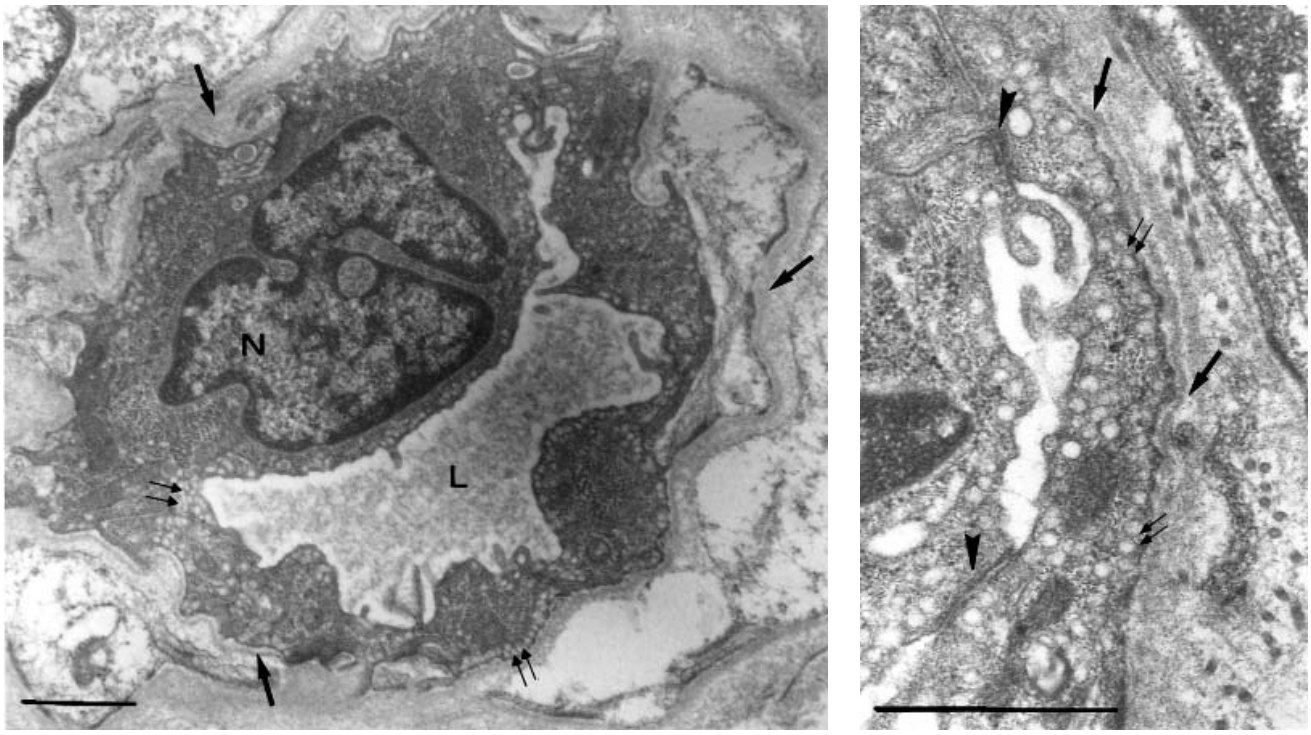


Figure 9. Electron micrographs of a dermal blood capillary comprised of a single layer of endothelial cells and surrounded by a basal lamina (single arrows). Numerous pinocytotic vesicles (double arrows) and cell junctions (arrowheads) between adjacent endothelial cells can be seen **b**. L, capillary lumen; N, endothelial cell nucleus. Bars: 1 μm.

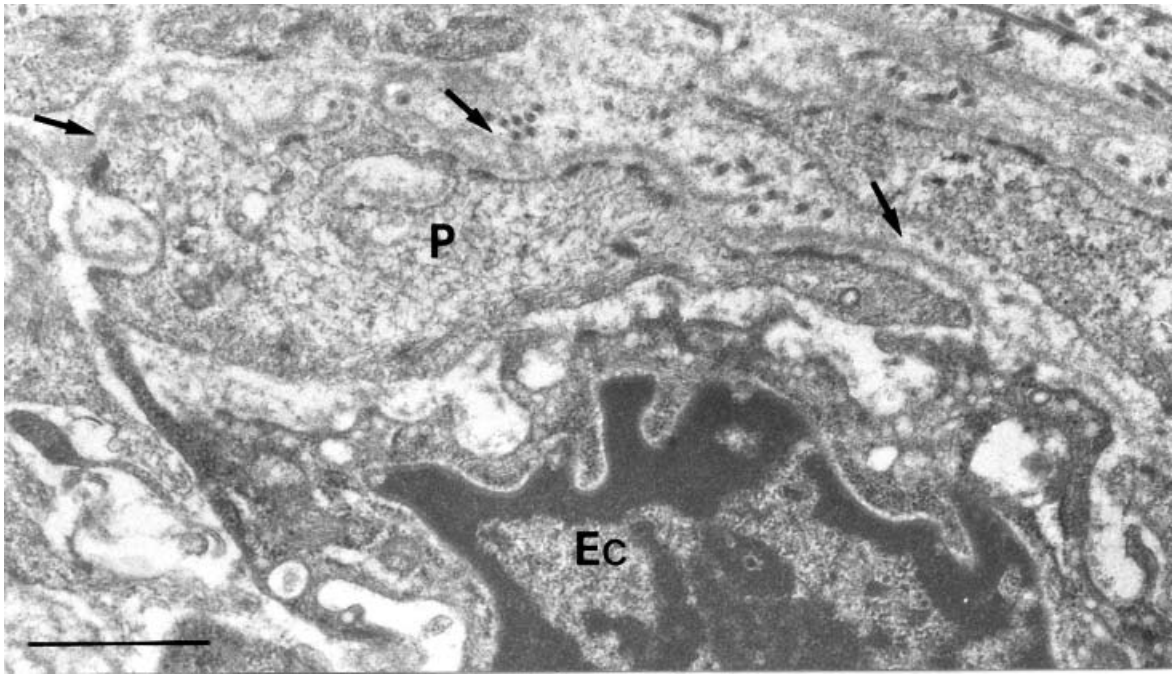


Figure 10. Electron micrograph of a pericyte (P) from a dermal blood vessel surrounded by the same basal lamina (arrows) as the endothelial cells (Ec). Bar: 1 μm.

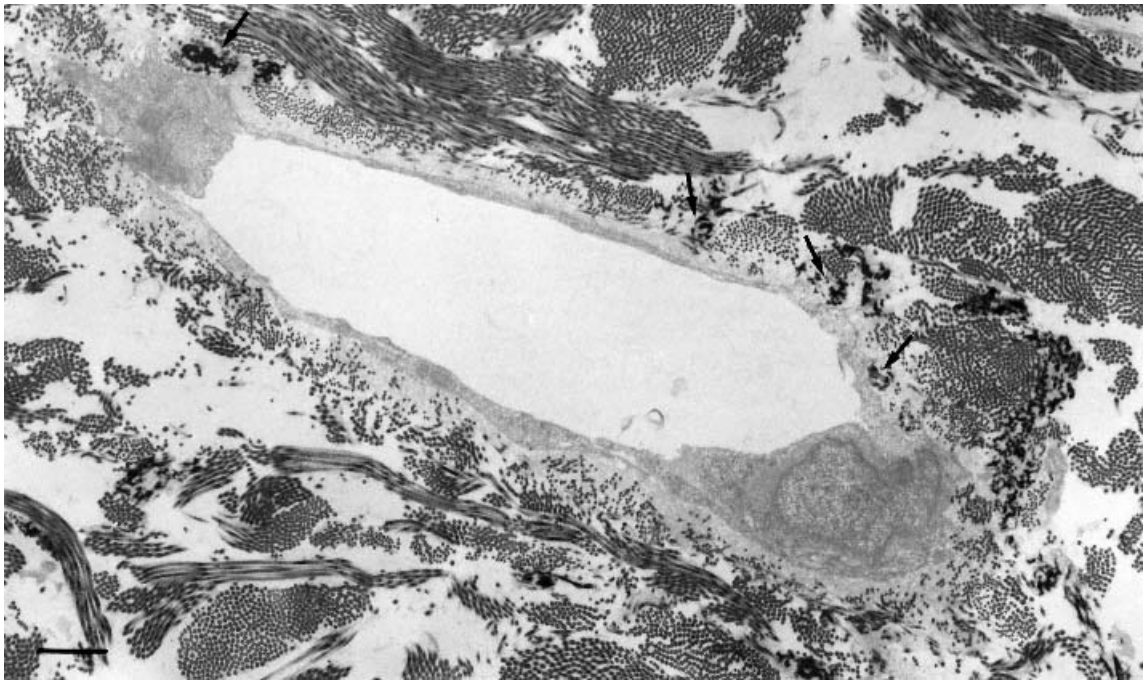


Figure 11. Electron micrograph of a dermal lymphatic vessel delimited by a single layer of endothelial cells and devoid of a continuous basal lamina. Numerous elastic fibers (arrows) can be seen at the periphery of the vessel. Bar: 1 μm.

vesicles, to molecular transport. Discontinuous capillaries, also called sinusoids, are typical of liver, spleen, and bone marrow. They are larger than the other types and characterized by the absence of a basal lamina and the presence of spaces between endothelial cells.

Lymphatic Vessels

These vessels transport fluid from the tissues back to blood vessels; they begin as blind-end tubes that form capillaries, thereafter becoming lymphatic vessels that terminate in the thoracic and right lymphatic ducts. The lumen of lymphatics is larger than that of blood vessels, is more irregular, and contains valves [8]. Due to their physiological role, lymphatic capillaries have to be highly permeable and therefore are devoid of a continuous basement membrane [9]. Moreover, unlike blood capillaries, they are almost always surrounded by a thin network of elastic fibers that may help in the propulsion of the lymph (Fig. 11). Going from the abluminal surface of these capillaries toward the extracellular matrix, oxytalan, elaunin and typical elastic fibers may be observed [10].

CELLULAR AND EXTRACELLULAR COMPONENTS

Endothelium

Endothelial cells constitute a monolayer of flat, expanded cells underlying the interior of the whole cardiovascular system (Figs. 3, 4, 5, 9, and 11) [11]. The biology as well as the pathological behavior of endothelial cells has been intensively studied, because they form the first and probably the most important barrier for the transport of nutrients to all organs [12]. Under physiological conditions, endothelial cells produce several substances involved in the regulation of vessel tone and permeability, hemostasis, immune and inflammatory reactions, fibrinolysis, and cell proliferation, such as nitric oxide, prostacyclins, bradykinin, acetylcholine, histamine, endothelin, thromboxane, adhesion molecules, tissue plasminogen activator and its inhibitor, angiotensin, von Willebrand factor, and several growth factors. Von Willebrand factor is synthesized and stored within the Weibel-Palade bodies of endothelial cells and is released when cells are damaged. It is involved in coagulation as it mediates platelet adhesion and fibrin formation and is required for factor VIII stability in the plasma. Metabolic characteristics and synthetic properties of endothelial cells as well as their products are mentioned in different sections of this chapter.

Myocytes

These cells constitute the heart tissue: they are very large with a centrally located nucleus and numerous mitochondria, are delimited by a cellular membrane called the sarcolemma, and exhibit numerous myofibrils (about 1 μm wide, 2 μm long) that represent 50% of cell volume. Their cross-striated appearance is due to the ordered arrangement of A-bands and Z-lines of contiguous myofibrils. The peculiar properties of myocytes, which contract or relax in response to changes in cell membrane potential, are a consequence of the sarcoplasmic reticulum forming a network of tubules that surround the myofibrils and are engaged in Ca^{++} release and uptake. The contractile proteins, in fact, are organized in sarcomeres and composed of myofilaments: the thicker are made of myosin and represent the A-bands, the thinner are made of actin and form the Z-lines at the ends of the sarcomeres [1]. In these areas is located a continuous coil of tropomyosin that interacts with actin and troponin in the complex that forms the contractile apparatus. In addition to these components, the sarcomeres contain other proteins

such as α-actinin, protein C, titin, and nebulin that can modulate sarcomere function [13].

Smooth Muscle Cells

Smooth muscle cells account for the major portion of the vessel wall and form concentric layers around the vessel lumen just beneath the intima. They are responsible for the homeostatic control of the organization of the vessel wall as major producers of elastin, collagens, proteoglycans, and glycoproteins [14]. Vascular smooth muscle cells *in vitro* are able to reproduce layers of cells intercalated by elastic fibers and collagen, mimicking the construction of a vessel wall. Recently, this property has been utilized to produce *in vitro* a vessel fulfilling the fundamental requirements for grafting [15]. In mature vessels, smooth muscle cells exhibit a contractile phenotype. They express mainly vimentin, as do other mesenchymal cells, but also smooth muscle cell α-actin, myosin, and desmin. In particular, desmin and vimentin are highly expressed in muscular arteries, whereas desmin is poorly represented in elastic arteries [16]. Interestingly, a few smooth muscle cells have been shown also to express cytokeratin, which is a typical marker for epithelial cells [16]. These data sustain the hypothesis that smooth muscle cells may have multiple origins; in fact, depending on vessel location, these cells originate from lateral mesoderm, cardiac neural crest, splanchnopleural mesoderm, dorsal mesocardium, and also endothelium [17]. Smooth muscle cells make contacts with each other and with elastic fibers (Fig. 6). Moreover, vessel smooth muscle cells promptly respond to endothelial injury by migrating towards the intima, proliferating and synthesizing a number of matrix molecules. These events are considered to be at the basis of intimal expansion and atherosclerotic plaque formation [18].

Fibroblasts

Fibroblasts are present in the connective tissue associated with the whole cardiovascular system (Fig. 3). In the vessel wall they are scattered among bundles of collagen fibrils and elastic fibers and are more strongly represented in the tunica adventitia (Fig. 5). Their presence is essential for the resistance of the vessel wall as they produce the collagenous extracellular matrix that confers tensile strength to the organ. No specific studies have been performed on adventitial fibroblasts; however, their presence has been considered of some importance in the production of engineered blood vessels in culture. Actually, artificial tubes of smooth muscle cells, seeded with endothelial cells on the internal lumen and coated by adventitial fibroblasts, were found to behave as good complete blood vessels [15]. Moreover, adventitial fibroblasts seem to be the major source of cell proliferation upon damage [19], and may differentiate into myofibroblasts [20,21].

Collagens

Apart from the elastic arteries, the major structural proteins of the cardiovascular system are collagens, in particular, types I and III (Figs. 3–8). In the adult human heart, collagen, measured by hydroxyproline, reaches an amount of 300–350 mg; of this, collagen type I accounts for about 85%, and type III for 10%, of the matrix produced by local mesenchymal cells. In vessels, the collagen content represents more than 30% of the total dry weight and varies along the vessel tree, being relatively more abundant in muscular arteries and in the adventitia of the vessel wall (Fig. 5). With age and in pathological conditions, collagen content may vary [22,23]; it usually increases relative to other vessel constituents as a result of reparative processes following injury [24] or hypertension [25,26]. As in other organs, the homeostatic balance between collagen synthesis and collagen degradation is regulated by several cytokines, either locally produced or transported by the blood stream.

Human heart fibroblasts have been shown to increase the production of type I collagen and TIMP mRNA in response to the addition of TGF-β, whereas collagenase secretion into the culture medium is stimulated by bFGF [27]. The presence of matrix metalloproteinases, MMP-2 in particular, in the myocardium, suggests that these enzymes may play a role in maintaining the integrity of the cardiovascular structure [28,29].

Types IV and V collagens are the major components of the basal lamina and the subendothelial space in the endocardium and beneath capillaries, acting as an antithrombotic barrier. Collagen type VI is present in the subendothelium, the media, and the adventitia. Moreover, collagen types VI, XII, and XIV are associated with laminin in different areas of the venous walls [30].

Elastin

Elastic fibers and lamellae confer extensibility and elastic recoil upon tissues, and are the major constituent of the largest arteries (see also Chapter 3, this volume). In human aorta, elastin represents about 20% of the total dry weight and its content per unit area decreases from the proximal to the distal aorta, there being a 58% decrease between the suprarenal and the infrarenal aorta [31]. Elastin synthesis is very active around birth and continues during body growth. The soluble monomeric precursor, tropoelastin, is rapidly and efficiently polymerized to mature insoluble elastin (Fig. 12a) by the copper-dependent enzyme lysyl oxidase (see also Chapter 3, this volume). Once laid down, insoluble elastin appears to turn over only very slowly, if at all [32,33]. Both *in vivo* and *in vitro,* elastin synthesis has been shown to be stimulated by TGF-β acting at both pre- and post-translational levels [34–36]. However, in spite of the clear evidence for developmental regulation of elastin production, and for altered elastin synthesis in pathological conditions, mechanisms controlling elastin production are not completely known [37,38]. Glucocorticoids have been reported to increase elastin synthesis, at least in the fetal period [39–41]. *In vitro*, elastin production is negatively influenced by lowering the pH and by increasing the concentration of sodium and potassium, whereas it is insensitive to several substances, including nonsteroidal anti-inflammatory agents, α- and β-adrenergic agonists, and histamine [42]. The degradation of elastin in vessels seems mainly to be influenced by local degrading enzymes, among which is elastase, and free radicals produced by local metabolic events, such as the radical species, nitric oxide, or by moderate inflammatory processes occurring as a consequence of lipid infiltration into the vessel wall. It has to be noted that elastin is a very hydrophobic protein that can bind unsaturated lipids rather efficiently, thus increasing its susceptibility to degradation by elastases and/or other proteolytic enzymes [43]. It is also possible that interactions with other extracellular components, such as glycosaminoglycans or proteoglycans, may modify the sensitivity of elastin to enzymatic degradation.

Glycoproteins

The glycoproteins most represented in the arterial wall are laminin, a constituent of the basal lamina of endothelial and smooth muscle cells, and fibronectin and vitronectin, which

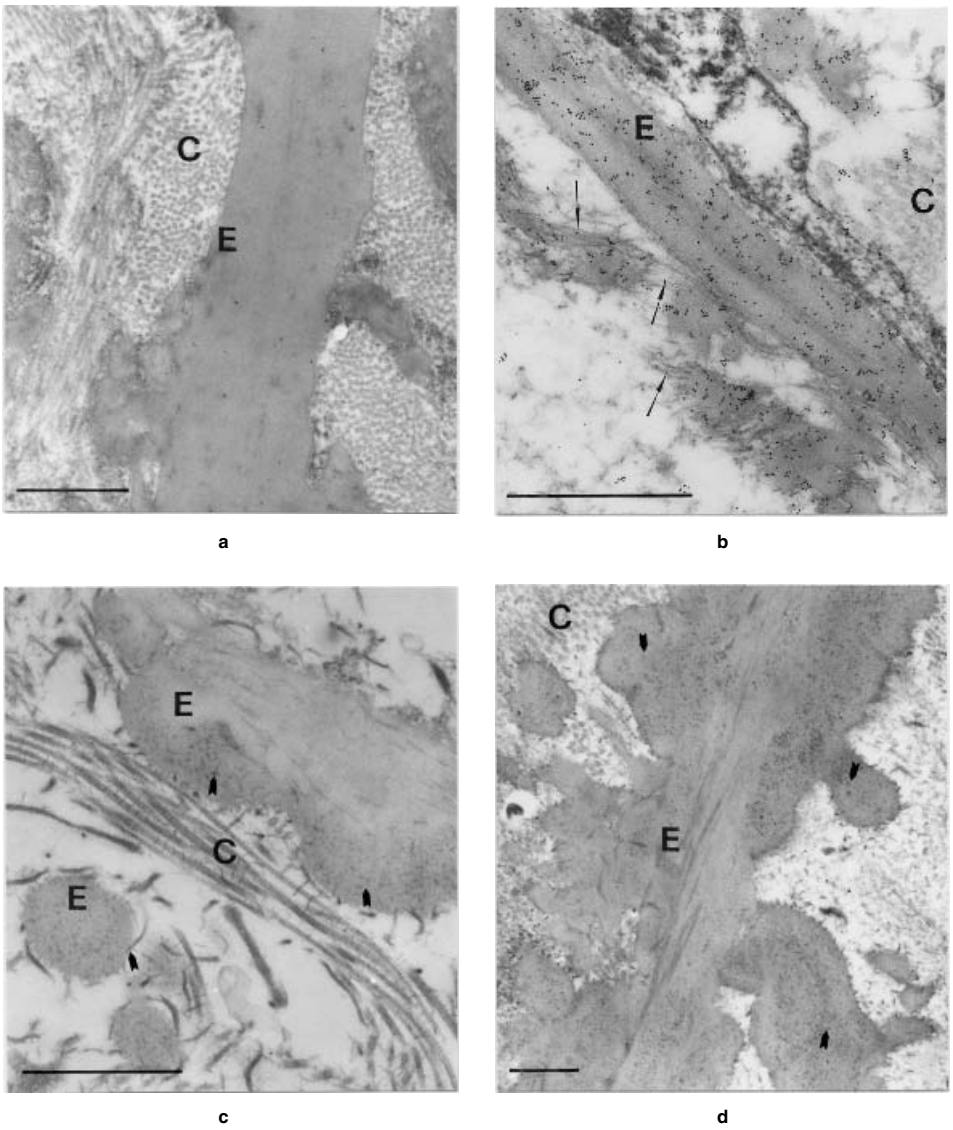


Figure 12. Electron micrographs of aortic collagen (C) and elastic fibers (E). In adult subjects (**panel a**) elastin (E) appears as lamellae of amorphous material surrounded by collagen fibrils (C). Microfibrils associated with the elastic lamellae are clearly visible in a longitudinal section from a fetal human aorta (**panel b**, arrows). Elastic fibers further contain several other molecules beside tropoelastin, such as osteopontin, that has been immunolocalized and visualized by means of gold particle-conjugated antibodies (**panel b**). Glycosaminoglycans are known to be major constituents of elastic fibers, and can readily be observed as alcian blue precipitates after the inhibition of lysyl oxidase in experimental lathyrism (**panel c**, arrowheads) as well as having been observed in a patient with Menkes disease (**panel d**, arrowheads). Bars: 1 μm.

serve to attach endothelial cells and adventitial fibroblasts to the matrix scaffold [44–46] (see also Chapter 5, this volume).

Laminin

The laminins are a family of molecules formed by the association of three from eight genetically distinct chains. Laminin 1 is the major constituent of basement membranes and plays a role in embryonic development, tissue homeostasis, and remodeling, whereas laminin 2 seems mostly to be involved in maintaining the integrity of muscle cell function [47].

Fibronectin

Fibronectin, by actively interacting with collagens and glycosaminoglycans (GAGs), is involved, for example, in the attachment and polarity of endothelial cells and in their migration during angiogenesis [45]. Its transcription is induced by serum, cAMP, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, and angiotensin II.

Vitronectin

Vitronectin modulates cell migration, tissue remodeling, and proteolytic degradation. Within the cardiovascular system it is mainly localized near elastic fibers, in the media, and adventitia of blood vessels [48]. Moreover, it has been suggested that vitronectin is also internalized and translocated to the subendothelial matrix by an integrin-independent mechanism that requires the presence of the

urokinase (u-PA) receptor [49]. In the subendothelial matrix, in association with fibronectin, vitronectin may play a role in platelet–vessel wall interactions [50] through Arg-Gly-Asp (RGD)-binding [51].

Osteopontin

Osteopontin, an extracellular matrix phosphoprotein that is produced in many tissues in response to inflammation and injury, is induced in both cardiac myocytes and microvascular endothelial cells by glucocorticoids and, in turn, reduces the expression of nitric oxide synthase in pretreated endothelial and cardiac cells [52]. We have recently shown that osteopontin is present in the normal arterial wall in humans, where it is associated with elastin fibers (Fig. 12b). Given the inhibitory effect of osteopontin on mineral crystal growth, we have suggested that its presence inside elastic fibers may prevent the occurrence of mineral precipitates over time [53].

Elastin-Associated Microfibrils

Elastic fibers are associated with arrays of microfibrils (Fig. 12b) that are composed of a series of proteins, including fibrillins, microfibril-associated glycoproteins (MAGP), emilin, and others (see also Chapter 3, this volume). The importance of fibrillins, in particular fibrillin 1, localized on human chromosome 15, has been recognized in the last decade by the discovery that mutations in the *FBN1* gene are the fundamental molecular defect in Marfan syndrome (see also Chapter 12, this volume). Microfibrils have been suggested to serve as a scaffold for elastin fibrillogenesis [54] and therefore may play a regulatory role during development. In developing aorta, for instance, fibrillins, in particular fibrillin 2, and tropoelastin are up-regulated simultaneously during elastogenesis, whereas MAGP mRNA levels remain constant. The peak of elastin and fibrillin gene expression in the human aorta is reached within the first year of life, following which there is an age-dependent progressive decline [55]. These data suggest that microfibril composition may change according to structural and/or physiological needs [56] and support the hypothesis that microfibrils have also anchoring functions, running from the surfaces of elastic fibers to plaque sites on smooth muscle cell plasma membranes [57].

Proteoglycans and Glycosaminoglycans

Proteoglycans, particularly those containing chondroitin sulfates, are present in great amount in the cardiac jelly and cardiac extracellular matrix during development and their relative content declines with age. The proportion of proteoglycans in adult aorta is relatively low (0.5–1% of the dry weight); however, their extended structure is sufficient to completely fill the aqueous spaces of the tissue and to allow multiple contacts with cells and other extracellular matrix constituents.

The vessel wall contains several proteoglycan species that are differently distributed from the intima to the adventitia, and that may function as structural elements of the extracellular matrix or modulators of cell adhesion or biohumoral processes [58]. Heparin, for example, may inhibit smooth muscle cell proliferation and migration. Moreover, it may negatively influence intimal thickening and elastin and collagen production, whereas, on the contrary, it seems to stimulate the synthesis of proteoglycan subclasses [59]. Very little is known about the structural role of proteoglycans within the vessel wall. By the use of cytochemical markers, proteoglycans have been shown to form a three-dimensional scaffold connecting all vessel constituents, to be more abundant in the intima, and to be strictly associated with elastin, especially when lysyl oxidase activity is chemically (lathyrism) (Fig. 12c) [60] or genetically (Menkes disease) (Fig. 12d) [61] inhibited. The biological role of these molecules may depend on both their protein and carbohydrate moieties. For example, thrombomodulin, a protein exposed on the endothelial surface, contains a chondroitin sulfate chain that enhances the affinity of thrombomodulin for thrombin, thus preventing coagulation [62]. The amount, as well as the nature and distribution of proteoglycans, has been widely studied in relation to their possible role in degenerative processes of the arterial wall, such as atherosclerosis [63]. Arterial proteoglycans have been shown to interact with circulating low density lipoproteins (LDL), inducing structural rearrangements of both the lipid and the protein moieties of the lipoproteins. This would lead to increased uptake of modified LDL by macrophages and arterial smooth muscle cells, the accumulation of lipids within cells [64], and increased smooth muscle cell proliferation [65]. However, recently, proteoglycans, and in particular heparan sulfate-containing proteoglycans, have been suggested to have a protective effect against the peroxidation of LDL. Peroxidated LDL would favor macrophage uptake and thrombotic events affecting both endothelial cells and platelets [66]. Other physiological roles of heparin-containing proteoglycans appear to be in the control of the release of growth factors affecting neighboring cells [67,68] and modulation of smooth muscle cell proliferation and migration [59].

CELL–CELL AND CELL–MATRIX INTERACTIONS

Given its role in the transport and controlled release of oxygen and nutrients to all parts of the body, the correct functioning of the cardiovascular system is strictly dependent on its structural organization, which, in turn, relies on cell–cell interactions on the endothelial side and cell–matrix interactions in the remaining vessel wall (Table 2). In the heart, cell–cell interactions are essential for stimulus transmission through the organ. Gap junctions, for example, play a crucial role in the synchronized contraction of the heart [69]. Their functional unit, the connexon, is composed of six identical transmembrane protein subunits, called connexins, which form a channel that mediates electrical and chemical coupling between cells. This channel is a dynamic structure that undergoes reversible conformational changes allowing its closure in response to changes within the cell [70]. Furthermore, contraction of both interacting and single myocytes may be modulated by N-cadherin, a cell-surface Ca^{2+}-dependent adhesion molecule, found in intercalated disks and extrajunctional sites of the myocardium. In addition, it has been hypothesized that N-cadherin may also promote the myofibril formation necessary for functional activity of the myocardium [71].

Different types of endothelial cell interactions have been described. Tight, gap, adherens junctions, desmosomes, and syndesmos are variously represented in the vessel tree in relation to the requirement for permeability control, coordination of cell migration and replication during angiogenesis, and injury repair [72,73]. In particular, tight junctions are mainly comprised of a transmembrane protein called occludin, which is directly or indirectly associated with cytoskeletal proteins. Adherens junctions appear in different shapes and sizes and contain the transmembrane glycoprotein

TABLE 2. Adhesion Molecules

Family	Names	Structure	Function
Cadherins	B-cadherin E-cadherin M-cadherin N-cadherin P-cadherin R-cadherin T-cadherin VE-cadherin	Polypeptides of 750 aa with an extracellular, a transmembrane and a small highly conserved cytoplasmic domain that interacts with proteins related to the 'armadillo' family (β-catenin, plakoglobin, and p120)	Cell–cell adhesion proteins developmentally regulated. Are involved in morphogenetic processes, embryogenesis, tumor invasion and metastasis, vascular remodeling
Integrins	$\alpha_1\beta_1$; $\alpha_2\beta_1$; $\alpha_5\beta_1$ $\alpha_6\beta_4$; $\alpha_7\beta_1$; $\alpha_4\beta_7$ $\alpha_X\beta_1$; $\alpha_L\beta_2$; $\alpha_M\beta_2$ $\alpha_{IIb}\beta_3$; $\alpha_V\beta_3$; and others	Glycoproteins comprised of two noncovalently associated subunits: α_n(150–200 kDa) and β_n (90–110 kDa) differently combined	Cell–cell and cell–matrix adhesion molecules. Involved in embryogenesis, platelet aggregation, inflammation, immune functions, wound repair, metastasis, angiogenesis
Ig superfamily	CD2; CD4; CD8; CD22; CD58; C-CAM; N-CAM; ICAM-1,-2; PECAM-1; CEA, MAG; contactin; and others	Proteins of 70–110 aa organized in two parallel beta sheets; the primary structure is highly variable, whereas the tertiary is quite constant	Cell–cell adhesion molecules. Involved in development and regulation of the immune system, contribute to the assembly of vascular structures
Selectins	E-selectin (ELAM-1) L-selectin P-selectin	Characterized by three different domains: a calcium-dependent lectin domain at the N-terminal; an EGF-like domain and several CRP repeats	Cell–cell adhesion molecules. Involved in the regulation of inflammatory and immunological events at the vessel wall, mainly promoting leukocyte extravasation

CAM, cell adhesion molecule; ICAM, intercellular adhesion molecule; PECAM, platelet and endothelial cell adhesion molecule; CEA, carcino-embryonic antigen; MAG, myelin-associated glycoprotein; ELAM, endothelial leukocyte adhesion molecule.

E-cadherin. The cytoplasmic portion of E-cadherin forms complexes with α-, β-, and γ-catenin and plakoglobin, which, together with other proteins such as vinculin, constitute a plaque at which actin microfilaments insert. Desmosomes are membrane domains traversed by the two types of desmosomal cadherins, the desmogleins and the desmocollins, the cytoplasmic tails of which contribute to the formation of a dense plaque containing plakoglobin and desmoplakin. Lymphatic endothelia exhibit junctions of variable size and shape called complexi adhaerentes, among which, syndesmos allow connections between the retothelial cells of lymph node sinuses. Syndesmos are almost negative for vinculin and α-actinin, and have a desmoplakin- and plakoglobin-rich plaque, the specific transmembrane proteins of which include the endothelial cadherin-5 (also called VE-cadherin). In contrast to desmosomes, the syndesmos are negative for the known desmosomal cadherins and also for E- and M-cadherin [74,75]. It has been hypothesized that adherens junctions and VE-cadherins, in particular, play a major role in vascular remodeling by controlling and limiting endothelial cell migration and growth [73].

Usually, the number of specialized endothelial junctions is greater in large vessels than in capillaries and postcapillary venules and, moreover, their presence and functioning are regulated by constitutive as well as local factors. In the brain microvasculature, for example, endothelial cells have unique metabolic and permeability properties induced by astroglial cell-released factors [76]. Furthermore, endothelial cells serve as a nonthrombogenic, selectively permeable boundary between the blood stream and the extravascular space. On the luminal side, endothelial cells present a series of ligands and receptors for cells and molecules circulating in the blood stream, namely CAM proteins, members of the immunoglobulin and selectin gene families [77,78], and heparan sulfate proteoglycans [79,80]. The expression of these receptors is finely regulated as they participate in bidirectional transmembrane signal transduction, lipid metabolism [81], and microvessel involvement in the inflammatory process in response to bacterial endotoxins [lipopolysaccharides (LPS)] through the up-regulation of interleukin (IL)-1 and tumor necrosis factor (TNF)-α [82]. Endothelial adhesion receptors clearly play an important role also in hemostasis and thrombosis as well as in cancer metastasis [83]. Leukocyte and LDL adhesion to the endothelium, for example, is mediated by several adhesion molecules, namely vascular cell adhesion molecule (VCAM)-1, ICAM-1, and ELAM-1, that in turn may activate the transcription factor NF-κB, a proinflammatory molecule able to up-regulate the gene expression of cytokines and cell-adhesion molecules [84].

On the basement membrane side, endothelial cells interact mainly with laminin, collagen type IV, and heparan sulfate proteoglycans through specific integrins (Table 2). These are heterodimeric membrane proteins mediating cell attachment to the extracellular matrix [85]. Following the binding of the integrin to its ligand in the matrix, the cytoplasmic domain of the β-chain interacts with talin, which, in turn, binds to vinculin, which is associated with α-actinin and is thus linked to an actin filament. Integrin-mediated

cell–matrix interactions control the formation of the matrix itself as well as the expression of the number and nature of integrin receptors on the cell membrane [86]. Resting endothelial cells and smooth muscle cells of the vessel wall express integrins α1β1, α5β1, α6β1, and αvβ3, which are receptors for collagens, fibronectin, laminin, and vitronectin, respectively [83]. The use of transgenic or "knockout" mice involving genes for adhesion or matrix molecules is revealing that integrins as well as cell–matrix interactions are of paramount importance in the development and maintenance of integrity of several organs, among which is the cardiovascular system [87,88].

Cell–matrix interactions seem also to regulate the phenotype of vessel smooth muscle cells; in fact, adhesion to laminin, through integrins α3β1 and α2β1, permits the persistence of the contractile nonproliferative phenotype, whereas adhesion to fibronectin through integrin α5β1 leads to *ras* activation and cell proliferation [89].

Vascular cells, as well as monocytes, neutrophils, and lymphocytes, express a multifunctional 67-kDa protein that differs structurally and functionally from other matrix adhesion molecules and is known as an elastin receptor, functioning as a chaperone that facilitates the secretion of tropoelastin and the assembly of elastic fibers. Similarly to the transmembrane selectins, the 67-kDa protein has lectin-like properties, but unlike integrins and cadherins, it is not a transmembrane molecule, although it can be immobilized on the cell surface by association with two other membrane-anchored proteins, mediating cell–matrix interaction in a calcium-independent manner and recognizing the secondary structure of matrix macromolecules. It has been hypothesized that the 67-kDa protein may play a role in the interactions between vascular smooth muscle cells, leukocytes and several components of extracellular matrix in physiologic as well as pathologic conditions [90].

STRUCTURAL CHANGES

Developmental Changes

The cardiovascular system develops quite early during embryogenesis [91]. The heart is already present as a tube in the human embryo at 19 days of gestational age, when cellular aggregates increase their mass and form a lumen, thus creating the first vascular network through the process of vasculogenesis. At the stage of 21–22 days, the vascular system is bilaterally symmetrical and the heart is organized into three different layers: an inner layer, also called cardiac jelly, poorly structured and devoid of cells; a middle layer comprised of numerous cells, some of which start to exhibit the contractile properties typical of the myocardium; and an outer layer that takes the form of a monolayer of mesenchymal cells. Cardiac septation is almost completed by the 27th day of embryonal age. One of the most well-known mammalian cardiogenic transcription factors is MEF2, which binds to regulatory DNA sequences in both early cardiogenic precursor cells and differentiated cardiomyocytes [92]. During the third and fourth weeks, venous and coronary vessels are formed from epicardial vascular buds. When the vascular tubes associate with pericytes, growth of blood vessels proceeds by angiogenesis, a phenomenon characterized by the disruption of basement membranes, and the migration and proliferation of endothelial cells to form new vessels. Thickening of the tunica media and adventitia of the vessel wall due to collagen deposition and the formation of the elastic laminae occur between the fourth month and birth, and continue during the first year of life [93,94]. The role of cell adhesion molecules in cardiovascular system development has been widely investigated. In particular, β1- and β3-containing integrins seem to play a major role, since impairment of binding to β1 integrins can block lumen formation of the dorsal aorta in chick embryos [95]; it has been suggested that these integrins are crucial for later development, whereas they do not seem to influence angioblast differentiation [96]. By contrast, VCAM-1 or α4 integrin mutant embryos reveal, beside placental abnormalities, loss of the epicardium and lack of subepicardial vascular development [96]. Furthermore, connexins seem to be critical for heart contraction, since alterations in connexin 43 cause blockage of the right ventricular outflow and consequent perinatal mortality [97]. During fetal and early postnatal life, the heart increases in mass through an increase in the number of myocytes. However, after birth, cardiac myocytes lose their ability to divide and, during life, the response to work overload is assured only by cellular size increase. The mechanisms involved in the terminal differentiation of myocytes are still under investigation.

Several growth factors are also involved in the induction of lateral plate mesoderm and subsequent cardiac organogenesis. Among these, TGF-β superfamily factors have been shown to display spatial and temporal expression suggestive of cardiac muscle induction, septation and valve formation [98–100]. TGF-β factors have been shown to stimulate cardiac differentiation of axolotl mesoderm explants [101] and to induce the expression of sarcomeric proteins, such as myosin heavy chain, titin, α-actinin, and troponin I, in splanchnic mesodermal cells *in vitro*. Moreover, antibodies to TGF-β or antisense oligonucleotides against TGF-βs have been demonstrated to inhibit the induction of valve-forming mesenchyme in the endocardial cushions [102]. However, the complex role of TGF-β family growth factors has not yet been completely elucidated. Interestingly, it has been demonstrated that mice deficient in TGF-β1 develop a normal cardiovascular system accompanied by an inflammatory cardiomyopathy [103]. Moreover, TGF-β1 seems to have either stimulatory or inhibitory effects on cardiac gene expression that differ depending on the target gene, being more active in up-regulating the array of fetal cardiac genes characteristic of myocardial hypertrophy, such as those for smooth muscle α-actin, SkA, β-myosin heavy chain (MHC), and atrial natriuretic factor (ANF) [104]. Furthermore, TGF-βs, in particular TGF-β1, are very much involved in the production of the extracellular matrix; however, very little is known about such involvement in the early stages of cardiovascular development [105], where the relationships between cells and between cells and matrix are fundamental in cell migration, cavitation, and modeling of the tubular organization of vessels, in the organization of the vessel wall, and, later, in the maintenance of the nonadhesive properties of blood cells in respect of endothelial cells [106,107]. It has been shown that fibrillar fibronectin and collagen type I are necessary for normal endothelial–mesenchymal interactions during early development and that alteration in the organization of both fibronectin and collagen type I by retinoic acid leads to transposition of the great arteries in a mouse model [108]. The role of connective tissue molecules (namely collagens, glycoproteins, glycosaminoglycans, and proteoglycans) during myocardial development is still under investigation. It has been demonstrated that myocardial growth proceeds from the epicardium toward the endocardium, with progressive

structural organization in strata. The interstitium grows in parallel with the myocardial growth, beginning with a thin network surrounding each fiber and progressively becoming more dense. Collagen fibers first appear at the epicardial level, especially around coronary vessels [109]. During heart development, vitronectin is present before endocardial cushion cell migration and remains until prevalvular structure formation [110].

Postnatal Changes

From birth and throughout life, the cardiovascular system undergoes continuous remodeling. Major changes occur at birth due to the transition from fetal to newborn circulation and consist of 1) closure of the foramen ovale as a result of increased blood flow and augmented left atrial pressure that becomes greater than that in the right atrium; 2) closure by collagen deposition of the ductus venosus sustained by an increase in the systemic vascular resistance; 3) closure of the ductus arteriosus, first by smooth muscle cell contraction and later by collagen deposition, related to high oxygen tension; and 4) dilatation of pulmonary vessels after separation of the pulmonary and systemic circulations [1].

During body growth, the vessel tree must increase its spread and ramifications through a series of controlled mitotic and apoptotic events [111], and by controlled matrix synthesis and degradation through a balance between the following three factors: the presence of growth factors, the regulation of receptors, and the control of growth inhibitors. These last include PF-4, thrombospondin-1, angiostatin, TGF-β, interleukins, and tissue inhibitors of metalloproteinases (TIMPs).

Age-Related Changes

In adults, the cardiovascular system has to overcome a series of mechanical, nutritional, and hormonal stresses that lead to a continuous rearrangement of its structural organization. Apart from cardiac myocytes, fibroblasts, as well as smooth muscle cells and endothelial cells, are capable of proliferation in the presence of growth factors, such as insulin-like growth factor (IGF)-1, basic fibroblast growth factor (bFGF), PDGF, and EGF, at least in part through activation of the p21-Ras/MAP kinase pathway [112,113]. TGF-β is also implicated in the hypertrophic growth of cardiac muscle cells upon mechanical load, where its release can be induced by the initial production of angiotensin II [114]. The pleiotropic functions of TGF-β isoforms have also been shown in the development of cardiovascular pathologies, such as hypertension, arterial restenosis, and infarct, in which these growth factors interact with others (IL-1, NO) in modulating cell growth and differentiation and matrix deposition [115].

With age, the heart, and in particular the left ventricle (Fig. 13), progressively increases in volume and weight, despite a numerical reduction of myocytes. The increased size of the aging heart, mainly as a consequence of peripheral vascular stiffening, is due to collagen deposition, increased myocyte size and the augmentation of adipose tissue especially in the subendocardial region [116,117]. These phenomena are more evident in men than in women. Moreover, there is an increased deposition of lipofuscin among myofibrils and of amyloid in the interstitial spaces in more than 50% of individuals over 70 years [118,119]. In the rat, collagen has been demonstrated to accumulate from the age of 3 months, the amount being doubled in 2-year-old animals [120]. Fibrosis is generally dispersed within the myocardium, although small areas of dense collagen bundles may be noted in the subendocardial and subepicardial areas [121].

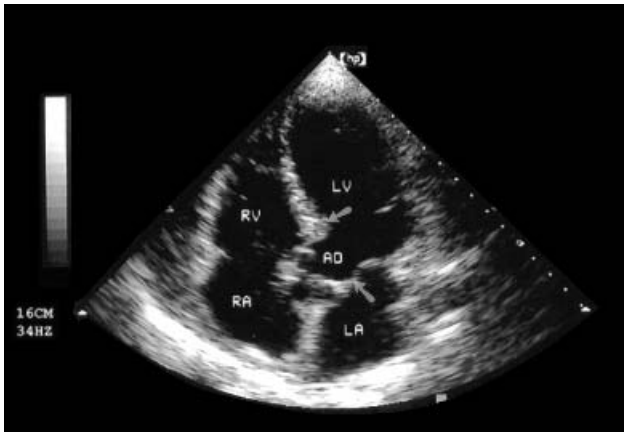


Figure 13. An apical ''4 chamber with aorta'' (AO) view of the heart by two-dimensional echocardiography of a 70-year-old asymptomatic subject. The thickness of the wall separating the right from the left ventricle and of the mitral valve leaflets is suggestive of a mild septal hypertrophy of the left ventricle (LV) and fibrosis of the valve (arrows). Compare Figure 2. LA, left atrium; RA, right atrium; RV, right ventricle (Figure courtesy of L.Targa, MD, Division of Cardiology, Este Hospital, Padova, Italy.)

Changes occur also in the morphological characteristics of the valves, such as fibrosis and degenerative calcification, which affect their function. Interestingly, whereas the relative content of elastin is independent of age, the amount of collagen decreases progressively, even though it becomes more cross-linked, and therefore less insoluble [3]. Furthermore, lipids, as well as amyloid, may also accumulate within valves and a fibroelastic hyperplasia may frequently be observed, especially at the insertion of mitral leaflets, the aortic cusp, and the anulus [122]. Aging is also associated with dramatic changes in the vascular system, i.e., vessel dilatation, vessel wall thickening, and reorganization of the cellular and extracellular compartments (Fig. 14) [123].

Several factors, including genetic and/or environmental components, contribute in human beings to the age-associated cardiovascular modifications [124]. It is therefore quite difficult to separate changes due to aging from those due to pathologic events and/or complications, such as hypertension and atherosclerosis [84,125]. We performed studies of vascular connective tissue components in the rat, in which it is much easier to investigate the role of the aging process itself (Fig. 14) [126,127], and how exogenous factors, such as diet, may influence these phenomena [128].

We have shown that the interactions between cellular and extracellular compartments as well as the relative proportions of collagen and elastin play a key role in conditioning the morphological and functional properties of the arterial wall. With time, endothelial cells become progressively more elongated, have reduced contacts, and form a more fragile barrier at the lumen surface. The subendothelial space consists of a coat of proteoglycans that changes quantitatively and qualitatively, thus contributing to alterations in the permeability and metabolism of the intima. *In vitro* studies, for instance, revealed that the concentrations of hyaluronic acid and chondroitin sulfate increase with time,

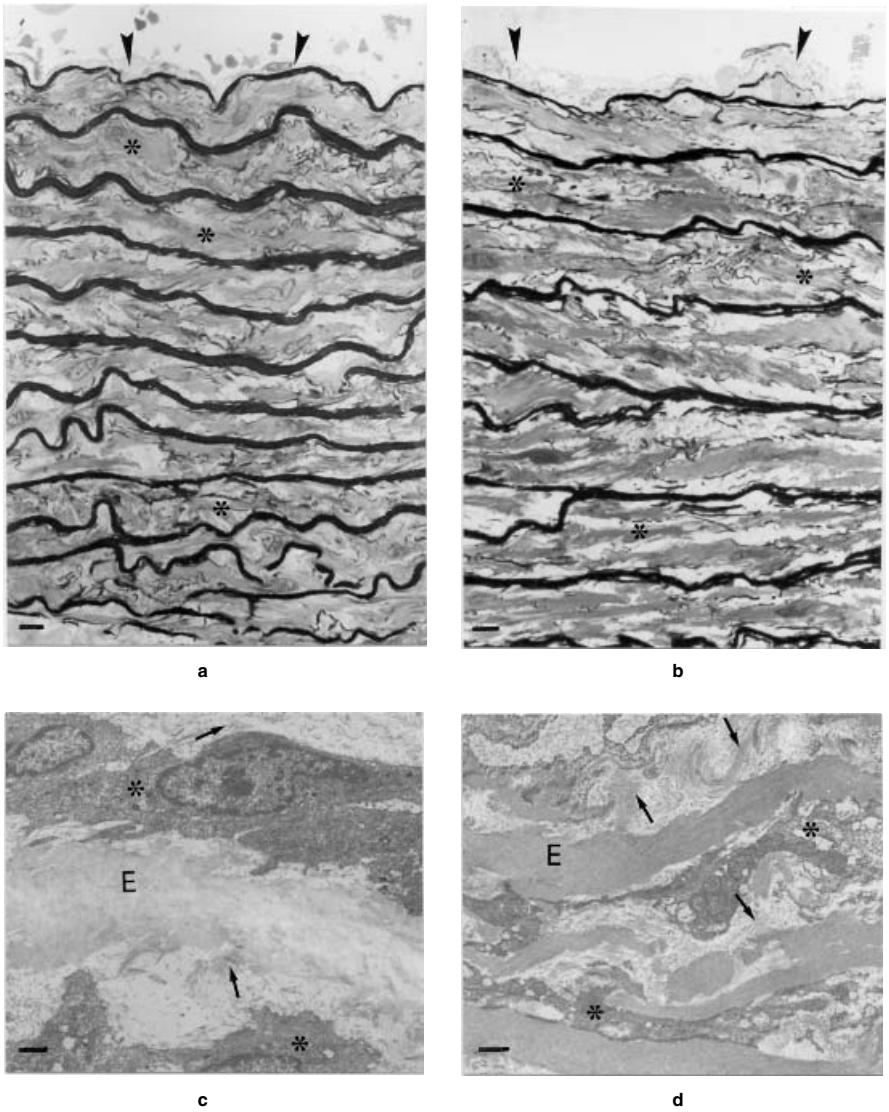


Figure 14. Light (**a, b**) and electron (**c, d**) micrographs of aorta from rats of 5 (**a, c**) and 29 (**b, d**) months of age. With time, there is an increase in the subendothelial space (arrowheads), elastic lamellae become less ordered (compare **a** and **b**; dark waved lines), and collagen increases in the extracellular matrix (compare **c** and **d**; arrows) contributing to the distortion of smooth muscle cells (asterisks) between elastic lamellae (E). Bars: **a, b**: 10 μm; **c, d**: 1 μm.

whereas that of heparan sulfate decreases. Ultrastructural studies have demonstrated that, with age, smooth muscle cells undergo several modifications with respect to shape, nucleus to cytoplasm ratio, abundance of cytoplasmic organelles, such as Golgi, endoplasmic reticulum, and mitochondria, and thickening of basal lamina [126,129]. Morphometric data indicate that the total number of smooth muscle cells is quite similar in young and old rats. On the contrary, with age, there is an increase of collagen volume density in the arterial wall due to the persistence of collagen type I synthesis, continuing lysyl oxidase activity [127] and the nonenzymatic glycosylation of amino-groups (Maillard reaction) [130,131] in collagen and other molecules, such as vitronectin. These changes may contribute to decreased adhesion and spreading of endothelial cells on matrix molecules and impaired endothelial function [132].

Vascular elastin metabolism has been shown to be age-related: elastin synthesis and deposition are particularly efficient in aortas of newborn and very young rats, then dramatically decrease [127], even though in some circumstances, as after surgical intimal injury or in the presence of atherosclerosis or hypertension, at least some smooth muscle cells seem to be able to produce large amounts of elastin together with other matrix proteins [133]. An increase, with age, of minor constituents of the cardiovascular extracellular matrix, such as fibronectin, has been shown by several authors [134,135]. Because fibronectin exhibits high affinity for LDL [136], this has been strongly related to some cardiovascular risk factors with age.

Even though the importance of growth factors in extracellular matrix regulation has been widely demonstrated in different experimental models, during development and

in pathologic conditions [137,138], little data is available regarding their role in the aging of the cardiovascular system. Cytokines, such as TGF-β, are able to stimulate matrix deposition also in systems aged *in vitro* [139]; moreover, vascular endothelial growth factor (VEGF) may significantly delay senescence of endothelial cells [140]. Interestingly, an age-related increase of PDGF-rβ and TGF-β1 expression has been observed in the rat aorta, whereas a decrease was noted in the heart [141]. The physiological role of these differences is not obvious.

INFLUENCE OF EXOGENOUS AND ENDOGENOUS FACTORS

Mechanical Loading

Mechanical stresses influence the structural and functional properties of cells at the molecular and genetic levels. In an *in vitro* model, static stretching has been demonstrated to stimulate tropoelastin synthesis by porcine aortic smooth muscle cells [142], sustaining the hypothesis that matrix secretion is regulated in response to hemodynamic forces. Collagen and elastin synthesis increase after elevation of intraluminal tension, but are inversely regulated along the aorta, elastin deposition diminishing proportionally from the arch toward the abdominal portion, whereas collagen secretion behaves oppositely [143,144]. Cell surface proteins and extracellular matrix are linked by transmembrane proteins to the cytoskeleton, and mechanical deformations can be converted to electrophysiological and biochemical responses. The vascular wall is continuously exposed to mechanical forces and reacts both acutely, by relaxation/contraction of smooth muscle cells, and chronically by the reorganization of the cellular and extracellular components. Endothelial cells respond to pressure and shear stresses with changes in ionic conductance [145], adenylate and guanylate cyclase activity (mediated by NO), inositol-triphosphate generation [146], and, within hours, with activation of genes for growth factors, endothelin and regulators of fibrinolysis. These events require the integrity of the cell membrane, cell–cell and cell–matrix interactions, and the cytoskeletal network [147,148]. At least some of these effects seem to be mediated by PDGF-B, as a stress response element has been found in its promoter region [149]. Mechanical loading also induces specific gene expression in cardiac myocytes leading to cell hypertrophy [150] and in vessel smooth muscle cells leading to the expression of muscle-specific myosin isoforms [151]. Also in these cells, mechanical stimulation is mediated by the extracellular matrix through cell–matrix interactions [152]. Chronic overload, such as hypertension, stimulates smooth muscle cells to produce large amounts of extracellular matrix, especially collagen, leading to a progressive fibrosis of the vessel wall.

Nitric Oxide

Nitric oxide is a free-radical gas produced by a family of enzymes (NOS) that catalyse the conversion of L-arginine to L-citrulline in the presence of oxygen and reduced NADPH. The enzymes are present in various organs and tissues, especially the nervous system, and have been immunolocalized to several areas of the heart and within endothelial cells. Both cardiac and endothelial NOS mRNAs increase *in vitro* upon the addition of IL-1β, TNF-α, or IFN-γ, and decrease in the presence of dexamethasone or TGF-β [153]. *In vivo*, NO production is induced by chronic exercise in dogs [154]; *in vitro*, NOS expression is induced by cyclic strain, shear stress [155], and a low concentration of oxidized LDL [156], whereas it is depressed by hypoxia, TNF-α, and high concentrations of LDL. Released from the endothelial cells, NO diffuses in a few seconds to smooth muscle cells, where it acts as a relaxant by binding to the heme iron at the active site of guanylate cyclase, thus generating cGMP. Thus, NO, formerly called endothelium-derived relaxing factor (EDRF) [157], is a powerful vasorelaxant, and is considered the major physiological regulator of basal blood vessel tone. Interestingly, basal release of NO may vary considerably among vessel types and diameters, being secreted more abundantly in smaller rather than larger arteries and veins, but its production may be increased in large arteries undergoing mechanical stress due to intense blood flow [158]. Moreover, NO has pleiotropic functions, and may act as a scavenger for superoxide anion in inflammatory reactions, and as an inhibitor of platelet aggregation, smooth muscle cell proliferation, and adhesion molecule expression [159,160].

Hypoxia

Chronic ischemia is an important factor in the process of angiogenesis. There is *in vivo* evidence that myocardial ischemia results in collateral development and the opening of preexisting vessels [161], and that chronic intrauterine hypoxia is associated with retinal neovascularization of the fetus. *In vitro*, endothelial cells cultured under hypoxic conditions show increased DNA synthesis and proliferation [162,163], very likely by up-regulation of the expression of VEGF [164]. Hypoxia, among a variety of other factors (thrombin, TGF-β, IL-1, angiotensin II, epinephrine), induces also the transcription, synthesis, and secretion of endothelin-1, a 21-amino acid peptide produced by endothelial cells, which acts as a transient vasoconstrictor by binding to specific receptors on smooth muscle cells. Endothelin secretion is regulated by cGMP- and cAMP-dependent mechanisms which are, in turn, activated by NO levels [165].

Low oxygen levels may also stimulate the secretion of adenosine, which is one of the most powerful vasodilators and one of the major regulators of coronary perfusion. Adenosine acts on the surface of vascular smooth muscle cells, activating specific receptors that block the entry of Ca^{++} into cells, causing vasodilatation; moreover, adenosine diminishes the atrioventricular conduction and myocardial contractility induced by adrenergic compounds [166].

Nutrients and Hormones

Among nutrients, the vascular system is particularly sensitive to copper, vitamin C, and vitamin A deficiencies. Copper is essential for the production of the active form of lysyl oxidase, the enzyme that catalyzes the first step towards the formation of stable intermolecular crosslinks in both collagen and elastin [167,168] (see also Chapters 2 and 3, this volume). Vitamin C is a cofactor of lysyl and prolyl hydroxylases, enzymes necessary in the biosynthesis of collagen molecules prior to their secretion, and increases collagen type I mRNA levels [169,170]. In contrast, vitamin C decreases elastin production by negatively influencing elastin mRNA stability and elastin gene expression [171]. Vitamin A deficiency during embryogenesis induces myocardial defects, suggestive of a role for the retinoid signaling pathway in cardiac gene regulation [172].

Homocysteine exerts a negative influence on endothelial cells, affecting their interactions with platelets and increasing thrombomodulin expression. Atherosclerosis is one of the major complications in patients with alterations in

homocysteine metabolism [173] (see also Chapter 13, this volume).

Nitrite ions, constituents of cigarette smoke, and meat preservatives, have been shown to react with insoluble elastin, inducing remarkable structural alterations, and could be responsible for some of the chronic vascular elastin damage associated with cigarette smoking [174].

Angiotensin I-converting enzyme (ACE) is a cell membrane peptidase constitutively present on vascular endothelial cells and inducible on smooth muscle cells and adventitial fibroblasts [175]. The product of the reaction is angiotensin II, a potent vasoconstrictor. ACE expression is stimulated *in vitro* by glucocorticoid hormones and bFGF; *in vivo*, a high level of ACE expression is mostly associated with renovascular or general hypertension. In humans, there is a considerable degree of ACE gene polymorphism. Although no conclusive evidence has been obtained for an association between high ACE levels, coronary artery disease, cardiac hypertrophy, and vascular wall thickening, the treatment of hypertension with ACE inhibitors has been proven to be highly beneficial in humans [176,177].

Among others, renin, aldosterone, norepinephrine and atrial natriuretic peptide (ANP) are known to regulate cardiovascular system homeostasis. Plasma ANPs, in particular, increase during rat growth and do not seem to change with age, whereas tissue immunoreactive ANPs are significantly increased in old animals [178]. Secretion of a series of hormones is altered in aged animals, thus contributing to the impairment of the senescent cardiovascular system [179].

RECENT DEVELOPMENTS

Response to Mechanical Stimulus

Additional information has been reported in relation to the modulation of connective tissue metabolism within the cardiovascular system in response to mechanical stimuli. The myocardium responds to mechanical stresses as a result of changes in blood pressure or volume by increasing both contractile and extracellular matrix protein synthesis [180].

Contractile Cells

Within seconds to minutes of onset, the unidirectional loading of myocytes stimulates signaling molecules. There is activation of membrane-associated phospholipases, an increase in inositol triphosphate, diacylglycerol, and arachidonic acid metabolites, stimulation of protein kinase C activity, and intracellular calcium release. Activated MAP-kinases then translocate to the nucleus and phosphorylate nuclear factors such as c-fos and c-jun, which form a heterodimer that binds to AP-1 sites in a number of gene promoters including those for matrix metalloproteinases and interleukins, as well as *COL1A1*, the gene for the α1(I) chain of collagen I [180].

Fibroblasts

Among the proteins and factors synthesized and released by cardiac fibroblasts are endothelin-1, TNF-α, and angiotensin II [181–183]. Cardiac fibroblasts *in vitro* have been shown not to respond to stretching by activating the Erk and Jnk signaling pathways when spread on collagen I, whereas they do show activation of both factors, or of Jnk alone, in response to stretching when cultured on fibronectin and on laminin or vitronectin, respectively. As there is increased expression of fibronectin, laminin, and vitronectin in pathologic conditions such as hypertension or ischemic injury, it may be that in the presence of such matrix glycoproteins, cardiac fibroblasts express Erk and Jnk, leading to increased collagen production [184].

Endothelial Cells

Endothelial cells transmit information regarding changes in shear, pressure, and tensile stresses to underlying cells by producing vasoactive substances and growth factors, such as PDGF-B, the promoter for which includes a shear stress response element [185]. A stretch response element involving the transcription factor Egr-1, and possibly Sp-1, has also been demonstrated in the promoter of the gene for PGDF-A [186]. Interestingly, the synthesis and release of PDGF-A chain in response to cyclic load can be blocked by RGD peptides, suggesting a crucial role for integrins in mechanical signal transduction [180].

Smooth Muscle Cells

Smooth muscle cells, which are relatively resistant to the antimitogenic effect of heparin, have been shown still to exhibit heparin-dependent inhibition of Erk activation and may develop c-fos-independent pathways for proliferation. Protein kinase C-dependent signaling through the Erk family of MAP-kinases activating c-fos transcription is selectively inhibited by heparin. Moreover, upstream of Erk, heparin decreases also the phosphorylation of Raf-1 in response to PDGF, without affecting PDGF receptor phosphorylation [187].

Integrins

There is growing evidence for the role of integrins as mechanosensors for both cardiac fibroblasts [180] and vascular smooth muscle cells [188]. Integrin-mediated cell adhesion and spreading lead to intracellular signalling and regulation of cell function similar to that observed in response to mechanical load, namely an increased focal adhesion kinase and phosphorylation and activation of a cascade of second messenger events. In particular, integrins α5β1 and αvβ3 are essential for the load-induced stimulation of procollagen synthesis and for the replicative response of aortic smooth muscle cells to mechanical load. Cells grown on fibronectin respond more dramatically, in terms of replication, than cells grown on collagen, laminin, or elastin, and the RGD peptides, the major extracellular binding domain of many integrins, partially inhibit the load-induced response.

It has been demonstrated that, in ventricular myocytes, voltage-dependent calcium channels are regulated in a cytoplasmic compartment close to the cell membrane and to the ryanodine-release channels of the sarcoplasmic reticulum, allowing a tight spatial control of the Ca^{2+}-induced Ca^{2+} release mechanisms [189]. Integrin β1-deficient cardiomyocytes display defective myofibrillogenesis and an absence of the typical electrical properties of ventricular-, atrial-, and nodal-like cells [190]. Therefore, integrins appear to play an important role during cardiac development and in the regulation of heart function.

Besides integrins known to link cell adhesion to intracellular signaling pathways, thereby determining whether a cell will survive, proliferate, or differentiate in response to soluble factors [191], several other proteins have been implicated in cell adhesion, migration, and proliferation, among which is galectin-1, a small β-galactoside-binding lectin, particularly abundant in cardiac smooth muscle. Galectin-1 has been shown to enhance DNA synthesis and to affect the adhesion

of smooth muscle cells *in vitro* by interacting with α1β1 integrin, laminin, and fibronectin [192].

Remodeling

Changes in the characteristics of newly deposited collagens may lead to alterations in the functional properties of the cardiovascular system. Li et al. have shown that an increase in the amount of collagen I and/or its enhanced cross-linking increase myocardial stiffness, whereas an increased amount of collagen III may facilitate myocardial compliance [193]. MMPs may facilitate the production of matrikines, peptides arising from the degradation of matrix components, and the tripeptide Gly-His-Lys, derived from the degradation of several matrix proteins, including α2(I), α2(V), and α2(IX) collagen chains, osteonectin, thrombospondin-1 and the fibrin α chain, actively stimulates new connective tissue formation [193].

Increased MMP activity may thus result in fibrillar collagen degradation, extracellular matrix synthesis and remodeling, and progressive structural changes in the cardiovascular system leading, for example, to ventricular dilatation. The interplay of MMPs, TIMPs, and their regulators, determines the progression of fibrotic processes in the heart. The level of extracellular MMP inducer (EMMPRIN) protein is very high in failing human hearts. Since mechanical stretch has been shown to induce neutral transmembrane MMPs in isolated cardiac fibroblasts, it has been suggested that EMMPRIN could be modulated *in vivo* by changes in the hemodynamic load [193].

With age, the structural architecture and composition of the extracellular matrix progressively and irreversibly change as a result of the fragmentation and loss of elastin, and the accumulation of GAGs, fibronectin, and collagen, accompanied by increased elastase and MMP activity, all of which contribute to a shift from an elastic to a stiff/fibrotic vessel. Both fibronectin and TGF-β expression are regulated by angiotensin II, and prolonged administration of ACE inhibitors is able to reduce some of these changes [194]. Advanced glycation end-products (AGEs), the products of irreversible, nonenzymatic glycation and oxidation of proteins and lipids, also exert an effect on vascular cells, indirectly, by cross-linking extracellular matrix proteins, thereby increasing vascular stiffness, and directly, by inducing pro-oxidant stress through cell surface receptors such as that for AGEs, RAGE, that is markedly increased in atherosclerotic lesions and after vessel injury [194].

The late events of angiogenesis, in which matrix degradation is associated with the proliferation of endothelial cells that migrate into the perivascular stroma, forming capillary buds and then tubes with a patent lumen, have been described by Deroanne et al. in an *in vitro* model of tubulogenesis [195]. The addition of native collagen to the matrigel substrate, leading to increased stiffness of the support, inhibits endothelial cells from forming tubes [195].

Stem Cells

The potential use and future applications of stem cells are currently under intensive investigation [196]. Not only hematopoietic stem cells [197,198], but also adult liver-derived stem cells [199], have been shown to respond to the microenvironment of the adult heart, *in vivo*, and to differentiate into mature cardiac myocytes. It has been shown that $Flk1^+$ cells derived from embryonic stem cells, and devoid of markers for endothelial cells or mural cells (smooth muscle cells and pericytes), can be stimulated to differentiate *in vitro* into endothelial cells under the influence of VEGF, and into smooth muscle cells under the influence of PDGF-BB [200].

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Chapter 1, Part V

Morphology and Chemical Composition of Connective Tissue: The Eye

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INTRODUCTION

The human eye contains a wide diversity of connective tissues that function in a coordinated manner both during the development of the eye and to ensure successful vision. It might, therefore, be anticipated that genetic diseases involving connective tissues generally throughout the body would also have profound effects on the eye and seriously impair vision. However, for reasons that are not entirely clear, this has rarely proved to be the case except for such notable examples as Marfan syndrome, which involves the zonular apparatus, often leading to ectopia lentis [1], or Stickler syndrome which features vitreous collapse due to mutations in either the type II or type XI collagen chains [2] (see also Chapters 12 and 23, Part II, respectively, this volume). In contrast, some genetic diseases of connective tissue specifically affect the eye. For example, various corneal dystrophies are now known to involve mutations in the product of the *BIGH3* gene, kerato-epithelin [3] (see Chapter 26, this volume).

In this limited chapter, it is not possible to provide a detailed morphological description of all of the connective tissues of the eye, and interested readers should consult a recent and highly readable textbook for details [e.g., 4]. Instead, this chapter specifically describes the connective tissue macromolecules present in each tissue of the eye together with some functional information and an indication of their potential involvement in genetic disease. A recent textbook [e.g., 5] should be consulted for more general information on the wide variety of genetic diseases that involve primarily the eye. Figure 1 shows the morphology of the human eye together with the location of the various connective tissues that will be discussed.

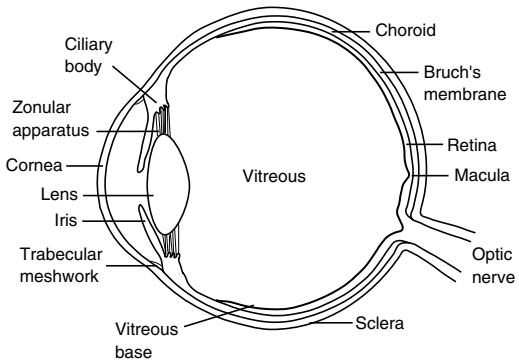

Figure 1. Diagram of the structure of the human eye.

CORNEA

The function of the cornea is to allow the clear passage of light. As such, the stroma contains highly ordered arrays of collagen fibrils of a constant small diameter that are associated with a number of small leucine-rich-repeat (LRR) proteoglycans [6]. In addition, the cornea also possesses an epithelial and an endothelial cellular layer, both of which are associated with highly specialized connective tissues known respectively as Bowman's layer and Descemet's layer (Fig. 2).

Stroma

For many years, the chicken cornea has provided a suitable model for studying both the morphology [7] and biochemical

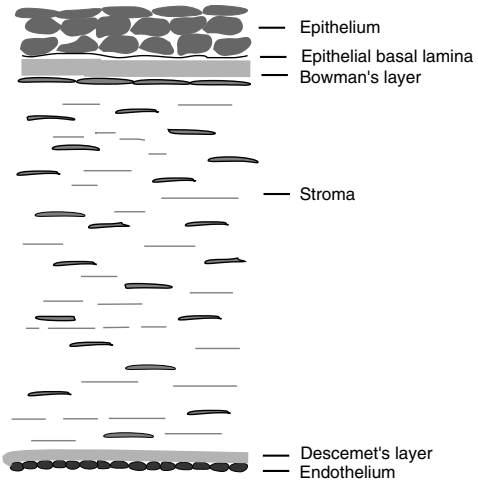

Figure 2. Diagram showing the different cell layers and connective tissues of the human cornea.

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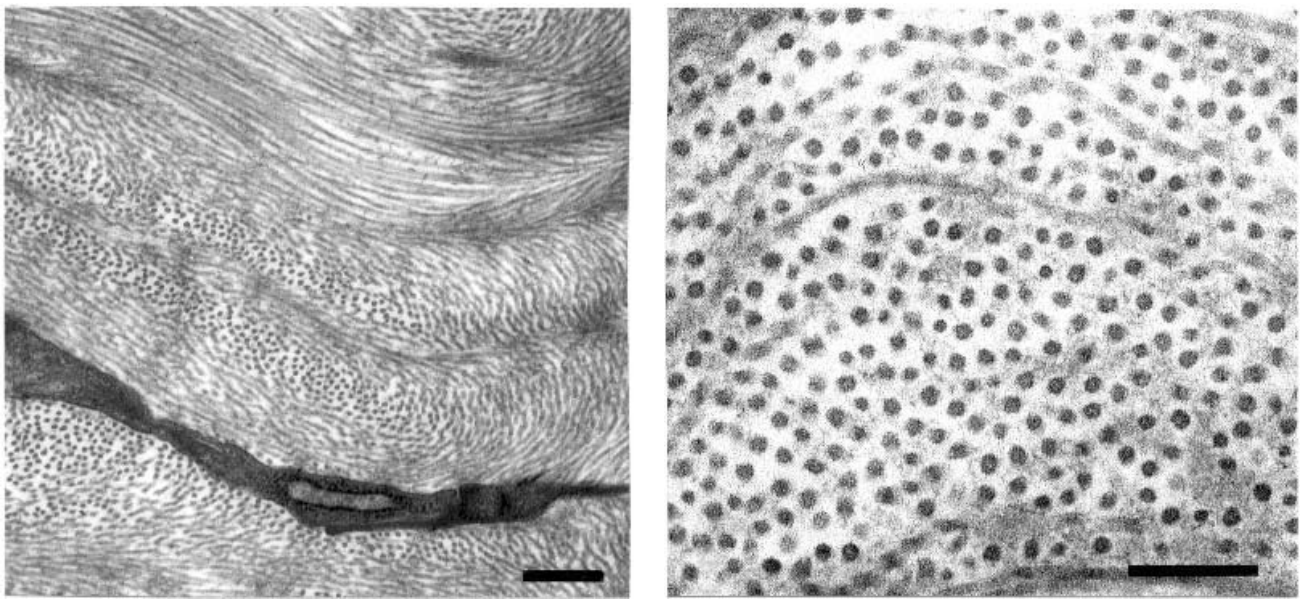

Figure 3. Electron micrographs of the adult mouse corneal stroma. **Panel A** represents a low magnification (bar = 500 nm) showing the lamellae of collagen fibrils. **Panel B**: higher magnification (bar = 200 nm) showing individual collagen fibrils and the extensive matrix between fibrils. Electron micrograph kindly provided by David Birk, Ph.D.

organization of the corneal stroma [8]. The latter review [8] emphasizes the structure of the primary stroma (which does not arise in development of the mammalian eye) and should be consulted for details of the potential mechanisms in birds by which the primary stroma swells and is replaced by the secondary stroma.

In this chapter, the structure and biochemical composition of the mammalian corneal stroma is reviewed in detail. Morphological observations illustrated in Figure 3 show that the collagen fibrils are of constant diameter and spacing and are organized into interweaving sheets as shown in Figure 4 [9]. Lateral associations between the collagen fibrils are also apparent from quick-freeze deep-etch electron microscopy (Fig. 5) [10]. How this highly ordered arrangement is initially achieved and subsequently maintained by the keratocytes remains unclear.

Collagen

The major collagen type of the adult mammalian cornea is type I but it is also reported that type V may constitute as much as 20% of the total collagen [11–14]. Mammals also differ from birds in exhibiting the presence of type III collagen in the corneal stroma [15,16]. The cornea contains the highest proportion of type V collagen of any tissue of the body and it is thought that this is directly related to the very tight control that must exist on fibril diameter within the stroma [8]. There is some evidence that supports this conclusion from experiments in which the synthesis of type V collagen during corneal development was selectively reduced, resulting in the formation of wider fibrils [17]. In addition, a transgenic mouse with a targeted deletion of a key exon of the α2(V) chain displayed a disruption in the organization of corneal collagen fibrils [18]. Remarkably, these results contrast with Ehlers-Danlos syndrome (EDS) I and EDS II patients who possess mutations in the α1(V) and α2(V) chains yet do not present with noticeably impaired vision (see Chapter 9, this volume). As described in Chapter 2, Part I, this volume, type V collagen is thought to be located such that its noncollagenous amino terminus projects from the surface of the fibril [19]. Both type XII and type XIV collagens are additionally present in the corneal stroma in appreciable amounts and often at discrete locations both in the chicken [20,21] and in mammals [22,23]. Their function there is unknown. Microfibrils of type VI collagen are also present throughout the corneal stroma [24–28], but this tissue and the eye in general appear unaffected in patients suffering from Bethlem myopathy (MIM 158810) (see Chapters 2, Part I, and 26, this volume). Another collagen of the corneal stroma is type XIII, which is an unusual collagen since it contains a transmembrane sequence, and does not form fibrils (see Chapter 2 Part I this volume). It is located in the posterior two thirds of the human cornea where it may play a role in cell–matrix or cell–cell interactions [29].

Proteoglycans

At least four small LRR proteoglycans have been isolated from the corneal stroma [30–32]. These are decorin, lumican, keratocan, and osteoglycin, the latter more recently having been renamed mimecan [33–35] (see Chapter 4, this volume). The organization of these LRR proteins between the surfaces of the collagen fibrils and the interfibrillar matrix is not precisely known, nor is the contribution of the chondroitin sulfate or keratan sulfate chains of each LRR proteoglycan to the staining patterns observed with cupromeronic blue [36]. Renewed interest in the function of these proteoglycans in the cornea originates from the recent knockout of lumican, in which disorganized collagen fibrils of the cornea led to a cloudy appearance [37]. It was also recently demonstrated that mutations in keratocan are responsible for at least one form of corneal dystrophy called

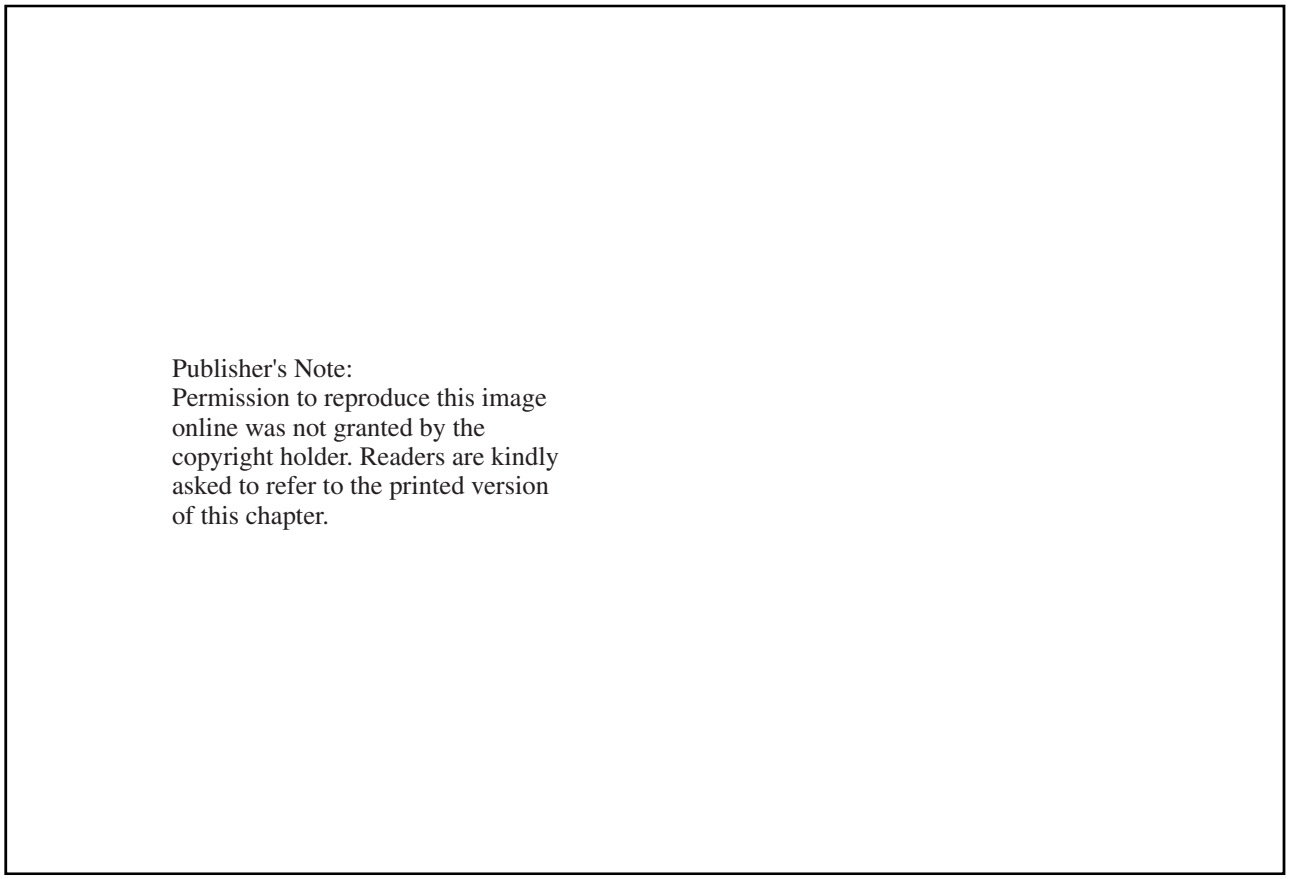

Figure 4. **A**. Scanning electron micrograph of the human corneal stroma (paracentral region). Note a small lamella (arrow) crossing through a larger lamella (arrowhead). **B**. Model for the proposed three dimensional arrangement of lamellae composed of collagen fibrils within the cornea. Figures kindly provided by Dr. Wolfgang Radner, and published previously [9] (with permission of the publishers).

cornea plana 2 (MIM 217300) [38]. However, a knockout of decorin did not appear to affect the cornea sufficiently to cause it to become opaque [39].

Epithelial Basement Membrane

This is a thin, discontinuous, basement membrane that separates the corneal epithelium from Bowman's layer. It appears to be a typical basement membrane and in both mouse and human contains type IV collagen, laminin, nidogen/entactin, and heparan sulfate proteoglycan [40–45]. It is connected to the epithelial cells by hemidesmosomes containing such structural proteins as α6β4 integrin, laminin 5, and type VII and type XVII collagen [46–50].

Bowman's Layer

This layer is a separate structure from the basement membrane of the epithelial cells. Bowman's layer in the human cornea contains collagen fibrils of types I, III, and V, together with microfibrils of type VI collagen but not type IV collagen [51,52]. By electron microscopy, the collagen fibrils are randomly organized in the form of a feltwork and are of slightly narrower diameter than those of the corneal stroma [53].

Descemet's Layer

Much of what is known concerning the structure of this tissue is derived from electron micrographs initially produced by Jakus, in which hexagonal arrays of fibrils were observed stacked on top of each other [54]. The consensus now is that these arrays are type VIII collagen arranged in an antiparallel manner [55,56] (see Fig. 6). However, it appears likely that other as yet unidentified components are also present that stabilize the arrays. Type IV collagen is certainly present and, for rabbit corneal endothelial cells in culture, type VIII and type IV collagen are the major secreted collagens [57].

SCLERA

Recently, the structure and composition of the sclera has been examined in considerable detail in an attempt to understand its role in establishing successful focusing on the retina during eye development (emmetropia) [58,59]. However, less is known concerning the different extracellular matrix macromolecules present within the sclera when compared to the cornea.

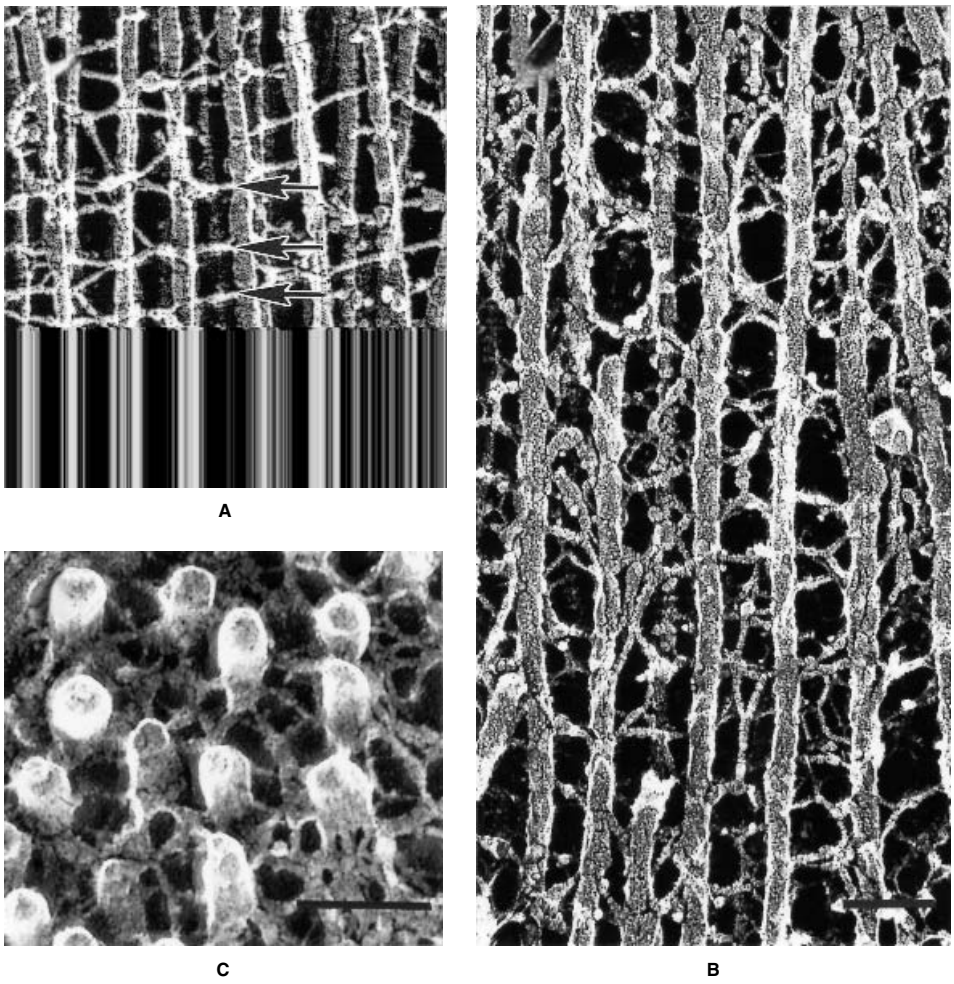

Figure 5. Structure of embryonic chicken corneal stroma as demonstrated by quick-freeze, deep etching at 10 days (panels **A** and **C**) and 15 days (panel **B**). (Bars = 100 nm). Note cross-linking struts between the collagen fibrils often decorated with a small globule (arrows). Figures kindly provided by Dr. Michel Hirsch and Professor Gilles Renard and published previously [10] (with permission of the publishers).

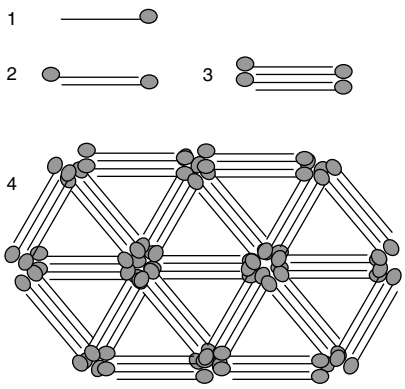

Figure 6. Diagram to show the proposed organization of type VIII collagen molecules to form the hexagonal arrays observed in Descemet's layer. Assembly is thought to occur by antiparallel associations of monomer (1), dimer (2) and tetramer (3) with final formation of an hexagonal array (4). Each monomer consists of a collagen triple helix (shown as a rod) with a globular domain at the carboxyl terminus.

Collagen

The fibrils are much larger in diameter when compared with those in the cornea. In addition, they are not so highly organized and increase in diameter towards the outer surface. Type I collagen is the major collagen of the sclera with smaller amounts of type III collagen and a little type V collagen [51]. It appears that the scleral fibrils are affected in some forms of osteogenesis imperfecta, often being thinner, and the sclera also appearing markedly blue in color (see Chapter 8, this volume).

Proteoglycans

The three major proteoglycans of the sclera are decorin, biglycan and aggrecan [59–62]. The smaller proteoglycans appear largely associated with the collagen fibrils as observed using cuprolinic blue at the critical electrolyte concentration [63,64].

TRABECULAR MESHWORK

It is thought that an accumulation of extracellular matrix within the trabecular meshwork may prevent aqueous outflow, leading to eventual glaucoma. Type VI

collagen is prominent in morphological observations of the trabecular meshwork [65], and often appears as "wide spacing collagen" with a typical banding pattern [66,67]. Other studies have localized collagen types I, II, III, and V to the human trabecular meshwork [68]

LENS CAPSULE

During development, the lens largely consists of epithelial cells which transform to lens fiber cells, elongate, and accumulate large amounts of crystallins [69]. The major extracellular matrix component of the lens is the capsule, which is located on the outer surface of the epithelial cells and contains many typical basement membrane components. The lens capsule is a good source of basement membrane material and played an important role in the initial identification of type IV collagen [70]. In addition to α1(IV) and α2(IV) chains, the presence of α3(IV), α4(IV), and α5(IV) chains has been identified in the lens capsule using highly specific antibodies [71]. These observations are particularly relevant to the etiology of Alport syndrome in which the lens and sometimes Bruch's membrane can be affected (see Chapter 25, this volume) [72]. During the development of the lens, extracellular matrix molecules and their ligands may, however, play critical roles. Thus, mice with a targeted deletion of SPARC (osteonectin, see Chapter 5, this volume) develop cataracts as the major phenotypic response [73–76] and it is considered that SPARC may play a critical role in directing the continuing development of the lens epithelial cells [77]. Developing lens fiber cells synthesize integrin α6β1 [78,79], although the ligand for this integrin has not yet been identified. N-cadherin has also recently been proposed to play a key role in the development of the lens cells [80].

ZONULAR APPARATUS

This tissue forms the suspensory ligament of the lens and consists largely of microfibrils assembled from fibrillin1 [81–83]. Similar microfibrils are found throughout the body and appear to be required for the deposition of elastin. Of particular importance is the failure of these filaments in the zonular apparatus resulting in ectopia lentis as observed in Marfan syndrome (see Chapter 12, this volume).

VITREOUS

The vitreous consists of a very dilute meshwork of collagen fibrils between which are extensive arrays of long hyaluronan molecules [81,84]. The collagens consist largely of type II collagen with smaller amounts of type IX collagen and type V/XI collagen, the latter molecule being assembled from α1(XI) and α2(V) chains [85]. Clinically, collapse of the vitreous can occur because of mutations in either the α1(II) or α1(XI) chains [2] and these results have recently led to the reclassification of Stickler syndrome into 4 subclasses (see Chapter 23, Part II, this volume). The major noncollagenous connective tissue protein of the vitreous is a LRR glycoprotein called opticin, which is found associated with the collagen fibrils [86,87]. Another novel protein of the vitreous is called VIT1, which likely becomes associated with the collagen fibrils during early development and is synthesized by the lens [88]. The large proteoglycan, versican, is also found in the vitreous [89] and may originate from the retina [90]. During the aging process, collapse or liquefaction of the vitreous often occurs with rapid separation of the vitreous from the inner limiting membrane (ILM) of the retina [84]. This can cause a retinal detachment and may also potentially lead to the development of a macular hole [91]. Nothing is known at present of the molecular mechanism by which the vitreous is relatively weakly attached to the ILM or why this attachment fails with increasing age. At the vitreous base attachment is much stronger and again the molecular mechanism is unknown.

RETINA

Several structures in the retina have connective tissue components that are involved in connective tissue disease. These include the ILM and Bruch's membrane, between which is located the retina. Several proteoglycans are present in the retina. The interphotoreceptor matrix contains two novel proteoglycans called either IPM 150 and IPM 200 or SPACR (sialoprotein associated with cones and rods) and SPACRCAN (chondroitin sulfate proteoglycan expressed exclusively by photoreceptors and pinealocytes) [92–95] whereas the large proteoglycan, versican (see Chapter 4 this volume), appears to be located throughout the retina [90]. In addition, decorin [96], neurocan [97], and phosphacan [98] are all expressed in the developing retina. An unusual collagen called type XIII (see Chapter 2, Part I, this volume), is located in the neural retina where it is expressed in the ganglion cell layer [29]. It is speculated that this collagen may be involved in cell–cell contacts within the retina and may contribute to the integrity of this tissue.

Usherin is an important connective tissue macromolecule of the retina. Mutations in the usherin gene are responsible for Usher syndrome type IIa (MIM 276901) which, in the eye, is characterized by progressive retinitis pigmentosa [99–101]. Molecular cloning of usherin shows that it contains laminin-like epidermal growth factor (EGF) domains and also fibronectin type III domains [100,101]. Retinitis pigmentosa is also caused by mutations in a human homologue of *Drosophila crumbs* called *CRB1* (MIM 604210) which is located at the RP12 locus on chromosome 1q31-q32.1 [102]. This is also an extracellular protein that contains EGF-like domains, laminin A-chain G-like domains and a C-type lectin domain [102].

Inner Limiting Membrane

This forms a typical basement membrane on the inner surface of the retina and contains all of the usual constituents of basement membranes, including laminin, type IV collagen (largely α3, α4, and α5 chains, with smaller amounts of α1 and α2 [103,104]), nidogen and agrin. Interestingly, in birds it appears that the ILM is not assembled by retinal tissue, but is derived from the vitreous, where it accumulates on specific receptors located on the retinal surface [105]. These results have not so far been confirmed for mammals in which, unlike the chicken, the retina is thicker and in some species, including man, extensively vascularized. This work is very relevant to recently developed methodology for the repair of macular holes in which, after initial removal of the vitreous, the ILM is peeled from the surface of the retina [106]. If the ILM originates from the vitreous this may explain why it does not reform after peeling and why it can be peeled so easily, especially in older individuals.

Bruch's Membrane

This is a complex connective tissue with basement membrane-like elements located between the retina and

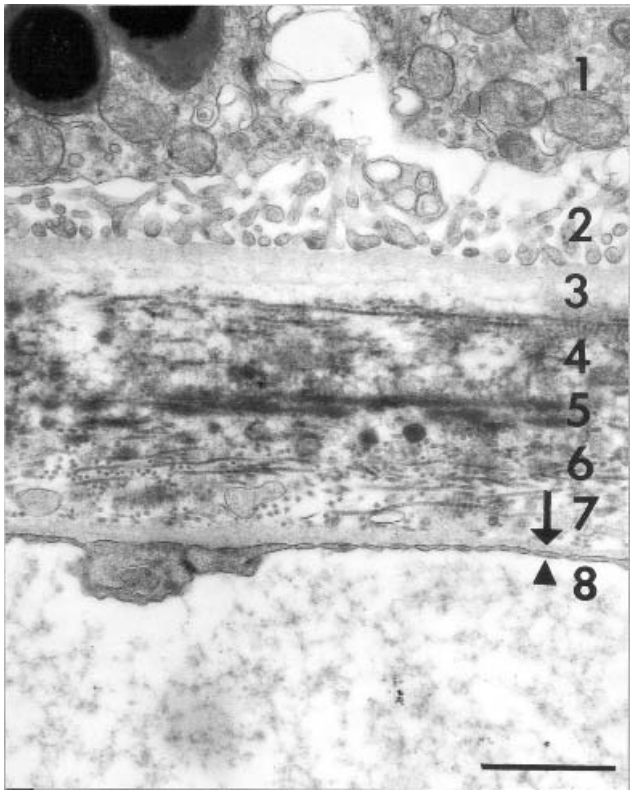

Figure 7. Bruch's membrane from the retina of an 87-year-old female. 1. RPE (retinal pigmented epithelial) cell, 2. basal location of extensive infolding of the plasmalemma of the RPE cell, 3. inner basement membrane. 4. inner collagen layer, 5. elastin layer, 6. outer collagen layer, 7. outer basement membrane, 8. choroidal capillary endothelial cell (note pores). (Bar = 1 μm). Electron micrograph kindly provided by Christine Curcio, Ph.D.

the choroid (Fig. 7) which serves as a support element for the retinal pigmented epithelium and also acts as a filtration barrier for nutrients and metabolites passing between the photoreceptors and the choriocapillaris. It shows marked changes with aging as material accumulates within Bruch's membrane that can eventually predispose the retina to age-related macular degeneration (AMD) [107]. There are five layers that can be distinguished (see Fig. 7); the innermost basement membrane associated with the retinal pigmented epithelial cells, the inner collagen zone, an elastic zone, an outer collagen zone, and an outer basement membrane associated with the endothelial cells of the choriocapillaris. The collagen fibrils consist of types I, III, and V collagen that are interwoven and appear to pass through the elastic layer [108]. Type IV collagen, laminin, and heparan sulfate are present in both basement membranes, while small proteoglycans are associated with the collagen fibrils. It is thought that Bruch's membrane is permeable to metabolites of the photoreceptor cells which are being continuously passed from the retinal pigmented epithelial cells to the choroid. With aging, drusen are observed that are poorly characterized deposits between the retinal pigmented epithelium (RPE)—basal lamina and Bruch's membrane. Interestingly, vitronectin has recently been identified as one component of drusen, but it is not known if it is derived from the retina or plasma [109], although gene expression for vitronectin occurs in the adult human retina [110]. Accumulations of long-spacing collagen commonly occur in Bruch's membrane with aging and recent results suggest that these may consist of lateral aggregates of type VI collagen [111]. Of particular interest is the recognition that the accumulation of deposits in Bruch's membrane in Sorsby fundus dystrophy (MIM 136900) is related to mutations in *TIMP3* [112–118] (see Chapter 7, this volume). Another observation of interest is the identification of a novel extracellular matrix protein called EFEMP1 (epidermal growth factor fibrillin-like extracellular matrix protein-1) in which a single mutation has been found for both the Malattia Leventinese and Doyne honeycomb (MIM 126600) forms of retinal dystrophy [119]. *EFEMP1* was previously cloned and shares sequences common to both fibulins and fibrillins [120] and presumably forms an important structural component of Bruch's membrane.

Pseudoxanthoma elasticum (PXE) (MIM 177850; 264800) (see Chapter 11, this volume) is a genetic disease in which all elastic structures of the body, including Bruch's membrane, are affected. Initially, it was considered that elastin or elastin-associated genes were likely to be involved. However, the condition arises from mutations in a gene called *MRP6*, which is a member of the ABC transporter gene family [121–123].

Interphotoreceptor Matrix

This represents the extracellular matrix that occupies the apical surface of the retinal pigmented epithelium and the external limiting membrane of the neural retina. Two novel chondroitin sulfate-containing proteoglycans have been identified in this structure, called IPM 150 and IPM 200 [92,93] or SPACR (sialoprotein associated with cones and rods) and SPACRCAN (chondroitin sulfate proteoglycan expressed exclusively by photoreceptors and pinealocytes) [94,95]. In addition, hyaluronan is present in the interphotoreceptor matrix [124] and interacts with SPACR [94] and SPACRCAN [95].

CONNECTIVE TISSUE MACROMOLECULES IN THE DEVELOPMENT OF THE EYE

In the rare autosomal recessive disorder called Knobloch syndrome, characterized by occipital encephalocele (MIM 267750) (see also Chapter 26, this volume), there is a failure of early eye development that results in severe myopia, and vitreoretinal degeneration often resulting in retinal detachment, as well as changes in macular structure [125]. In one form of the syndrome, a mutation has been identified in the gene for collagen XVIII, *COL18A1*, which was also found to be expressed in the adult human retina [125]. These results suggest a key role for collagen XVIII in early eye development. The powerful inhibitor of angiogenesis called endostatin is potentially derived from the carboxyl terminus of collagen XVIII by proteinase activity [126–128]. It is possible that endostatin plays a key role in the degeneration of the hyaloid artery present in the primary vitreous and this is supported by analysis of transgenic mice with a targeted deletion of the α1(XVIII) gene (B.R. Olsen, personal communication). Interestingly, in the adult chicken eye, collagen XVIII is a major constituent of the inner limiting membrane where it appears to be derived from the vitreous [129]. Thrombospondin-1 may also play a key role in controlling angiogenesis in the eye. It is synthesized by human retinal epithelial cells [130], is present in the vitreous

and aqueous humor [131], and can give rise to peptides that inhibit retinal angiogenesis [132]. However, other factors such as pigment epithelium derived factor (PEDF) are also likely to be important in controlling angiogenesis in the mammalian eye [133].

RECENT DEVELOPMENTS

Cornea

Macular Corneal Dystrophy I and II (MCDCI and MCDC2; MIM 217800)

Although it was long speculated that MCDC1 and MCDC2 arose from a defect in glycosaminoglycan metabolism [134], this has now been confirmed by the discovery of mutations in a novel carbohydrate sulfotransferase gene (*CHST6*; MIM 605294) which shows considerable specificity for the cornea [135]. The levels and activity of CHST6 enzyme in macular corneal dystrophy-affected cornea can easily account for the low- or nonsulfated keratan sulfate chains commonly seen in patients [136–138]. Similar mutations in *CHST6* have also been found in MCDC patients from the Icelandic population, showing that mutations in the *CHST6* gene commonly occur in diverse populations [139].

Vitreous

Further studies have now determined the structural organization of the human and mouse opticin genes [140]. In addition, *in situ* localization of opticin expression in the mouse shows that the molecule is largely synthesized by the nonpigmented posterior ciliary epithelium [140].

Retina

Interphotoreceptor Matrix

SPACR and SPACRCAN. The role of these molecules in the structure and development of the interphotoreceptor matrix has now been studied in detail [141–143]. In the present model, hyaluronan is considered to be the primary scaffold of the interphotoreceptor matrix, with SPACR and SPACRCAN interacting directly with it [144].

Retinitis Pigmentosa

Usher syndrome type IIa (USH2A; MIM 276901). Further patients with the clinical symptoms of USH2A have been examined for mutations in usherin [145–149]. Although many different mutations have been found, a single mutation (2299delG) commonly occurs among many different races and clearly represents a frequent cause of the disease. It appears that this mutation represents between 16% to 44% of all mutations detected to date in various populations [149].

Crumbs Homologue 1 (CRB1; MIM 600105). It has been shown that mutations in the *CRB1* gene, in addition, cause Leber congenital amaurosis (LCA) in a small proportion of patients [150,151].

Congenital Stationary Night Blindness, Type 1 (CSNB1; MIM 310500). Mutations in a novel small leucine-rich proteoglycan called nyctalopin (NYX; MIM 300278) have been demonstrated in X-linked complete CSNB [152,153].

Bruch's Membrane

Malattia Leventinese and Doyne Honeycomb Forms of Retinal Dystrophy

Further investigations have been made into the mutations in *EFEMP1* (epidermal growth factor fibulin-like extracellular matrix protein) which are associated with premature, autosomal dominant drusen [154,155]. A single mutation, Arg345Trp accounts for all the mutations in EFEMP1 so far detected [155]. Interestingly, EFEMP1 is identical to a protein called fibulin-3 [156]. In addition, a second gene, *EFEMP2*, has been identified [157], the product of which is fibulin-4 [156,158]. This is a potential candidate gene for several retinopathies.

Pseudoxanthoma Elasticum (PXE)

The gene involved in PXE was originally called *MRP6*, but more recently the abbreviation *ABCC6* has been used extensively. Further mutations in *ABCC6* have been described [159–161], and a general review of the disease has been published which describes the morphological changes observed in the eye [162].

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Chapter 2, Part I

The Collagen Family: Structure, Assembly, and Organization in the Extracellular Matrix

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INTRODUCTION

The evolution of multicellular organisms was associated with the emergence of a framework in which cells could survive in a controlled environment where cell contact was retained and cell specialization could occur. This fundamental requirement was met by the formation of a network of intercellular connecting elements that not only permitted the inflow and outflow of materials essential for life but also provided protection from external physical stress on the organism. Thus it was that the connective tissues evolved to provide the strength and shape required by higher animals, and the simple basis on which these tissues are designed has been maintained throughout much of evolution. Two main elements fulfill the needs of the structures now described as the extracellular matrix of connective tissues, namely, the insoluble fibers that resist tensile forces and the interfibrillar polymers that inflate the fibrous network, thereby providing resistance to compressive forces but also allowing diffusion of small molecules in and out of the tissue. The proteinaceous fibers are normally composed of collagen, although elastin too can provide a significant portion of the insoluble fibers in elastic tissues, and the soluble interfibrillar carbohydrate-protein complexes occur primarily as proteoglycans, although the glycosaminoglycan hyaluronan can be an important constituent in tissues such as cartilage, vitreous, and skin. Also contributing to the stability of the matrix are the noncollagenous adhesive glycoproteins such as fibronectin, laminin, tenascin, and entactin, which provide a potential bridge between the matrix and the cells embedded within it. Tissue matrices comprising collagens, elastic fibers, glycoproteins, and proteoglycans form diverse composite structures that determine organ histoarchitecture and provide cells with a mechanical scaffold for adhesion and migration. Indeed, almost all aspects of cell function are influenced, often profoundly, by the surrounding matrix, and the dynamic interplay between cells and matrix regulates tissue remodeling during growth, differentiation, morphogenesis, and wound healing, and in pathological states such as arthritis, fibrosis, atherosclerosis, and tumorigenesis.

New methodologies developed between 1940 and 1970 established the detailed chemical structures of the major constituents of the matrix. Proteoglycans (previously described as mucopolysaccharides) and collagen were shown to be the predominant components of the extracellular matrix, and noncollagenous glycoproteins (originally referred to as mucoproteins), which were initially considered to be impurities or insignificant constituents possibly of cellular or plasma origin, were subsequently shown to have a fundamental role in the matrix. Matrix–matrix interactions between modular motifs within extracellular matrix (ECM) molecules drive fibril or lamina formation and are integral to connective tissue structure and function. The relative proportions of the collagenous and proteoglycan components were generally viewed as the key determinants of the mechanical properties needed by a tissue to fulfill its biological function. Thus, a tissue such as tendon, which needs to be resistant to extension, comprises primarily tough collagen fibers, whereas a tissue like cartilage must be resistant to compression and, therefore, needs to comprise approximately 50% proteoglycan. Other tissues must be rigid and resistant to deformation, and bone achieves its function by combining calcium salts with the collagen fibers, but in tissues such as aorta and lung, where elastic recoil is a crucial feature of tissue function, the collagen fibers are accompanied by elastic fibers containing the protein elastin.

This essentially simple view of connective tissue is still valid today, although over the last 30 years it has become apparent that these important tissues that provide the framework or scaffolding of higher animals are far more complex than previously realized. The basic building blocks — collagens, proteoglycans, and glycoproteins — are now each recognized to occur as large families of matrix macromolecules, with many representatives occurring in one tissue. For example, cartilage is known to contain at least five different collagen types, several proteoglycans, and an ill-defined number of matrix glycoproteins. Similarly, bone is known to contain at least three collagen types, two or three proteoglycans, and several noncollagenous glycoproteins. Some of these components represent very minor constituents of the extracellular matrix and their roles remain unknown, but their importance to tissue integrity

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is likely to be critical, as is well illustrated by the specific absence of functional type VII collagen, quantitatively a very minor component of skin, in patients with the severe recessive dystrophic type of epidermolysis bullosa ([1–3] and Chapter 15, this volume). In this chapter, we discuss the current understanding of the family of matrix macromolecules that are classified as collagens. Wherever possible, reference will be made to reviews and recent papers at the expense of original observations, for it is intended that the reader be provided with a way into the relevant literature rather than an exhaustive survey of the bibliography.

TABLE 1. The Family of Genetically Distinct Collagen Types

Collagen Type	Chains	Molecular Assembly	Supramolecular Structure	$M_r \times 10^{-3}$/ α chain	Examples of tissue location
I	$[\alpha1(I)]_2\alpha2(I)$	Monomers staggered by 67 nm	Large-diameter, 67-nm banded fibrils	95	Bone, cornea, dermis, tendon
I trimer	$[\alpha1(I)]_3$		67-nm banded fibrils		Tumors, dermis
II	$[\alpha1(II)]_3$	Monomers staggered by 67 nm	67-nm banded fibrils	95	Cartilage, vitreous
III	$[\alpha1(III)]_3$	Monomers staggered by 67 nm	Small-diameter, 67-nm banded fibrils	95	Dermis, aorta, uterus, intestine
IV	$[\alpha1(IV)]_2\alpha2(IV)$; also α3(IV), α4(IV), α5(IV), α6(IV)	Association of 4N- and 2C-termini	Nonfibrillar meshwork	170–180	Basement membranes
V	$[\alpha1(V)]_2\alpha2(V)$ $[\alpha1(V)\alpha2(V)\alpha3(V)]$ $[\alpha1(V)]_3$	Monomers staggered by 67 nm	9-nm diameter banded fibrils	120–145	Placental tissue, bone, dermis, cornea
VI	[α1(VI) α2(VI) α3(VI)]	Association into tetramers that aggregate end to end	5–10-nm diameter beaded microfibrils, 100-nm periodicity	α1 (VI) 140 α2 (VI) 140 α3 (VI) 340	Uterus, dermis, cornea, cartilage
VII	$[\alpha1(VII)]_3$	Lateral aggregation of antiparallel dimers	Anchoring fibrils	170	Skin, amniotic membrane, mucosal epithelium
VIII	$[\alpha1(VIII)]_2\alpha2(VIII)$	?	Nonfibrillar, hexagonal lattice?	61	Descemet's membrane, endothelial cells
IX	[α1(IX) α2(IX) α3(IX)]	Covalently cross-linked to surface of type II collagen fibrils	FACIT[1]; nonfibrillar	68–115	Cartilage, vitreous
X	$[\alpha1(X)]_3$	?	Nonfibrillar, hexagonal lattice?	59	Calcifying cartilage
XI	[α1(XI)α2(XI)α3(XI)]	Monomers staggered by 67 nm	Fine fibrils similar to those of type V collagen	110–145	Cartilage, intervertebral disc
XII	$[\alpha1(XII)]_3$	Probably associates with surface of collagen fibrils	FACIT; nonfibrillar	220,340	Dermis, tendon, cartilage
XIII	α1(XIII)	?	Membrane-intercalated	62–67	Endothelial cells, epidermis
XIV	$[\alpha1(XIV)]_3$	?	FACIT; nonfibrillar	220	Dermis, tendon, cartilage
XV	α1(XV)	?	MULTIPLEXIN[2]; nonfibrillar	125	Placenta, kidney, heart, ovary, testis
XVI	α1(XVI)	?	FACIT; nonfibrillar	150–160	Heart, kidney, smooth muscle
XVII	$[\alpha1(XVII)]_3$	?	Membrane-intercalated	180	Hemidesmosomes of specialized epithelia
XVIII	α1(XVIII)	?	MULTIPLEXIN; nonfibrillar	200	Kidney, liver, lung
XIX	α1(XIX)	?	FACIT; nonfibrillar	Unknown	Fibroblast cell lines

[1] FACIT collagens are fibril-associated collagens with interrupted triple helices.

[2] MULTIPLEXIN collagens have multiple triple helical domains and interruptions.

The Collagen Family of Proteins

Collagens are the most abundant proteins in the animal kingdom, and are even found in the earliest forms of metazoan life [4,5]. The term "collagen" has been in the English language since at least 1865, and its derivation from the Greek word γξλλξζνξ for "glue" is reflected in its early definition in the *Oxford Dictionary* (1893 edition) as "that constituent of connective tissue which yields gelatin on boiling." The economic importance of collagen has been recognized for thousands of years, for collagen is the main constituent of skin, the raw material of leather, and gelatin, which have long been commercially important commodities. Indeed, much of our current knowledge of collagen structure derives from important work conducted by chemists in the leather and gelatin industries. Subsequently, interest in the biochemistry of collagen focused on the unusual properties of this protein and its involvement in many of the chronic diseases of humans, e.g., atherosclerosis, liver fibrosis, and rheumatoid arthritis, where the excessive deposition of connective tissue elements compromises tissue structure and function. Latterly, the application of the techniques of gene cloning to the study of collagen genes and their involvement in inherited diseases such as osteogenesis imperfecta (see Chapter 8, this volume) and the Ehlers-Danlos syndrome (see Chapter 9, this volume) has provided new insight into normal collagen synthesis, structure, and function.

Modern research on collagen at the molecular level began in the 1950s, with the application of rapidly developing physicochemical techniques, e.g., X-ray crystallography and electron microscopy. The triple-helical arrangement of collagen was recognized using helix diffraction theory [6,7], and models of supercoiled triple-helical arrays were subsequently developed [8–10]. Using collagen-like peptides, high-resolution crystal structure determinations of triple-helices have been achieved [11–13], which confirm the triple-helical model obtained from fiber diffraction. Further advances in protein separation techniques (see introductory chapter by Piez, this volume) led to the discovery, in 1969, that cartilage collagen (type II) was genetically distinct from the well-studied fibrous collagen (type I) of skin, bone, and tendon — and so provided the first evidence for the existence of a family of collagenous proteins associated with the connective tissues of vertebrates [14]. Shortly after this, another unique collagen (designated type III) was detected in skin [15].

Studies over the last two and a half decades have now led to the identification of approximately 33 different collagenous polypeptides believed to be encoded by a minimum of 33 different gene sequences [4,5,16–20]. These polypeptides occur in the extracellular matrix in at least 19 different collagen types (designated I to XIX, see Table 1 and Recent Developments), and further unique collagenous polypeptides will undoubtedly be identified following completion of elucidation of the human genome sequence. The collagenous triple-helix conformation has also been found as a domain in an increasing number of secreted proteins, including host defense proteins such as complement component Clq and serum mannan-binding lectin [21–32] (see Table 2). The collagenous sequences in these proteins clearly contribute to their distinctive structures and functions, but the fact that they have no known structural role in the extracellular matrix precludes their classification as collagens.

Classification of Collagen Types

Early definitions of collagen were conditioned by our knowledge being restricted to the major fibrous collagen

TABLE 2. Non-Extracellular Matrix Molecules with Homologies to Collagen Types VIII and X

Molecule	Homologous regions	Molecular organization	Tissue distribution	Biological role
Collectin subgroup of C-type animal lectins				
Mannan-binding lectin (MBL)	Collagen-like domain, carbohydrate recognition domain (CRD), homologous to NC1	Triple-helical homotrimers, oligomeric (serpiform, 6 helices)	Liver, serum	Lectins, host defense factors involved in complement activation
Surfactant protein-A (SP-A)	Collagen-like domain, CRD homologous to NC1	Triple-helical homotrimers, oligomeric (serpiform, 6 helices)	Lung, gut	Mediator in lung, innate immune defense
Surfactant protein-D (SP-D)	Collagen-like domain, CRD homogolous to NC1	Triple-helical homotrimers, oligomeric (cruciform, 4 helices)	Lung, gut	Mediator in lung, innate immune defense
Conglutinin	Collagen-like domain, CRD homologous to NC1	Triple-helical homotrimers, oligomeric (cruciform, 4 helices)	Liver, serum	Lectin, conglutination, inhibits haemagglutination by influenza A virus
Collectin 43 (CL-43)	Collagen-like domain, CRD homologous to NC1	Triple-helical homotrimers ? Non-oligomeric	Serum	Role in innate immune defense against microorganisms
CL-L1	Collagen-like domain, CRD homologous to NC1	Triple-helical homotrimers ? Oligomers	Liver, placenta	Unknown (binds mannan weakly).

(*continued overleaf*)

TABLE 2. (*continued*)

Molecule	Homologous regions	Molecular organization	Tissue distribution	Biological role
Complement and related factors				
C1q	Collagen-like domain, NC1-like sequence	Heterotrimeric triple-helical, oligomeric	Serum	Complement component, recognition of microbes, antibody-antigen complexes
C1q-related factor (CRF)	Collagen-like domain, NC1-like sequence	Triple-helical homotrimers, ?Oligomeric	Brain	?Role in motor function
Ficolins (L- and M-forms)	Collagen-like domain, CRD homologous to NC1	Triple-helical ?Homotrimers ?Oligomeric	Liver, plasma, monocytes	Binds sugars, enhances bacterial phagocytosis.
Metabolic molecules				
Adipocyte complement-related protein of 30 kDa (ACRP30)	Collagen-like domain,NC1-like sequence	Homotrimeric, triple-helical, oligomeric	Fat cells	Energy homeostasis, obesity
Hib27	Collagen-like domain, NC1-like sequence	?Homotrimeric triple-helical, oligomeric	?? (Chipmunk)	Energy homeostasis, hibernation
Acetylcholinesterase (AchE)	Collagen-like domain	?Homotrimeric triple-helical	Neuromuscular junction	Hydrolyses released acetylcholine, controls duration of receptor activation
Cytokines and related molecules				
Tumor necrosis factor (TNF)-α	Gly, Pro-rich sequences, NC1-like sequence	?Homotrimeric triple-helical	Widespread	Roles in immunity, energy homeostasis
Tumor necrosis factor (TNF)-β	Gly, Pro-rich sequences, NC1-like sequence	?Homotrimeric triple-helical	Widespread	Roles in immunity, energy homeostasis
CD40L	NC1-like sequence		Widespread	Roles in immunity, energy homeostasis
FAS ligand (FASL)	NC1-like sequence	?Trimeric	Widespread	Membrane-bound, energy homeostasis, apoptosis in immune system
Ectodysplasin	Collagen-like domain, NC1-like sequence	Trimeric	Epithelia	Membrane bound, role in cell adhesion, epithelial- mesenchymal interactions
Elastic fiber-associated molecules				
Emilin-1	NC1, short collagenous sequence	Homotrimeric triple helical	Elastic tissues	Elastic fibre formation
Emilin-2	NC1, short collagenous sequence	?Homotrimeric triple helical	??	??

found in skin, bone, and tendon (i.e., type I collagen). Accordingly, such definitions were based on type I collagen in terms of its histological properties, electron microscopic features, chemical composition, physical properties including X-ray diffraction pattern, and behavior toward enzymes. As knowledge of the structure of the new collagens has accumulated, it has become increasingly difficult to provide a satisfactory general definition other than to state that such proteins comprise three polypeptides that fold to form triple-helical domains (determined by the high glycine and imino acid contents in specific repeating triplets of Gly–X–Y, where X is often proline and Y is often hydroxyproline), which assemble into supramolecular aggregates in the extracellular space [33]. Clues to the evolution of the different collagen types are revealed in the detailed analysis of collagen gene structure (see this Chapter, Part II), but, in functional terms, it appears that the various collagens have developed to provide a

variety of structures (fibers, microfibrils, microfilaments, and meshworks) necessary to adapt connective tissue to its numerous physiological and mechanical functions in the body.

As the number of collagen types defined in the literature has increased, attempts have been made to discuss them in terms of groups or classes. The collagen numbering system (roman numerals for each collagen type and arabic numerals for individual α chains) to some extent reflects the relative abundance of the various collagens, in that generally the more abundant collagens were identified earliest. Thus, in terms of the whole body collagens, types I, II, and III are quantitatively the most important and probably account for over 70% of the total [34]. These fibril-forming collagens were originally referred to as the interstitial collagens on the basis of their widespread distribution and structural roles within the connective tissues supporting the major organs of the body. However, this terminology is no longer favored, in as much as several other less abundant collagens (both fibril-forming and with different macromolecular arrangements) have similar tissue distributions. Early classifications often referred to major and minor collagens on the basis of their quantitative abundance in connective tissues. However, quantification is fraught with difficulties, and a major collagen in one tissue may be a minor component in another (e.g., the ubiquitous microfibrillar type VI collagen is a major collagenous component of skin but a relatively minor element of cartilage). Moreover, such a simple classification says little about the structure/function relationships of the collagen types that derive from their primary amino acid sequences.

Current classifications are based largely on primary structure, and collagenous molecules can be grouped into three categories on the basis of the respective sizes of their collagenous triple helices [35,36] (Fig. 1). Group 1 molecules are characterized by an uninterrupted helical domain of approximately 300 nm. These collagens are synthesized as procollagens comprised of three proα chains that undergo processing to α chains and subsequently assemble into collagen fibrils and fibers. Collagens in Group 1 (i.e., types I, II, III, V, and XI) exhibit several common structural features that reflect the highly conserved exon-intron structure of the genes for many of the chains involved (see this Chapter, Part II). Group 2 comprises collagen types IV and VII, whose component polypeptides are distinguished by their large M_r (>160,000) and especially by their extended triple helices (>350 nm), and whose primary structures are characterized by imperfections in the Gly–X–Y triplet sequence. These imperfections are a particular feature of type IV collagen, in which the helical domain contains more than 20 short stretches of non-helix-forming amino acids. In contrast, Group 3 comprises short-chain collagens. In this review, this grouping has been subdivided into Group 3A, which comprises short-chain collagens with continuous triple-helical domains (type VI, VIII, and X collagens), and Group 3B, which includes collagens with interrupted helical domains such as the fibril-associated collagens with interrupted triple helices (FACIT collagens) types IX, XII, XIV, XVI, and XIX, the transmembrane collagens XIII and XVII, and the MULTIPLEXIN collagens (multiple triple-helix domains and interruptions) types XV and XVIII. In Group 3B, the individual polypeptides vary in size from 623 to 3063 residues as a consequence of large nonhelical domains at the termini of some of the α chains.

This type of grouping of collagenous molecules, based largely on size and physicochemical properties, is not totally satisfactory because it provides no clues as to the biological roles of individual collagens. An alternative approach would be to classify the collagens according to particular structural features that might be related to their physiological function; e.g., the possession of intrahelical sites susceptible to cleavage by different vertebrate collagenases (as in collagen types I, II, III, IV, VII, VIII, and X) might indicate an involvement in matrix turnover and tissue (re)modeling. Further classifications based on gene structure seem likely to emerge as evidence for alternative splicing of some of the collagen genes indicates that individual collagen types may themselves represent a family or group of related collagenous structures in the extracellular matrix (e.g., type VI collagen).

Homology between the gene and protein structures of the different collagens can provide evidence of evolutionary relationships and functional activities. Thus, types IX, XII, XIV, XVI, and XIX collagens exhibit fascinating similarities in their interrupted triple-helical structures; based on structure/function considerations, the grouping or subclass of FACIT collagens, containing these types, has arisen [16,37,38]. Similarly, the remarkable homology of collagen types VIII and X suggests that they probably have similar roles in the tissues in which they are located [16,37,39].

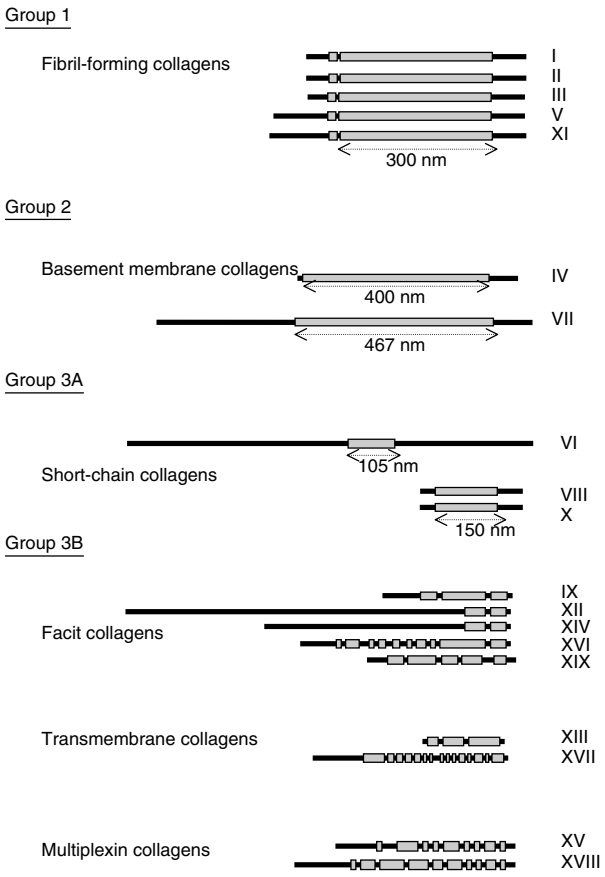


Figure 1. Schematic representation of the domain structures and relative dimensions of the different molecules of the classified collagen family (types I–XIX; groups 1–3). Triple-helical domains are represented by shaded bars and non-helical sequences as solid lines. Lengths of triple-helical domains (nm) are indicated by arrows.

In this chapter, it has been considered appropriate to discuss the biochemistry of the collagen types in relation to their known structure, organization, and function in the extracellular matrix. Where individual collagens fall into well-defined subclasses, they are discussed together. A detailed description of the structure, assembly, and supramolecular aggregation of collagen type I is presented below, for it provides the prototype against which background has developed our appreciation of the other fibrillar collagens and the nonfibrillar "minor" collagens.

COLLAGEN TYPE I: THE CLASSICAL FIBROUS COLLAGEN

Collagen is probably the most abundant protein in the human body and, while the classical fibrous collagens (types I, II, and III) occur in the greatest amounts, type I collagen is, quantitatively, by far the most important. Accordingly, it is not surprising that much of our current understanding of the structure, synthesis, and assembly of collagens is derived from studies on this collagen, which provides the major mechanical strength of tissues such as skin, tendon, bone, dentine, cornea, and sclera.

Fibrous collagen is classified as a quasi-crystal, or crystalloid, because of its highly symmetrical insoluble structure built up of essentially identical subunits. Gross visual examination of a tissue such as tendon demonstrates that type I collagen (accounting for approximately 90–95% of the dry weight of the tissue) is present as large, highly orientated fibers. More detailed examination under the electron microscope, after appropriate fixation and staining, reveals the fibers to comprise bundles of smaller, identical, and parallel microfilaments having a characteristic pattern of cross-striations or bands (Fig. 2). There is, as yet, no common nomenclature for collagen fibers and fibrils. For the sake of clarity, we refer to the structures that can be seen with the naked eye as "fibers" and the smallest structures visible by light microscopy as "fibrils."

Although not all collagen types are organized into classical cross-striated fibrillar structures, the major fibrillar collagen types form distinctive long, unbranched, banded fibrils. Low-angle X-ray diffraction studies have revealed a characteristic

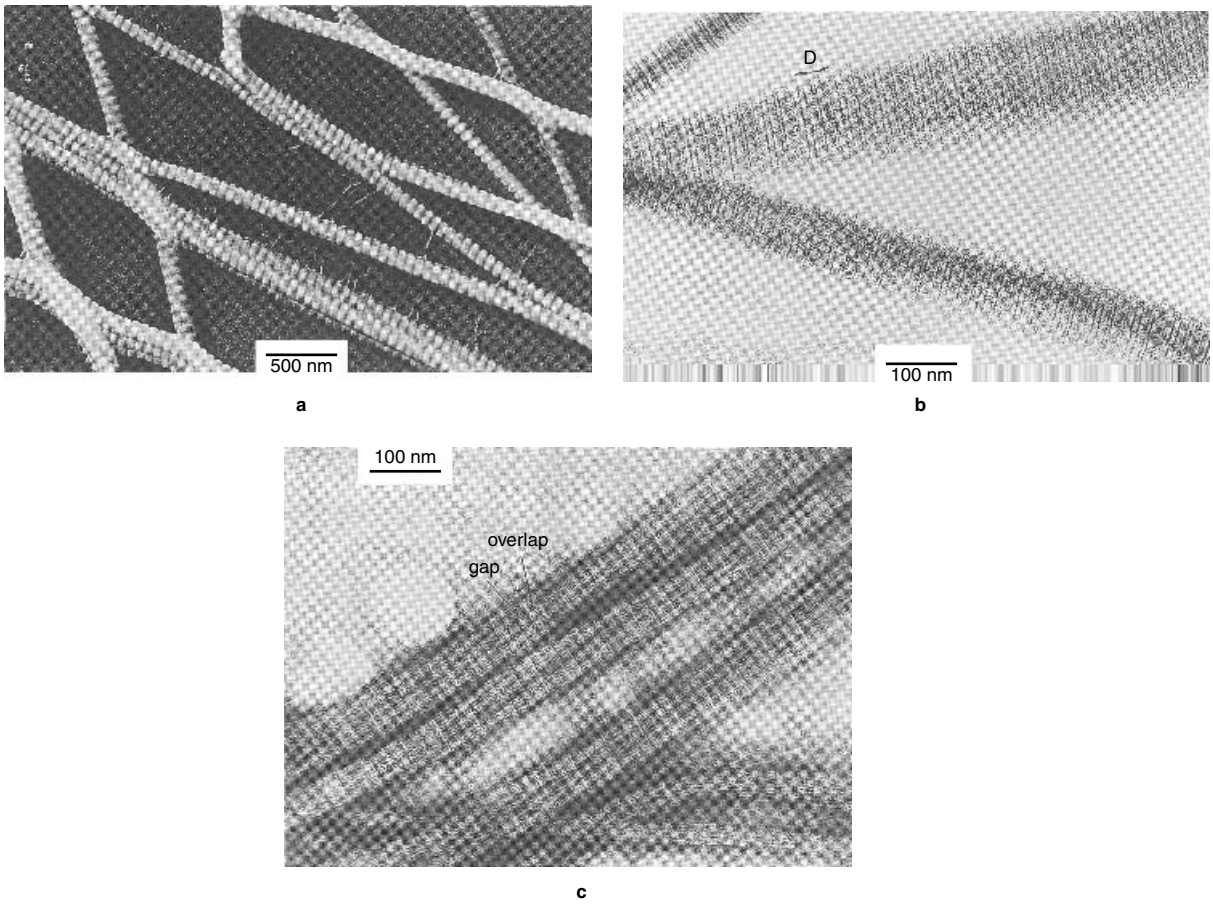


Figure 2. The appearance of collagen fibrils in the electron microscope. In (**a**), fibrils (extracted from human Achilles tendon) have been unidirectionally shadowed with gold-palladium. The D-periodic banding is strikingly revealed, but only low-resolution surface contours, accentuated by the shadowing, are detectable. In (**b**), fibrils have been positively stained by exposure to aqueous solutions of phosphotungstic acid (PTA, 1%, pH 3.4) and uranyl acetate (UA, 1%, pH 4.2). In (**c**), fibrils have been negatively stained with phosphotungstic acid (1%, pH 7.0), which reveals the characteristic alternation of dark (stain-penetrable) and light (stain-excluding) zones, the so-called "gap "and "overlap "zones. In (**b**) and (**c**), the fibrils are of type I collagen from calf skin, initially solubilized in citric acid solution and then reconstituted into fibrils. (Reprinted from ref. 53 with permission from Pergamon Press.)

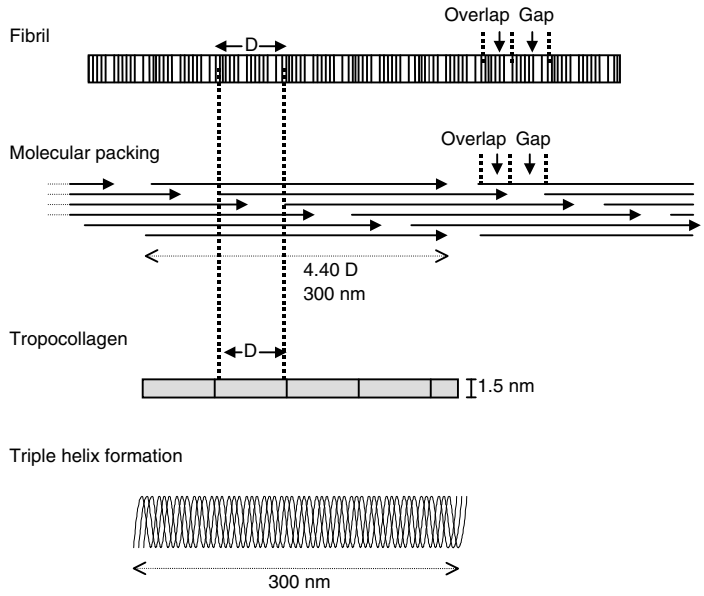


Figure 3. Schematic diagram illustrating in two dimensions the relationship between collagen chains and assembled fibrils. The right-handed triple-helical molecule of 4.4D periods in length that constitutes tropocollagen is made up of three individual collagen chains (two α1(I) chains and one α2(I) chain), each coiled in a left-handed helix. The staggered arrangement of tropocollagen molecules in the fibril gives rise to one overlap zone and one gap zone per D period. Note: 0.15D (10.4 nm) represents one turn of the superhelix.

periodicity (D) close to 67 nm in the native hydrated state [40], although dehydration and shrinkage during conventional sample preparation for electron microscopy results in lower values of around 64 nm. These fibrillar structures are accounted for by the specific parallel and mutually staggered alignment of the basic molecular units of collagen — the collagen fibril monomers (frequently described as "tropocollagen") — which is directed by the sequence of the interactive hydrophobic and charged residues distributed along the triple helix to provide the maximum electrostatic and hydrophobic interaction of the neighboring molecules. A variety of physicochemical studies, including direct visualization of the individual molecules by rotary shadowing techniques in the electron microscope, have demonstrated the rodlike nature of the collagen fibril monomer. These molecules of type I collagen have a length of slightly less than 300 nm and a diameter of about 1.4 nm. The rod is neither rigid nor randomly flexible, but appears to possess an intermediate level of semiflexibility that probably varies along its length. The individual molecules consist of three polypeptide chains (designated α chains) that are coiled into a unique type of semirigid helical structure (Fig. 3). In type I collagen, the helical molecule is a heterotrimer comprising two identical α1(I) chains and one α2(I) chain. The α1(I) and α2(I) chains are very similar, but their primary sequences, coded for by separate genes, are sufficiently different for the chains to be separable by ion-exchange chromatography and SDS-polyacrylamide gel electrophoresis. The α chains each contain just over 1,000 amino acids and have molecular weights of approximately 95,000.

The three-dimensional structure of the collagen triple helix was originally established by X-ray diffraction techniques [6–10,40–43], and more recently confirmed by crystallographic studies on small peptide models [11–13]. The occurrence of glycine as every third residue throughout 95% of the α chains and the presence of large amounts of proline and hydroxyproline (together accounting for approximately 22% of the amino acid residues) allow each polypeptide to be arranged in a stretched polyproline helix, the mobility of which is restricted by the pyrrolidine rings. This helical structure of collagen differs from the α helix characteristic of many globular proteins in that the axial distance between one amino acid and the next is 0.286 nm instead of the axial distance of 0.15 nm found in the α helix. The overall helical symmetry is left-handed, with 10 residues in three turns and a pitch of approximately 3 nm, and the three helical chains are further coiled about a central axis to form a right-handed helix (Fig. 4) with a repeat distance of approximately 10 nm [44].

The high content of glycine in fibrillar collagens (33% of all amino acid residues and therefore approximately 333 amino acids per α chain) and its occurrence as every third residue gives rise to a polymer of tripeptide units with the formula $(Gly{-}X{-}Y)_n$. The helical conformation of the individual α chains arises largely as a result of steric repulsion between the proline (approximately 120 residues per α chain in the X position) and 4-hydroxyproline residues (approximately 100 residues per α chain in the Y position). These residues also have a stabilizing effect on this conformation, as the five-membered rings of the imino acids are rigid and limit rotation about the peptide N–C bond. Collagen stability appears further to rely on inductive effects that favor the requisite *trans* conformation of the hydroxyproline peptide bond since $(GlyFlpPro)_{10}$ (where Flp is an electronegative 4(*R*)-fluoroproline residue that does not form hydrogen bonds) is more thermally stable than $(GlyProHyp)_{10}$ [45]. Glycine is the smallest possible amino acid and, as such, the only one that can pack tightly at the center of the triple-stranded collagen fibril monomer. It will immediately be appreciated that any mutation occurring within the sequences coding for the triple-helical domains of the α chains is likely

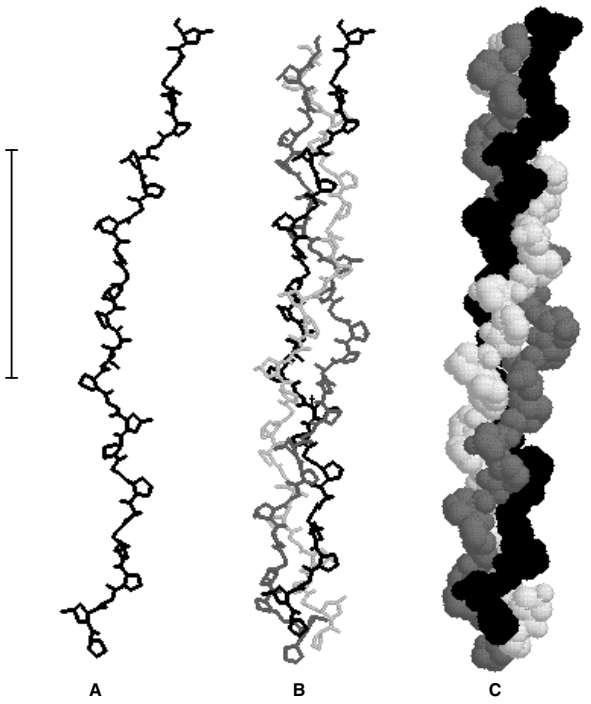


Figure 4. Three-dimensional representation of the conformation of the collagen triple helix. The repeating sequence shown is Gly–Pro–Hyp. **A**: Conformation of a single strand of the collagen helix illustrating the left-handed stretched polyproline helix with 10 residues (indicated by bar) in three turns and a pitch of approximately 3 nm. **B**: Skeletal model of the triple-stranded collagen helix in which the three helical polypeptides are coiled about a central axis to form a right-handed triple helix. **C**: Space-filling model of the assembled triple helix. Chains are shaded to demonstrate the right-handed symmetry along the longitudinal axis of the helix.

to have dramatic consequences, especially if substitution of a glycine residue occurs with consequent disruption of the helical conformation. Indeed, a glycine substitution at any site along the collagen helix of a fibril-forming collagen is the cause of a variety of diseases of bone, cartilage, and blood vessels [46,47] (see also Chapters 8, 9, 15, 23, and 25, this volume).

A major stabilizing force within collagen molecules is hydrogen bonding, and the trimerization of chains is favored by close packing and hydrogen bonding between the three chains. There is only one NH–O=C bond per Gly–X–Y triplet, involving the residue in position X. The glycine provides the HN-group for hydrogen bonding to this O=C group, and the remaining two backbone C=O groups in each triplet and any backbone NH groups of X and Y residues are not involved in peptide hydrogen bonding. The side chains of amino acids in the remaining X and Y positions protrude from the chain and this arrangement allows a variety of amino acid residues (acidic, basic, hydrophobic, etc.) to be accommodated in the molecule. These side chains have relatively minor effects on triple helix stability, but are highly exposed to the surrounding milieu and available for intermolecular interactions. The presence of an appropriate number of 4-hydroxyproline residues in the Y position is, in contrast, a crucial determinant of the stability of the triple helix under physiological conditions. Indeed, the transition temperature (thermal denaturation temperature, melting temperature, T_m) of triple helices formed *in vitro* by nonhydroxylated collagen is notably below 37°C [48]. It has long been thought that this extra stability arises from hydrogen bonds mediated by a network of bridging water molecules. Indeed, high-resolution crystal structures of triple-helical peptides show that a highly ordered hydration network satisfies hydrogen bonding with all remaining backbone peptide groups, and confirm that hydroxyproline plays a pivotal role in this network [12,13]. Nucleation of the triple helix has been studied using Gly–X–Y peptides, and folding rates have been shown to be strongly sequence dependent [49]. Gly–Pro–Hyp triplets promote rapid folding, but single amino acid substitutions in the Y positions affect the nucleation rate.

In the fibrillar collagens, the triple-helical conformation occurs throughout 95% of the length of the rodlike monomer. Thus, of the 1,057 residues in the α1(I) chain of human collagen, 1,014 occur in the repeating Gly–X–Y triplets essential for triple-helical packing. The N-terminal 17 residues and the C-terminal 26 residues (referred to as telopeptides) do not have glycine as every third residue and exist in a less regular conformation. N-terminal and C-terminal extrahelical peptides occur at the ends of the 1,039-residue-long α2(I) chain but are shorter.

The telopeptides are susceptible to proteolytic attack, whereas the intact triple-helical domain is generally resistant to most proteolytic enzymes. However, when heated above physiological temperatures, it undergoes a helix-to-coil transition and, once melted, becomes susceptible to degradative enzymes. As discussed later, the majority of covalent cross-links stabilizing the fibrillar collagens involve the telopeptides and, consequently, pepsin treatment of insoluble cross-linked fibers tends to release the triple-helical domain, which can be recovered in its native conformation. Specific collagenases of bacterial and animal origin act directly on the collagen triple helix, but whereas bacterial collagenases yield many small peptides, the animal collagenases (matrix metalloproteinases MMP-1, MMP-8, MMP-13) are specific for only one or two sequences within the triple helix and yield large, defined proteolytic fragments [50] (see also Chapter 7, this volume).

The Collagen Fibril and Its Self-Assembly

Similar fibrils and microfibrils of collagen type I are seen in different connective tissues, although their organization and arrangement are subject to considerable tissue-specific variation. Thus, in tendon, all the fibrillar units are arranged in large parallel bundles (Fig. 5a), whereas in skin, where type I collagen accounts for 80–90% of the collagenous proteins, the fibrils form a coarse network partially oriented in the plane of the skin (Fig. 5c). Further variations include the orthogonally arranged and precisely packed fibrils in corneal stroma, which form the basis of tissue transparency (Fig. 5b), and concentric circles of collagen fibrils in cortical bone. In tendon, the average diameter of the fibrils varies between 50 and 500 nm, while in skin it is between 40 and 100 nm, but in the cornea, which also has a high type I collagen content, the collagen fibrils have a uniform diameter of approximately 25 nm. Fibril formation is an entropy-driven process controlled to a large extent by the amino acid sequence of the collagen and, in particular, by the distribution of polar and hydrophobic residues. However, the fact that collagen I can be organized in these different macromolecular

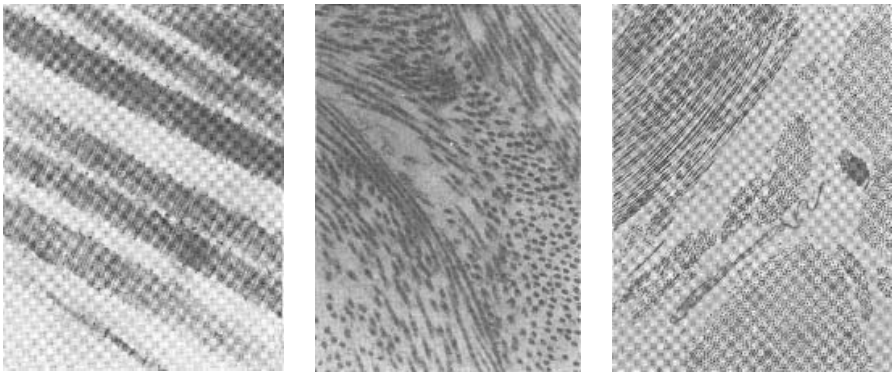


Figure 5. Electron micrographs of sections through arrays of collagen fibril bundles from different type I collagen-containing tissues, demonstrating the diversity of the fibril dimensions and arrangements that exist. (**a**) Parallel fibrils in tendon; (**b**) horizontal crisscrossing layers in cornea; (**c**) layers in skin. (Micrographs courtesy of Professor J. Scott.)

structures indicates the involvement of additional tissue-specific regulatory elements.

The collagen fibrils provide long-range structures extending over tens of microns and their assembly requires that billions of collagen molecule subunits assemble to make a structure that is both space-filling and mechanical over these long distances. The mechanisms by which these long cylindrical aggregates form are not fully understood and remain a focus of investigation, hypothesis, and controversy [51–55]. Stringent control over lateral growth of the cylindrical fibers *in vivo* is obvious from assessments of the sharp diameter distributions exhibited by collagen fibrils in a wide range of developing connective tissues. How this control is exerted and how the fibrils extend in length and their diameters grow *in vivo* are ill defined. However, the discovery that fibrils will spontaneously self-assemble from solutions of extracted collagen when the pH, temperature, and ionic strength are adjusted to physiological values led to the idea that this process provided an explanation of fibril formation *in vivo*. The reconstituted fibrils exhibit a classical cross-striated banding pattern in the electron microscope, demonstrating conclusively that the aggregation of collagen molecules into axially ordered fibrillar structures is basically a self-assembly process where the information for association is contained within the assembling molecules themselves [55]. However, the process of fibrillogenesis *in vivo* is far more complex, involving tissue-specific factors influencing lateral and axial growth, fibril diameters, and interfibrillar spacings, and these factors are clearly crucial in determining the precise supramolecular architecture of the collagen fibrils in the different tissues. Part of the complexity of the process can be revealed by investigating the highly irregular fibrils that occur in some genetic disorders of collagen, notably dermatosparaxis in cattle and certain variants of Ehlers-Danlos syndrome in humans (see Chapter 9, this volume).

Nonetheless, the concept of self-assembly remains important, for there has to be a recognition phenomenon to promote aggregation and alignment of the collagen fibril monomers. Detailed analysis of the interactions involved became possible when the full details of the primary structures of the α1(I) and α2(I) chains became available from sequencing at both protein and cDNA levels. The fact that the collagen molecule is essentially a one-dimensional molecule with its near-constant axial separation between amino acid residues throughout over 95% of its tertiary structure permitted a direct correlation of structural data obtained by electron microscopy with chemical sequence data. Thus, it has been recognized for over 30 years that the cross-striated periodic structure of the native collagen fibril is a consequence of the assembly of molecules in a parallel array, but mutually staggered (i.e., axially displaced with respect to one another) by approximately one-quarter of their length — often referred to as the "quarter-staggered array." The periodicity of the cross-striations in the fibril is explained by the fact that each collagen fibril monomer, or tropocollagen unit, has five highly charged regions 67 nm apart, detectable under appropriate conditions as stained bands. This repeat period (the D-period) is confirmed by low-angle X-ray diffraction of rat tail tendon fibrils, and the overall length of a collagen fibril monomer is 4.4 D units (when D = 234 amino acids, the length of one cross-striation period of 67 nm). Within a collagen fibril, the molecules are staggered by multiples of the common distance D. Figure 6 illustrates in two dimensions 1D and 4D staggered contacts, but in a three-dimensional fibril, 0D, 2D, and 3D staggers will also be present between adjoining molecules. As the ratio of molecular length to D-stagger is nonintegral, as noted above, each D-period can be seen to be divided into an overlap zone that includes the N- and C-termini of molecules and a gap zone that does not. Thus, for every five molecular segments in an overlap, there are only four in a gap zone, and the different protein density in these zones is reflected in the negative staining patterns observed in the electron microscope.

Procollagen, the Soluble Precursor

The well-recognized phenomenon of tropocollagen self-assembly under physiological conditions prompted the question of how fibril monomers are prevented from forming insoluble fibrils prior to secretion. The answer came with the discovery in 1970 of the synthesis by fibroblasts of the precursor, procollagen, which is soluble under physiological conditions [56,57]. The term "procollagen" has often been used rather liberally to refer to the soluble precursors of all the different collagen types that have been identified since the initial discovery of procollagen type I. However, today the description of a molecule as a procollagen tends to be restricted to the fibrillar collagens, where a precursor-to-product relationship is well defined and a

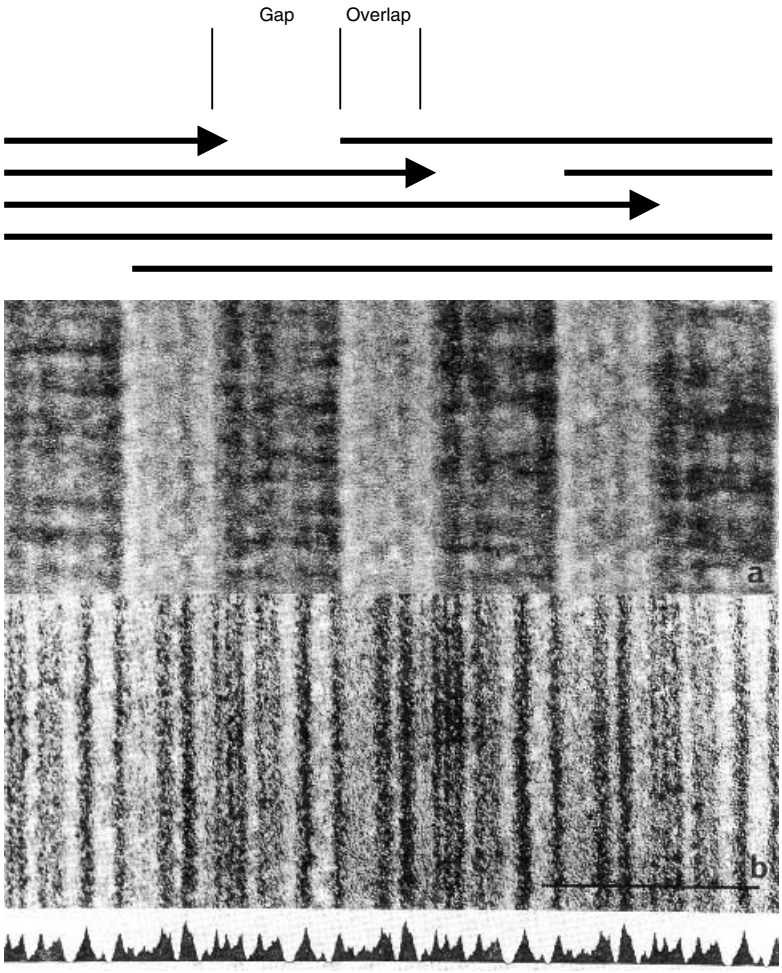


Figure 6. Matching of (**a**) the negative staining pattern with (**b**) the positive staining pattern of a reconstituted fibril of calf skin collagen. The positive stain was phosphotungstic acid (1%, pH 3.4) and uranyl acetate (1%, pH 4.2) both in aqueous solution. The negative stain was uranyl acetate (1%, pH 7.0). The broad stain-penetrable / stain-excluding zoning seen in (**a**), arising from the ''gap/overlap'' staggering of molecules in a fibril, is the most conspicuous feature of the negative staining pattern. Superimposed on this broad staining are finer dark staining bands that appear to align with the staining bands of the positive staining pattern below. The smoothed distribution of charged residues in the fibril, represented at the bottom of the picture, can be aligned with the staining patterns of the fibril, since the charge-rich (−ve and +ve) regions preferentially take up staining ions along the collagen molecule. The bar indicates one D period. (Micrographs courtesy of Dr J. A. Chapman.)

specific physiological cleavage of the procollagen to the collagen fibril monomer (tropocollagen) is known to occur.

The precursor polypeptides of the major fibrillar collagens (types I, II, and III) all have molecular masses about 50% larger than their corresponding α chains, owing to the presence of peptide extensions (propeptides) at both N- and C-termini [58]. Thus, the molecular weights of these complete proα chains are approximately 150,000. In early studies on collagen synthesis, the extension peptides were lost because the susceptibility of procollagen to proteolytic degradation during isolation was not appreciated. However, careful studies, particularly on type I procollagen, established the existence of propeptides at both the N-terminus and the C-terminus (Fig. 7A). cDNA analyses for human procollagen proα1(I) and proα2(I) chains have established that the N-propeptides contain 139 and 57 amino acids, respectively, and the corresponding C-propeptides comprise 246 and 247 residues. The structures of these propeptides were quickly shown to differ from that of the triple-helical domain and to be more typical of globular proteins. Of particular interest was the observation that cysteine and tryptophan residues, which are not present in collagen types I and II, were both present in the precursor molecules. The specific location of these amino acids in the extension peptides proved useful in the development of early assays of the proteinases effecting the extracellular conversion of procollagen into collagen. However, attention focused primarily on the role of the cysteine residues and their potential for forming intra- and interchain disulfide links, and specifically on the role of this type of cross-link in the intracellular assembly of the triple helical precursor (see below).

Studies conducted with matrix-free cells (i.e., those isolated from tissues by collagenase/trypsin digestion and then incubated in a cell suspension) from a variety of embryonic chick tissues permitted a detailed study of helix assembly, disulfide-bonding of precursor polypeptides, and procollagen secretion [59]. Such studies (see below) identified a role for the carboxyl propeptides in helix

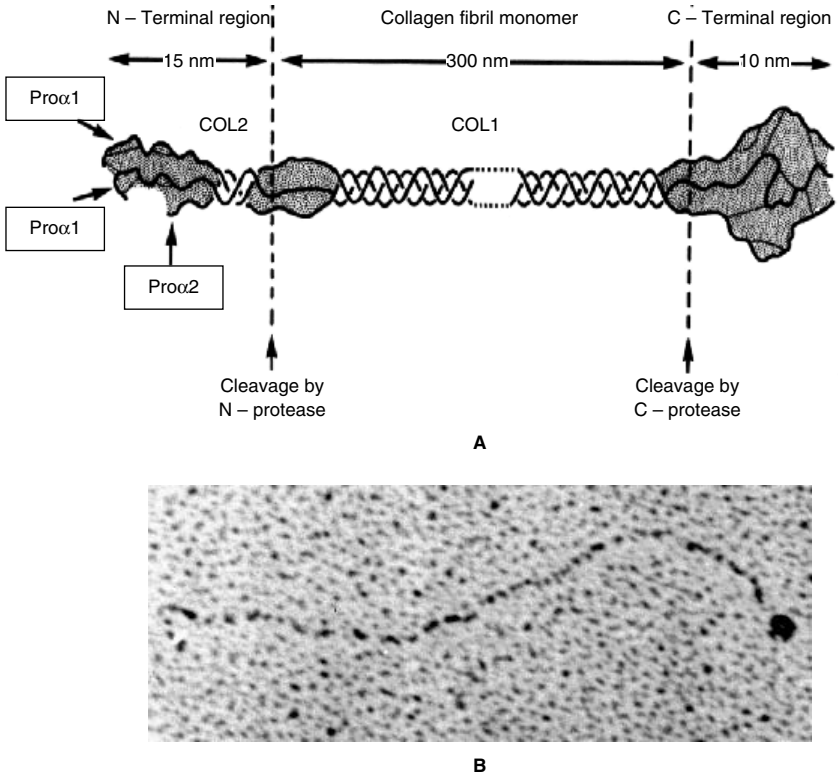


Figure 7. The structure of a type I procollagen molecule. **A**: Diagrammatic representation of procollagen I showing the relative dimensions of the rod-like triple-helical domain and the largely globular N- and C-terminal propeptides contributed by two proα1(I) chains and one proα2(I) chain. Intramolecular disulphide bridges are present in both N- and C-propeptides, but stabilizing intermolecular disulphide bonds are present in the C-propeptide only. **B**: The major structural features of type I procollagen are revealed in rotary shadowing images. The length of the rodlike triple helix is 300 nm. The C-terminal propeptide is observed as a distinct globular domain, while at the other end of the molecule is the characteristic ''hook ''arrangement of the N-propeptide that arises as a consequence of the short helical sequence it contains.

assembly. The presence of the globular extensions on the procollagens also confers solubility under physiological conditions and prevents aggregation and fibril assembly occurring intracellularly. It therefore follows that controlled cleavage of the propeptides is likely to be a prerequisite for collagen fibril formation in the extracellular space. Much research has focused on the processing of procollagen by specific procollagen proteinases [60] and a brief discussion of the relevance of the self-assembly processes of collagen fibril formation to events *in vivo* is presented below. The removal of the large globular domain at the carboxyl-terminus is apparently a necessary step in fibril assembly. This extension is clearly visible in rotary shadowed preparations of type I procollagen (Fig. 7B), and biochemical studies have shown that it is further distinguished by its content of sugar residues occurring at a single Asn–X–Ser/Thr carbohydrate attachment site in both the proα1(I) and proα2(I) chains [61]. The N-terminal extension of procollagen I can also be visualized in the electron microscope, but it appears more like a small, flexible hook than as a globular domain.

Detailed studies of the structure of the N-propeptide of type I procollagen were made possible by the abundance of pN-collagen in cattle suffering from dermatosparaxis — a heritable condition in animals, in which failure to cleave off the N-propeptide but in which there is normal removal of the C-propeptide gives rise to an accumulation, especially in the skin, of collagen molecules retaining the N-terminal extensions [62–65] (and Chapter 9, this volume). Intensive study of the N-terminal propeptides of this material showed that cysteine residues are confined to pNα1(I) chains and are involved in intrachain disulphide bonds in the very N-terminal region (approximately 100 residues) of the propeptide. Adjacent to this region of the molecule is a short helical segment rich in Gly, Pro, and Hyp (the NC2 domain), which is separated from the main body of the collagen helix by a further nonhelical segment, the amino telopeptide [66] (Fig. 7). The N-terminal propeptide of the pNα2(I) chain is shorter than that of the pNα1(I) chain, as it lacks the very N-terminal globular domain. Confirmation of this organization of the propeptides of procollagen I has come from cDNA sequence analysis of the respective genes. However, much remains to be elucidated about the detailed three-dimensional structures of these extensions, and their physiological roles associated with the assembly and processing of procollagen molecules continue to be investigated. Recently, the secondary structure of type I collagen N-telopeptide, as demonstrated by Fourier transform infrared spectroscopy and molecular modeling, has been reported. At 30°C, the structure is β sheet with β turns, and is stabilized by hydrogen bonds [67].

The subsequent fate of the propeptides is also of great interest for a role in feedback control of collagen synthesis has been suggested, and the deposition of the cleaved propeptides in the matrix cannot be excluded [68]. Detection

TABLE 3. Collagen Post-translational Processing Enzymes

Enzyme	Event Catalyzed	Substrate Requirement
A. Intracellular		
Signal peptidase	Cleavage of signal peptide of pre-proαchains	Wide range of newly synthesized proteins
Prolyl 4-hydroxylase	4-hydroxylation of proline	−X−Pro−Gly−
Prolyl 3-hydroxylase	3-hydroxylation of proline	−Pro−4Hyp−Gly−
Lysyl hydroxylase	Hydroxylation of lysine	−X−Lys−Gly−
Hydroxylysyl galactosyl-transferase	O-Glycosylation of hydroxylysine	UDP-galactose; hydroxylysine in peptide linkage
Hydroxylysyl glucosyl-transferase	O-Glycosylation of galactosyl hydroxylysine	UDP-glucose Galactosyl-hydroxylysine in peptide linkage
Protein disulphide isomerase	Native disulphide bond formation by thiol:disulphide interchange	Newly synthesized polypeptide chains
Prolyl-peptidyl *cis/trans* isomerase	Interconversion of *cis* and *trans* isomers of the peptide backbone around the planar imide bond in−X−Pro−sequences	Newly synthesized polypeptide chains
B. Extracellular		
Procollagen N-proteinase	Removal of N-propeptides	Types I and II procollagens
Procollagen C-proteinase	Removal of C-propeptides	Types I, II and III procollagens
Lysyl oxidase	Cross-link formation	D-periodic arrangement of molecules in fibril; telopeptidyl lysyl and hydroxylysyl residues

of the propeptides in the serum and urine offers a means of monitoring collagen synthesis and degradation [69,70]. The value of such clinical assays is clearly apparent with respect to the measurement of increased levels of pNα1(I) peptide in metabolic bone and cartilage diseases [71] and diseases associated with fibrosis [72], α1(II) N-propeptide associated with altered cartilage metabolism [73], α1(III) N-propeptide following severe rejection after cardiac transplantation [74], α1(III) N-propeptide and collagen IV fragments in hepatic fibrosis and abdominal aortic aneurysm [75,76], hydroxylysine glycosides and pyridinium cross-links [77], and C-telopeptide in multiple myeloma [78]. Type I collagen C-telopeptide breakdown products have also been used as markers of menopause and osteoporosis [79] (see also Appendix, Extracellular Collagen Metabolites in Body Fluids; this volume).

Biosynthesis and Assembly of Procollagen I

The biosynthesis of the collagen molecule is a complex, multistage process that begins with the transcription of the individual collagen genes and culminates in the maturation of the fibrous collagen component of the extracellular matrix [58,80,81]. This biosynthetic process is characterized by a number of complex co- and post-translational modifications, many of which are unique to collagens and collagenous sequences. Intracellular modifications of the newly synthesized polypeptide chains result in the formation of triple-helical procollagen molecules, and extracellular processing converts these into collagens and incorporates them into stable, cross-linked fibrils (Fig. 8). At least 10 different enzymes have been implicated in the post-translational processing of the collagen molecule (Table 3). The biosynthesis of type I collagen can be regarded as a useful model exemplifying many of the common features of collagen biosynthesis, although it will be noted that nonfibrillar collagens exhibit deviations from this general scheme, particularly in their macromolecular assembly.

Pre-Procollagen mRNA Translation

Translation of the mRNAs encoding the pre-proα(I) chains begins on free ribosomes with the synthesis of homologous and highly hydrophobic N-terminal signal peptide extensions of 22–26 amino acid residues, similar to those in most other secreted proteins [82]. The signal peptides are essential for the efficient and selective targeting of nascent protein chains to the endoplasmic reticulum. These sequences interact first with an intermediate cytoplasmic signal recognition particle (SRP)

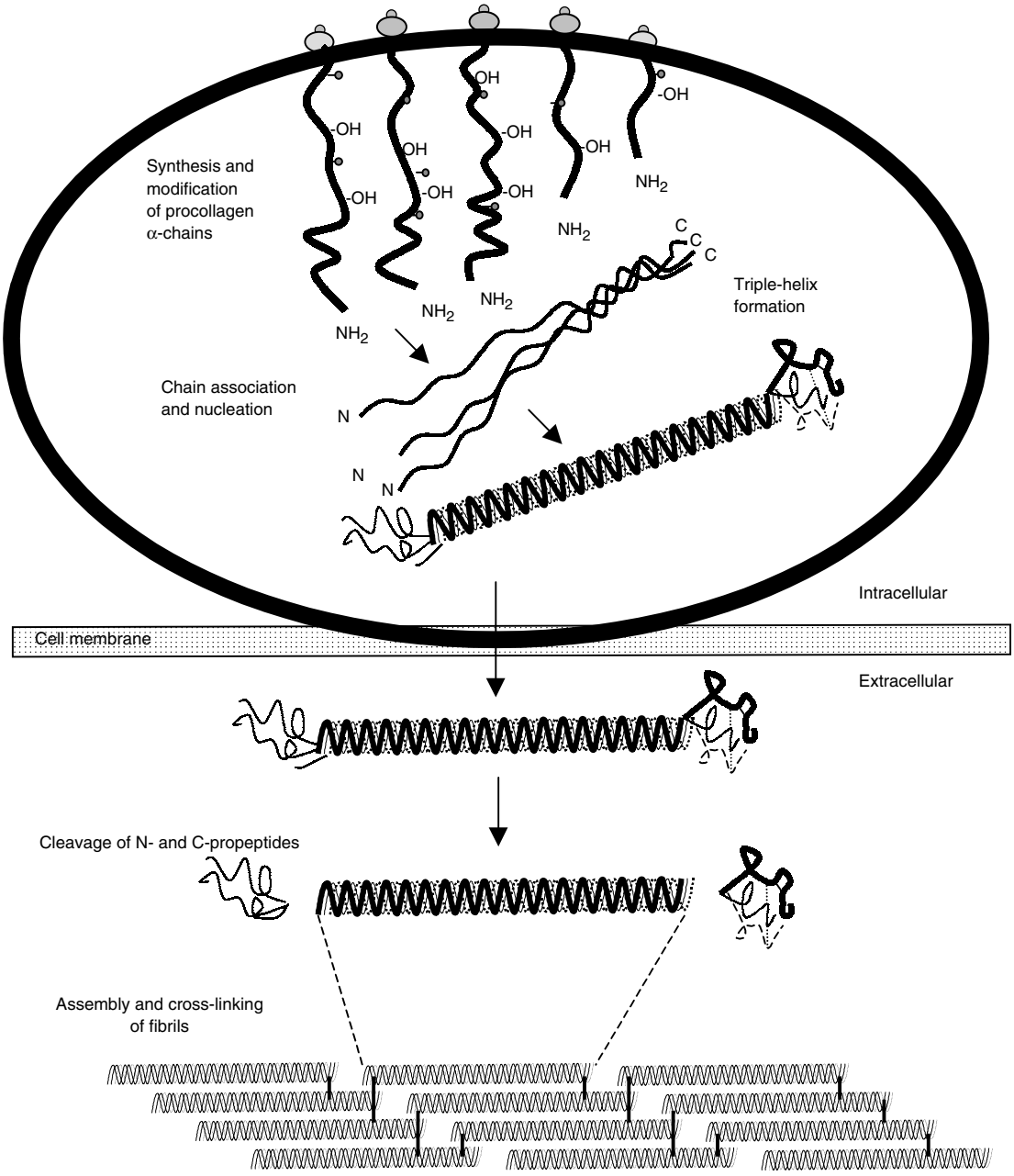


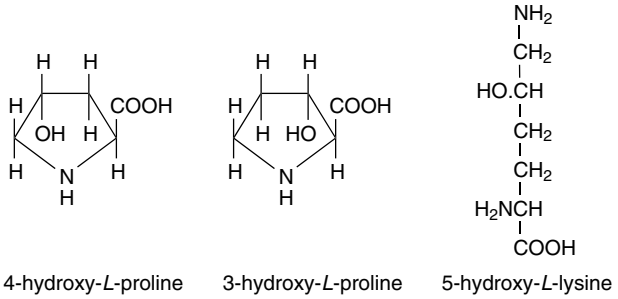
Figure 8. Schematic representation of the intracellular and extracellular steps involved in the synthesis, processing, and assembly of type I collagen molecules into fibrils. The individual collagen polypeptide chains are synthesized on the ribosomes of the rough endoplasmic reticulum and secreted into the lumen, where they undergo complex enzymic modifications prior to chain association and triple helix formation. The newly formed procollagen molecules are then secreted via secretory vacuoles originating from the Golgi apparatus, and once in the extracellular space undergo further modification by specific proteinases which cleave off the N- and C-propeptides. The tropocollagen molecules thus formed may participate in fibril formation by aligning in a characteristic staggered array that is subsequently stabilized by the formation of covalent cross-links. —OH represents prolyl and lysyl hydroxylation. —○ represents hydroxylysyl glycosylation.

to produce an SRP/polypeptide complex able to associate either with a proteinaceous receptor component or directly with membrane lipids of the endoplasmic reticulum [83]. The signal sequences also play a central, although poorly understood, role in the translocation of the pre-proα collagen chains across the endoplasmic reticulum. During or shortly after translocation, a recognition event occurs between the signal sequence and the signal peptidase on the luminal side of the endoplasmic reticulum membrane, which culminates in the cleavage of the signal peptide to give pre-proα chains. The signal peptidase involved in this reaction exhibits a wide substrate specificity, and is able to cleave the signal peptide from a number of unrelated newly synthesized proteins within the same cell [84].

Intracellular Post-Translational Modifications

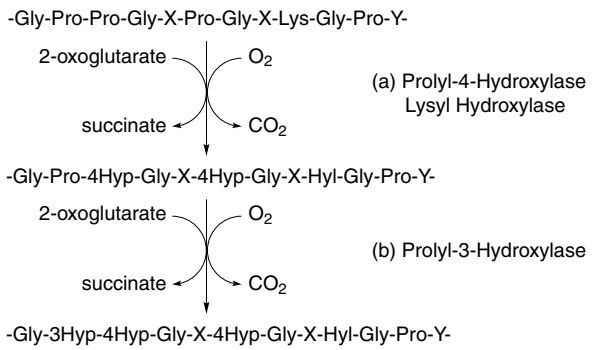
Intracellular modifications of the newly synthesized polypeptide chains result in the formation of procollagen molecules containing an array of hydroxyproline and hydroxylysine residues, which derive not from insertion into the polypeptide chains by translation of specific codons but which arise entirely as a consequence of collagen-specific intracellular enzymic modifications within the cisternae of the rough endoplasmic reticulum. The principal modifications are the hydroxylation of prolyl and lysyl residues and the glycosylation of hydroxylysyl residues, and these are brought about by the actions of three hydroxylases and two glycosyl transferases. Most of these events occur as cotranslational modifications while the nascent polypeptide chains are growing on the ribosomes, but the reactions are continued as posttranslational modifications until triple-helix formation of the proα(I) chains occurs, which prevents any further processing.

Hydroxylation of prolyl and lysyl residues. The hydroxylation of prolyl and lysyl residues in peptide linkage is catalyzed by three separate glycoprotein members of the 2-oxoglutarate dioxygenase family of enzymes, *viz.* prolyl 4-hydroxylase, prolyl 3-hydroxylase, and lysyl hydroxylase, to yield the hydroxy amino acids shown below.



All three enzymes require a nonhelical substrate and the minimum substrate requirements of these enzymes are the triplets X–Pro–Gly, Pro–4Hyp–Gly, and X–Lys–Gly, respectively. Prolyl 4-hydroxylase and lysyl hydroxylase are influenced by adjacent amino acids and by peptide chain length. All three hydroxylases are inactive when presented with a triple-helical substrate, and therefore the required modifications must be completed before triple-helix formation of the newly synthesized proα chains occurs [59,85–91].

The reaction mechanisms of all three hydroxylase enzymes are similar and each requires as cosubstrates Fe^{2+}, 2-oxoglutarate, molecular O_2, and ascorbate.



The 2-oxoglutarate is stoichiometrically decarboxylated during hydroxylation, with one atom of the O_2 molecule being incorporated into succinate and the other into the hydroxyl group formed on the proline or lysine residue. Ascorbate is also a specific requirement for the collagen hydroxylases, although it is not consumed stoichiometrically, and the hydroxylation reaction may even proceed for a few cycles in its absence. The main biological function of ascorbate in the collagen hydroxylase reaction *in vivo* may well be to act as an alternative oxygen acceptor in uncoupled decarboxylation cycles (decarboxylation of 2-oxoglutarate without subsequent hydroxylation of a proline or lysine residue) [90]. The iron chelator, α,α′-bipyridyl, is a potent inhibitor of these hydroxylase reactions, and it is frequently employed *in vitro* for the synthesis of nonhydroxylated collagen (sometimes called protocollagen), which may be used as a substrate in the assays for prolyl 4-hydroxylase and lysyl hydroxylase [86].

Prolyl 4-hydroxylase. The enzyme prolyl 4-hydroxylase (proline, 2-oxoglutarate 4-dioxygenase, EC 1.14.11.2) catalyzes the formation of 4-hydroxyproline in collagen by the hydroxylation of proline residues in X–Pro–Gly sequences [86–89,91]. These 4-hydroxyprolyl residues are essential for the folding and stabilization of the newly synthesized procollagen polypeptide chains into triple-helical molecules at physiological temperature, a crucial function which renders prolyl 4-hydroxylase a target for pharmacological modulation as a potential means of controlling fibrotic diseases characterized by uncontrolled overproduction of collagen [72]. The active prolyl 4-hydroxylase in vertebrates is a tetramer ($\alpha_2\beta_2$) of M_r 240,000 and consists of two different types of subunits (α subunits, M_r 64,000 and β subunits, M_r 60,000). It has two catalytic sites, one per pair of dissimilar subunits.

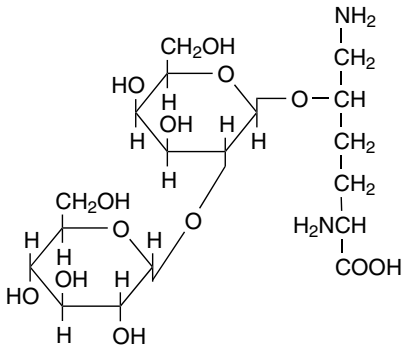
A number of inactivators and substrate analogs of prolyl hydroxylase have now been identified [89], and binding studies using several of these have demonstrated that the peptide- and 2-oxoglutarate-binding sites are located on the α subunit, whereas the ascorbate-binding sites may be built up of both α and β subunits. Thus, the principal contribution to the catalytic sites is from the α subunits, but some parts of the large catalytic sites may be cooperatively built up of both the α and β subunits. The avian and mammalian α subunit sequences revealed by cDNA analyses are highly conserved [88]. Two highly homologous isoforms, α(I) and α(II), have now been identified and both are expressed in various human tissues [92,93]. The α subunit of prolyl 4-hydroxylase from *Drosophila* has been cloned, and the corresponding enzyme tetramer characterized [94]. The β subunit has proved to be a highly unusual multifunctional polypeptide involved in two entirely different enzymic reactions. The human β subunit has been shown to be the same protein as the enzyme protein disulfide isomerase (PDI) [95–100], which catalyzes disulfide bond formation in the *in vitro* biosynthesis of collagens and other secretory and cell surface proteins, and acts as a molecular chaperone during the assembly of procollagen [96,98,101–104]. It also serves as a subunit in the microsomal triacylglycerol transfer protein [105]. Recently, it has become apparent that PDI is the archetype of a family of sequence-related PDI-like proteins [106–108].

Prolyl 3-hydroxylase. The enzyme prolyl 3-hydroxylase (procollagen proline, 2-oxoglutarate 3-dioxygenase, EC

1.14.11.7) exhibits very specific substrate sequence restraints, catalyzing the hydroxylation of prolyl residues only at the X position of X–Y–Gly triplets, and then only when the Y position is occupied by a 4-hydroxyprolyl residue [109]. It is known that prolyl 3-hydroxylase requires the same specific cofactors as are involved in prolyl 4-hydroxylation, and on this basis related reaction mechanisms are postulated [85]. Few structural details of this enzyme have so far emerged, although it is known to be a glycoprotein of M_r 160,000 [109,110]. The function of the major product of the reaction, 3-hydroxyproline, is unknown and its relative abundance varies markedly in the different collagens — the individual chains of type I collagen each contain a single residue, whereas the 3-hydroxyproline content of type IV collagen can be as high as 20 residues per 1,000 amino acids [110].

Lysyl hydroxylase. The enzyme lysyl hydroxylase (procollagen lysine, 2-oxoglutarate 5-dioxygenase, PLOD1, EC 1.14.11.4) catalyzes the hydroxylation of lysyl residues in X–Lys–Gly triplet sequences by a mechanism similar to that described for prolyl 4-hydroxylase [85,111]. The hydroxyl groups of the hydroxylysyl residues have two important functions: they act as attachment sites for carbohydrate residues (either galactose or glucosyl-galactose) and they play a crucial role in the formation of intra- and intermolecular collagen cross-links. In view of the functional significance of these residues, there is surprising variation in the hydroxylysine content of the α chains of the different collagen types (5 to 70 residues per 1,000 amino acids) [85,86,89]. The importance of hydroxylysine in collagen cross-link formation may be inferred from the inherited connective tissue disorder, Ehlers-Danlos syndrome type VI, in which lysyl hydroxylase deficiency results in impaired cross-link formation and consequent susceptibility to mechanical disruption of tissues [112–114] (see also Chapter 9, this volume). The active enzyme in vertebrates is a glycoprotein containing asparagine-linked carbohydrate moieties which are essential for enzymic activity [115]. It exists, probably, as a dimer (M_r 190,000) comprising two monomers (M_r 85,000) that may differ in their carbohydrate content. Elucidation of the cDNA sequence for chick lysyl hydroxylase revealed a polypeptide of 710 amino acids with a 20-residue signal sequence, but, surprisingly, only low homology with the catalytically important α subunit of prolyl 4-hydroxylase with which it shares certain enzymic properties with respect to cofactor and substrate specificities [116]. Other human lysyl hydroxylase isoforms have recently been identified [117,118]. The absence of X–Lys–Gly triplets within the telopeptides, the tissue specificity of Ehlers-Danlos syndrome type VI, and reports that highly purified lysyl hydroxylase fails to hydroxylate telopeptidyl lysyl residues all suggest the involvement of at least one other enzyme. Evidence has been obtained for a bone-specific telopeptide lysyl hydroxylase that directs hydroxylation of lysyl residues specifically in the nonhelical regions [119].

Glycosylation of procollagen chains. Vertebrate collagen molecules contain the monosaccharide galactose and the disaccharide glucosyl-galactose, which utilizes an unusual α1 → 2-O-glycosidic linkage. These glycosides are associated in covalent linkage with hydroxylysine residues within the triple-helical domains [120]. The structure of 2-O-α-D-glycopyranosyl-O-β-D-galactopyranosylhydroxylysine is shown below.



The extent of this glycosylation is very variable, both between collagen types and within the same collagen in different tissues and at different ages [121–123]. The biological role of the collagen-specific carbohydrate units remains unclear, although they may have some role in fibril organization. Indeed, it has been suggested that the inverse relationship that exists between carbohydrate content and collagen fibril diameter could reflect steric hindrance to the formation of highly ordered fibrils by these bulky groups [121]. However, this is only one of a number of possible factors likely to participate in determining fibril diameter.

The glycosylation of hydroxylysine residues within the triple helix requires the activity of two enzymes of the rough endoplasmic reticulum, hydroxylysyl-galactosyltransferase (UDP galactose: 5-hydroxylysine-collagen galactosyltransferase, EC 2.4.1.50) and galactosyl-hydroxylysyl-glucosyltransferase (UDP glucose: 5-hydroxylysine-collagen glucosyltransferase, EC 2.4.1.66), which transfer galactose to some of the hydroxylysines and glucose to some of the galactosyl-hydroxylysine residues, respectively [121–123]. The carbohydrate is donated in both reactions by the appropriate UDP-glycoside, and both enzymes require a bivalent cation (preferably Mn^{2+}) for activity. A free ε-amino group in the substrate hydroxylysyl residue and a nonhelical polypeptide conformation are absolute requirements for both transferases, and the reactions are further influenced by chain length (longer peptides are more effective substrates) [121,124,125]. Hydroxylysyl-galactosyl transferase can bind at least two manganese ions per molecule of active enzyme. Its molecular weight is less certain, with values of between 50,000 and 450,000 reported for impure preparations. In contrast, the molecular size of the glucosyl transferase is well defined (M_r 72,000–78,000), and it exists as a single polypeptide chain.

The propeptides of procollagen (only the C-terminal propeptide in type I procollagen but both N- and C-propeptides in procollagen II) contain asparagine-linked oligosaccharide units that are not found in the collagenous domain. These N-linked carbohydrate units are synthesized on carrier lipids and then transferred as a whole via a lipid oligosaccharide carrier protein intermediate to asparagine residues in Asn–X–Thr(Ser) sequences in the nascent proαI(I) and proα2(I) chains [126,127]. These glycosylation reactions take place within the rough endoplasmic reticulum but the function of these asparaginyl-linked oligosaccharide units of procollagen are unknown.

Helix Formation and Secretion

The assembly of the type I procollagen molecule is a complex helical folding process initiated by association of

the three C-terminal propeptides and presumed to proceed through a series of events involving chain alignment, nucleation, and propagation. Among the more poorly understood aspects of this assembly process are the mechanisms of the selection process, the locus of the initial chain registration event, and the effect of the post-translational modifications on helix assembly. The propeptides begin to fold into their appropriate secondary and tertiary structures soon after synthesis and these conformations are likely to be intimately involved in determining the association of the three polypeptide chains. As the polypeptide chains are translocated across the endoplasmic reticulum membrane, intrachain disulfide bonds are formed within the amino and carboxyl terminal propeptides, and hydroxylation of proline and lysine residues occurs within the collagenous domains. Chains then associate via their C-propeptides to form homo- or heterotrimeric molecules. This allows the triple helical domain to form a nucleation point at its carboxyl terminal end, ensuring correct alignment of the chains. The triple helix then folds in a carboxyl to amino terminal direction, with the N-propeptides finally associating and, in some cases, forming interchain disulfide bonds. The formation of the triple helix precludes further enzyme-linked post-translational modification, and, once folded, the procollagen is readily secreted into the extracellular space.

It has been proposed that the chain selection event is associated with the attachment of the cytosol-initiated signal peptide sequences of each chain to the endoplasmic reticulum surface, such that the appropriate nascent proα chains (two proα1(I) chains and one proα2(I) chain in the case of type I procollagen) might be inserted into the endoplasmic reticulum at a common entry site [128]. This recognition process may involve membrane surface component(s) of unknown identity [129]. Chain association and alignment takes place only as the chains near full elongation, such that folding may proceed in the C- to N-terminal direction, a process that may be facilitated by the organization of the attachment of the ribosomes on the rough endoplasmic reticulum [130]. This association of proα chains almost certainly is initiated by noncovalent interactions between the C-terminal propeptides.

Several sequences in α1(III) that are crucial for recognition and trimerization have recently been defined. By exchanging the C-propeptides of proα1(III) and proα2(I) chains, a discontinuous sequence of 15 amino acids in proα1(III) was identified that directs procollagen self-association [131–133]. The last ten amino acids of the proα2(I) carboxyl terminal region were shown to be important for the assembly of stable type I collagen [134,135]. A study of disproportionate micromelia (*Dmm*) in mice, caused by a three nucleotide deletion in the C-propeptide coding region of *Col2a1*, results in decreased assembly and folding of type II collagen molecules and their retention in the endoplasmic reticulum [136]. An important initial force for association is the entropy-driven association of strategic surface aromatic and hydrophilic residues within the C-terminal regions [137]. The association of C-termini is then stabilized by disulphide bond formation catalyzed by the enzyme PDI (EC 5.3.4.1), which is located within the lumen of the endoplasmic reticulum [135,138]. PDI also acts as a molecular chaperone during the assembly of procollagen [96]. Helix formation may be interrupted by the random occurrence of *cis* peptide bonds which are converted to the *trans* configuration by a separate cytosolic (and potentially rate-limiting) enzyme, prolyl-peptidyl *cis-trans* isomerase (PPI) [139–141].

It is generally believed that folding of the triple-helical domain occurs by nucleation and propagation. The existence of a stable nucleation site was first postulated some years ago when it was discovered that there was a short stretch of between 3 and 10 Gly–Pro–Hyp triplets (known from thermal stability experiments to form one of the most stable triple helices) at the very C-terminus of the collagenous domain of type I procollagen α chains. This concept has now been generally accepted with the identification of similar features in all collagens for which the sequence is known. The sequence Gly–Pro–Arg has been shown to be of similar stability to Gly–Pro–Hyp. Variations in a central guest triplet within the framework $[Gly–Pro–Hyp]_3–Gly–Xaa–Yaa–[Gly–Pro–Hyp]_4$ have highlighted the sequence dependence of the folding of collagen-like peptides, with half-times for refolding varying from 6 to 100 minutes [142]. Such information on the relative propensities of different tripeptide sequences to promote nucleation of the triple helix in peptides will aid in identification of nucleation sites in collagen sequences.

Stable triple-helix formation is dependent not only on the presence of glycine as every third residue, but also requires 4-hydroxyproline in the Y position of a high proportion of Gly–X–Y triplets (at least 100 of the 1,000 amino acids of the helical domain of each chain), and assembled proα chains that do not contain the appropriate number of hydroxyproline residues accumulate in the lumen of the endoplasmic reticulum. Some insights into the process of triple-helix formation have been provided by analysis of naturally occurring mutant collagens in inherited connective tissue diseases such as osteogenesis imperfecta and Ehlers-Danlos syndrome ([47,143–145], and Chapters 8 and 9, this volume). For example, a mutation involving the substitution of a glycine residue by another amino acid has drastic consequences for triple-helical assembly and stability. The rate of triple-helix formation is retarded, allowing post-translational enzymes the opportunity to overmodify the collagenous domain [145a]. The relationship of these biochemical modifications to the clinical phenotype, however, is not well understood.

The mechanism of procollagen secretion is also poorly understood, but it is known that procollagen follows the classical secretion route for extracellular proteins, passing through the Golgi complex *en route* to the extracellular space [59]. The normal rate of procollagen secretion is apparently dependent upon the triple-helical conformation, for if triple-helix formation is prevented by inhibiting prolyl hydroxylase (by the use of α,α′-bipyridyl or by limiting the availability of the cofactors Fe^{2+} or O_2) then the nonhelical proα chains first accumulate within the cisternae of the rough endoplasmic reticulum and are then secreted at a slower rate [59,146,147].

Extracellular Processing and Fibril Formation

The factors that control fibril assembly and lateral growth, and which account for the widely differing fibril diameters, are not well understood. Although the aggregation of collagen molecules into axially ordered fibrillar structures is basically a self-assembly process, factors extrinsic to these molecules must also be implicated in determining the sharp diameter distributions of collagen fibers *in vivo*. Fibril formation involves the specific enzymic cleavage of the procollagen N- and C-terminal propeptides in the extracellular space, and the enzymes involved must therefore play a key role in this ordered aggregation. Consequently, the study of factors involved in regulating their activity has been

an area of intense interest. It should be noted, however, that such processing is essentially a characteristic of the fibrillar collagens and is not a universal prerequisite for the assembly of all collagen types.

The cleavage of the N- and C-terminal propeptides from the procollagen I molecule at specific peptide bond cleavage sites is achieved by two specific neutral metalloproteinases, procollagen N-proteinase (EC 3.4.24.14) and procollagen C-proteinase (E.C 3.4.24.19), both of which require Ca^{2+} for activity and are members of the zinc-binding metalloproteinase superfamily [16]. These enzymes are therefore inhibited by metal chelators but not by inhibitors of serine or cysteine proteinases. The procollagen proteinases initially proved difficult to purify [148–153], but the primary structures of both enzymes have now been determined [154,155].

Purified type I procollagen N-proteinase (M_r 500,000) cleaves the N-terminal propeptides of types I and II procollagens between a proline and a glutamine residue, only if the proteins are in a native conformation, and not if they are partially unfolded so that the N-telopeptides are no longer in a hairpin conformation. It resolves into four polypeptide components on SDS-polyacrylamide gel electrophoresis, the two larger of which exhibit "N-proteinase" activity. The full-length cDNA for bovine N-proteinase encodes 1,205 residues (equivalent to a polypeptide of $M_r \sim 140,000$). An active human N-proteinase of M_r 70,000 has recently been characterized, which may have arisen by alternative splicing of a larger gene [155]. A separate enzyme is involved in the processing of the N-propeptide of type III procollagen [150], and this N-propeptidase has been purified from fetal aortic smooth muscle cell cultures and human placenta [151,152].

Type I procollagen C-proteinase was first isolated as a $M_r \sim 70,000$ enzyme from the medium of cultured fibroblasts and from organ cultures of chick embryo tendons [148,153,154]. This enzyme cleaves native and denatured C-terminal propeptides of both the proα1(I) and proα2(I) procollagen chains at an Ala–Asp bond, those of the α1(III) procollagen chain at a Gly–Asp bond, and those of type I procollagen homotrimer and type III procollagen, all at identical rates. The C-proteinase can also specifically cleave precursors of lysyl oxidase and laminin 5. A possible mechanism of stimulation of C-proteinase activity by a copurifying enhancer protein (M_r 55,000) has been described [153]. Analysis of cDNA for procollagen C-proteinase [154] revealed open reading frames for three proteins — an M_r 70,000 polypeptide identical to bone morphogenetic protein-1 (BMP-1), a similar protein with an additional histidine-rich sequence, and a polypeptide of $M_r \sim 100,000$ which has a high degree of similarity to the protein encoded by the *Drosophila* gene *tolloid*, and is thus referred to as mammalian tolloid (mTld). BMP-1/procollagen C-proteinase may also play an essential role in development in addition to its role in the processing of procollagens.

It has been shown that human mast cell chymase, but not tryptase, can cleave the proα1(I) collagen carboxyl terminus in the propeptide region at Leu-1248–Ser-1249, to create a novel C-telopeptide 20 amino acids longer than that generated by procollagen C-proteinase [156]. Turbidometric fibril formation assays demonstrated *de novo* formation of chymase-generated collagen fibrils that had characteristic lag, growth, and plateau phases. Thus, mast cell chymase may play a role in regulating collagen fibrillogenesis in fibrosis.

The precise role of the N- and C-terminal propeptides in controlling fibril formation, diameter, and general morphology is far from resolved, and even the sequence of events leading to their controlled cleavage is less straightforward than originally thought. For example, the classical pulse-chase experiments that demonstrate a precursor-to-product relationship indicative of a predominant processing sequence, primary precursor→pC-collagen→collagen, fail to explain either the presence or the proportions of pN-collagen[1] and pC-collagen[2] in extracted collagen fibrils. It has been possible to study the role of procollagen I processing *in vitro* using highly purified N- and C-terminal procollagen proteinases in an elegant, cell-free assay system [157–159] in which mixtures of type I procollagen and pC-collagen (prepared by digestion of type I procollagen with N-proteinase) are used as a substrate for C-proteinase and the fibrils formed examined ultrastructurally. These experiments demonstrate that persistence of the N-propeptide causes distortion of the morphology of the fibrils from cylindrical to fluted and sheetlike structures. A study of dermatosparactic calf skin collagen fibrils has confirmed that the persistence of the N-propeptide distorts fibril shape but does not inhibit the binding of gap-associated macromolecules (e.g., decorin) [64]. However, immunoelectron microscopic analyses of normal fibrils have demonstrated the presence of both pN-collagen and pC-collagen on fibril surfaces [160,161]. These studies have also demonstrated the coexistence of type I and type III collagens within individual fibrils, and it now appears that heterotypic fibrils containing molecules of type III collagen and possibly type V collagen, in addition to type I collagen molecules may, in fact, predominate *in vivo* [162,163]. The presence of the N-propeptide of type III procollagen, in particular, at the surface of collagen fibrils has led to the suggestion of a fibril diameter-limiting role for this peptide. Although pC-collagen can be persuaded to assemble *in vitro* into flat sheets by manipulating the kinetics of the system [159,164], the bulky C-propeptide probably precludes a major physiological role for this as an incorporated intermediate of fibril formation *in vivo*. The key role of telopeptides in collagen fibrillogenesis is now being elucidated [165].

Linear and Lateral Fibril Growth

The formation of very long and near-uniform diameter collagen fibrils is fundamental to the assembly of the extracellular matrix of animals. However, the mechanisms by which linear and lateral growth are regulated are incompletely understood. Regulated tip growth provides an attractive explanation for how cells are able to synthesize long fibrils during extracellular matrix formation. *In vitro* collagen fibrillogenesis following procollagen cleavage by purified proteinases results in fibrils that initially have a near paraboloidal pointed tip and a blunt end, and growth is exclusively from the pointed tip with the N-terminus closest to the fibril tip end [53]. Subsequently, a new N-terminal pointed growth tip appears in the other direction. Examination of developing chick embryonic metatarsal tendon fibrils by scanning transmission electron microscopy (STEM) mass mapping revealed that these physiological fibrils had smoothly tapering C- and N-terminal tips leading to an abrupt stop in lateral growth and a local plateau in

[1] pN-Collagen: Intermediate in the conversion of procollagen to collagen, which contains the N-propeptide but not the C-propeptide.

[2] pC-Collagen: Intermediate in the conversion of procollagen to collagen, which contains the C-propeptide but not the N-propeptide.

diameter [166]. The distance from the end of the fibril to the abrupt stop occurred at multiples of 5 D-periods, showing that D-periods at the ends of fibrils are not equivalent sites for accretion, that diameter regulation depends on surface structural features which repeat every 5 D, and that coarseness of the fibril tip is independent of fibril length.

Collagen fibrils from developing tendons have been shown to grow through deposition of early fibril segments that subsequently fuse [167,168]. The growth of these fibril segments and their intercalation into fibrils results in linear and lateral growth during matrix development and is an important determinant of tissue architecture, stability, and mechanical attributes. Fibril growth has also been found to be associated with a significant decrease in fibril-associated decorin [169,170], a member of the small leucine-rich proteoglycan (SLRP) family, supporting an earlier proposal [171] that fusion of fibrils occurs in tendon with possible regulation by a surface coat of decorin.

The role of decorin and other SLRPs (fibromodulin, lumican) in regulating fibril fusion has recently been confirmed in genetically manipulated mice models. Mice harboring a targeted disruption of the decorin gene are viable but have fragile skin with markedly reduced tensile strength; collagen fibers in skin and tendon appear coarser with irregular fiber outlines [172]. STEM mass mapping shows abrupt increases and decreases in mass along their axes. These data indicate uncontrolled lateral fusion of collagen fibrils in the decorin-deficient mice, provide a partial explanation for the reduced tensile strength of the skin, and demonstrate a fundamental role for decorin in regulating collagen fibril formation *in vivo*. Mice homozygous for a null mutation in lumican display skin laxity and fragility resembling that in certain types of Ehlers-Danlos syndrome, and the mice also develop corneal opacification [173]. The underlying defect is deregulated growth of collagen fibrils with numerous abnormally thick collagen fibrils in skin and cornea. Other studies have demonstrated that fibromodulin-type I collagen interactions are also crucial for regulating fibrillogenesis [174,175]. The relative contribution of each SLRP to regulated fibrillogenesis in different tissues remains to be determined.

Thrombospondin-2 also appears to be involved in collagen fibrillogenesis, because thrombospondin-2–null mice exhibit abnormal collagen fiber patterns in skin, abnormally large fibrils with irregular contours, and fragile skin with reduced tensile strength [176]. Thrombospondin-2 may function as a collagen fibril-associated molecule that regulates fibril diameter, and/or the disordered fibrillogenesis may reflect altered cell surface properties within extracellular assembly crypts formed by fibroblast cell surface folding. Cartilage oligomeric matrix protein (COMP), which is homologous to the thrombospondins, has been shown to participate in a high affinity zinc-dependent interaction with triple helical cartilage collagen type II, and may influence the organization of cartilage collagen fibrils [177].

Molecular Packing of Fibrils

Collagen fibrils are approximately cylindrical, with diameters in the range10–1000 nm, but the packing arrangement of monomers in fibrils is not well defined [178–181]. The lateral packing of monomers in fibrils is currently being investigated using electron microscopy and X-ray diffraction approaches. Monomers may be laterally packed on a triclinic unit cell lattice, in the form of microfibrils comprising monomers coiled into a rope-like pentameric structure [180]. Collagen molecules contain kinks between the gap and overlap regions that make the molecules tilted by about 5° relative to the fibril axis. The initial stages in fibril assembly are thought to involve the formation of structures that correspond to pentameric microfibrils. *In vitro* assembly systems have demonstrated that fibrils probably grow from pointed tips; a proposed mechanism for increase in diameter is by adding microfibrils or individual molecules to form microfibrils. The left-handed Gly–Pro–Pro helix is stabilized by a right-handed triple-helical conformation, and this in turn may be stabilized by a left-handed microfibril structure that may form cross-links to maintain the integrity of the fibrils [180].

Studies of the relative strengths of hydrophobic and electrostatic interactions in fibrillogenesis of acid soluble collagen molecules show that hydrophobic interactions, but not electrostatic interactions, maximize D-periodic banding [182]. The process of self-assembly of type I collagen monomers into fibrils also appears to depend on the interactions of specific binding sites in different regions of the monomer. Synthetic peptides with sequences found in the telopeptides were found to inhibit self-assembly of purified type I collagen in an *in vitro* system [183]. A strong interaction between the α2(I) N-telopeptide in the overlap zone and residues 781–794 of the α1(I) chains was identified, which would place the molecules in a quarter D stagger; this binding event could thus initiate assembly of a pentameric microfibril. Molecular modeling of the binding sites demonstrated favorable hydrophobic and electrostatic interactions between the α2(I) telopeptide and the α1(I) chains.

The importance of glycosylation to fibrillogenesis has been demonstrated in a study of human recombinant type II collagen expressed using an insect system, in which low and high levels of hydroxylysine and its glycosylated form resulted in marked differences in fibrillogenesis *in vitro* [184]. Marked differences in fibril formation were found between the collagen preparations with low hydroxylysine and high hydroxylysine, respectively, in that the maximal turbidity of the former was reached within 5 minutes under the standard assay conditions, whereas the absorbance of the latter increased until about 600 minutes. The morphology of the fibrils was also different, in that the high hydroxylysine collagen formed thin fibrils with essentially no interfibrillar interaction or aggregation, whereas the low hydroxylysine collagen formed thick fibrils on a background of thin ones.

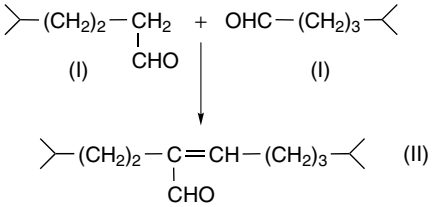
Cross-Linking and Fibril Stabilization

The spontaneous aggregation of processed collagen molecules into fibrils with a characteristic D-periodicity is followed almost immediately by the formation of covalent cross-links within and between the collagen molecules. These cross-links, which are formed from specific lysyl and hydroxylysyl residues, are essential in providing the tensile strength and mechanical stability of the collagen fibrils which their structural roles demand [185]. The only step in the cross-linking process that is known to be under direct control is the initial oxidative deamination of specific lysine or hydroxylysine residues in the nonhelical telopeptide regions. Thus, lysyl and hydroxylysyl aldehydes are generated by lysyl oxidase (protein lysine 6-oxidase, EC 1.4.3.13) [186,187], and an assortment of di-, tri-, and tetrafunctional cross-linking amino acids can form spontaneously by the intra- and intermolecular reactions of these lys/hyl aldehydes within the newly formed collagen polymers. It is noteworthy that disulfide bonding cannot play a part in the stabilization of

collagen I (or II) fibers because of the absence of cysteine residues, but a significant role for disulfide bonding in the stability of other collagen aggregates (e.g., collagens IV, VI, and IX) is well defined (see below).

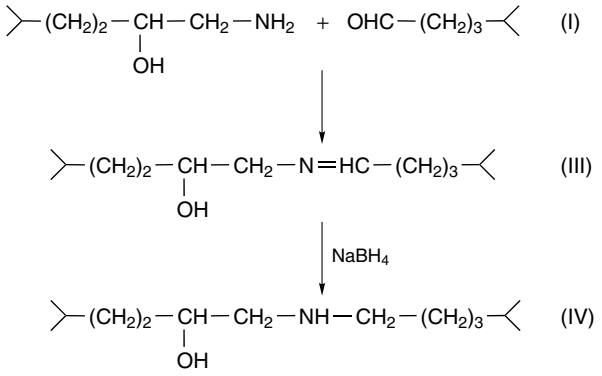
Lysyl oxidase-initiated cross-linking. Much of our early knowledge of collagen cross-links and the role of lysyl oxidase derived from studies of the tetrafunctional cross-links in elastin [36,188,189] (see also Chapter 3, this volume). In particular, the elucidation of the structure of the desmosine cross-links and the demonstration that they were derived from lysine residues provided the initial evidence for a unique mechanism for the stabilization of fibrous proteins of the extracellular matrix. However, marked differences exist between elastin and collagen in terms of their primary structures, their lysine-containing sequences involved in cross-linking, and the nature of the cross-links formed [190,191]. The catalytic activity of lysyl oxidase is dependent on strict steric requirements (in the case of collagen, the quarter-stagger arrangement of molecules in the fibril) and on the sequence of amino acids surrounding the "target" lysyl/hydroxylysyl residues [186,187,192,193]. The enzyme has been purified from several avian and mammalian sources, and its primary structure has been elucidated [192]. It is a copper-dependent amine oxidase requiring molecular oxygen and a novel aromatic carbonyl compound recently identified as lysyltyrosine quinone [194,195]. Lysyl oxidase is synthesized as a preproprotein, secreted as an M_r 50,000 N-glycosylated proenzyme, and then proteolytically cleaved to the M_r 32,000 catalytically active, mature enzyme. New lysyl oxidase-like genes have also been identified and cloned, indicating the existence of a multigene family [196–199]. Lysyl oxidase may have important roles to play in cellular homeostasis in addition to the oxidation of peptidyl lysine in the extracellular matrix [187,200]. Lysyl oxidase binds to the fibril surface and cannot penetrate to its inner domains [201], implying that the oxidation of lysyl groups must occur at an early stage of fibrillogenesis and/or that cross-links are continuously manufactured at the surface of growing fibrils.

The nature of the lysyl oxidase-derived collagen cross-links has been extensively reviewed [191,202–206]. Two related pathways can be distinguished for the fibrillar collagens, one based on lysine aldehydes, the other on hydroxylysine aldehydes. The lysine aldehyde pathway occurs primarily in adult skin, cornea, and sclera, whereas the hydroxylysine aldehyde pathway predominates in bone, cartilage, ligament, most tendons, embryonic skin, and most major internal connective tissues of the body. The first step in both pathways is the oxidative deamination of the ε-amino group in telopeptidyl lysine and hydroxylysine residues to form their corresponding aldehydes, often referred to by their trivial names as allysine and hydroxyallysine. In the lysine aldehyde pathway, two allysines (I) may condense to form the aldol condensation product (ACP) (II).

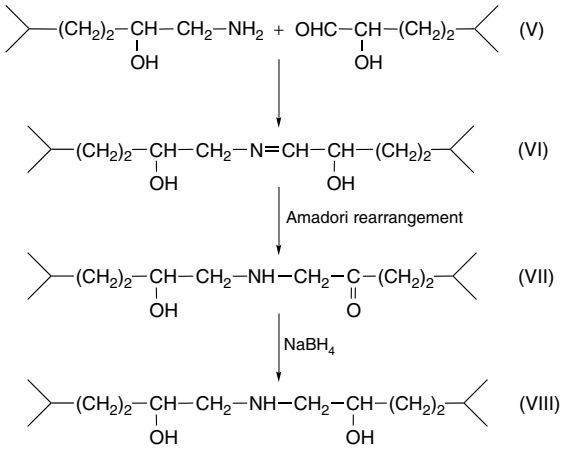


This structure was the first collagen cross-link to be identified as an intramolecular bond forming a dimer, or collagen β component, between α1(I) and α2(I) chains [207]. Its physiological significance is still not certain since it is the intermolecular, and not the intramolecular, cross-links that confer stability and strength on collagen fibers.

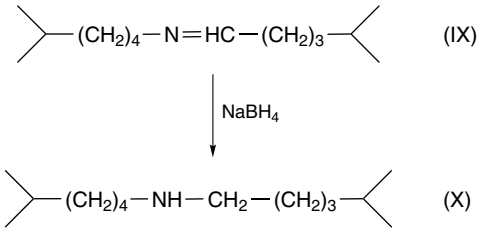
Much importance has been assigned to the aldimine (Schiff base) cross-links formed when allysine in the telopeptides reacts with lysine or hydroxylysine residues in adjacent helices to provide covalent cross-links between collagen molecules. These aldimine cross-links are acid-labile and their detection has depended on their prior reduction with $NaBH_4$ (sodium borohydride), which permits their recovery from acid hydrolyzates of collagen and their characterization by chromatographic techniques. In this way, the reduced cross-link hydroxylysinonorleucine (HLNL) (IV), derived originally from allysine and hydroxylysine, has been demonstrated as the reduction product of the aldimine cross-link, dehydro-hydroxylysinonorleucine (III).



In tissues such as bone, cartilage, and tendon, where hydroxyallysine rather than allysine residues are involved in cross-linking, the second, hydroxylysine aldehyde-derived pathway becomes important. Here, hydroxyallysine (V) can condense with a hydroxylysine residue to form the reducible cross-link dehydro-hydroxylysinohydroxynorleucine (VI) which can undergo an Amadori rearrangement *in vivo* to form hydroxylysino-5-oxo-norleucine (VII), which is resistant to heat, dilute acid, and also penicillamine (see below), and which on reduction yields hydroxylysinonorleucine (VIII).



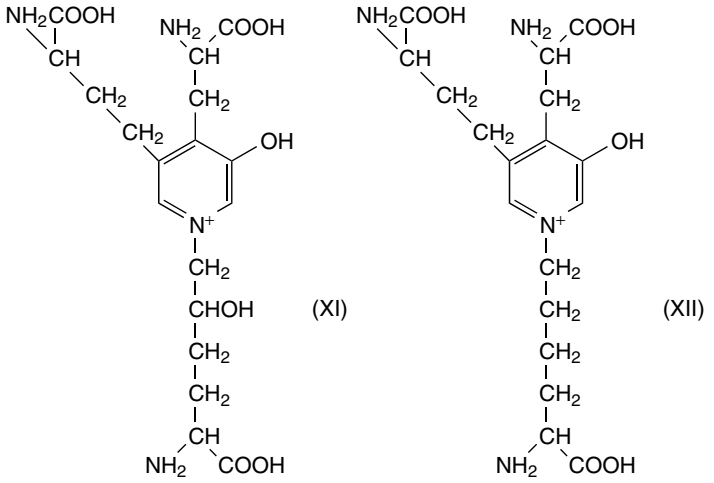
Both of these aldimine cross-links (III and VII) can occur as galactosyl or glucosylgalactosyl derivatives, demonstrating that O-glycosylation of Hyl residues does not preclude their involvement in cross-linking. A third reducible component occurs in small amounts, especially in skin, and derives from the condensation of lysine and allysine to form the dehydro-lysinonorleucine (IX), which on reduction *in vitro* is recovered as lysinonorleucine (X).



Quantification of the absolute concentration of the individual cross-links in collagens is not well documented and is complicated by the knowledge that in most tissues, with the possible exception of bone and dentine, the number of reducible cross-links decreases with age. Thus, the reducible cross-links are said to mature as they are modified to become stable, nonreducible cross-links, the nature and number of which remain to be fully defined. In the case of the hydroxylysine aldehyde pathway, however, the major mature cross-links appear to be based on trivalent 3-hydroxypyridinium residues. Two chemical forms of the cross-link have now been identified, hydroxylysyl-pyridinoline (XI), derived from 3 Hyl residues, and lysyl-pyridinoline (XII), derived from 2 Hyl and 1 Lys. These two cross-links are naturally fluorescent and can be assayed directly in tissue hydrolysates.

The highest concentrations of hydroxylysyl-pyridinoline are found in the nucleus pulposus of human intervertebral disk and in human articular cartilage [204,208], and measurement of this cross-link in body fluids has been proposed as a potential marker for the catabolism of cartilage collagens in pathological conditions such as joint and metabolic bone disease [209–211] (see also Risteli and Risteli, Appendix, this volume). Aberrant cross-link formation due to decreased activity of lysyl oxidase can occur both in inherited connective tissue diseases such as Menkes disease and the occipital horn syndrome (see Chapter 14, this volume), and in acquired disorders such as nutritional copper deficiency and lathyrism in animals [212]. The latter is a defect in collagen metabolism associated with the ingestion by animals of β-aminopropionitrile (βAPN) (derived from the sweet pea *Lathyrus odoratus*), which is a potent and irreversible inhibitor of lysyl oxidase and prevents cross-linking of newly synthesized collagen molecules into insoluble fibers. D-penicillamine is also an inhibitor of cross-linking that is able to interact with and block the newly formed lysine aldehydes and, at higher doses, inhibits lysyl oxidase activity by chelating copper. Inhibitors of cross-link formation have been used to treat fibrotic disorders such as scleroderma, and can successfully prevent excessive accumulation of collagen. Such treatment is not without risk, however, for prolonged use of βAPN, for example, may not only induce a lathyritic condition but may also interfere with elastogenesis, by inhibiting cross-linking of elastin monomers [212].

Nonenzymic glycosylation of collagen and sugar-derived cross-links. The nonenzymic glycosylation (glycation) of extracellular proteins has been of interest ever since the detection of glycosylated hemoglobin molecules in patients with diabetes mellitus [206,213]. The prolonged association of collagens with reducing sugars produces glycation end products that are implicated as factors in aging and diabetic complications. Indeed, glycation is a major cause of dysfunction of collagenous tissues in old age, and the process is accelerated in diabetic subjects due to the higher levels of glucose [206,214–216]. Glycation can affect a number of properties of collagen including its biomechanical functioning and its ability to form precise supramolecular aggregates, and result in an alteration in its charge profile and interaction with cells, and, in addition, glycated collagen can act as an oxidizing agent. The most damaging effects, however, are caused by glucose-mediated intermolecular cross-links which decrease flexibility and permeability, and reduce turnover. In virtually all extracellular proteins it is possible for a sugar group (frequently glucose) to react nonenzymically with the free ε-amino group of a lysine (or hydroxylysine) residue. This form of glycation (the Maillard reaction) involves the chemical reaction of a sugar aldehyde or ketone with a free amino group to form a Schiff base, which may undergo a rearrangement to form a fairly stable ketoamine (the Amadori product) [214]. These



structures may go on to form advanced glycation end-products (AGEs) by condensing to form imidazole-based Maillard products. Alternatively, they can be degraded into reactive α-dicarbonyl groups that react with other free amino groups to form cross-linked, pyrrole-based, pigmented, and fluorescent adducts. The occurrence and accumulation of these groups on stable low-turnover macromolecules such as collagen has received considerable attention [206]. The possibility that these cross-links could influence the resistance of collagen to being broken down or remodeled cannot be excluded and, accordingly, the contribution of nonenzymic glycosylation to normal aging and to events associated with hyperglycemia and collagen involvement in diabetic microangiopathy is under investigation [206,215,216].

ISOLATION AND CHARACTERIZATION OF COLLAGEN TYPES

The recognition of many genetically distinct collagen types was achieved initially through classical protein separation techniques [217]. The screening of cDNA libraries with individual collagen cDNA probes has now led to the identification of new collagen types (*viz.* types XII–XIX) as a consequence of the cross-hybridization of related collagen probes at low stringency. In this latter approach, the identification of amino acid sequences derived from cDNA sequences has permitted the synthesis of appropriate oligopeptides which, in turn, have been used to generate antibodies which recognize the new collagens in tissues and in cells in culture. Both classical protein separation techniques and cDNA cross-hybridization procedures rely on the distinctive features of the collagenous molecules and their respective DNA coding sequences. The application of rotary shadowing techniques to collagenous molecules has provided a visualization of the shape of individual collagen molecules [218] (Fig. 9) and, consequently, an insight into their macromolecular structure/function *in vivo*.

The key feature of collagenous macromolecules is that they perform a structural role in the extracellular matrix, where the fibrils and networks which the individual types form are stabilized by covalent cross-links. Consequently, it is virtually impossible to extract the intact subunits of these structures from mature tissues and, for this reason, the majority of work on the isolation of collagen types has been done on young animals or fetal tissues [219]. In such tissues, the biosynthetic rate is high and approximately 5–10% of the total collagens can be solubilized in dilute salt solutions and/or dilute acid solutions. By rendering animals lathyritic it is possible to increase this yield, particularly of the Group 1 collagens stabilized by lysyl oxidase-dependent cross-linking. Lathyrogens have also been found useful in increasing the solubility of collagens in two transplantable tumors — an EHS (Engelbroth-Holm-Swarm) sarcoma used in the isolation of type IV collagen [220] and a rat chondrosarcoma used in the isolation of type IX collagen [221] — and in bone for the isolation of type V collagen [222]. However, in the case of many of the minor collagens that contain substantial noncollagenous domains, it is not clear to what extent the stability of their aggregates is dependent on lysyl-derived cross-links. Indeed, in most of these collagens, disulfide bonding may predominate, and in the heavily disulfide-bonded type VI collagen aggregates, for example, no lysyl cross-links have been identified. Here it has been found appropriate to use chaotropic agents such as guanidinium chloride and urea in the presence of reducing agents to effect the solubilization of collagen VI from tissues [223–225].

Other strategies for the isolation of intact native soluble collagens and their precursors include the use of appropriate cell culture systems that permit the recovery of intact collagens from the culture medium. Care is always required to avoid proteolysis, especially of the nonhelical domains, by the inclusion of proteinase inhibitors at all stages of protein isolation and fractionation. A range of procedures, including salt fractionation followed by various chromatographic, electrophoretic, and/or immunoprecipitation procedures, is normally used to identify and characterize the collagen types. Although the yield of material from cell cultures is very limited, there are a number of advantages to the cell culture system in conjunction with radiolabeling techniques, not least among which are the ready supply of labeled precursors for the analysis of processing phenomena and the possibilities for manipulating collagen gene expression by extrinsic factors. However, for the most part, interest in collagen types relates to their occurrence in normal and diseased tissues, and consequently it has been important to develop techniques to solubilize collagens from tissues in order to quantify the proportions of types present. To this end, the most widely used approach is pepsin digestion at pH 1–2 of tissues at 4° to 15°C, which relies on the resistance of the collagen triple helix to enzymic degradation below its denaturation temperature, but the susceptibility of virtually all other proteins to digestion. The collagen triple helix is unique in being both resistant to pepsin and soluble at acid pH, so that the end product of digestion is a mixture of solubilized collagen types. This procedure rarely proceeds to complete solubilization, for, with increasing age of the tissue, the fibrillar collagens become more resistant to pepsin as a result of increased stabilization of the cross-links. In addition, it will be appreciated that those collagens (such as FACIT and MULTIPLEXIN) with interrupted helices will give rise to multiple triple-helical digestion products when pepsin cleaves the noncollagenous domains. Consequently, a complex mixture of pepsin-derived peptides can arise from the digestion of tissues such as placenta, skin, or cartilage, and salt fractionation procedures of the type shown in Figure 10 are adopted to separate the collagen types. The identification of the collagen types is initially achieved through their characteristic mobilities on SDS-polyacrylamide gel electrophoresis. Further characterization can be achieved by peptide mapping of the individual chains following cyanogen bromide (CNBr) digestion [226] or by immunoblotting with chain- or type-specific antiserum. Type VI collagen is resistant to degradation by bacterial collagenase, and it has been possible to extract microfibrils from elastic and non-elastic tissues using a combination of bacterial collagenase and size fractionation approaches [227].

Recombinant approaches to generating collagen molecules and domains have generally overtaken tissue purifications as the method of choice for generating molecules for structure: function analyses [228–235]. In these studies, it has often been necessary to coexpress the collagen genes with recombinant prolyl 4-hydroxylase to ensure stable triple helix formation [231,233].

FIBRIL-FORMING COLLAGENS

Self-assembly (assembly of the triple helix through to fibrils) is central to collagen biochemistry, but the process of assembling the characteristic fibril organization of each tissue is clearly influenced by the various cell types responsible for the biosynthesis and maintenance of each particular

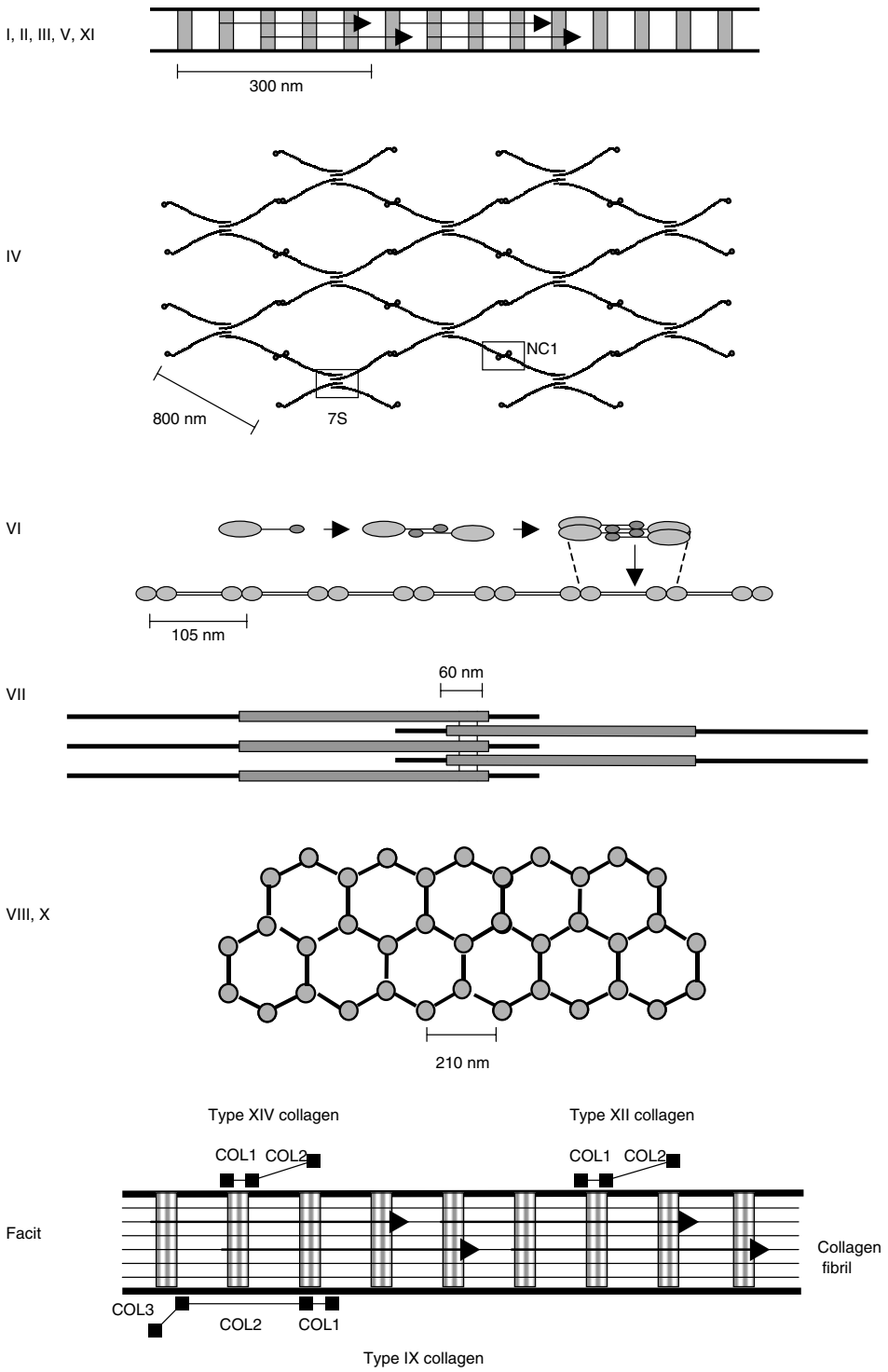


Figure 9. Diagrammatic representation of the molecular morphology of the macromolecular organization of the fibrillar and nonfibrillar collagens. Collagens I, II, III, V, and XI form fibrils as a consequence of the staggered alignment of molecules. Type IV collagen forms a flexible open network through N- and C-terminal and helical interactions, and this is the basic structural framework of basement membranes. Thin microfibrils of type VI collagen represent the end-product of a complex pathway of assembly, whereby monomers associate to form dimers, which, in turn, come together to form tetramers able to associate end to end. Type VII collagen anchoring fibrils are created as a result of monomers overlapping at their C-termini to form centrosymmetric dimers capable of aligning in limited parallel arrays. Triple-helical domains are shown as shaded bars, and non collagenous domains as lines. The closely related types VIII and X collagens form open hexagonal arrays. The FACIT collagens (fibril-associated collagens with interrupted triple helices) (types IX, XII, XIV) do not form defined assemblies on their own but instead are thought to associate with the surface of fibrils. Type IX collagen molecules are cross-linked to the outermost fibrillar collagen molecules of type II collagen fibrils.

matrix. Although virtually nothing is known of how fibroblasts, chondroblasts, and osteoblasts generate the fine architectural arrangements found in skin, cartilage, and bone, it seems likely that the packing, orientation, and organization may involve a complex interplay between cells, external forces, and the macromolecules (both collagenous and noncollagenous) of the extracellular matrix. It would appear that the cells are capable of producing discrete compartments in the adjacent extracellular space where assembly of discrete fibril bundles can take place prior to incorporation into the growing fibril [54,167,168]. Physicochemical studies demonstrating the influence of proteoglycans on collagen fibril self-assembly *in vitro* and histochemical demonstration of the close association of proteoglycans and fibronectin with collagen fibers *in vivo* provide evidence for macromolecular interactions as determinants of tissue architecture [172–175]. However, the situation is much more complex than initially thought, for at least five collagen types (I, II, III, V, and XI) are now recognized to form fibrils in the extracellular matrix (Fig. 11). Indeed, most individual fibers are very likely to be comprised of more than one of these collagen types and there is evidence to suggest that other minor collagens are also associated with these major fiber-forming collagens.

Collagen Types I, II, and III

Piez and co-workers in the 1960s demonstrated that denatured collagen molecules could be separated by carboxymethyl-cellulose chromatography into two components (see Introduction to this volume by Piez), and thus provided the description of the commonest form of collagen (now designated type I) as a heterotrimer comprising two α1 chains and one α2 chain [236]. The nonidentity of these two chains was further demonstrated after characterization of their CNBr cleavage profiles, which reflect both the abundance and position of the methionine residues contained within each polypeptide [237]. Knowledge of the CNBr-derived peptide patterns of the α1 and α2 chains was the key to the discovery of collagen type II by Miller and Matukas [14], who generated a distinct CNBr-derived peptide map for collagen extracted from lathyritic chick cartilage. This genetically distinct collagen was designated type II, and is now known to be the major collagenous component of hyaline cartilage and vitreous humor. The type II collagen molecule is a homotrimer consisting of three identical α1(II) chains, and therefore has a molecular conformation of $[\alpha1(II)]_3$ in its native triple-helical state. Subsequently, a further collagen was identified after pepsin treatment of fetal calf skin, which released the triple-helical domain of an α-chain distinct both in its chromatographic behavior on CM-cellulose and in its CNBr-derived peptide map [15]. This type III collagen was found to be of composition $[\alpha1(III)]_3$ and it has a widespread distribution, accompanying type I collagen in many connective tissues and being the principal collagenous component of the thin, so-called "reticulin" fibers present in the loose collagenous networks of highly differentiated organs such as the liver, lung, or lymphoid tissues.

Amino acid analysis reveals differences between these collagen types, a particularly notable feature being the higher content of hydroxylysine and hydroxylysine-glycosides in type II collagen; type III collagen is distinguished by its increased level of 4-hydroxyproline and the occurrence of cysteine (involved in disulfide bonds) within the triple helix. Amino acid sequence data indicate a close homology among these collagen types and explain the different CNBr-derived peptide maps on the basis of the distribution of methionine residues in the individual α chains [226]. Comparison of collagen types I, II, and III by electron microscopy of segment long spacing (SLS) crystallites also indicates a close homology [238–240]. This latter technique, in which ordered aggregates of collagen molecules are achieved at low pH in the presence of polyanions such as ATP, results in the individual molecules aligning themselves in parallel array with their ends in register rather than in the staggered array of the native fibril. These structures give a laterally extended picture of a single collagen molecule and allow the different collagen types to be compared, their staining patterns reflecting the charge distribution along the molecule.

The advent of recombinant DNA technology facilitated the determination of the complete amino acid sequences of collagen types I, II, and III, and descriptions of their gene structures provided an insight into the evolutionary relationship between these and other collagen types (Fig. 11) (see this Chapter, Part II). It has become clear that they are all translated from mRNAs coding for pre-proα chains with similar, but not identical, N- and C-terminal extensions. Thus, collagen types I, II, and III are synthesized as procollagens, which are then processed to fibril monomers in which more than 97% of each of the molecules consists of a continuous triple-helical domain along which the main information for an ordered parallel and D-staggered self-assembly is located. The formation of the continuous helical structure, the length of which varies between 1,014 residues in the α1(I) chain and 1,023 residues in the α1(III) chain, is dependent on the uninterrupted tripeptide sequence Gly–X–Y [16]. The rigidity or flexibility of distinct areas along the molecule is a reflection of the varying amounts of Pro and Hyp, in that the three types of tripeptide containing imino acids contribute to helix stability in the following order: Gly–Pro–Hyp > Gly–Pro–Y > Gly–X–Hyp [239]. Thus, at the C-terminal end of the triple-helical domain of the α1(I) and α2(I) chains is a very stable fivefold repeat of the triplet, Gly–Pro–Hyp, which probably seals the helix as it folds from the C-terminus following alignment of the three proα chains of type I procollagen during intracellular assembly. In contrast, the single vertebrate collagenase cleavage site within these fibril-forming collagens is a much more relaxed and flexible structure with a relatively low content of imino acids [241] (see also Chapter 7, this volume).

The C-propeptides of all the proα chains are strongly homologous [16], as might be expected on the basis of their similar function in helix assembly. Noteworthy differences occur between the N-propeptides, however, except for the highly conserved signal peptide sequences. Figure 7 illustrates the domain organization of type I procollagen, in which the proα1(I) N-propeptide contains a globular region of 66 residues stabilized by five intrachain disulfide bonds adjacent to a helical sequence of 48 residues, whereas in the proα2(I) N-propeptide the globular domain is absent. The N-propeptide of the proα1(III) chain is similar to that of the proα1(I) chain in having a 68-residue globular domain and a short triple helix of 39 residues, whereas in that of the proα1(II) chain the globular domain is missing and the helical region is 82 amino acids (i.e., 26 tripeptides plus a short interruption) long. There is also evidence for alternatively spliced variants of the type II collagen N-propeptide [16].

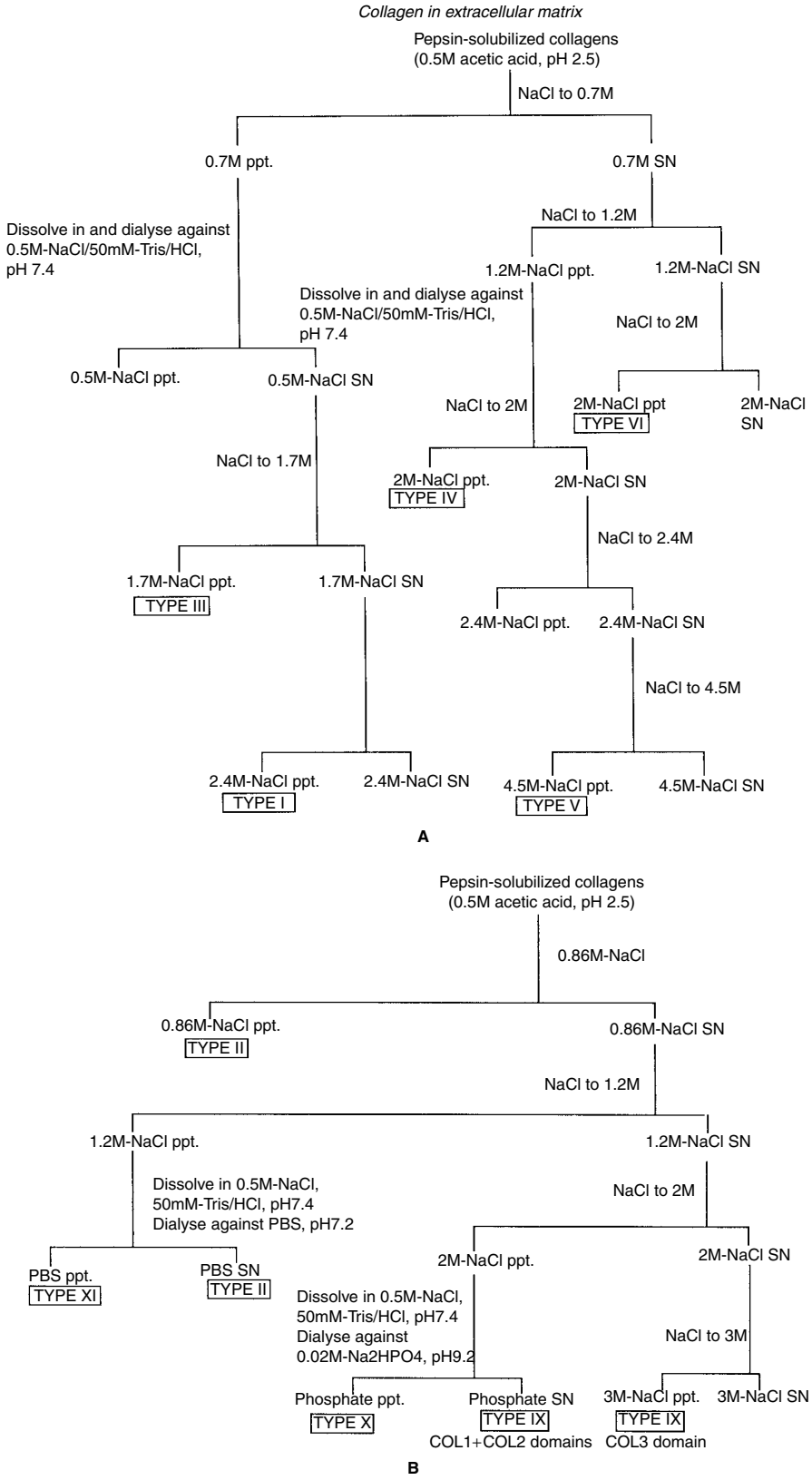


Figure 10. Fractionation procedures for the isolation of pepsin-derived tissue collagens. **A**: Flow sheet outlining the fractionation protocol for the purification of the various collagen types present in extracts of noncartilaginous tissues such as skin or tendon. **B**: Flow sheet outlining the fractionation protocol for the purification of the various collagen types present in extracts of cartilage.

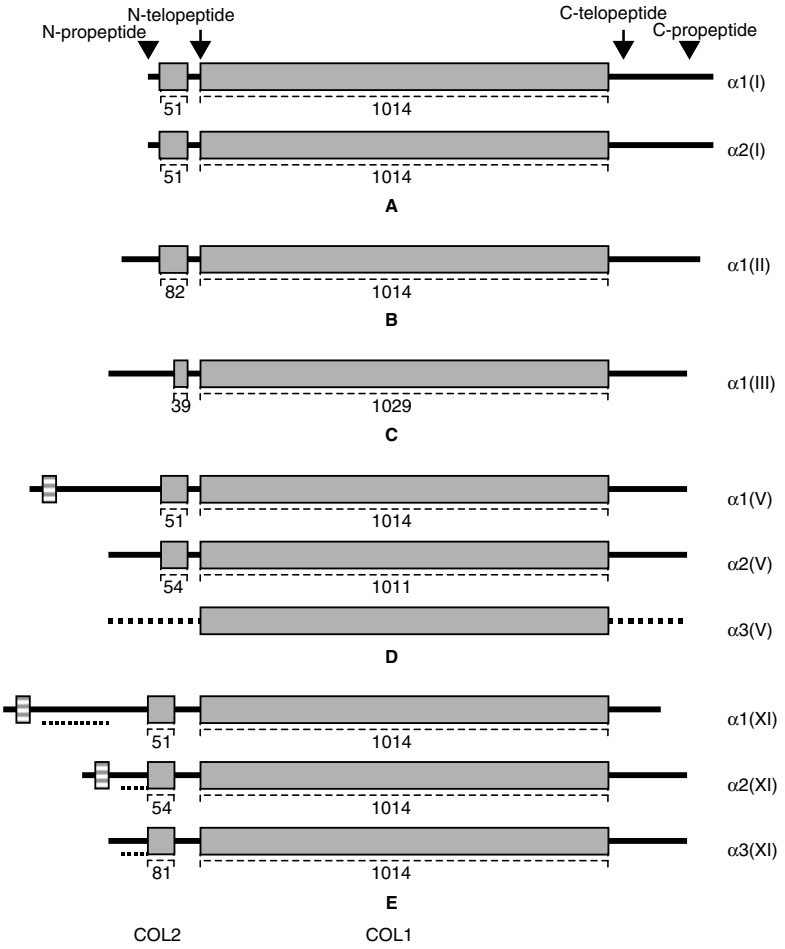


Figure 11. Molecular organization of the fibrillar collagens. Line diagrams illustrating the dimensions and domain structures of the α chains of triple-helical and non-collagenous regions of the fibrillar collagens. (**A**) Type I collagen, (**B**) type II collagen, (**C**) type III collagen, (**D**) type V collagen, (**E**) type XI collagen. Triple-helical domains (COL1 and COL2) are shown as dark shaded bars, and noncollagenous domains as lines. PARP (proline-and arginine-rich protein) domains that occur in the amino-terminal noncollagenous sequences of types V and XI collagen α chains are shown as vertical rectangles (▯) within the individual chains. The numbers of amino acid residues comprising each domain are indicated beneath the individual chains. Sequences outside the central triple helical domain of α3 (V), indicated by interrupted solid lines, are not yet known.

The removal of the N- and C-propeptides is a prerequisite for fibril formation, and cleavage of the N-propeptide from type III procollagen requires a specific N-proteinase distinct from that capable of processing procollagens I and II [150–152]. The flexible junction regions that connect the N- and C-propeptides to the main triple helix of all these α chains contain not only the cleavage sites for the specific proteinases but also nonhelical cross-link sites [242]. The rates of processing of the propeptides and the sequences in which they are removed from the procollagen are known to vary from tissue to tissue and may be a determinant of fibril size. It is fairly clear, however, that procollagen III is preferentially cleaved at the C-terminus, for a substantial proportion of the type III molecules incorporated into fibrillar elements appear to retain their N-propeptide sequences [160,161]. Such observations have led to the proposal that the N-propeptide specifically prevents the formation of thick collagen type III fibrils, and thereby offers an explanation for the occurrence of the relatively small (reticulin-like) fibrils seen particularly in elastic tissues.

No clear explanations can be offered in regard to the requirements of particular tissues and their contents of the fibril-forming collagens. Nevertheless, several generalizations seem to hold: fibrils enriched in type I collagen are often large, in terms of their cross-sectional diameter and length, are often packed in parallel bundles, and afford physical strength to tissues likely to experience extensive forces; type II collagen forms narrow fibrils and is the major collagen of cartilaginous tissues and vitreous humor; and type III fibrils are thinner and especially prevalent (up to 30% of total tissue collagen) in tissues exhibiting a degree of elasticity, e.g., skin, aorta, gut, and lung. Immunoelectronmicroscopy has demonstrated the close association of thick type I and thin type III collagen fibrils in tissues [241a], and there is chemical evidence that different collagens can be present in the same fibril [242]. Where type III collagen levels are reduced, as in patients with Ehlers-Danlos syndrome (EDS) type IV (see Chapter 9, this

volume), the tendency of the aorta or gut to rupture provides clear, if indirect, evidence of type III collagen having an important role in tissue elasticity and integrity [113].

Collagen Type I Homotrimer

Although type I collagen generally occurs as the heterotrimer $[\alpha1(I)]_2\alpha2(I)$, there is good evidence for the existence of low levels (no more than 0.5% of the fibrillar collagens) of a type I homotrimer $[\alpha1(I)]_3$ in both cells [243] and tissues [244,245]. The α chains of the type I homotrimer are the same gene products as the α1 chains of type I collagen, but in α1(I) trimer the 3-hydroxyproline content is elevated and the hydroxylation of lysyl residues is more extensive. The potential role of these hydroxylysine residues in stabilizing intermolecular collagen cross-links has led to speculation that α1(I) trimer might contribute to the stability and tensile strength of various connective tissues [245].

Early tissue analyses failed to demonstrate any major differences between fibers comprised of predominantly type I homotrimer and those of the heterotrimeric form, although the former are slightly thinner. In contrast, homotrimeric type I fibers generated *in vitro* appear not only to be thicker than corresponding heterotrimeric type I fibers, but also to differ in the kinetics of fibril formation, in that they are formed at a significantly slower rate [16]. This observation attests, perhaps, to a primary role for α2(I) chains in driving fibril formation. Further evidence for the importance of the α2(I) chain is derived from observations on a patient with a severe form (type III) of osteogenesis imperfecta [246,247]. Although fibroblasts derived from this patient synthesized proα2(I) chains, they were not incorporated into heterotrimers, and only α1(I) homotrimers were assembled. A naturally occurring mouse model (*oim*) with osteogenesis imperfecta with a mutation that also results in nonfunctional proα2(I) chains has presented a unique opportunity to explore changes in mouse tail tendon collagen fibril organization in homozygous and heterozygous mice [248]. The total absence of α2(I) chains results in type I collagen homotrimer formation, and is associated with a decrease in the order of axial packing and a loss of crystalline lateral packing. Thus, the presence of α2(I) appears to be crucial in long-range axial order in type I collagen–containing tissues [249].

Collagen Types V and XI

The genetically distinct collagen types V and XI are relatively minor components of the extracellular matrix but they share a close structural relationship with the major fibrillar collagens (types I–III) and form a distinct subset of fibril-forming collagens with functions ancillary to those of the major tissue collagens (Fig. 11) [250]. Types V and XI collagens are immunologically masked within fibrils and require partial fibril disruption for immunological detection, leading to the idea that they form a core within the major collagen fibrils (I, II and III). They can be further distinguished from the major fibrillar collagens by the complex processing they undergo, which yields monomers retaining a portion of the N-terminal globular domains [226,250–263]. These features suggest that they participate in the regulation of collagen fibril diameters. Type V collagen is a widespread component of virtually all noncartilaginous tissues (including bone, tendon, cornea, skin, blood vessels) which contain type I and types I/III collagen fibers. Type XI collagen is expressed mainly in cartilaginous tissues but is also expressed by nonchondrogenic tissues, particularly during embryonic development, and in these tissues heterotypic triple helical molecules comprising types V and XI collagen α-chains occur. Thus, these two collagens can be considered a single collagen type that is sometimes referred to as type V/XI collagen [250].

The molecular structure and assembly of type V/XI collagen shows many parallels with the major fibrillar collagens. The intact molecules are characterized by a single, continuous triple helix with N- and C-terminal nonhelical sequences, and visualization by rotary shadowing after pepsin digestion reveals rodlike molecules of approximately 300 nm, comparable in size to those of collagens I, II, and III. They can, however, be distinguished from the major collagens in thermal stability experiments that demonstrate the presence of unfolding intermediates in their triple helix to coil transitions [255]. SLS aggregates of collagen V/XI confirm the length of its helix, and demonstrate a cross-striation pattern similar to those of the major fibrillar collagens. Type V/XI collagens are synthesized as procollagens, with collagenous domain structures equivalent to those of the major fibrillar collagen precursors. cDNA-derived sequences have confirmed the homology and classification of these two collagens as fibril-forming species [16,256–258], although the N-terminal propeptides are longer than those of collagens I, II, and III, and the short helical regions therein have interruptions. The N-terminal propeptide of α1(XI) is, in fact, the longest N-propeptide described for a fibrillar collagen, with an extended and, in part, highly acidic globular region (Fig. 11). The size of the human proα1(V) chain (predicted M_r 183,415; apparent M_r 220,000–240,000) is significantly greater than that of both the proα2(V) chains (M_r 150,000–170,000) and other procollagen chains, as a consequence of a larger N-terminal propeptide of M_r 70,000–85,000. In contrast, sequences of the C-terminal propeptides of the precursors of collagen types V and XI derived from cDNA clones show very close homology in terms of residue number and organization with the C-propeptides of proα1(I), proα2(I), proα1(II), and proα1(III) chains; it is concluded that these propeptides are involved in directing triple-helix formation in a manner analogous to that in collagens I–III. Monomer assembly proceeds via disulphide-bonded precursors, although the processed chains have no interchain disulphide bonds.

Type V collagen was initially described as AB collagen following its isolation and identification in pepsin digests of chorionic amniotic membranes [257]. Ultrastructural analysis of such tissue reveals that aggregates of collagen V are frequently concentrated in pericellular zones, in close association with the basal lamina, and in association with larger fibrous elements throughout the interstitium [250]. Type V collagen is synthesized in both homotrimeric and heterotrimeric procollagen forms. The major form comprises two proα1(V) chains and one proα2(V) chain, but in some tissues (e.g., uterus and placenta) a second form comprising proα1(V), proα2(V), and proα3(V) chains is present. A homotrimer of proα1(V) chains has been observed in cell culture and the fully processed homotrimer has been identified in tissues. It has recently been shown that the carboxyl terminal propeptide of proα1(V) homotrimers is cleaved by the subtilisin-like prohormone convertase furin, while procollagen C-proteinase (BMP-1) actually cleaves the N-propeptide [259]. In addition, the proα1(XI) and proα2(XI) chains can substitute for the proα1(V) and proα2(V) chains, respectively, forming cross-type heterotrimers [250]. The procollagen forms are processed extracellularly at the C-terminus by procollagen C-proteinase. The

N-terminal domain of proα2(V) is apparently not processed, and in heterotrimers, therefore, a globular domain persists at the N-terminus that could sterically inhibit the accretion of collagen molecules. However, partial or complete processing occurs at the N-terminal end of the proα1(V) chain depending on whether it forms heterotrimers or homotrimers. The cleavage of N-terminal propeptides is particularly slow, which has a bearing on the function of type V collagen in influencing fibrillogenesis. In a recombinant study, stable homotrimeric $[\alpha1(V)]_3$ assemblies formed that were processed at the C-terminus; some of these molecules also underwent N-terminal trimming into a form corresponding to the main triple helix plus the major part of the N-terminal noncollagenous domain [234].

Differential processing of type V collagen isoforms could fine-tune fibril diameter modulation. *In vitro* studies have demonstrated that highly purified collagen V will form fine fibrils with or without a 67-nm D-periodic banding pattern [264]. It is not clear whether collagen V exists *in vivo* as independent fine fibrils, but it is often seen in intimate association with type I collagen fibrils and controls the diameter of type I collagen fibrils as a copolymerized constituent. The type V collagen molecules are staggered by 4D (D = 67 nm) and are primarily cross-linked to each other by head-to-tail bonds involving the N-telopeptides and the C-terminus of the helix. The α1(I) C-telopeptide is also cross-linked laterally to the N-terminus of the α1(V) helix. Native type V collagen resists mammalian collagenase but is cleaved by 92-kDa gelatinase (MMP-9) at two sites, one near the N-terminus and the other in the triple helix [265]. Mutations in the *COL5A1* and *COL5A2* genes have been linked to EDS types I and II, which are characterized by joint hypermobility, fragile skin, atrophic scars, and general abnormality in the architecture of the major collagen fibrils [266,267] (see also Chapter 9, this volume). Reduction of the amount of α1(V) using a dominant negative strategy in transgenic mice has confirmed the role of type V collagen in regulating fibril diameter; affected mice had altered fibrillogenesis resulting in assembly of large diameter fibrils with a broad size distribution and loss of corneal-specific fibril morphology [260]. Mice homozygous for mutations in the *COL5A2* gene produced structurally abnormal type V collagen and disorganized type I collagen fibrils, and had a high rate of perinatal mortality due to spinal deformities and both skin and ocular (especially corneal) abnormalities.

The term "type XI collagen" is used to describe the cartilage-specific chains originally designated lα, 2α, and 3α, or κ collagen, which were first isolated from pepsin digests of human hyaline cartilage [16,18,20,261]. This collagen can account for 8–10% of the collagens in most cartilages, has been localized to the territorial matrix where fine fibrils predominate, and forms heterotypic fibrils with types II and IX collagens. Type XI collagen is synthesized as a heterotrimeric procollagen comprising three distinct chains, proα1(XI), proα2(XI), and proα3(XI), although alternative assemblies with proα1(V) and proα2(V) may occur [250]. The proα3(XI) chain appears to be identical in sequence to the alternatively spliced form of the proα1(II) chain lacking the region coded for by exon 2, but its triple helical domain has more hydroxylysyl glycosides than the α1(II) chain. The proα1(XI) chain is also cleaved by procollagen N-proteinase and mammalian collagenase, indicating differences between these two chains either in amino acid sequence or enzyme accessibility. The procollagen forms are processed extracellularly at the C-terminus. Complex alternative splicing occurs in the mRNAs encoding a variable region between the PARP (proline and arginine-rich protein) and the large collagenous region (COL2) domains of both proα1(XI) and proα2(XI) chains (see Fig. 11). Differently spliced forms of α1(XI) undergo uniform N-terminal processing [267a], partial processing of the proα2(XI) chain occurs at the N-terminus, and the proα3(XI) chain is not processed further.

The bulk of the type XI collagen triple-helix is copolymerized in the interior of type II collagen-containing fibrils [250]. However, the portion of N-propeptide that remains (see above) after processing projects from the fibril surface, and is available for specific interactions with other extracellular matrix components, depending on the alternatively spliced forms present. Type XI collagen molecules are staggered by 4D (D = 67 nm) and are primarily cross-linked to each other by head-to-tail bonds involving the N-telopeptides [16]. The α1(II) C-telopeptide is also linked to the N-terminus of the α1(XI) helix. Mutations in the *COL11A1* and *COL11A2* genes have been linked to Stickler syndrome, Kniest dysplasia, and Marshall syndrome, characterized by numerous skeletal deformities [268–270] (see also Chapter 23, Part II, this volume); mutations in the *COL11A1* gene also cause ocular abnormalities [47,145]. *Cho/cho* mice homozygous for a mutation in the *Col11a1* gene that causes autosomal recessive chondrodysplasia are functionally equivalent to homozygous knock-outs for *Col11a1* [262]. These mice die at birth with numerous skeletal defects: a shortened snout, cleft palate, protruding tongue, short limbs with widened metaphyses and a shortened spine; growth plate chondrocytes are also highly disorganized.

Types V and XI collagens are implicated in regulating the diameter of types I and II collagen fibrils, respectively, a function presumably arising out of their characteristically slow N-terminal propeptide processing. Type XI collagen may participate in collagen–proteoglycan interactions and type V collagen has been shown to interact *in vitro* with a number of extracellular macromolecules including thrombospondin, heparin, heparan sulfate, and biglycan [263].

BASEMENT MEMBRANE COLLAGENS

Type IV Collagen

Basement membranes are widely distributed, sheet-like structures that underly almost all epithelia and endothelia in the body and surround many cell types, including smooth and skeletal muscle cells, Schwann cells, and adipocytes. They require molecular continuity extending over long distances, mechanical stability to resist high shearing forces or hydrostatic pressures, flexibility to allow tissue movement, and the ability to self-seal to allow the passage of immune cells. These properties are imparted mainly by type IV collagen, a large, distinctive form of collagen found only in basement membranes and which constitutes their major insoluble scaffold. Type IV collagen was originally described by Kefalides in 1971 [271,272], although the occurrence of a collagen in basement membranes had been indicated by X-ray studies of intact basement membranes some 20 years earlier [273]. Later, hydroxyproline and then hydroxylysine were detected in amino acid hydrolyzates of whole basement membranes, and the use of proteases to release the triple-helical collagen from glomerular basement membrane and anterior lens capsule led to the identification of collagen chains distinct from those of the fibrillar collagens I, II, and III [274,275].

The type IV collagen family is now known to comprise six genes, the translation products of which associate to form distinct type IV collagen molecules [16] (Fig. 12)(see also Chapter 25, this volume). The presence of distinct type IV collagen molecules, together with many additional components including the laminins and heparan sulfate proteoglycans, highlights basement membrane complexity and the considerable variation that can exist between basement membranes of the body in terms of molecular composition, organization, and function. It has proved difficult to investigate the chemistry of specialized basement membrane extracellular matrices, for they are essentially insoluble and not readily separated from underlying connective tissues. Accordingly, much of our knowledge of the chemistry and composition of basement membranes is derived from those that can be prepared in high purity and reasonable yield, i.e., lens capsule, glomerular basement membrane, seminiferous tubule basement membrane, and the extraembryonic membranes such as rodent Reichert's membrane [272,276–278]. A particularly useful source of type IV collagen is the transplantable EHS mouse tumor, which elaborates a basement membrane-like matrix that can be prepared in large amounts [279,280]. This material has been valuable in identifying the characteristics of basement membrane collagen type IV, as well as those of other basement membrane components [281–284].

Type IV collagen was the first collagen to be identified lacking the capacity to undergo a regular lateral aggregation of molecules into fibrillar structures [276,285]. It does not even form well-ordered SLS crystallites, but rotary shadowing has revealed the intact monomers to be approximately 390 nm long with a large globular domain at the carboxyl terminus (NC1 domain), an extended collagenous domain of approximately 350 nm, and a short noncollagenous amino-terminal domain. A series of kinks occur within the triple-helical domain, which arise as a result of numerous (>20) short imperfections in the Gly–X–Y repeat sequence. These noncollagenous sequences confer a high degree of flexibility on the individual monomers and, therefore, on their assembled aggregates. Newly assembled type IV collagen monomers do not undergo processing before incorporation into the matrix. Details of the macromolecular assembly of type IV collagen into a filamentous network were established largely as a result of detailed enzyme digestion experiments and rotary shadowing analyses of aggregation phenomena *in vitro*. Bacterial collagenase digestion experiments on EHS tumor and other basement membrane-containing tissues identified a stable resistant fragment that turned out to be a basic structural unit of type IV collagen, the tetrameric 7S domain, which comprises parallel and antiparallel overlapping 30 nm-long amino-terminal helical and nonhelical segments from four collagen IV molecules. Acid extractions of these tissues yielded dimers of two 330 nm-long triple-helices connected to each other by

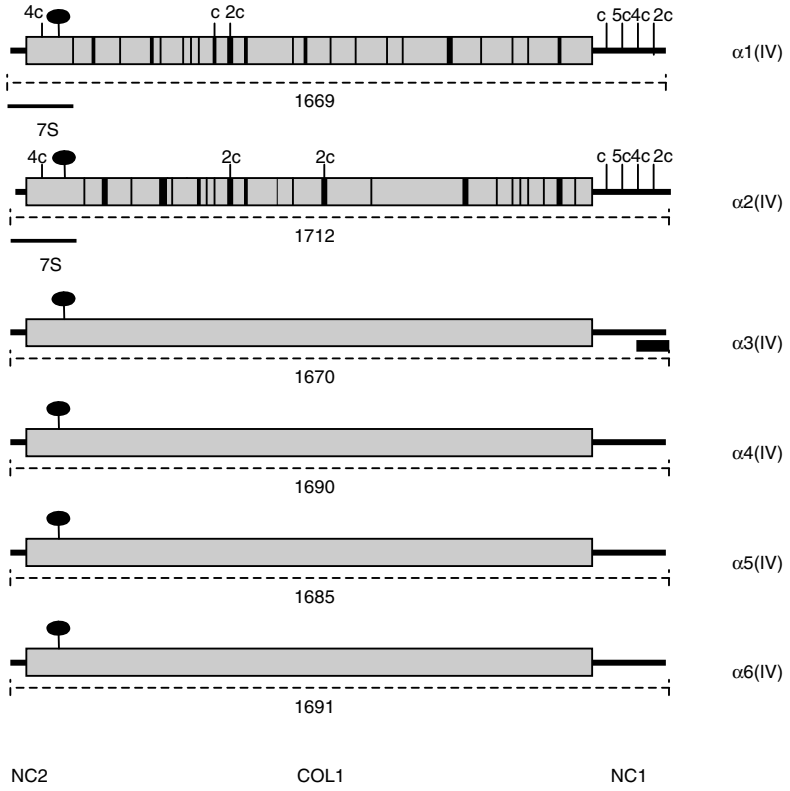


Figure 12. Molecular organization of basement membrane type IV collagen. Line diagrams illustrating the dimensions and domain structures of the collagenous and noncollagenous regions of the α1(IV) –α6(IV) chains of type IV collagen. Imperfections within the triple-helical regions are indicated by vertical lines and solid vertical bars: these endow flexibility on the triple-helical domains of the assembled molecules. The 7S region (—), alternatively spliced sequences (▬), positions of cysteine residues (c) and N-linked carbohydrate attachment sites (🍍), are marked. The numbers of amino acid residues comprising each chain are indicated below the individual chains.

multiple disulfide interactions in the NC1 domains after cleavage of the 7S domain, and pepsin digestion of type IV collagen resulted in the destruction of the NC1 domains and concomitant release of a large tetrameric fragment in which the molecules were held together by their amino-terminal ends. The identification of these interactions at both amino and carboxyl termini led to the proposal of there being an open network structure in which four molecules are bound to one another by their amino-terminal 7S domains and at their opposite ends by the joining of their carboxyl terminal NC1 domains, with each strand running between 7S domains being 800 nm long and a single triple helix thick (Fig. 9) [286–288]. Stabilization of this alignment takes place through the formation of reducible and nonreducible intermolecular cross-links. This arrangement may be repeated over many microns and is compatible with the formation of polymers of infinite size and with the mechanical properties and amorphous appearance of basement membranes. These predictions were confirmed *in vitro* by reconstituting polygonal networks from type IV collagen dimers [289,290]. However, the distances between NC1 domains were irregular, indicating a much tighter network with extensive lateral associations between central segments of the triple helix. Such laterally associated networks have also been visualized in the basement membranes of amnion and the EHS tumor, and are predicted from X-ray diffraction of lens capsule [272]. The molecular basis for this lateral association is unclear, but appears to involve low-affinity binding, with the carboxyl terminal globular domain possibly having an important role. A degree of interfilament superhelical twisting along many of the type IV collagen strands of EHS and amniotic networks has been implicated in stabilizing the laterally associated network [290–292]. The final collagen IV networks are stabilized by disulfide and/or lysine aldehyde-derived cross-links between their interacting NC1 and 7S domains and are highly insoluble. Thus, during tissue remodeling, type IV collagen must be cleaved, initially by matrix metalloproteinases, to accommodate structural changes [50] (see also Chapter 7, this volume).

Biochemical, immunohistological, and molecular biological data have shown that the most abundant form of type IV collagen molecules exists as heterotrimers with the major molecular formula $[\alpha1(IV)]_2\alpha2(IV)$ [16]. The genes encoding α1(IV) and α2(IV) chains are in a head-to-tail arrangement on chromosome 13, with common bidirectional promoters and transcription start sites separated by only 130 bp. The complete primary structures of the α1(IV) and α2(IV) chains elucidated from man and mouse display a highly conserved carboxyl terminal NC1 globular domain (229 and 228 residues, respectively) that consists of two homologous parts (C4 modules) each stabilized by three disulphide bonds and containing a high proportion of hydrophobic residues (Fig. 12). Their characteristic interrupted triple-helical domains are also of similar lengths (1,398 and 1,427 residues, excluding non-triple helical interruptions, respectively). The reducible and nonreducible intermolecular cross-links located within the 7S domain render this portion of the molecule so stable that it can easily be isolated as a bacterial collagenase-resistant fragment [293]. The NC1 domains of type IV collagen α chains are thought to play a key role in their alignment and in chain selection and the initiation of triple-helix formation in the rough endoplasmic reticulum, in a manner analogous to the C-propeptides of the fibrillar collagens with which they share limited homology. Recombinant expression studies have shown that neither α1(IV) nor α2(IV) is able to form homotrimers, and that trimer formation requires the presence of both α chains. A major focus of current research is to determine the molecular basis of specific α chain NC1 associations during trimerization [294,295].

The identification of four additional type IV collagen α chains has greatly increased the molecular complexity of basement membrane chemistry (Fig. 12) [16]. The α3(IV), α4(IV), α5(IV), and α6(IV) chains have collagenous sequences of 1396, 1415, 1415, and 1404 residues and carboxyl terminal noncollagenous (NC1) domains of 232, 229, 229, and 241 residues, respectively. As with the α1(IV) and α2(IV) genes, those encoding α3(IV) and α4(IV) chains on chromosome 2 and those on the X chromosome encoding α5(IV) and α6(IV) chains are each arranged in a head-to-tail manner and are functionally related and coordinately regulated (see also this Chapter, Part II). Their unique tissue-specific expression patterns are illustrated in immunolocalization studies using α1(IV) to α6(IV) chain-specific monoclonal antibodies [295–298] (see also Chapter 25, this volume). In kidney, all basement membranes in glomeruli and tubules stain for α1(IV) and α2(IV), whereas the α3(IV) and α4(IV) chains are confined to the glomerular basement membrane and some parts of the tubules. In stark contrast, the expression patterns of the α5(IV) and α6(IV) chains are different in the glomerulus — α6(IV) is not present in the glomerular basement membrane but occurs within the basement membranes of Bowman's capsules and some distal tubules, whereas α5(IV) is densely positive in the glomerular basement membrane where its distribution parallels that of α3(IV) and α4(IV) [295,296]. Smooth muscle cells in the urinary bladder and uterus are surrounded by basement membranes containing α1, α2, α5, and α6 type IV chains, and these four chains are also present around smooth muscle cells in the muscular layer of the fundus of the stomach, whereas those in the antrum and distal side of the gastrointestinal tract express only α1(IV) and α2(IV). In the vasculature, most smooth muscle cells are positive for α1(IV) and α2(IV), but α5(IV) and α6(IV) are also expressed in the aorta and some arteries where blood pressure changes significantly. In skin, which lacks α3(IV) and α4(IV), α1(IV) and α2(IV) are present in all epidermal basement membranes and capillary basement membranes, while α5(IV) and α6(IV) colocalize only in the epidermal basement membranes [298]. The temporal and spatial expression of the α1, α2, α5, and α6 type IV chains have also been demonstrated during the formation of the basal lamina in an *in vitro* skin model [298]. These localization studies suggest that type IV collagen molecules comprising not only α1(IV)/α2(IV) but also α3(IV)/α4(IV)/α5(IV) and α5(IV)/α6(IV) may occur.

Kidney has been a particular focus for investigating the role of type IV collagen in basement membranes due to the pathologies associated with its specialized basement membranes (see Chapter 25, this volume). The primary lesion in one such disease, the Goodpasture syndrome (a rare form of glomerular nephritis) has been localized immunologically to the α3(IV) NC1 domain [47,145,299]. Ultrastructural changes in the glomerular basement membrane started to be recognized in cases of hereditary Alport syndrome in the late 1960s. The pathogenesis of this X-linked progressive nephritis associated with hearing loss and characterized by the splitting of the glomerular basement membrane is caused by mutations in the α5(IV) gene resulting in an absence of functional α5(IV) chains [145,275]. Linkage to

chromosome Xq22-26, the locus for the genes encoding α5(IV) and α6(IV) was established in 1990, and, to date, more than 200 different mutations have been reported in the *COL4A5* gene. Moreover, deletions at the 5′ end of *COL4A6* extending to the neighboring *COL4A5* gene have been reported to cause diffuse leiomyomatosis associated with Alport syndrome, and it has been speculated that α5(IV) and/or α6(IV)-containing basement membranes that enclose smooth muscle cells of the aorta and other tubular organs might have some particular function related to mechanical stress or tensile strength during their characteristic contractile activity [300]. Several kindreds with autosomal forms of Alport syndrome have mutations in *COL4A3* and *COL4A4* genes.

Disease and structural studies have shed light on the molecular composition of type IV collagen molecules. In cases of X-linked Alport syndrome, α3(IV), α4(IV), and α5(IV) chains are virtually undetectable in the glomerular basement membrane, indicating that all three probably associate in this tissue to form a triple helical molecule. Moreover, in mouse models of Alport syndrome in which α3(IV) is lacking, there is absence also of α4(IV) and α5(IV). When pseudolysin digestions were used to excise truncated triple-helical molecules from glomerular basement membrane, two distinct assemblies were isolated, one at 4°C which comprised α1(IV) and α2(IV) chains, and the other at 25°C which contained mainly α3(IV), α4(IV), and α5(IV) chains with a few α1(IV) and α2(IV) chains [295]. The 25°C assemblies appeared as a network containing loops and supercoiled triple helices and were stabilized by disulfide cross-links between the α3(IV), α4(IV), and α5(IV) chains. Several lines of evidence suggest that heterotrimeric molecules composed of α5(IV) and α6(IV) chains also exist [275]: α5(IV) and α6(IV) chains are codistributed in basement membranes of the Bowman's capsule and some kidney tubules, and in epidermal basement membranes; in typical Alport cases in which mutations in *COL4A5* cause a lack of the α5 chains, the α6 chain is also missing from dermal basement membranes; primary sequence comparisons suggest that the α5(IV) and α6(IV)-containing molecules could have the molecular formula $[\alpha5(IV)]_2\alpha6(IV)$; loss of both α5(IV) and α6(IV) chains has been demonstrated in basal cell carcinomas, invasive prostate carcinomas, and diffuse leiomyomatosis associated with Alport syndrome [300].

As the major structural scaffold of all basement membranes, the insoluble type IV collagen network not only accommodates and interacts with many other noncollagenous and collagenous basement membrane molecules, but also directly supports cell adhesion [16,277,301]. Human mesangial cells bind avidly via α1β1 and α2β1 integrins to type IV collagen $[\alpha1(IV)]_2\alpha2(IV)$ through the major triple-helical domain and the NC1 domain, and a major, specific site ~100 nm from the amino-terminus of α1(IV) has been identified that mediates α2 integrin binding. The other molecules that make a major contribution to the repertoire of functions ascribed to basement membranes include laminin, entactin (nidogen), heparan sulfate proteoglycan, types XV, XVIII, and XIX collagens, BM-40 (SPARC), fibulins, and dystroglycan. For example, laminin, a large and abundant noncollagenous basement membrane glycoprotein (see Chapter 5, this volume), can form a noncovalent quasi-hexagonal network and has the capacity to bind to type IV collagen, heparan sulfate proteoglycans, and entactin, to create an integrated structure enmeshed within the basement membrane [276]. Entactin occurs in intimate association with laminin, and helps to integrate laminin stably in the basement membrane matrix. The laminin-entactin complex contributes to the cell-binding activity of basement membranes. Heparan sulfate proteoglycans, in contrast, provide polyanionic sites that serve as selective charge barriers that make the matrix impermeable to proteins. The roles of BM-40 and the many minor basement membrane components remain largely obscure. A clear understanding of how all these molecules interact should reveal the molecular basis of basement membrane processes such as tissue differentiation, angiogenesis, and metastasis.

Type VII Collagen and Anchoring Fibrils

Type VII collagen is one of the largest of the known mammalian collagens and constitutes a distinct fibril-forming collagen subset with a number of unique structural features that set it apart from the classical fibril-forming collagens (Fig. 13) [16,302]. Whereas type VII collagen exhibits a restricted tissue distribution and can be classified as a minor collagen, it is the major component of the anchoring fibrils, specialized extracellular structures found underlying the basement membrane of numerous external tissues such as skin, oral mucosa, and cervix, the function of which is to anchor the basement membrane to the stroma [303–305]. This collagen was first observed as a minor component of pepsin-solubilized type V collagen preparations of chorioamnion, and was subsequently identified as a triple-helical molecule comprising apparently identical α chains and with an extended helical domain almost 1.5 times the length of the type I collagen helix [306]. Native, pepsin-digested type VII collagen monomers appear rod-like by rotary shadowing, with a length of 467 nm, but the major isolated form of the molecule is a dimer of 785 nm, in which the two monomers are associated with an overlap of 60 nm (Fig. 9). The dimers then associate laterally to produce "segment long spacing (SLS)-like" structures that insert into the basement membrane via the large NC1 domains. SLS crystallites prepared from type VII collagen dimers reveal a distinctive centrosymmetrically banded aggregate with a unique cross-striation pattern [307].

Type VII collagen resembles the fibrillar collagens in that it is synthesized and secreted as a procollagen, with a broadly similar domain structure [16,302,308]. There the similarities end, however, for this procollagen species is not only significantly larger than the corresponding fibrillar procollagens, with a molecular mass of about 1×10^6 (3× 2,944 amino acids), but it also has numerous fine structural differences and, via a novel assembly pathway, forms a strikingly different fibrillar structure. Type VII collagen is synthesized and secreted as a homotrimeric molecule with the molecular formula $[\alpha1(VII)]_3$. Secreted type VII procollagen monomers aggregate extracellularly into antiparallel assemblies of two monomers that overlap each other at the amino-terminus by 60 nm [306,307,309]. These form centrosymmetric aggregates that are thought to be stabilized by intermolecular disulphide bonds within the overlap, and with the large amino-terminal NC1 domain extending outward from both ends. This aggregation is probably specified by the carboxyl terminal NC2 domain prior to its removal by proteolytic cleavage [311]. This model for the macromolecular assembly of type VII collagen predicts that these dimers associate laterally in a nonstaggered array to form a tightly packed, fibrous SLS-like structure, in which the NC1 domains are embedded at one end in type IV collagen and laminin-5 in the lamina densa and at the

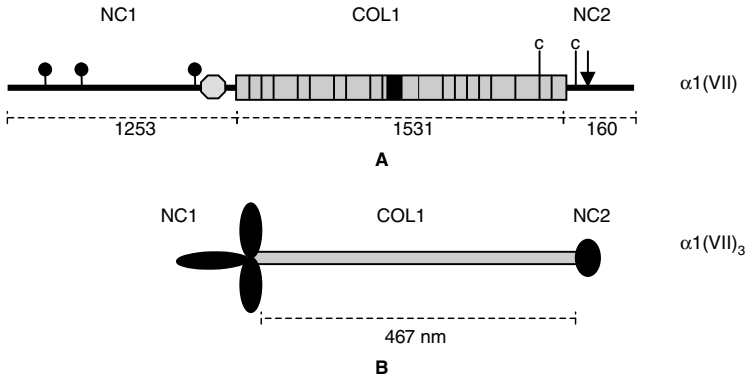


Figure 13. Molecular organization of type VII collagen. **A.** Line diagram illustrating the dimensions and domain structures of the collagenous and non-collagenous regions of the α1(VII) chain of type VII collagen. The triple-helical domain is represented as a dark shaded bar, with approximate positions of the deduced interruptions of, and imperfections within, the triple-helical region indicated by vertical lines and solid vertical bars, and non-collagenous domains shown as horizontal lines. The A-domain that occurs within the NC1 domain is indicated (●), together with the positions of cysteine residues (c) and N-linked carbohydrate attachment sites (Y). The numbers of amino acid residues comprising each domain are indicated beneath the chains. **B**. Line diagram illustrating the dimensions and structures of the triple-helical and noncollagenous domains of the intact type VII procollagen molecule, as deduced from rotary shadowing images.

other end in anchoring plaques containing type IV collagen within the stroma, with the triple-helical zone extending between them. These structures are further stabilized through the formation of transglutaminase-2-mediated cross-links, with both triple-helical and noncollagenous domains serving as substrates for tissue transglutaminase-2 [312]. Such anchoring fibrils have been identified in human skin by immunogold labeling, although some adopt a U-shaped structure in which both ends are inserted into the lamina densa [311]. Anchoring fibrils thus exist as an extensive scaffolding, capable not only of providing support to the stromal matrix elements but also of reinforcing the attachment of epithelial cells to the subjacent stroma. Such a role for type VII collagen is supported by the clinical manifestations of severe recessive dystrophic epidermolysis bullosa, in which the principal lesions are blisters caused by separation of the dermal stroma from the epithelium immediately below the basement membranes in response to normal mechanical friction, and which is characterized by a lack of ultrastructurally identifiable anchoring fibrils [1–3,145,313] (see also Chapter 15, this volume).

The α1(VII) chain comprises a central collagenous region flanked by non-collagenous terminal sequences (Fig.13) [16,308]. The small C-terminal NC2[3] domain (M_r 100 kDa, 160 amino acids) is nonhelical and in rotary shadowing images appears as a small globule. It is cysteine-rich and contains a Kunitz proteinase inhibitor-type repeat similar to that found in the α3(VI) chain of type VI collagen. The processing of type VII collagen is thought to contribute to correct association and folding of the polypeptide subunits [311]. The putative cleavage site is within amino acids 2814–2843 in the proximal NC2 domain. This 29 amino acid stretch contains a consensus sequence for procollagen C-proteinase [314], and this enzyme may therefore cleave the C-propeptide of procollagen VII. This cleavage site falls within the sequence that brackets the junction of the COL1 and NC2 domains; this region also contains the two cysteine residues that stabilize the pro-α1(VII) antiparallel dimers. The central triple-helical domain (1531 amino acids) contains 19 nonhelical discontinuities (Gly–X–Y imperfections, or insertions) with the major disruption (39 amino acids in length) occupying a central position, and also possesses cleavage sites for vertebrate interstitial and type IV collagenases [50] (Fig. 13) (see also Chapter 7, this volume).

The amino-terminal NC1 domain of type VII procollagen contains a very large and unusual sequence of M_r 450 kDa (1237 amino acids), which consists of submodules with homology to known adhesive proteins, including a partial von Willebrand factor type A domain, nine consecutive fibronectin type II repeats, a complete von Willebrand factor type A domain, and a cysteine/proline-rich domain [16]. Because these modules reside at the ends of the assembled anchoring fibrils and are thought to interact with basement membrane structures, their adhesive properties probably play a critical role in providing integrity to the lamina densa/papillary dermis interaction. This NC1 domain has been visualized as a three-armed structure linked by interchain disulfide bonds within the junctional region, and it provides potential targets for interactions with other extracellular matrix molecules including type IV collagen, the recently identified GDA-J/F3 antigen (a small 45–50 kDa globular protein), and possibly fibrillar collagens [16,315]. Type VII collagen also interacts with fibronectin via a unique subdomain within the collagenous triple helix [316], and with laminin 5 via the NC2 domain [317,318]. The deduced primary structure of human α1(VII) contains four RGD (Arg–Gly–Asp) sequences that may mediate interactions with cells.

Recent studies on the recessive dystrophic forms of epidermolysa bullosa (EB) have disclosed several mutations in the type VII collagen gene that have been highly instructive toward understanding the structure-function relationships of the various type VII collagen

[3]Historically, nonhelical, noncollagenous (NC) domains have been numbered from the carboxyl-terminal domain [16]. In the cases of collagen VII and collagen XIII, the NC1 domain is now accepted to be the amino-terminal noncollagenous region and numbering is from the amino-terminal domain.

domains [1–3,47,145,313,319,320] (see also Chapter 15, this volume). Many of the mutations consist of premature termination codons that predict the synthesis of truncated α1(VII) polypeptides. In the homozygous state these mutations result in the absence of anchoring fibrils, thus explaining the fragility of skin. A compound heterozygote for two premature termination codons indicated that the polypeptide segment between the upstream and downstream termination codons (von Willebrand factor A domain and cysteine/proline-rich region), is a critical region for fibril assembly. The intact triple-helical conformation is required for the function of type VII collagen, because substitutions in the Gly–X–Y repeat structure result in dominant dystrophic EB. However, recent data demonstrate that type VII collagen is a remarkable exception among collagens in that not all glycine substitutions within the triple helix exert dominant-negative effects [3]. Thus, the biological consequences of these substitutions seem to depend on their position within the triple helix. The NC1 domain is one of the most highly conserved sequences in the molecule, yet mutations here cause a relatively mild form of recessive dystrophic EB.

SHORT-CHAIN COLLAGENS

Type VI Collagen and Collagenous Microfibrils

Type VI collagen is essentially a glycoprotein with a short triple-helical core, which forms a distinct subclass of microfibril-forming collagen and is characteristically organized into an extensive filamentous network in the extracellular matrix of virtually all connective tissues (Fig. 14) [19,321]. It was originally isolated as a disulfide-bonded collagenous fragment in pepsin digests of vascular tissues, and designated "intima collagen." Many difficulties were initially encountered in isolating intact collagen VI because of its insolubility in most physiological buffers, and this led to the assumption that it constituted a minor component of connective tissues. However, the use of improved extraction procedures showed that this collagen represents a significant component of many tissues, notably skin and cornea [225,322–324], and the increased yields also facilitated the elucidation of the structure and details of the molecular assembly of intact type VI collagen.

Type VI collagen is unusual among the collagen family in possessing a particularly short triple-helical domain (105 nm) and exceptionally large N- and C-terminal globular domains that account for more than two-thirds of the total mass of the molecule [325,326]. It occurs as a heterotrimer composed of three genetically distinct subunits, [α1(VI)α2(VI)α3(VI)]. A number of studies have shown the synthesis of the three chains of collagen VI to be differentially regulated in cell culture and to be influenced by exogenous cytokines [327,328].

The three genetically distinct constituent chains of collagen VI show significant variation in their molecular masses (Fig. 14) [223,321,325,326]. The α3(VI) chain with an M_r of 260,000–280,000 is much larger than the partially homologous αl(VI) and α2(VI) chains (both M_r 140,000) (Fig. 14A) [321,329,330]. Despite this discrepancy in molecular mass, the lengths of the triple-helical sequences of all three chains are virtually identical (335–336 residues). The elucidation of the cDNA sequences of the chick and human collagen VI chains has revealed the molecule to have a multidomain structure with a number of potentially functional motifs, including 11 potential cell-binding RGD (Arg–Gly–Asp) sequences within the helical region [16,331–337]. The globular domains of each α chain contain three homologous motifs encoded by 200-base repetitive domains and designated N1, C1, and C2 in the human molecule. These exhibit significant sequence identity with the collagen-binding A domains of von Willebrand factor (vWF), cartilage matrix protein, some integrins, and complement components. The α3(VI) chain is the largest and one of the most novel collagen chains sequenced to date, with an extended N-terminal globule (1,804 residues, deduced M_r 343,000) containing nine additional motifs similar to the type A repeats of von Willebrand factor (encoded by 200-base repeats), and a C-terminal globule with three additional unrelated motifs (C3 to C5) in the most distal sequence. The C3 domain, a lysine and proline (KP)-rich region in the human, but not the chick, shares some homology with several proline-rich salivary proteins, and contains threonine repeats that may be utilized for O-linked glycosylation. The C4 domain has features typical of the structurally important type III repeat of fibronectin, and although overall sequence identity is rather low, this domain could form analogous rod-like elements with binding sites for heparin and cells. The most C-terminal domain (C5) is highly homologous to a variety of serine protease inhibitor sequences, and exhibits 42% identity with aprotinin and 50% identity with human urinary trypsin inhibitor.

Multiple alternative splicings of the N-terminal vWF type A repeats of the α3(VI) chain, apparently generated by exon skipping, have been detected at the cDNA level, which are presumed to give rise to the multiple forms of α3(VI) detected at the protein level as a ladder of polypeptides upon electrophoresis [338–340]. In addition, three α2(VI) variants with distinct C-termini arise because of an alternative splicing mechanism involving both mutually exclusive utilization of two exons and selective usage of an internal splice site. The spliced collagen VI isoforms exhibit distinct tissue-specific patterns and may impart subtly different functional properties [340].

The process of type VI collagen polymerization follows a route quite dissimilar to that of any of the other known collagens. The assembly of type VI monomers and their association into dimers and tetramers occurs intracellularly [321,341,342], and there is no evidence that processing of the α chains is a necessary prerequisite for assembly [343]. However, discrepancies between the deduced M_r (340,000) and the electrophoretically determined M_r (280,000) of α3(VI) suggest partial processing, and this is confirmed by the demonstration of the loss of the Kunitz module and possibly other adjacent modules from the α3(VI) chain [16].

The intracellular association of the three constituent α-chains into monomers is probably stabilized by the formation of interchain disulfide bridges. Dimer formation is brought about by the lateral aggregation of two antiparallel monomers with their helical rods staggered and the C-terminal regions overlapping by about 75 nm. The subsequent parallel alignment of two dimers with their N-terminal ends in register gives rise to the tetramer, the secreted form of type VI collagen and the basic unit of type VI collagen microfibrils (Fig. 9). The dimers are thought to be stabilized by disulfide bonds formed between the single cysteine residues present within the helices of α1(VI) and α2(VI), while a corresponding cysteine in the helix of α3(VI) may participate in tetrameric associations [333]. In addition, disulfide bonds formed between the clusters of cysteines found at the junctions between the N- and C-termini and the triple helix contribute to the high thermal stability and protease

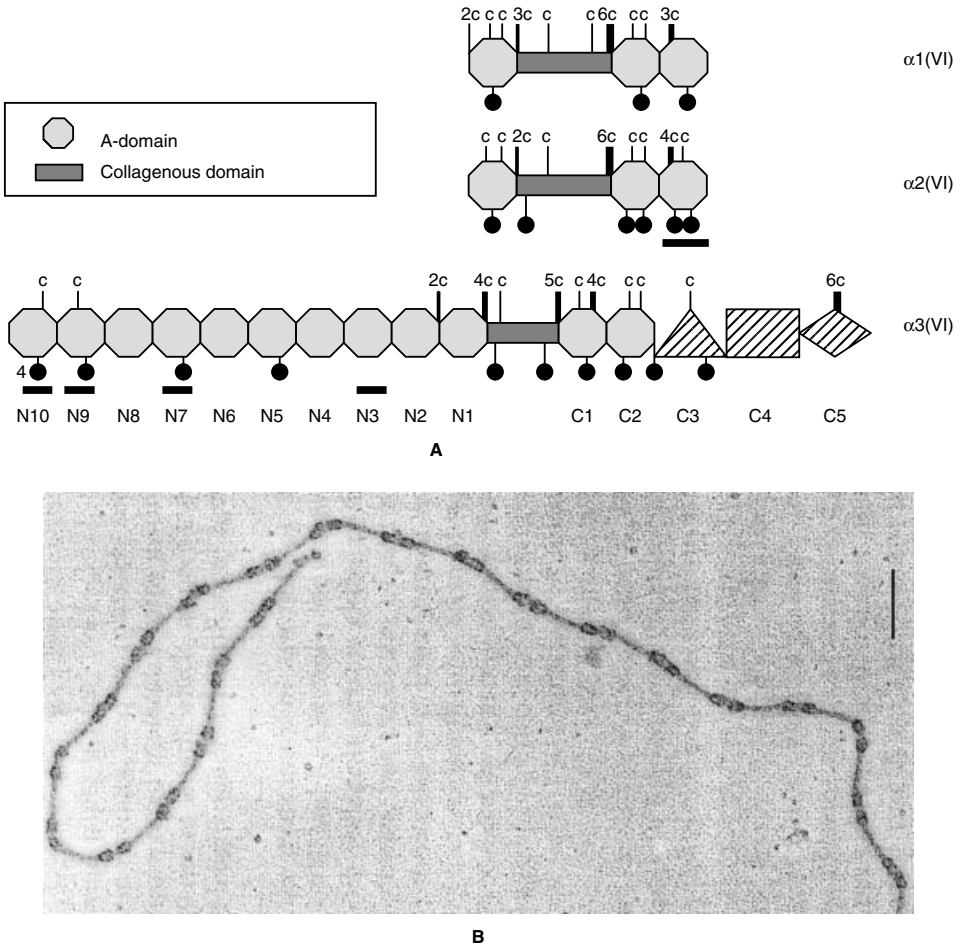


Figure 14. Molecular organization of type VI collagen. **A**. Line diagrams illustrating the dimensions and domain structures of the collagenous and noncollagenous regions of the α1(VI), α2(VI), and α3(VI) chains of type VI collagen. The noncollagenous regions of all three chains comprise a number of motifs designated N1, C1, and C2 in the case of α1(VI) and α2(VI) chains, and N1–N10 and C1–C5 in the case of the α3(VI) chains. Triple-helical domains are represented as dark shaded bars. The A-domains that flank the collagenous regions are indicated as shaded octagons (⬡), and the positions and numbers of cysteine residues (c) and N-linked carbohydrate attachment sites (🍡) are shown. The unique domains C3-C5 in the α3(VI) chain are indicated as lightly shaded motifs. Underlying solid bars indicate alternatively spliced A-domains **B**. Rotary shadowing image of long, thin (4 nm) type VI collagen microfibril of characteristic 105 nm periodicity that comprises tetrameric units in end-to-end association. The type VI collagen was isolated from fetal calf skin by bacterial collagenase digestion under non-reducing conditions, and subjected to a subsequent gel filtration step. The bar represents 100 nm.

resistance of the collagen VI helix. These assembly details were established by electron microscopic studies of pepsin-solubilized and intact collagen VI molecules synthesized in culture, and the various intermediate molecular forms have been visualized [341]. Once in the extracellular space, the tetramers aggregate end-to-end by the interaction of the outer N-terminal globular domains, giving rise to characteristic long, thin, beaded filamentous structures with a periodicity of 100 nm and a diameter of 3–5 nm. Such intact collagen VI microfibrillar macroassemblies have now been isolated from various fetal and adult tissues and visualized by rotary shadowing [227] (Fig. 14B). Ultrastructural analysis of tissues has also provided evidence for alternative collagen VI aggregates occurring as broad-banded fibrils of 100 nm periodicity, and these have been variously described as "zebra collagen," "Luse bodies," and fibrous long-spacing (FLS) collagen. Such structures apparently arise as a result of lateral aggregation of tetramers (in register), and have been identified in a number of normal tissues such as intervertebral disk and synovium and in pathological tissues including tumors and osteoarthritic cartilage [344–346].

Mutations in the *COL6A1*, *COL6A2*, and *COL6A3* genes encoding the three α chains have recently been linked to the rare autosomal dominant disorder, Bethlem myopathy (MIM 158810) (see also Chapter 26, Part VI, this volume), which is characterized by contractures of multiple joints and generalized muscular weakness and wasting [347–350]. One pathogenetic mechanism proposed is haploinsufficiency of the affected α chain and reduced production of structurally normal type VI collagen molecules. The presence of appropriate levels of type VI collagen in the pericellular matrix of muscle cells thus appears to be essential for normal myotubule stability and function. Type VI collagen interacts with basement membrane type IV collagen, disturbance of which may also contribute to the pathogenesis of Bethlem myopathy [351,352].

Several studies have demonstrated that type VI collagen interacts with cells through both integrin- and nonintegrin-mediated receptors [353–358]. For example, it promotes neural crest cell attachment and migration through multiple cooperative binding sites within triple-helical and globular domains. Type VI collagen also regulates normal and transformed mesenchymal cell proliferation *in vitro* and both increases cell survival and prevents anti-β1-integrin-mediated apoptosis [358,359]. Colocalization and ligand binding studies *in vitro* indicate that type VI collagen can interact with a number of other matrix molecules including hyaluronan, types II and XIV collagens, decorin, biglycan, and the cell membrane-bound chondroitin sulphate proteoglycan NG2 receptor [16]. The widespread distribution of collagen VI between collagen fibers and near the surface of cells, together with its cell-adhesion [353–358,360–364] and matrix-binding [352,353,360,365–367] capacities, have led to the proposal that type VI collagen microfibrils perform a crucial bridging function between cells and matrix, which may be of particular significance in influencing cellular phenotype and function, and directing tissue (re)modeling [19,321,364].

Collagen Types VIII and X

Types VIII and X collagens are true structural collagen members of a family of short-chain collagen-like proteins sharing a condensed gene structure, a triple helical domain and a highly conserved carboxyl terminal noncollagenous (NC1) domain (Fig. 15) [16,39]. This family also includes the C1q component of complement, conglutinin, serum mannose-binding protein, hibernation proteins, cerebellin, and ACRP30, an abundant serum protein implicated in energy homeostasis and obesity [21–32]. Types VIII and X collagens possess striking structural similarities at nucleotide and amino acid levels, and are approximately half the size of fibrillar collagens. A number of immune defense and cytokine molecules that are not structural elements of extracellular matrices, and are therefore not classified as "collagens", have collagenous sequences and NC1-like sequences that are homologous to collagens VIII and X (see Table 2).

Type VIII collagen was first detected as a product of bovine aortic and rabbit corneal endothelial cells and designated EC collagen [368,369]. It is also expressed by proliferating vascular smooth muscle cells, mast cells, and certain epithelial and tumor cell lines [368–371]. Type VIII collagen is present in subendothelium and medial layers of the vasculature, embryonic heart and placental capillaries, Descemet's membrane of the eye, renal glomerulus, and several structures of neural crest origin [372–376]. It is markedly up-regulated following vascular injury and in atheroma [377,378]. In contrast, type X collagen has a very restricted tissue distribution, being primarily a product of hypertrophic chondrocytes in that part of the endochondral growth plate destined for matrix mineralization [379–383]. It is also expressed at the margins of epiphyseal cartilage during fracture repair and in osteoarthritis and in the intervertebral disc [384–386].

Type VIII collagen comprises two distinct chains, α1(VIII) and α2(VIII) (Fig. 15) [16,39]. The human α1(VIII) primary translation product comprises 744 amino acids; it contains a triple-helix-forming collagenous sequence of 454 residues with eight imperfections in the Gly–X–Y sequence, a large globular noncollagenous carboxyl terminal (NC1) domain of 173 amino acids, and a noncollagenous amino-terminal (NC2) domain of 117 amino acids. The human α2(VIII) chain has a triple-helical domain of 457 residues, with eight imperfections in similar positions to those of α1(VIII), and a carboxyl terminal NC1 domain of 167 residues; the full primary structure of the amino-terminal NC2 domain has not yet been reported. The human α1(X) collagen chain is 680 amino acids in length; it has a short NC2 domain of 56 amino acids followed by a collagenous domain with 463 residues and eight imperfections, and an NC1 domain of 161 amino acids [16]. Bovine type X collagen has two cysteine residues within the fourth imperfection of the collagenous domain, and these participate in disulfide bonding in the assembled homotrimer.

The chain composition and molecular organization of type VIII collagen remain unresolved. Recent *in vitro* studies have demonstrated that recombinant α1(VIII) and α2(VIII) chains each form highly stable homotrimers (T_ms of 45°C and 42°C for $[\alpha1(VIII)]_3$ and $[\alpha2(VIII)]_3$, respectively), and heterotrimers $[\alpha1(VIII)]_2\alpha2(VIII)$ and $\alpha1(VIII)[\alpha2(VIII)]_2$ can also assemble [387,387a]. Available biochemical evidence from tissue extracts suggests that in Descemet's membrane, type VIII collagen molecules are heterotrimeric, with two α1(VIII) chains and one α2(VIII) chain [19,39]. By contrast, type X collagen is a homotrimeric molecule $[\alpha1(X)]_3$ with a melting temperature of 47°C. The triple-helical stabilities of collagens VIII and X are due largely to their high hydroxyproline contents.

The importance of the NC1 domains of types X and VIII collagens is illustrated by their high degree of conservation between family members and species, and reinforced by the fact that Schmid type metaphyseal chondrodysplasia (MIM 156500) is caused by mutations in *COL10A1* that are clustered in the NC1 domain [47,145,388,389] (see also Chapter 23, Part II, this volume). The amino-terminal one quarter of the NC1 domain is quite different between the three α chains, but the carboxyl three-quarters is highly homologous at both nucleotide and amino acid levels and includes a number of conserved tyrosine residues [16]. *In vitro* translation studies have demonstrated that the intact NC1 domain spontaneously trimerizes, and that calcium or microsomal membranes and associated chaperone proteins prevent the not yet folded collagenous domain from interfering with the folding and trimerization of the nascent NC1 domain. New information regarding the three-dimensional structure of monomeric and trimeric NC1 has emerged from 2.1 Å resolution crystal structure studies of ACRP30 (adipocyte complement-related protein of 30 kDa, or AdipoQ) and the subsequent molecular modeling of the NC1 domain of types VIII and X collagens (Fig. 15), which show putative asymmetric trimers composed of three 10 β-sheet jellyroll motifs [389,390]. The high thermal stability of trimerized α1(X) NC1 domains appears to reflect the increased numbers of hydrophobic contacts formed upon trimerization, with additional contributions from a hydrophobic region at the base of the NC1 domain and the 27 amino acid sequence present at the amino terminus of the NC1 domain. The NC1 domains of α1(VIII) and α1(X) each have an unpaired cysteine residue, while α2(VIII) NC1 has two cysteine residues; these residues may participate in disulfide bonds that stabilize supramolecular aggregates. The α1(X) NC1 domain also has a putative N-glycosylation site. The NC2 domains of α1(VIII) and α1(X) are structurally quite distinct, and thus may confer on each molecule unique interactive features. Both domains have an alkaline pI (~10.0 and 10.4, respectively) and could interact electrostatically with polyanionic molecules.

Full-length recombinant α1(VIII) and α2(VIII) chains have an electrophoretic mobility consistent with molecules of

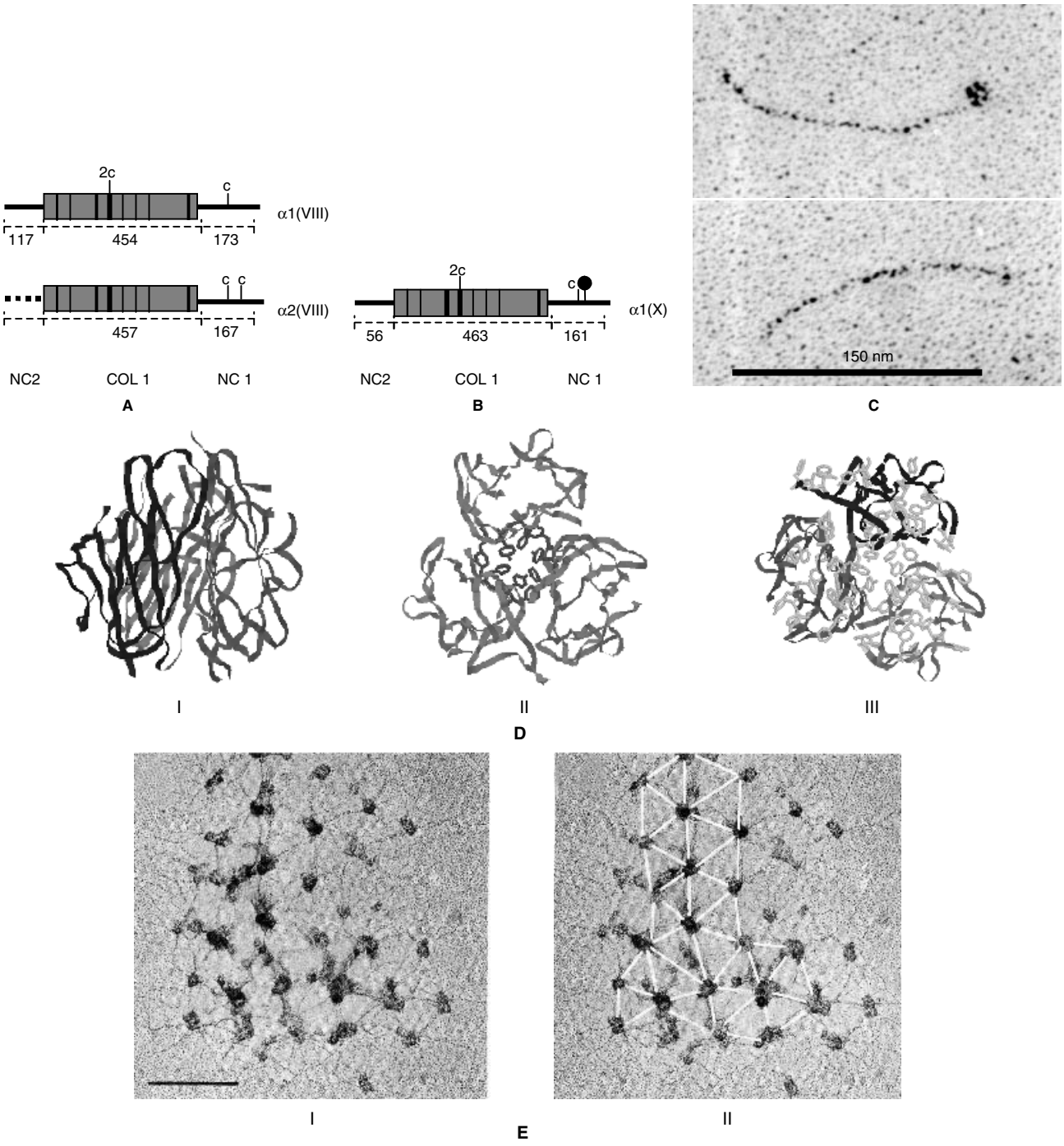


Figure 15. Molecular organization of types VIII and X collagens. **A** and **B**. Line diagrams illustrating the dimensions and domain structures of the collagenous and noncollagenous regions of the α1(VIII) and α2(VIII) chains of type VIII collagen and the α1(X) chain of type X collagen. Triple-helical domains are represented as dark shaded bars and noncollagenous domains as lines. Imperfections within the triple-helical regions are indicated by vertical lines. The positions and numbers of cysteine residues (c), and an N-linked carbohydrate attachment site (🍍), are indicated. The numbers of amino acid residues comprising each domain are indicated below the individual chains. **C**. Rotary shadowing images of pepsinized (lower) and intact (upper) type X collagen monomers showing the length of the helical domain to be approximately 150 nm (bar). The C-terminal non-collagenous (NC1) domain is clearly visible in the intact monomer as a small globule that is removed by pepsin digestion. **D**. Three-dimensional models of the trimeric form of the NC1 domain of the α1(VIII) chain of type VIII collagen, highlighting the cluster of core hydrophobic residues that are important in stabilizing the trimers. I = exterior face; II = vertical view of interior showing hydrophobic core residues. III = vertical view showing internal and external hydrophobic residues. **E**. Rotary shadowing image showing an extended network of type X collagen aggregates formed *in vitro* by incubating purified chick type X collagen in PBS at 34°C. The regularly spaced nodules of aggregated carboxyl terminal noncollagenous domains are interconnected by filamentous structures formed via interactions between triple helices. The hexagonal nature of the type X collagen lattice is highlighted in micrograph (**b**). The bar represents 200 nm. (Reprinted from ref. 396, by copyright permission of the Rockefeller University Press.)

~80 kDa, and trypsin and chymotrypsin resistant fragments have an M_r of ~60 kDa [387]. However, "intact" type VIII collagen chains isolated from Descemet's membrane have a reported M_r of ~61 kDa, and the presence of additional dimeric β forms of 120 kDa and trimeric γ forms of 170 kDa suggest possible intermolecular cross-linking. Pepsin treatment of tissue-derived type VIII collagen yields ~50 kDa fragments. Full-length recombinant α1(X) chains expressed in underhydroxylated form in mammalian cells migrate with an apparent M_r of 75 kDa, and after pepsin treatment, a 62 kDa band remains [235]. In contrast, SDS-PAGE studies of intact α1(X) chains extracted from chick and mammalian tissues indicate a relative molecular mass of 59 kDa with an additional related form of 49 kDa, while disulfide-bonded chains of ~120 kDa have been observed in long-term cultures [381]. Pepsin treatment of these chains generated ~45 kDa resistant fragments. The molecular basis of these size discrepancies is unknown, but could reflect processing events. Type VIII collagen is degraded by neutrophil elastase [391], and type X collagen is cleaved by matrix metalloproteinase 1 (MMP-1) at two sites [392].

Type VIII collagen is a major structural component of the hexagonal lattice-like network structure characteristic of Descemet's membrane, the specialized basement membrane laid down by corneal endothelial cells [372–374,393]. Similar type X collagen-containing network-like structures have been localized to pericellular regions of hypertrophic chondrocytes, and are disrupted in transgenic mice with a dominant interference phenotype for this collagen [394,395]. It remains unclear whether these collagens can adopt other supramolecular forms. Rotary shadowing visualization of native intact type X collagen molecules has revealed a relatively short triple-helical segment (132 nm) with globular "knobs" at each end corresponding to NC2 and NC1 domains (Fig. 15) [396]. Thermal aggregation products of type X collagen molecules have been shown to include unique hexagonal lattice structures *in vitro*, while similar type VIII collagen-based hexagonal lattices have been observed in cell layers of cultured bovine corneal endothelial cells. It is not known how molecules of such dimensions relate to the hexagonal lattice-like structures of Descemet's membrane with reported dimensions of ~160 nm unit structure (Fig. 9). Supramolecular assembly is thought to be driven, in part, by interactions between NC1 domains that, in the case of type X collagen, have long been known to have the potential to aggregate [379,396]. Other molecules of this short-chain collagen-like family (e.g., conglutinin, mannose-binding lectin) can interact via their carboxyl terminal (NC1-like) domains to form biologically active oligomers (frequently triple-helical hexamers).

The function of type X collagen is not known. Because the hypertrophic zone is structurally the weakest point within the growth plate, type X collagen may provide reinforcement as the matrix weakens. Type X collagen apparently also occurs in association (largely surface) with type II collagen fibrils, which probably represents a secondary association of newly synthesized type X collagen molecules with pre-existing type II collagen fibers [397,398]. It remains unclear whether type X collagen plays a role (direct or indirect) in the initiation of matrix mineralization. Certainly, modulation of collagen X gene expression in hypocalcemic and normocalcemic rickets with agents that promote mineralization (e.g., calcium β-glycerophosphate) or inhibit it (e.g., levamisole, an inhibitor of alkaline phosphatase) implies a functional relationship between type X collagen deposition and the mineralization of cartilage [399]. It is conceivable that type X collagen provides an appropriate structural support that is permissive for calcification and allows for the remodeling phases associated with vascular invasion and bone development. Little is known of the biological role of type VIII collagen. In the vasculature, it is associated with elastic fibers [376,400], and may serve as a structural and adhesive support for vascular smooth muscle cells, and it may also be involved in mineralization associated with vascular disease. The specific molecular interactions of types VIII and X collagens with cells and their surrounding matrix remain to be defined.

FACIT COLLAGENS

Collagen types IX, XII, XIV, XVI, and XIX are partially homologous, multidomain molecules, possessing structural features that have resulted in their classification as fibril-associated collagens with interrupted triple helices, or FACIT collagens (Fig. 16) [16,37,38,401,402]. Type XX collagen is a newly identified member of the FACIT family of collagens [402a]. These genetically and immunologically distinct collagens are characterized by short triple-helical domains interrupted by noncollagenous sequences. They share regions of high sequence homology and distinct structural characteristics, but they also exhibit extreme divergence in molecular mass (60 kDa to at least 220 kDa), in the size and sequence of their amino and carboxyl termini, and in the proportion of the molecule devoted to the interrupted collagenous domains. For example, 10% of α1(XII), 16% of α1(XIV), and 59% of α1(XVI) polypeptides are collagenous. The COL1 domain of type IX collagen has been used as a "signature" to identify other FACIT collagens, and is characterized by a pair of precisely conserved cysteine residues and by replicated imperfections in the triple helix [16,403]. Conservation of this triple-helical domain is a critical feature that facilitates lateral association with the surface of collagen fibers. Chain selection, registration, and assembly of FACIT collagens are directed by the COL1 domain and the adjacent five residues of the NC1 domain. Rotary shadowing analysis has revealed large structural differences in the domains projected into the fibrillar space, which reflects the low homology between the noncollagenous domains of these collagens and, presumably, the fine function of these species within a specified tissue [401,402,404,405]. The FACIT collagens are not capable of forming fibers or other supramolecular structures. Instead, types IX, XII, and XIV collagens have been shown to decorate the surfaces of interstitial collagen fibers and to act as molecular couplers that organize and anchor the fibrils in defined three-dimensional patterns within the matrix by means of interactions with other ECM components [406–410].

The tissue distribution of each of the FACIT collagens is unique and often highly tissue-specific. Type IX collagen is a fairly major component of type II collagen-containing tissues such as cartilage (up to 5% of pepsin-extracted collagen), intervertebral disc, and the vitreous humor [402]. Type XII collagen is localized in type I collagen-containing dense connective tissues such as tendons, ligaments, perichondrium, and periosteum [411]. Type XIV collagen is found mainly in tissues rich in type I collagen, particularly in regions in which high mechanical stresses are involved, such as bone-ligament junctions [412–414]. It is also present in basket-like structures around hair follicles and in cartilaginous tissues that contain type II collagen. Type XVI collagen has a broad tissue distribution, being localized predominantly in heart, kidney, intestine, ovary, testis, eye, arterial wall, and smooth

muscle [16,415]. Type XIX collagen is expressed in human rhabdomyosarcoma and fibroblast cell lines [416,417].

Type IX Collagen

Type IX collagen is the prototype molecule of the FACIT collagens (Fig. 16) [16,402]. It was first identified as disulfide-bonded triple-helical fragments in pepsin digests of mammalian cartilage [418–421], which could be separated from type II collagen by differential salt fractionation. A combination of biosynthetic studies and cDNA sequence data [422] subsequently revealed the complex structure of the intact form of the molecule to have an M_r of 300,000 and a length approximately two-thirds that of types II and XI collagens. It exists as a heterotrimer composed of three

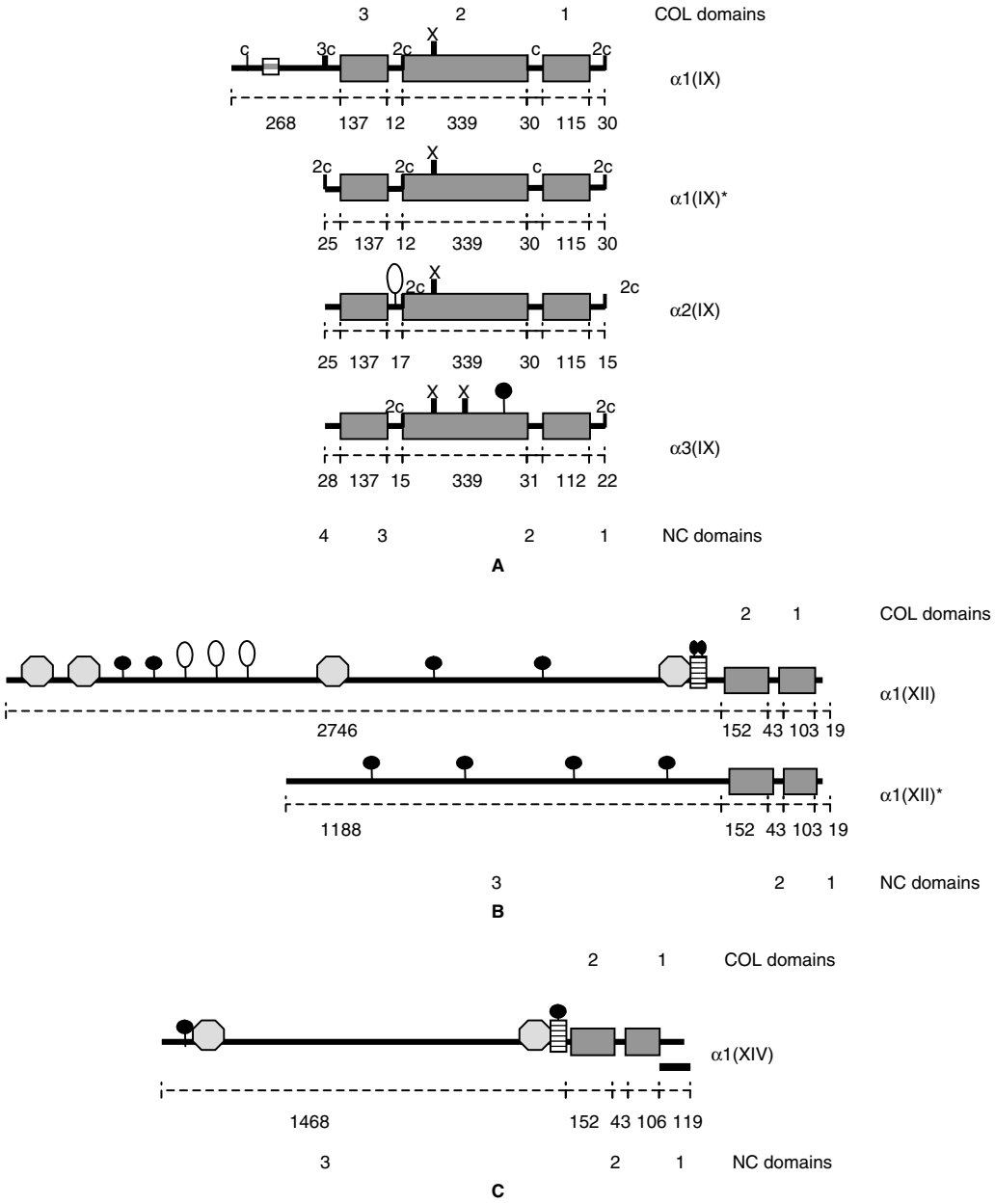


Figure 16. Molecular organization of the FACIT collagens. **A–E**. Line diagrams illustrating the dimensions and domain structures of the component chains of type IX collagen (**A**), type XII collagen (**B**), type XIV collagen (**C**), type XVI collagen (**D**), and type XIX collagen (**E**). The triple-helical (COL) domains are represented as dark shaded bars, and the noncollagenous (NC) domains as lines. The sequences of the COL1 domains of the FACIT collagens are homologous. Alternatively spliced sequences are highlighted as α1(IX) and α1(IX)*, and α1(XII) and α1(XII)*, and, in the case of type XIV collagen, as a heavy line below the diagram. The positions and numbers of cysteine residues (c), N-linked carbohydrate attachment sites (♀), A-domains (octagons) (◯), PARP (proline and arginine-rich protein) domains (▯), and glycosaminoglycan attachment sites (♀) are indicated. The numbers of amino acid residues comprising each domain are indicated beneath the individual chains. **F**. Rotary shadowing images of type XII collagen monomers. The very large NC3 domain is apparently arranged in a trident shape reminiscent of the type VII collagen NC2 domain. Bar = 100 nm. (Courtesy of Dr B. Dublet and Prof. M. van der Rest.)

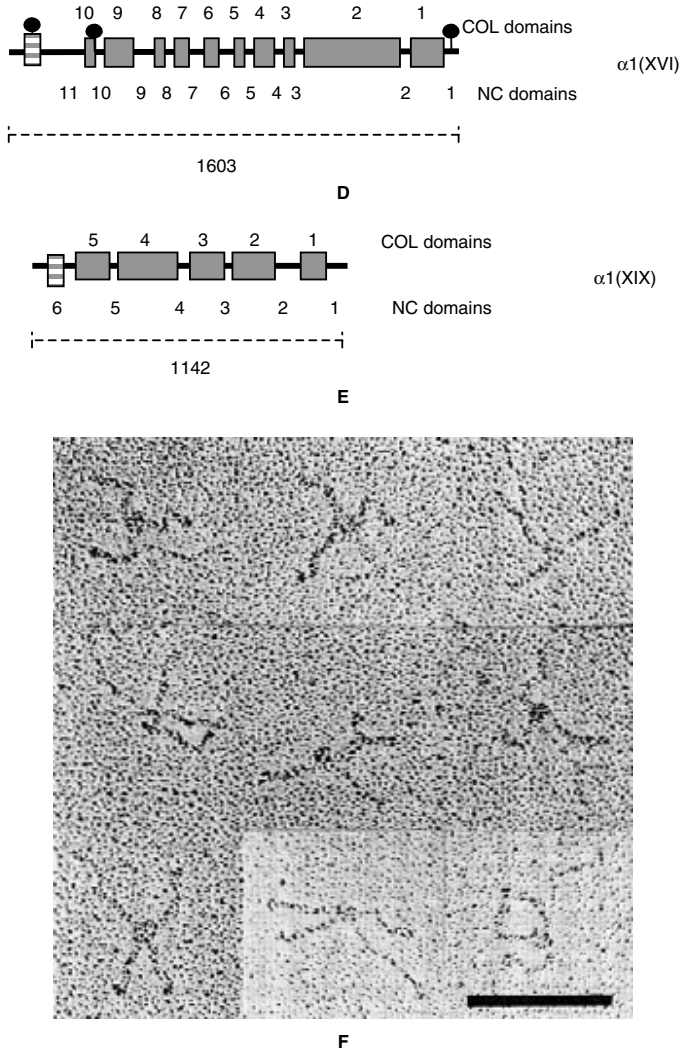


Figure 16. (*Continued*)

genetically distinct chains designated α1(IX), α2(IX), and α3(IX), which are associated into an interrupted triple helix that comprises a short arm and a long arm connected by a flexible "hinge" (see Fig. 17A). There is no evidence that any processing of collagen IX occurs as a necessary prerequisite for molecular association with the fibrillar collagens. Type IX collagen has three collagenous domains (COL1–COL3) that can be recovered as pepsin-resistant fragments, and four noncollagenous domains (NC1–NC4), and is stabilized by interchain disulphide bonds located within NC1, NC2, and NC3 (Fig. 16A). The α1(IX) chain is the longest chain, with the large NC4 domain at the amino-terminus of the short arm, which is seen as a globule in electron micrographs. Type IX collagen was the first collagen to qualify for the classification of proteoglycan, with the α2(IX) chain being able to act as a core protein for a covalently attached chondroitin sulfate (or dermatan sulfate) side chain [406,423,424]. Serine 167 of the NC3 domain of α2(IX) has been identified as the glycosaminoglycan attachment site (see Figs. 16A and 17). Type IX collagen can exist in both proteoglycan and nonproteoglycan forms *in vivo*, and the length of the glycosaminoglycan chain is both species- and tissue-dependent. In developing chick, the expression of much shorter type IX collagen molecules has been demonstrated in nonchondrogenic, mesenchymal regions of the developing limb, notochord, and various ocular tissues including cornea and vitreous [425–428]. These molecules contain an α1(IX) collagen chain that lacks the amino-terminal globular NC4 domain characteristic of the cartilage form and instead contains a short alternative sequence and a significantly longer glycosaminoglycan chain [429]. These differences arise as a consequence of the use of widely separated alternative transcription start sites in the α1(IX) gene. Homologous α1(IX) transcripts have also been detected in human fetal tissues, and in mouse the short form has been detected in the heart and eye [401,410,427,428]. mRNAs for the three type IX collagen α chains are generally coordinated in cartilage, but differences are apparent in noncartilaginous tissues in which type IX collagen is present at low levels.

The association of type IX collagen with type II collagen fibrils in cartilaginous tissues has been well studied [16].

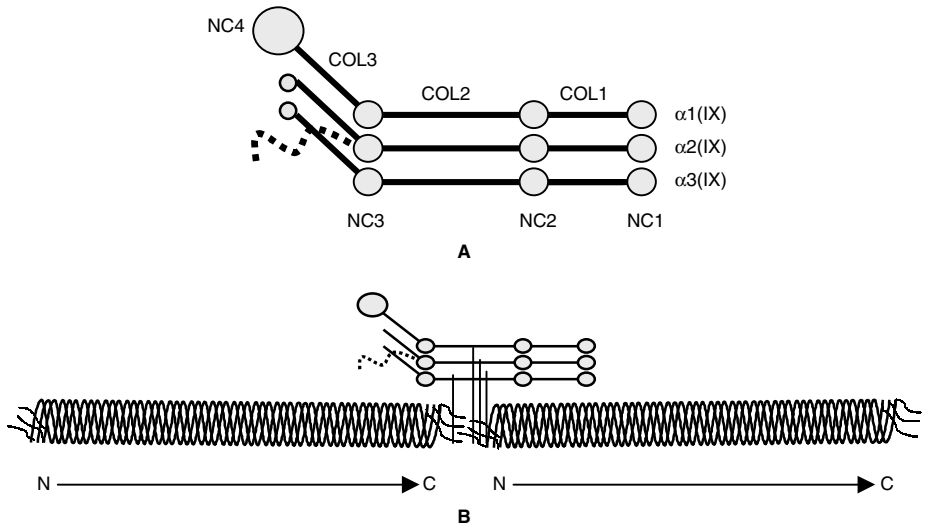


Figure 17. Molecular organization of type IX collagen, and its association with type II collagen fibrils. **A**. The three α chains of type IX collagen are aligned such that the COL1 and COL2 domains can form triple helices. Serine 168 of the NC3 domain of α2(IX) is an attachment site for glycosaminoglycans (•••••). The nonhelical NC3 region forms a 'hinge'; it contains 12,17, and 15 residues in the α1(IX), α2(IX) and α3(IX) chains, respectively, which causes a sharp kink. **B**. The long arm comprising NC1, COL1, NC2, and COL2 lies along the surface of the type II collagen fibril, and is covalently linked by lysine/hydroxylysine-derived cross-links (shown by vertical lines) to the N-telopeptides in the fiber via sites on all three chains in the amino-terminal region of COL2; the α3(IX) COL2 domain is also cross-linked to the C-telopeptide of type II collagen. The short arm comprises COL3 and NC4, and projects into the interfibrillar space. Cross-links between type IX collagen and type II collagen probably help stabilize the COL2 triple helix. COL1 may be partially stabilized by interactions with type II collagen to which it lies adjacent and runs antiparallel.

The long arm comprising NC1, COL1, NC2, and COL2 lies along the surface of the type II collagen fibril (Figs. 9 and 17) and is covalently linked by lysine/hydroxylysine-derived bonds to the N-telopeptides in the fiber via sites on all three chains in the amino-terminal region of COL2; the α3(IX) COL2 domain is also cross-linked to the C-telopeptide of type II collagen [16]. The short arm comprises COL3 and NC4, and projects into the interfibrillar space. The nonhelical NC3 region forms the "hinge"; it contains 12,17, and 15 residues in the α1(IX), α2(IX), and α3(IX) chains, respectively, which causes a sharp kink. Recently, the association of recombinant collagen IX α chains into homotrimeric and heterotrimeric molecules has also been described [430,431].

Studies on the thermal stabilities of the COL domains have shed new light on the structural organization of type IX collagen [432]. Melting curves reveal that unfolding of COL1 occurs at 40.6°C, that of COL2 at 39.6°C, and that of COL3 at 49.0°C. The high thermal stability of COL3 may be due to the presence of a high proportion of hydroxyproline residues (186/1000 residues). COL3 may be further stabilized by the cross-linking of the adjacent NC4 region (which has a basic pI) to nonfibrillar matrix material such as glycosaminoglycan chains in the extracellular matrix (perhaps glycosaminoglycans attached to an NC3 domain of an adjacent type IX collagen molecule). Disulfide links between the three α chains also affect the thermal stability of the trimer; for example, cysteine residues in the NC3 region of each α chain probably participate in disulfide bonds that hinder the unzipping of α chains between long and short arms. It has been suggested on the basis of the lower thermal stabilities of the COL1 and COL2 domains that type IX collagen may be stabilized by the presence of type II collagen fibrils [432]. Cross-links between type IX collagen and type II collagen probably help stabilize the COL2 triple helix, while COL1 may be partially stabilized by interactions with type II collagen to which it lies adjacent and runs antiparallel, and by cross-linking the α3(IX) NC1 domain to α1(IX) or α3(IX) sites on adjacent type IX collagen molecules also cross-linked to the fiber.

Type IX collagen locks onto specific sites on the surface of the quarter stagger lattices of type II collagen fibers, thereby sterically hindering the insertion of additional type II collagen molecules in its vicinity [406]. It competes with type II collagen for lattice positions, having the effect of progressively reducing type II collagen fiber diameter when fibrillogenesis *in vitro* is carried out at increasing type IX collagen concentrations. In tissues, type IX collagen appears to be nonuniformly distributed along fibers, and may play a role at fibril intersections. A new model of type IX collagen association with type II collagen fibrils has been proposed on the basis of the different thermal stabilities of the COL domains [432]. It has been suggested that type IX collagen could fold through 180° at NC2, allowing COL1 to run along an adjacent gap zone in a parallel direction. This would place the NC1 domain close to a further type IX collagen molecule to which it could cross-link. This scheme is consistent with the location of previously described cross-links, provides a structural reason for the NC interruptions in the Gly−X−Y sequence, a purpose for NC2 in terms of equipping COL1 with more flexibility for weaving into the gap region, and a reason for the precise lengths of COL2 and COL1 (454 residues). Moreover, it allows speculation that this structural alignment would allow COL1 to fill the gap region and inhibit nucleation of mineralization at this site, and consequently prevent any calcification of articular and other cartilage. If the COL1 were to be inserted into the gap region of an adjacent fiber, this would lead to a fibrillar network structure.

Naturally occurring mouse mutants lacking α1(IX) are viable and show no detectable abnormalities at birth, but show signs by 4 months of joint disease and, in time, develop a degenerative osteoarthritis with flattened articulating surfaces and epiphyses [433–435]. Transgenic mice expressing mutant α1(IX) chains with an in-frame deletion of COL2 progressively develop joint and intervertebral disc degeneration with age [434]. These animal models confirm an important role for type IX collagen in the long-term mechanical stability and function of the type II collagen fibril framework of cartilage. Moreover, mutations in the genes for α1(IX) and α2(IX), respectively, have been linked to multiple epiphyseal dysplasia [436–438] (see also Chapter 23, Part II, this volume).

Type XII Collagen

Type XII collagen was discovered through the isolation and sequencing of cDNAs from an embryonic chick tendon library that revealed a multidomain species exhibiting remarkable homology to the COL1 domain of the type IX collagen α chains (Fig. 16) [16,411,439,440]. It is the product of a single gene and exists as a homotrimer, $[\alpha1(XII)]_3$, the individual α1(XII) chains having an M_r of 220,000. Each α chain contains non-triple-helical sequences of relative molecular mass 190,000 arranged in a very large amino-terminal domain (NC3) and two shorter domains (NC2 and NC1), and only two short triple-helical domains (COL2 and COL1), which can be observed after pepsin digestion as unreduced fragments of M_r 46,000 and 32,000, respectively [404,405]. While the homology between the COL1 domains of types IX and XII collagens corresponds to an overall amino acid sequence identity of greater than 50%, no identity is apparent between the remaining collagenous sequences of the two species [405]. Within the noncollagenous sequences, partial homology is observed between the NC3 domain of type XII collagen and NC4 of type IX collagen, although the NC3 domain of type XII collagen is much larger than the corresponding type IX collagen NC3 domain. Large and small spliced forms of type XII collagen occur that share a common signal peptide but differ in the size of the NC3 domain (Fig. 16) [16]. The large form of NC3 is ~320 kDa and comprises 18 fibronectin type III repeats, four von Willebrand factor-like repeats, and a PARP (proline- and arginine-rich protein) motif. The short form is ~220 kDa and lacks the first two von Willebrand factor A repeats and the first eight fibronectin type III repeats. Three potential glycosylation sites are present in the fourth, fifth, and sixth fibronectin type III repeats, and in some tissues such as fetal bovine cartilage, the larger spliced form can carry a glycosaminoglycan and thus act as the core protein of a proteoglycan. The large form has a more restricted expression in embryonic tissue than the smaller form, which is particularly abundant in the dermis. Heterotrimers comprising short- and long-form chains are also possible, which, together with variable glycosylation of the long form, allows a large number of possible isoforms. Structural variants of the carboxyl terminal NC1 domain of collagen XII generated by alternative splicing have been described [441]. A new nomenclature has now been proposed, with type XIIA-1 and XIIB-1 collagens having a long NC1 form, and types XIIA-2 and XIIB-2 a short alternative NC1 domain.

Rotary shadowing images reveal type XII collagen molecules to have a thin (often kinked) collagenous tail of 75 nm attached through a central globule (NC2) to three extended arms, each 60 nm in length and corresponding to the NC3 domain (Fig. 16F) [19]. The role of type XII collagen remains to be defined, but it is envisaged as associating with type I collagen fibrils through its triple helical tail, with the NC3 domain being free to project outward from the fibril surface and to interact with other matrix components [401]. Indeed, immunohistochemical analysis of embryonic chick tendon using immunogold labeling has revealed type XII collagen molecules to be distributed along type I collagen-containing fibrils. This arrangement remains to be verified, however, and it should be noted that there is no strict codistribution of types I and XII collagens in some tissues such as bone, cornea, and scleral ossicles. Moreover, experiments have failed to reveal the existence of covalent cross-links between type XII collagen and type I collagen. Indeed, type XII collagen does not possess a domain homologous to COL2 (IX) and therefore is not cross-linked to either itself or other collagens via lysine/hydroxylysine-derived bonds, and it can readily be extracted from tissues by low-molarity NaCl solutions. No direct interactions of NC3 with monomeric fibrillar collagens or with collagen fibers have been demonstrated *in vitro*. However, the NC3 domain does contribute significantly to the contraction of collagen gels, a model system for studying the interaction of mesenchymal cells with a fibrous collagen substrate; perhaps by moderating interfibrillar interactions, these molecules influence extracellular matrix deformability [442]. Type XII collagen can be incorporated *in vitro* into acid-soluble type I collagen fibrils during, but not after, fibril formation. Type XII collagen interacts with the glycosaminoglycan chain of decorin and the protein core of fibromodulin [16]. The smaller chains resulting from NC3 alternative splicing interact weakly with heparan sulfate via their carboxyl terminal domains, while the larger forms have additional, stronger heparin-binding sites. Like type IX collagen, there is no evidence that any processing of type XII collagen occurs as a necessary prerequisite to matrix deposition.

Type XIV Collagen

Type XIV collagen was first isolated as disulfide-bonded triple-helical fragments of its COL1 domain from pepsinized fetal bovine skin and tendon; its amino acid sequence is strikingly homologous to the corresponding domains of homotrimeric type XII collagen (64% primary structure identity) and, to a lesser extent, of type IX collagen (Fig. 16) [16,405,413,414]. Each chain consists of two collagenous triple-helical domains (COL1 and COL2) and three noncollagenous domains (NC1–NC3). The NC3 domain comprises eight fibronectin type III repeats, two von Willebrand factor-like domains, and a PARP repeat. The NC3 domain is closely related to the noncollagenous protein undulin, which may be a proteolytic fragment or an alternatively spliced variant of type XIV collagen. As with collagen XII, the NC3 domain influences human fibroblast migration in collagen gels [442]. Two variants of type XIV collagen exist that differ in their NC1 domains and that may arise by alternative splicing [441]. In addition, alternative 5′ untranslated regions suggest differential splicing at the 5′ end of α1(XIV) transcripts. Type XIV collagen can exist in a proteoglycan form in some tissues (e.g., fetal bovine cartilage) [16]. This collagen can be extracted from tissues with low-molarity NaCl solutions because it is not covalently cross-linked within the extracellular matrix.

Type XIV collagen is thought to bind to collagen fibers via its NC1 and COL1 domains, while COL2 and NC3 are thought to extend into the extracellular space and to participate in interactions with other components of the interstitial matrix. There is no direct evidence for the binding of type XIV collagen to cells or to types I, III, or V collagens *in vitro*, but associations with type VI collagen, heparan sulfate proteoglycan, and a variant of the CD44 cell surface receptor have been demonstrated [16]. Type XIV collagen also binds decorin, a proteoglycan associated with collagen fibrils, and procollagen I N-proteinase, which may be important during fibrillogenesis, as discussed earlier.

A physiological role for gelatinases (specifically MMP-2 and MMP-9; see Chapter 7, this volume) has been indicated in degradation of the COL1 domain of type XIV collagen, and possibly in structural remodeling associated with other FACIT collagen-associated matrix changes in development [443].

Type XVI Collagen

Type XVI collagen was first identified as a novel collagenous polypeptide encoded by cDNA clones isolated from a human fibroblast cDNA library [415,444]. Biosynthetic studies using stably transfected kidney cell clones, and human fibroblasts and smooth muscle cells, indicate that type XVI collagen is a disulfide-bonded homotrimer comprising 200–220 kDa α1(XVI) chains [444]. The overall structural arrangement of α1(XVI) differs significantly from that of other known collagen chains, although it does possess several structural features characteristically seen in members of the FACIT collagen family (Fig. 16) [16,445]. The complete primary structure consists of 10 collagenous domains, most of which are short (15–138 amino acids), with the exception of COL2, which is relatively large (422 amino acids), interspersed among 11 noncollagenous domains. The collagenous domains contain numerous non Gly–X–Y imperfections that could make the molecule rather flexible. Except for a large amino-terminal NC11 domain of 312 residues that contains a PARP domain, most of the NC domains are short (11–39 residues) and cysteine-rich. In particular, two cysteine residues regularly located in the amino-terminal region of each noncollagenous segment may contribute to triple-helix formation of the adjacent amino-terminal collagenous domain by forming stabilizing disulphide bonds in the following carboxyl terminal noncollagenous region, and to stabilizing the three-dimensional structure of the molecule. The two cysteine residues separated by four amino acids at the COL1/NC1 junction, and the size of the COL1 domain, are characteristic of the FACIT collagens. There is also some homology between the α1(XVI) NC11 domain, the NC4 domain of type IX collagen, and the NC3 domain of type XII collagen.

Similarities between type XVI collagen and other FACIT collagens raise the possibility that this collagen may also serve as a molecular bridge responsible for maintaining the structural integrity of the extracellular matrix. One interesting difference is that the NC11 domain, homologous to the NC4 domain of type IX collagen, which has a pI of ~10 and is thought to interact with polyanions in the extracellular matrix, has a pI of ~7 and probably has unique interactive properties. Studies of recombinant NC11 suggest that type XVI collagen may be processed with the release of the PARP domain, resulting in a protein comprising chains of 150–160 kDa [444,445]. Type XVI collagen expression by human skin fibroblasts is enhanced in fibrotic skin diseases [446], and modulated by basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β) [447].

Type XIX Collagen

Type XIX collagen was originally discovered through cDNA cloning of a human rhabdomyosarcoma cell line [416]. The predicted polypeptide comprised 1,142 amino acid residues including a 23-residue signal peptide and five collagenous domains (COL1–COL5, varying between 70 and 224 amino acids, and all together comprising 832 residues) interspersed and flanked by six noncollagenous (NC) domains of 19, 31, 23, and 44 residues (Fig. 16) [16,417]. It is a relatively poorly characterized member of the FACIT collagens with limited homology to the others. The COL1 domain is smaller than the COL1 domains of types IX, XII, XIV, and XVI collagens, although the arrangement of two cysteine residues near its carboxyl terminus and two imperfections are conserved. The amino-terminal region of COL1 contains only the PARP domain similar to types IX and XVI collagens and differs from those of types XII and XIV collagens.

The expression pattern has recently been established in developing embryonic and adult organisms [448]. Type XIX collagen mRNA can be detected as early as 11 days of gestation and in all embryonic tissues, except the liver, of 18-day embryonic mouse. In contrast, only a few adult tissues (brain, eye, testis) accumulate this mRNA, but transcripts are at least ten times more abundant in adult than in fetal brain [449]. Biochemical and immunohistochemical characterizations indicate that human collagen XIX constitutes a novel class of basement membrane collagen [450]. The chain composition and molecular organization of type XIX collagen is unknown. However, polyclonal antibodies against α1(XIX) collagen react with a 150 kDa protein in neutral salt extracts of adult mouse brain tissue. After digestion with bacterial collagenase, this protein migrates under nonreducing conditions with an apparent M_r of ~400 kDa, probably representing a homotrimeric form of type XIX collagen [450]. An unusual number of alternatively spliced variants of type XIX collagen in human rhabdomyosarcoma and glioma cell lines have been reported [16]. However, transcripts from normal tissues consistently yield a single hybridizing species, and multiple forms of type XIX collagen may be a feature only of tumor cell lines.

Molecular modeling of the conserved cysteine residues at the COL1/NC1 junction indicate that intrachain disulfide bonds probably form, unlike the situation with other FACIT collagens for which experimental evidence demonstrates that their carboxyl termini participate in intermolecular disulphide interactions. Thus, the same sequence motif, $Cys–(Xaa)_4–Cys$, may result in two distinct protein structures depending on the arrangement of disulfide bonds.

Type XIX collagen expression in the embryo does not follow the patterns of the major fibrillar collagens (types I/III and II, respectively), for it occurs in both cartilaginous and noncartilaginous tissues. Its abundance in brain and expression by glial cells suggests a potential role as a connecting bridge within proteoglycan networks or as an anchor for the surrounding cells. Type XIX collagen thus appears to play a role distinct from that of other FACIT collagens in the assembly of specialized structures in developing embryonic matrices and in the maintenance of specific adult tissues.

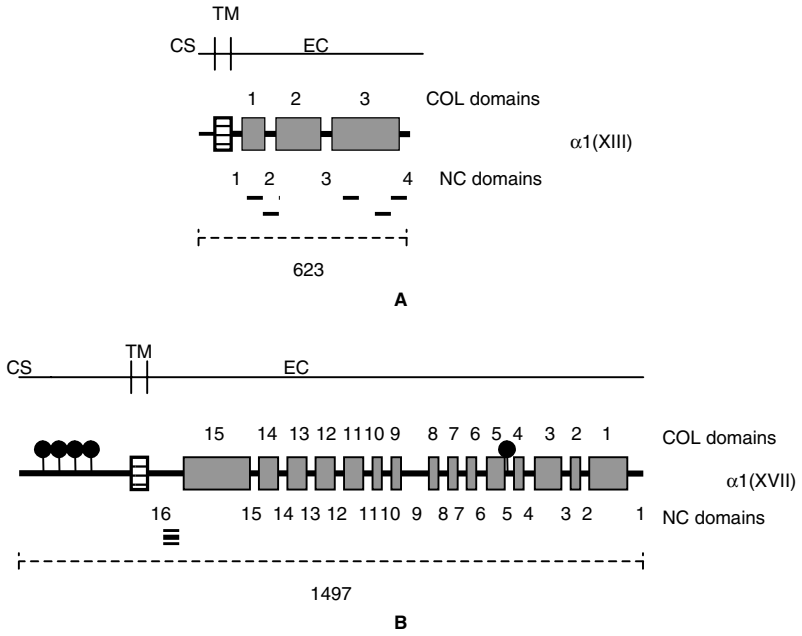


Figure 18. Molecular organization of the transmembrane collagens. Line diagrams illustrating the dimensions and domain structures of the component α1 chains of type XIII collagen (**A**) and type XVII collagen (**B**). The triple-helical (COL) domains are represented as dark shaded bars, and the noncollagenous (NC) domains as lines. Alternatively spliced type XIII collagen sequences are highlighted as single lines below the diagram. In the case of type XVII collagen, the major epitope recognized by autoantibodies in the sera of patients with both bullous pemphigoid and herpes gestationalis is in the extracellular domain immediately after the transmembrane domain, which is highlighted as triple lines below the diagram. The positions of N-linked carbohydrate attachment sites (♆), and PARP (proline- and arginine-rich protein) domains (▯) are shown, and the numbers of amino acid residues comprising the chairs are indicated beneath each CS, cytosolic domain; TM, transmembrane domain; EC, ectodomain.

TRANSMEMBRANE COLLAGENS

Types XIII and XVII collagens are genetically and immunologically distinct nonfibrillar collagens that each contain a hydrophobic transmembrane segment and are integral plasma membrane proteins (Fig. 18) [16,451–468,471–474]. These collagens are not homologous in sequence or in general molecular appearance but, because both are membrane-associated collagens expressed in mesenchymal tissues, they have been grouped together to comprise a new subgroup, transmembrane collagens. Several other transmembrane molecules with collagenous sequences have also been described; these include a bacteria-binding plasma membrane protein (MARCO; macrophage receptor with collagenous structure) and types I and II macrophage scavenger receptors [469,470], which have a broad polyanion-binding ability and participate in macrophage-associated functions such as host defense and inflammation. The presumed or confirmed orientation of all these plasma transmembrane molecules is with their amino-terminal regions situated intracellularly and their collagenous domains in the extracellular space [459,463]. This so-called "type II" orientation occurs in only 5% of plasma transmembrane proteins, and the presence of collagenous sequences may dictate such an arrangement. The direction of triple-helical folding in the transmembrane collagens remains to be determined. Unlike the fibril-forming collagens, in which three α chains associate through carboxyl terminal interactions after release into the endoplasmic reticulum lumen then form triple-helices proceeding toward the amino-terminus, the transmembrane collagens have their amino termini inserted into the rough endoplasmic reticulum before carboxyl terminal association and triple helix formation. Thus, formation of the triple helix might involve carboxyl to amino folding, with the resulting torsional tension relieved somehow by rotation of the transmembrane domains of the three polypeptides in the membrane, or the three chains might associate at their amino termini and then fold in an amino to carboxyl terminal direction. There are as yet no experimental data to resolve these possibilities.

Type XIII Collagen

Type XIII collagen is a short-chain collagen that is expressed at low levels in virtually all tissues examined to date, including fetal bone, cartilage, intestine, skin, striated muscle, and placenta. This distribution suggests that type XIII collagen may serve a general function in connective tissue [16,454,455,459]. Human type XIII collagen has been extensively characterized through human cDNA and genomic clones, but until the recent cloning of mouse type XIII collagen [455,456], the extreme amino-terminal sequence had not been predicted. The polypeptide consists of three collagenous domains (COL1–COL3) and four noncollagenous domains (NC1–NC4) (Fig. 17) [16][4]

[4]Historically, nonhelical, noncollagenous (NC) domains have been numbered from the carboxyl-terminal domain [16]. In the cases of collagen VII and collagen XIII, the NC1 domain is now accepted to be the amino-terminal noncollagenous region and numbering is from the amino-terminal domain.

A striking feature of type XIII collagen is that sequences corresponding to ten exons of the human gene undergo freely combinatory alternative splicing during the processing of the primary transcripts, and are generated by exon skipping (of exons 3B, 4A and 5 in COL1; exons 12 and 13 in NC2; exons 29 and 33 in COL3; exon 37 at the COL3/NC4 junction) [451–455]. Splicing is predicted to affect the structures of the NC4, COL3, NC2, and COL1 domains, and at least 12 mRNA species exist through the alternative expression of exons 3B-5, 12, and 13, and distinct differences in the proportions of the variant mRNAs have been observed in cultured cell lines and tissues. Furthermore, four combinations of alternatively spliced exons 29–37 have been found among cDNA clones isolated from human endothelial cells and HT1080 fibrosarcoma cells, and additional combinations are predicted to occur. The length of human α1(XIII) collagen chains may vary between 697 and 609 amino acids depending on the composition of alternatively spliced exons involved. The functional significance of this complex pattern of alternative splicing is not known.

The complete primary structure of mouse type XIII collagen shows that mouse cDNA extends further in the 5′ direction than the previously identified human clones and contains a new in-frame ATG codon for translation initiation that results in elongation of the amino-terminal noncollagenous domain by 81 amino acids [16,456]. These amino-terminal sequences lack a typical signal sequence but include a highly hydrophobic segment that clearly fulfils the criteria for a transmembrane domain. The longest possible coding region of mouse α1(XIII) represents 739 residues, but the length of the deduced polypeptide can vary markedly depending on the complex alternative splicing of mRNAs, and the calculated M_r of mouse type XIII collagen is between 58.3 and 69.7 kDa. An expressed sequence tag (EST) database search provided the corresponding sequence of the human upstream 83 amino acids that are ~90% similar to the extreme amino-terminal mouse sequence [456]. The human sequence also lacks a signal peptide, but the presence of a highly hydrophobic region of 23 residues located at residues 37–59 that fulfils the criteria for a transmembrane domain indicates that human type XIII collagen is probably located on the plasma membrane with a short cytosolic amino-terminal portion and a long collagenous extracellular portion. Western blotting of human HT1080 whole-cell extracts using peptide antibodies generated to unspliced type XIII collagen NC1 and NC3 domains revealed bands of >180 kDa representing disulfide bonded multimeric polypeptide forms that resolved upon reduction to 85–95 kDa bands that are probably a mixture of splice forms of monomeric α1(XIII) chains [453]. Although type XIII collagen appears to be synthesized as a homotrimer of three α1(XIII) chains, it is not known whether the three chains within a molecule exhibit identical splicing. The chains were susceptible to bacterial collagenase digestion, and contained the predicted amino-terminal extension and the transmembrane segment. Immunoprecipitation of biotinylated type XIII collagen from surface-labeled HT1080 cells, subcellular fractionation, and immunofluorescence labeling have confirmed that human type XIII collagen molecules are, indeed, located in the plasma membrane of these cells. The presumed cytosolic amino-terminal part of the NC1 domain includes a putative protein kinase C phosphorylation site (threonine, residue 6).

Type XVII Collagen

Type XVII collagen is the 180 kDa bullous pemphigoid antigen (also known as BPAG2 or BP180), a hemidesmosomal component expressed in stratified squamous epithelia of the skin, oral cavity, and uterine cervix [16,460,462–464,471], and α1(XVII) mRNA has also been detected in the heart [472]. This protein was originally recognized as an autoantigen in bullous pemphigoid and herpes gestationalis [47,145,465,466]. Its deduced primary structure predicts a "type II" orientation integral transmembrane protein of 1497 amino acids, with an amino-terminal intracellular domain of 466 amino acid residues, a transmembrane domain of 23 residues, and a highly interrupted collagenous carboxyl terminal extracellular domain of 1008 amino acid residues. The α1(XVII) chain comprises 15 collagenous domains (COL1–COL15) ranging from 15 to 242 amino acid residues in length, and 16 noncollagenous domains (NC1–NC16) (Fig. 17) [16]. The large, mainly cytosolic amino terminal NC16 domain contains 566 amino acids. Type XVII collagen has been solubilized from a bovine mammary gland epithelial cell line using detergent and purified by immunoaffinity chromatography using a monoclonal antibody to the bullous antigen, and shown to be a homotrimer [461]. Rotary shadowing images of trimeric type XVII collagen molecules show a quaver-like molecule consisting of a globular head (partially cytosolic NC16 domain), a central 60–70 nm rod (large COL15 domain), and a flexible tail (constituting ~66% of the molecule and comprising an interrupted collagenous domain) [461,462]. Moreover, recombinant extracellular fragments of human type XVII collagen show a low-M_r form with an elongated conformation. Immunoelectron microscopy demonstrates that antibodies against recombinant ectodomain fragments bind to structures outside the keratinocyte plasma membrane along the skin basement membrane.

Molecular genetic studies in an epithelial cell line indicate that the amino-terminus plays a role in targeting and polarizing type XVII collagen within the hemidesmosome [474], while a short noncollagenous extracellular region at the cell membrane is important for interaction with other hemidesmosomal components, including the α6 subunit of α6β4 integrin. This region also contains the major epitope recognized by autoantibodies in the sera of patients with both bullous pemphigoid and herpes gestationalis [465,466,475]. The use of domain-specific antibodies has shown that in skin and epithelial cells, at least, this collagen occurs in two triple-helical forms — a full-length homotrimeric transmembrane protein (3 × 180 kDa chains) and a soluble extracellular domain that is a specific proteolytic cleavage product of the full-length molecule [467,476]. The intact form has a globular intracellular domain that is disulfide bonded intramolecularly and an N-glycosylated extracellular domain that is triple-helical at physiological temperatures. The soluble form is a triple helical N-glycosylated molecule of three 120 kDa chains. Because only a single 6 kb mRNA has been detected, it appears that the 120 kDa polypeptide is generated post-translationally. Moreover, keratinocytes harboring a homozygous nonsense mutation in *COL17A1* synthesize neither the 180 kDa polypeptide nor the 120 kDa form. Treatment of these cells with a synthetic furin propeptide convertase inhibitor prevents the formation of the 120 kDa form. These data indicate that the soluble 120 kDa form is a specifically cleaved extracellular domain of type XVII collagen generated through furin-mediated proteolytic processing [476]. Type XVII collagen is thus the first collagen with

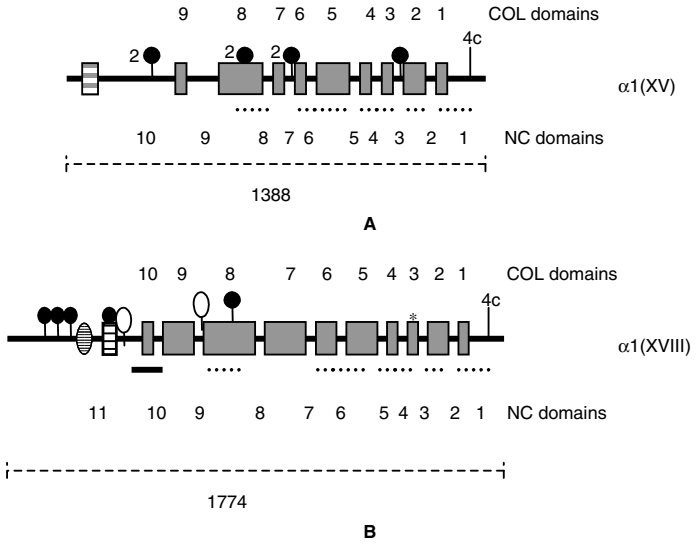


Figure 19. Molecular organization of the MULTIPLEXIN collagens. Line diagrams illustrating the dimensions and domain structures of the component α1 chains of type XV collagen (**A**) and type XVIII collagen (**B**). The triple-helical (COL) domains are represented as dark shaded bars, and the noncollagenous (NC) domains as lines. An alternatively spliced sequence of type XVIII collagen is highlighted as a heavy line beneath the diagram. Regions of high homology between types XV and XVIII collagens are shown as dotted lines beneath each diagram. The positions and numbers of cysteine residues (c), N-linked carbohydrate attachment sites (🌳), PARP (proline and arginine-rich protein) domains (▯), the amino-terminal frizzled motif (🌰), an RGD putative cell attachment sequence (✻), and two glycosaminoglycan attachment sites of type XVIII collagen (♀) are indicated. The numbers of amino acid residues comprising the individual chains are indicated beneath each.

a specifically processed, soluble triple-helical extracellular domain to be described. The role of this soluble extracellular domain is not known, but its loss from the cell surface may influence signal transduction and/or cell attachment to the basement membrane during proliferation and differentiation.

The functions of type XVII collagen are not known. As a transmembrane component of hemidesmosomes, it is likely to play a role in maintaining linkage between intracellular and extracellular structural elements, and in anchoring epithelia to underlying basement membranes. This concept is supported by observations made in pathological skin conditions [475]. In bullous autoimmune skin diseases, the presence of autoantibodies reactive against type XVII collagen is associated with diminished epidermal–dermal cohesion. Heritable skin blistering disorders of the junctional epidermolysis bullosa group are associated with mutations in the gene for type XVII collagen (see Chapter 15, this volume). Several mutations in the *COL17A1* gene (all in regions encoding the extracellular region) have been identified that result in premature chain termination and absence of the protein [465,466]. These mutations have been linked to the nonlethal junctional blistering disorder, generalized atrophic benign epidermolysis bullosa, in which the tissue separates within the cutaneous basement membrane zone at, or just below, the level of hemidesmosomes. Genotype-phenotype correlations and the molecular mechanisms underlying the phenotypes remain unclear. However, the recent elucidation of collagen XVII molecular interactions in hemidesmosome plaques with keratin [471], catenin [477], and bullous pemphigoid antigen 1 (BPAG1) [478], has provided new insights into the role of this collagen in skin.

MULTIPLEXIN COLLAGENS

Types XV and XVIII collagens are homologous members of a subfamily of collagens designated MULTIPLEXINS (multiple triple-helix domains and interruptions) due to the occurrence of multiple collagenous and noncollagenous sequences (Fig. 19) [16,479–484]. Types XV and XVIII collagens are highly homologous in seven of their collagenous domains, which are similar in size and in the positions of triple-helical imperfections within them. However, the two collagens also differ in the number of collagenous domains they possess. Moreover, the different lengths of the dissimilar collagenous domains make it impossible to align fully the two homologous polypeptides, and thus it is unlikely that they are different α chains of the same collagen. The most striking homology between these collagens is found in the most carboxyl terminal noncollagenous NC1 domains, the structures of which are unique to these two collagens [16,484]. These domains contain two regions of sequence homology, separated by a variable region. The second of these homologous regions includes a unique pattern of four conserved cysteine residues that is regarded as a signature of MULTIPLEXIN collagens. Both collagens possess a number of Ser–Gly sequences within noncollagenous sequences that are potential attachment sites for glycosaminoglycan chains, as well as a number of N-linked oligosaccharide attachment sites, suggesting these collagens may be extensively glycosylated *in vivo*. Mouse type XVIII collagen exists as three polypeptide variants with three N-terminal noncollagenous domains of differing lengths, and two N-terminal variant human α1(XVIII) chains have also been identified [485].

Types XV and XVIII collagens are both found in most basement membrane zones, but some tissue-specific differences exist [486–490]. Type XV collagen is expressed widely in basement membrane zones of the vasculature and many internal organs, including placenta, adrenal gland, kidney, heart, skeletal muscle, ovary, and testis, and the main producers of type XV collagen are mesenchymally derived

cells (fibroblasts, muscle cells, endothelial cells) [490]. Type XVIII collagen is a ubiquitous basement membrane component [485]. The short variant is the most common form, being found in conventional basement membranes of blood vessels and epithelia in heart, kidney, placenta, prostate, ovary, skeletal muscle, and small intestine. The long variant is very strongly expressed in liver, while its strongest signals in fetal tissues occur in kidney and liver; minor expression of both variants is observed in other tissues. The most obvious differences in distribution between the two molecules are the strong type XV collagen expression in muscle tissues, and the high levels of type XVIII in liver. The functions of types XV and XVIII collagens are not yet known, but in view of their homology, may be similar. They may contribute to the structural and functional integrity of basement membranes.

Type XV Collagen

Type XV collagen was first identified by screening a human placental library with a DNA fragment encoding a chick fibrillar collagen sequence [16,479–482]. The NC1, COL1–COL6, and COL8 domains, and an amino-terminal PARP motif in NC10 are homologous to those of type XVIII collagen [16] (Fig. 19). The complete deduced primary structure of human type XV collagen comprises 1388 residues, including a putative signal peptide of 25 residues, and has a calculated M_r of 140 kDa. It is characterized by a 577-residue collagenous sequence with nine collagenous domains of Gly–X–Y triplets (COL1–COL9) separated by eight interruptions of 7–45 residues (NC2–NC9), flanked by an amino-terminal noncollagenous NC10 domain (530 residues) and a carboxyl terminal NC1 domain (256 residues). The NC10 domain possesses a segment of ~200 residues that is homologous to the extreme amino-terminal portion of thrombospondin-1; this sequence has two conserved cysteine residues and is also found in the amino-terminal noncollagenous domains of several other collagen α chains, these being α1(V), α1(XI), α2(XI), α1(XII), α1(XIV), α1(XVI), and α1(XIX). The only function mapped to this sequence in thrombospondin-1 is heparin binding, but the residues critical for this function are not conserved among the above collagens. The NC10 domain of type XV collagen also has four tandem repeated amino acid segments homologous to rat cartilage proteoglycan core protein.

The molecular structure of type XV collagen is unknown and the protein has not yet been isolated. However, polypeptides of 125 kDa and 116 kDa have been identified by Western blotting of HeLa cell lysates and placenta homogenates using antisera raised against bacterially expressed recombinant protein representing the first half of the noncollagenous carboxyl terminal NC1 domain [16,490]. Differences between the molecular mass of the α1(XV) chain calculated from sequence data and that estimated from the Western blots suggest that the amino-terminal domain may be processed.

Type XVIII Collagen

Type XVIII collagen was first cloned from a mouse liver cDNA library, and the full deduced human and mouse primary structures have recently been determined [16,483–488] (Fig. 19). Two variant forms of the human α1(XVIII) chain (1336 and 1516 residues, respectively) have been reported, with predicted M_rs of 136 and 154 kDa, respectively. The short and long human forms also have different signal peptides. The 1336 residue variant contains a 33 residue signal peptide and the 1516 residue variant a 23 residue signal peptide. The NC11 domain of the short variant comprises 303 residues, and that of the long variant 493 residues. The collagenous sequence in both variants is 688 residues in length and consists of ten collagenous domains (COL1–COL10), varying in length from 18 to 122 residues, and separated by noncollagenous domains (NC2–NC10) of between 12 and 26 residues. The region of human NC11 common to both variants contains two conserved cysteine residues also found in the mouse polypeptide, and has a 200-residue sequence that is homologous with an amino-terminal segment of thrombospondin-1.

Two sequences conforming to a consensus sequence for the attachment of glycosaminoglycans occur in the NC11 and NC9 domains and type XVIII collagen has been shown to exist as a basement membrane heparan sulfate proteoglycan in human kidney and placenta, and in several chick tissues [489]. As such, it is the first member of the collagen family known to have heparan sulfate side chains. COL3 also contains an Arg–Gly–Asp (RGD) putative cell attachment motif. However, only weak adhesion promoting activity has been observed, and current evidence indicates that type XVIII collagen does not serve as a dominant cell adhesion molecule. An alternative splicing event encompassing the last amino acid of human NC11, COL10, and most of NC10 has been identified [485–487]. In the mouse, three α1(XVIII) variants have been detected, with a second "long" variant generated by alternative splicing of the primary transcript. An alternative upstream promoter directs the expression of the murine long forms. The longest form, which has not been identified in humans, contains a segment of 110 residues with 10 cysteine residues that is designated a "frizzled" motif (fz); such motifs are found in frizzled proteins that resemble the G-protein-coupled membrane receptors needed for the establishment of cell polarity in epidermis and which are found in the *Wingless* signaling pathway in *Drosophila* [491]. The fz domain of type XVIII collagen may be involved in transmembrane signaling.

In human type XVIII collagen, the NC1 domain is 312 residues long and contains four cysteine residues. A peptide comprising the most carboxyl terminal 184 amino acids of type XVIII collagen was isolated from a murine hemangioendothelioma cell line as a 22 kDa inhibitor of angiogenesis called endostatin [492]. This sequence corresponds to amino acid residues 1154–1336 in the human α1(XVIII) chain, and is the best conserved sequence between the two species. A related structure (54% sequence identity) exists at the same location in collagen XV. The crystal structure, function, and tissue forms of the NC1 domain of type XVIII collagen containing endostatin have been reported [493–495]. Recombinant NC1 aggregates noncovalently into a globular trimer (38 kDa) that can be partially cleaved by proteolysis into several monomers (25–32 kDa) related to endostatin. The amino-terminal part of NC1 associates to form stable trimers that may be important in triple helix formation (~50 residues per chain; appear ultrastructurally as stalks). This is the first direct evidence that type XVIII collagen can form homotrimers. There is also a central proteinase-sensitive hinge region, and a carboxyl terminal globular endostatin domain (~3 nm diameter). Proteolytic release of endostatin can occur through several pathways (11 positions in the hinge region are sensitive to proteolysis), which may facilitate a switch from a matrix-associated to a soluble endocrine form. Endostatins have also been obtained as highly soluble

monomers with compact folding, and form autonomous folding units. Both intact NC1 and endostatins bind fibulin-1, fibulin-2, and heparin (the latter probably through the endostatin sequence), but NC1 also binds sulfatides, laminin-1, and perlecan. Circulating forms of endostatin may be involved in the hemostatic control of angiogenesis. Thus, the NC1 domain serves disparate functions in oligomerization, in association with extracellular matrix components, and as a potent regulator of endothelial cell proliferation.

The molecular structure of type XVIII collagen is unknown. Type XVIII collagen protein has been detected in Western blots of extracts of mouse embryonic stem cells as a 200 kDa band, which corresponds to the short form.

COLLAGEN — THE FUTURE

The complexity of the family of collagenous proteins has become increasingly apparent over recent years, and numerous aspects of these molecules still remain to be revealed. Many new family members have now been identified by cDNA cloning techniques, but yet more molecules with collagenous domains will undoubtedly be identified on completion of the elucidation of the human genome. Several of the more recently identified collagens have been classified because they contain one or more Gly–X–Y collagenous domains, although their molecular and macromolecular forms and potential structural roles remain undefined. A number of other molecules that contain collagenous domains, e.g., members of the macrophage scavenger receptor family, are not classified as collagens. It may now be appropriate to reappraise the original definition of a collagen as a triple-helical molecule that contributes structurally to the extracellular matrix.

Structure-function determination is a major goal in current collagen research. In the past few years, recombinant expression approaches and mouse models have increasingly been employed to generate new insights into structure-function relationships. Studies of mutant collagen molecules and their macromolecular organization in the heritable collagen diseases are not only helping to elucidate genotype-to-phenotype relationships, but also their physiological structural and functional properties. Detailed knowledge of the biological properties and roles of the different collagens may, in time, form a basis for the functional classification of collagens.

The diagnosis and treatment of fibrotic, diabetic, and aging conditions is a major focus of interest. The discovery of the key fibrillar procollagen N- and C-proteinases, the actions of which lead inexorably to the deposition of insoluble collagen fibrils, has opened the way for development of new generation synthetic inhibitors to manage fibrosis. Proteolytic fragmentation of collagens forms the basis of a burgeoning pharmaceutical industry in the development of assays for collagen fragment detection in body fluids.

Much remains to be understood about the complex pathway of synthesis and assembly of collagen molecules, which is one of the most complex of all protein assembly mechanisms and is, therefore, potentially the most susceptible to genetic mutation. Recent advances in cell biology approaches are leading to major new insights into how cells direct and regulate chain selection and triple-helix formation within the secretory pathway, and how misfolded forms are recognized and removed. Molecular understanding of these fundamental cellular and molecular mechanisms forms the foundation for the development and production of a new generation of "designer" collagens for a host of therapeutic, biotechnological, and cosmetic applications [496].

RECENT DEVELOPMENTS

Basic Principles of Triple-Helix Conformation and Stability

Guest triplets of the form Gly–X–Hyp and Gly–Pro–Y have been used to quantify the conformational propensities of all 20 amino acids for the X and Y positions in the context of a $(Gly–Pro–Hyp)_8$ host peptide, highlighting the highly stabilizing nature of imino acids and the destabilizing effects of Gly and aromatic residues. The high propensity of ionizable residues in the X position indicates the importance of interchain hydrogen bonding, directly or through water, to backbone carbonyl or hydroxyproline residues [497].

Type VI Collagen

Mutations in the α2(VI) chain have been shown to cause Ullrich syndrome, a recessive congenital muscular dystrophy associated with severe reduction of collagen VI [498]. This disease shares some clinical features with Bethlem myopathy, but displays a more severe course. (In respect of both conditions, see also Chapter 26, Part VI, this volume).

Type VIII Collagen

The endothelial corneal dystrophies cause endothelial cell dysfunction and corneal decompensation. Causative mutations have been identified in *COL8A2*, which encodes the α2 chain of type VIII collagen, in familial and sporadic cases of Fuchs corneal endothelial dystrophy (FEDC; MIM 136800) and posterior polymorphous corneal dystrophy (PPCD; MIM 122000) [499].

Type XVIII Collagen

Mutations have been identified in the *COL18A1* gene that result in Knobloch syndrome (MIM 267750), an autosomal recessive disorder characterized by the occurrence of high myopia, vitreoretinal degeneration with retinal detachment, macular abnormalities and occipital encephalocele [500] (see also Chapter 26, Part VII, this volume). Thus, collagen XVIII has a major role in determining retinal structure and in closure of the neural tube.

Novel Collagens

α1(XX) is a new member of the FACIT collagen subfamily, which has been identified in chick [501]. It has sequence similarities to collagen types XII and XIV, but the cDNA predicts that the α1(XX) polypeptide is smaller than the short forms of collagens XII and XIV. It is present in cornea, skin, cartilage and tendon, and is expected to bind to collagen fibrils, the amino-terminal domains projecting away from the fibrillar surface. The cloning of collagen type XXIII, present in the cornea, has been reported [502].

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Chapter 2, Part II

Collagen: Gene Structure

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INTRODUCTION

Collagens are now known to constitute a family of more than 19 proteins. On the basis of the structure and function of the proteins, the collagens are generally divided into two categories: fibrillar collagens that form discrete fibrils with a characteristic cross-striated pattern, and nonfibrillar collagens that do not form cross-striated fibrils. The structures of the genes for collagens also appear to divide the proteins into two categories in that the genes for fibrillar collagens have similarities that distinguish them from the genes for nonfibrillar collagens and from the genes for most other proteins.

The first collagen gene clones isolated were for the proα2(I) chain of type I procollagen from chicken by de Crombrugghe and associates [1] and by Boedtker and her associates [2]. Work in their laboratories over several years defined the unusual structural features of the genes for fibrillar procollagens, including the 54-bp motif seen in the exons coding for the triple-helical domains. Boedtker and her associates completely defined the structure of the chick proα2(I) gene, the first collagen gene so defined [3]. Strategies modeled after those of de Crombrugghe et al. and Boedtker et al. were used by our own research group to prepare the first genes for human collagens [4]. Subsequently, a series of different and powerful strategies became available and were used to isolate genes for other collagens. At least 33 collagen genes coding for various collagen types have been completely or partially characterized to date (Table 1).

The large amount of information now available about the structures of collagen genes has a number of important applications. One of these is to provide the necessary database for identifying mutations in the genes that can cause human diseases [5]. In this chapter, we concentrate primarily on the structures of collagen genes in humans. The extensive literature about collagen genes in other vertebrates is not discussed except when data for human collagen genes have not been reported.

CHROMOSOMAL LOCATIONS OF COLLAGEN GENES

The chromosomal locations of most collagen genes have now been defined in both human and mouse genomes. Most genes are widely scattered throughout the genome but a few are clustered (Table 1). For example, the proα1(I) gene of type I collagen, *COL1A1*, is found on chromosome 17, while the proα2(I) gene, *COL1A2*, is on chromosome 7 [6,7]. However, there are several chromosomal loci where two or more collagen genes are clustered. The genes for the α1(IV) and α2(IV) chains of type IV collagen, *COL4A1* and *COL4A2*, are found at the tip of chromosome 13 [8–11]. The genes are coded for on opposite strands of the DNA with the first exons separated by no more than about 130 bp [12,13]. This unusual clustering of two genes for two polypeptides of the same protein means that the regulation of expression of the two genes must involve some complex mechanism. Similar head-to-head arrangements are found for the α3(IV) and α4(IV) collagen genes, *COL4A3* and *COL4A4*, on chromosome 2, and for the α5(IV) and α6(IV) collagen genes, *COL4A5* and *COL4A6*, on chromosome X [14–16]. The distances between the *COL4A3* and *COL4A4* genes and between the *COL4A5* and *COL4A6* genes are reported to be 5 and 442 bp, respectively [17,18].

Similarly, the genes for the α1(VI) and α2(VI) chains of type VI collagen, *COL6A1* and *COL6A2*, are in a head-to-tail orientation on the long arm of chromosome 21, approximately 150 kb apart [19,20]. The gene for type XVIII collagen, *COL18A1*, is also located close to the two genes for type VI on chromosome 21 [21]. Two additional collagen genes, for types X and XIII, are found in this gene cluster on mouse chromosome 10B5-C1, although the type X and XIII genes are scattered on human chromosome 6 and 10, respectively [22,23]. The order of these genes on mouse chromosome 10 is (cen-*Col10a1*-*Col13a1*-*Col6a1*-*Col6a2*-*Col18a1*-tel). In addition, the genes for type III collagen, *COL3A1*, and the α2(V) chain of type V collagen, *COL5A2*, are closely associated in the long arm of human chromosome 2 [24–26]. The 3′-ends of these two genes have been mapped to a 35 kb region [27], but their orientation has not yet been established. Also, the genes for the α1 chain of type IX collagen (*COL9A1*), type XII collagen (*COL12A1*), and type XIX collagen (*COL19A1*) are found in the long arm of chromosome 6. The gene order appears to be *COL19A1*-*COL9A1*-*COL12A1* [28–30].

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TABLE 1. Sizes, Total Number of Exons, and Chromosomal Locations of Collagen Genes[1]

Type	Gene	Size (kb)	No. of Exons	Human Chromosome	Mouse Chromosome	Features[4]	References
I	*COL1A1*	18	51	17q21.3–q22	11A2-3		[7,38,133,134]
	COL1A2	38	52	7q21.3–q22	6		[6,39,43,135–137]
II	*COL2A1*	30	54	12q13–14	15	Alt. splicing	[138–143]
III	*COL3A1*	44	52	2q24.3–q31	1		[25,40,44,141,144]
IV	*COL4A1*	>160	52	13q33–q34	8		[8,10,11,58]
	COL4A2		47[2]	13q33–34	8		[9,65,145]
	COL4A3			2q35–37		Alt. splicing	[17,62–64]
	COL4A4	>113	48	2q35–37		Alt. promoter	[66,67]
	COL4A5	230–250	51	Xq22		Alt. splicing	[14,60,146]
	COL4A6	425	46	Xq22		Alt. promoter	[16,18,59]
V	*COL5A1*	>750	66	9q34.2–q34.3	2A2-B		[47,147–149]
	COL5A2			2q24.3–q31			[24,26]
	COL5A3			19p13.2	9		[149a]
VI	*COL6A1*	30	36	21q22.3	10B5-C1		[19,71,77,150,151]
	COL6A2	36	30	21q22.3	10B5-C1	Alt. promoter & Alt. splicing	[19,71,73,75,151]
	COL6A3	>50	43	2q37	1	Alt. splicing	[74,76,152]
VII	*COL7A1*	30.5	118	3p21	9		[81,153,154]
VIII	*COL8A1*		4[3]	3q12–q13.1			[86,155]
	COL8A2			1p32.3–34.2			[88]
IX	*COL9A1*	90	38	6q12–q13	1	Alt. promoter	[29,96,156]
	COL9A2	15	32	1p32			[96,157]
	COL9A3	23	32	20q13.3			[97,158]
X	*COL10A1*	7	3	6q21–q22	10B1-B3		[22,23,83,85]
XI	*COL11A1*			1p21		Alt. splicing	[159]
	COL11A2	28	66	6p21.2		Alt. splicing	[48,49,160–162]
	COL2A1	30	53	12q13–14	15	Alt. splicing	
XII	*COL12A1*			6q12–13	9	Alt. splicing	[28]
XIII	*COL13A1*	140	42	10q22	10B4	Alt. splicing	[117,118,163,164]
XIV	*COL14A1*			8q23	15D	Alt. splicing	[165,166]
XV	*COL15A1*	145	42	9q21–q22	4B1-3		[124,167,168]
XVI	*COL16A1*			1p34–p35			[106]
XVII	*COL17A1*	52	56	10q24.3	19		[128,130,169]
XVIII	*COL18A1*	102[2]	43[2]	21q22.3	10B5-C1	Alt. promoter & Alt. splicing	[21,125,170]
XIX	*COL19A1*	>250	51	6q12–13			[109,171]

[1]Where data are available on the human genes, only these are presented.
[2]For the mouse gene;
[3]for the rabbit gene;
[4]Alt.: Alternative.

GENES FOR THE THREE MAJOR FIBRILLAR COLLAGENS

The Proteins for Which They Code

The three major fibrillar collagens are types I, II, and III (see Part I of this chapter). Type I collagen is the most abundant structural protein in the body and is the major protein constituent of tissues such as skin, bones, and tendons. Type II collagen is the characteristic collagen of articular cartilage, but it is also found in the eye and it appears transiently in many tissues during embryonic development. Type III collagen is generally associated with type I collagen, but is found in lesser amounts. In addition, the aorta and other large blood vessels appear to be particularly rich in type III collagen.

The three major fibrillar collagens show a great deal of similarity at the protein level. Each is first synthesized as a procollagen containing N- and C-terminal propeptides

Figure 1. Schematic drawing of a molecule of type I procollagen. (Reproduced from Prockop and Kivirikko [132] by permission of the New England Journal of Medicine.)

(Fig. 1). The separate domains of the proteins are very similar, but there are some differences (Table 2). The N-propeptide of the proα2(I) chain is considerably shorter than that of the other chains. The N-propeptide of the proαl(II) chain has a larger triple-helical region than the other N-propeptides; its Gly–X–Y sequence is interrupted by a short globular sequence. Type III procollagen differs from type I and type II in that its major triple-helical domain is 15 amino acids longer.

The Triple-Helical Domains

The gene structures of the three major fibrillar collagens also show a great deal of similarity (Table 1). The basic structure consists of 52 exons (Fig. 2). In the *COL1A1* gene, exons 33 and 34 found in the other genes are fused, and this fused exon is usually referred to as "33/34." In the *COL2A1* gene, two additional exons called 2A and 3B (Fig. 3) code for the N-terminal domain. Exon 2A is alternatively spliced in the processing of mRNA [31]. In the *COL3A1* gene, exons 4 and 5 are fused into a single exon called "4/5." One feature of the genes is that most of the major triple-helical domain of each protein is coded for by 42 exons that have striking similarities (Fig. 2). Another striking feature of the exon structure is that most of the exons are 54 bp and the others are either twice 54, three times 54, or combinations of 45 and 54 bp exons (Fig. 2 and Table 3). In all cases, each exon begins with a complete codon for glycine, and the exon codes for a discrete number of Gly–X–Y tripeptide units. Still another feature is the conservation of exon structures among the different genes and throughout evolution. Except for the fused 33/34 in *COL1A1*, the four genes for the three fibrillar collagens have the same exon sizes. In addition, the sizes of the exons are identical in human, rodent, and chick. Hence, there appears to be some common ancestry in evolution of the gene structures and probably selective pressure on maintaining the features of the gene structure.

The Propeptides

The similarities among the gene structures for the three fibrillar collagens extend to the propeptides. As indicated in Figure 3, the basic structure of the N-propeptides is coded for by six exons. In the various collagens, exon 1 contains 119–156 bp of noncoding sequences plus 70–103 bp of coding sequences, including sequences coding for the signal peptides. Exons 2A and 2 code for a cysteine-rich domain and appear to be the most variable among the gene structures. Exon 2 of the *COL1A2* gene is only 11 bp. Exon 2A in *COL2A1* is similar to exon 2 in the *COL1A1* and *COL3A1* genes. However, the transcribed RNA is alternatively spliced so that the codons of exon 2A are missing from some of the mRNA for type II procollagen [32–36]. The mRNA containing this exon, α1(IIA), is preferentially expressed in differentiating cartilage and noncartilaginous tissues during embryonic development, whereas the mRNA lacking it, α1(IIB), is primarily expressed in chondrocytes. Exons 3 to 5 code for a short triple-helical domain. Exon 6 in all four genes for fibrillar collagens is a junction exon containing the site at which the propeptides are cleaved by either a procollagen N-proteinase specific for type I and type II procollagen or a separate procollagen N-proteinase specific for type III procollagen. Exon 6 also contains a few codons for the initial Gly–X–Y sequences of the major triple-helical domain of the protein.

The gene structures coding for the C-propeptides also show a great deal of similarity (Fig. 4). Exon 49 is the junction exon in which the first 45 or so bp code for the last repeating Gly–X–Y sequences of the triple-helical domain. Exon 49

TABLE 2. Comparison of the Sizes of the Domains of Human Type II Procollagen with the Same Domains of Type I and Type III Procollagens[1]

Domain	Amino Acid Residues			
	Proα1(I)	Proα2(I)	Proα1(II)	Proα1(III)
N-Terminal Domain	161	79	112	153
Signal peptide	22	22	21,23, or 25	24
N-Propeptide	139	57	91,89, or 87	129
Cys in N-propeptide	10	2	1	11
Triple helix in N-propeptide	48	42	16 + 63	39
Collagen Domain	1,057	1,039	1,060	1,068
N-Telopeptide	17	11	19	14
Triple helix	1,014	1,014	1,014	1,029
C-Telopeptide	26	15	27	25
C-Terminal domain				
C-Propeptide	246	247	245	246
Carbohydrate sites	1	1	1	1

[1]Data for type I procollagen are from Bernard et al. [172], Kuivaniemi et al. [173], and Tromp [144]. Data for type II procollagen are from Baldwin et al. [174]. Data for type III procollagen are from Ala-Kokko et al. [175].

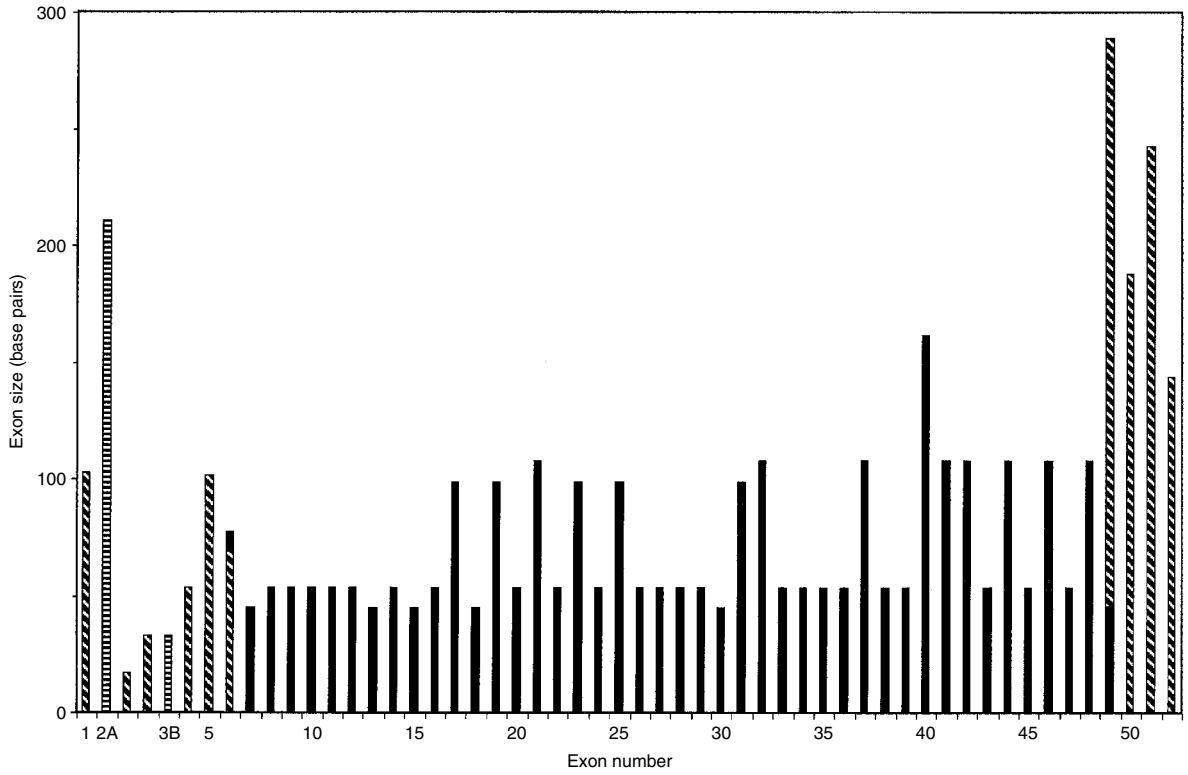
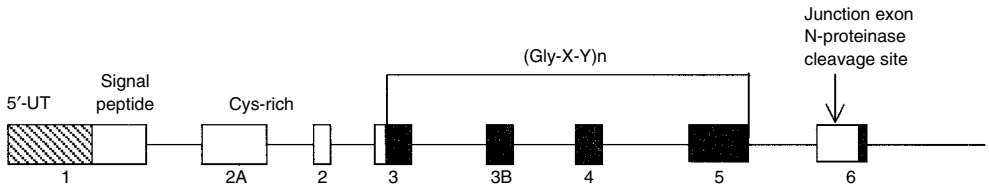


Figure 2. Prototypic structure of exons in genes for fibrillar collagens. The exon sizes shown apply to the human type II procollagen gene as defined by the references cited in Table 1. With minor modifications, the exons for other fibrillar collagens are the same size. The differences among the four genes for types I, II, and III procollagens are: (1) type II procollagen has two exons (2A and 3B) not found in the other genes; (2) exons 4 and 5 are fused in the gene for type III procollagen; (3) exons 33 and 34 are fused in the gene for the proα1(I) chain of type I procollagen (Table 3). Symbols: Solid bars, exons for the major triple-helical domain of the α1(II) chain; hatched bars, exons coding for the N- and C-propeptides. As discussed in the text, exon 2A of the type II procollagen gene is alternatively spliced in transcripts of the gene.



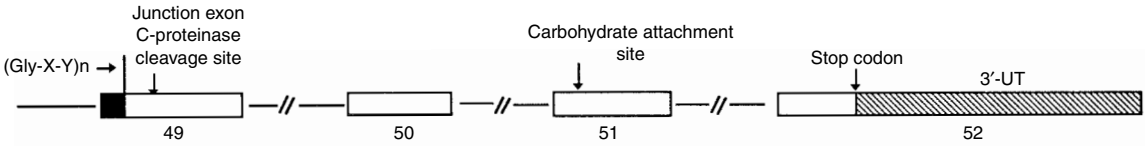
Gene	Exon 1 (bp) (5′-UT +Coding)	Exon 2A (bp)	Exon 2 (bp)	Exon 3 (bp) Globular + (Gly-X-Y)n	Exon 3B (bp)	Exon 4 (bp)	Exon 5 (bp)	Exon 6 (bp) Globular + (Gly-X-Y)n
α1(I)	119 + 103	---	195	26 + 9	---	36	102	63 + 9
α2(I)	135 + 70	---	11	15	---	36	93	45 + 9
α1(II)	156 + 85	213	17	6 + 27	33	54	102	69 + 9
α1(III)	120 + 79	---	203	24 + 27	---	144		54 + 27

Figure 3. Prototypic structure of exons coding for the N-propeptides of the major fibrillar collagens I, II, and III. The top of the figure presents the exon structure of type II procollagen. Variations of the structure for type I and type III are presented below. Data are from references indicated in Table 1.

TABLE 3. Exons that Code for the Triple-Helical Regions of the Major and Minor Fibrillar Procollagens

Major Fibrillar Procollagens (types I, II and III)[1]			*Minor Fibrillar Procollagens [α1(V) and α2(XI) chains]*[2]		
Exon No.	Size (bp)	First Amino Acid Encoded[5]	Exon No.	Size (bp)	First Amino Acid Encoded[6]
7	45[4]	4			
8	54	19	15	54	574
9	54	37	16	54	592
10	54	55	17	54	610
11	54	73	18	54	628
12	54	91	19	54	646
13	45	109	20	45	664
14	54	124	21	54	679
15	45	142	22	45	687
16	54	157	23	54	712
17	99	175	24	45	730
			25	54	745
18	45	208	26	45	763
19	99	223	27	54	778
			28	45	796
20	54	256	29	54	811
21	108	274	30	108	829
22	54	310	31	54	865
23	99	328	32	54	883
			33	45	901
24	54	361	34	54	916
25	99	379	35	45	934
			36	54	949
26	54	412	37	54	967
27	54	430	38	54	985
28	54	448	39	108	1003
29	54	466			
30	45	484	40	90	1039
31	99	499	41	54	1069
32	108	533	42	108	1087
33[3]	54	568	43	108	1123
34[3]	54	586			
35	54	604	44	54	1159
36	54	622	45	54	1177
37	108	640	46	108	1195
38	54	676	47	54	1231
39	54	694	48[7]	108	1249
40	162	712	49	54	1285
			50	108	1303
41	108	766	51	54	1339
42	108	802	52	54	1357
			53	54	1375
43	54	838	54	54	1393
44	108	856	55	108	1411
45	54	892	56	54	1447
46	108	910	57	54	1465
47	54	946	58	108	1483
48	108	964	59	54	1519
			60	36	1537
			61	54	1549

[1]Summary of data from Tromp [144].
[2]Data for the human α1(V) procollagen gene are from Takahara et al. [47] and for the human α2(XI) procollagen gene from Vuoristo et al. [48].
-[3]Exons 33 and 34 are fused in the proα1(I) gene.
[4]54 bp in the proα1(III) gene.
[5]Amino acid positions are numbered from the beginning of the α chains.
[6]Amino acid positions are numbered from the beginning of the signal peptide.
[7]Split into two exons of 54 bp in the proα2(XI) collagen gene.
Exons that differ in size between genes are shaded.



Gene	Exon 49 (bp) (Gly-X-Y)n + Globular	Exon 50 (bp)	Exon 51 (bp)	Exon 52 (bp) Globular + 3′-UT
α1(I)	45 + 238	191	243	144 + 280, 1280
α2(I)	45 + 214	185	243	144 + 210, 310, 645, 842
α1(II)	45 + 244	188	243	144 + 414
α1(III)	63 + 235	188	243	144 + 273, 872

Figure 4. Prototypic structure of exons coding for the C-propeptides of the major fibrillar procollagens I, II, and III. The structure at the **top** is type II procollagen. Variations on this structure are shown **below**. Three of the four genes have more than one polyadenylation site. Data are from references indicated in Table 1.

also contains 214–244 bp coding for globular sequences that include the C-terminal telopeptide and the site at which a procollagen C-proteinase cleaves the propeptides. Exons 50 and 51 and the coding region of exon 52 are very similar in size among the four genes. Exon 51 contains a coding sequence for Asn–X–Thr, which is a single site for the attachment of a mannose-rich carbohydrate to the C-propeptide of each of the proα chains. In each of the four genes, Exon 52 contains 144 bp of coding sequence plus a variable amount of 3′-noncoding or 3′-untranslated sequences.

5′- and 3′-Noncoding Regions

Analysis of mRNAs for the proα1(I) chain of chick type I procollagen and the proα1(III) chain of chick type III procollagen demonstrates that the 5′-untranslated regions contain two or more AUG triplets preceding the AUG that serves as a site for initiation of translation [37]. A sequence of about 50 nucleotides surrounding the latter AUG is conserved in the mRNAs, and contains an almost ideal inverted repeat structure that can form an apparently stable stem-loop structure. The structure initially suggested that the conserved sequences may play a role in determining expression or translation of the mRNAs by varying the extent to which stem-loop formation occurs, thereby permitting or preventing use of the critical AUG sequence for translation. The same sequences are conserved in the three genes for type I and type III procollagens in man [38–40], but the critical structure is not found in the 5′-untranslated region of type II procollagen [41]. Subsequent studies suggested, however, that the highly conserved sequences in the 5′-untranslated region do not generally function in the regulation of either transcription or translation [42].

The three major fibrillar collagen genes (*COL1A1*, *COL1A2*, and *COL1A3*) have more than one polyadenylation site in the 3′-end of the genes [38,43,44]. As a consequence, multiple mRNA transcripts with varying lengths of 3′-untranslated region are produced from each gene (Fig. 4). The two major transcripts of the *COL1A1* gene differ by as much as 1 kb in the 3′-noncoding region. It is not clear whether the multiple mRNA species differ in stability or translational efficiency.

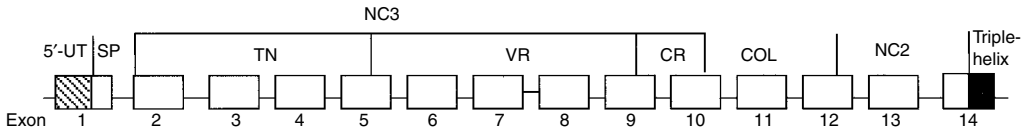
GENES FOR MINOR FIBRILLAR COLLAGENS: TYPES V AND XI

At least two other types of collagen, V and XI, belong in the class of fibrillar collagens [45]. Type V collagen is found in small amounts, usually as a heterotrimer of $[\alpha1(V)]_2\alpha2(V)$ chains, in tendon and other tissues that are rich in type I collagen. However, it is also found in even smaller amounts in tissues that produce type II collagen. Type V collagen also exists as an $[\alpha1(V)]_3$ homotrimer or an α1(V)α2(V)α3(V) heterotrimer (see Part I of this chapter). Type XI collagen is a heterotrimer of α1(XI), α2(XI), and α3(XI) chains, in which the α3(XI) chain is identical to the α1(IIB) variant of type II collagen. It is found in relatively small amounts in cartilage and other tissues rich in type II collagen, but is also found in tissues synthesizing type I and III collagens. Because heterotypic molecules consisting of both type V and XI chains are found *in vivo* [46], these two collagen types are thought to constitute a single collagen type.

The gene structures of type V and XI collagens show considerable divergence from those of the major fibrillar collagens. The genes for the α1 chain of type V collagen, *COL5A1*, and the α2 chain of type XI collagen, *COL11A2*, each contain 66 exons [47–49]. The sizes of the two genes, however, differ markedly. The *COL5A1* gene appears to be greater than 750 kb in size with a large first intron over 500 kb long, whereas the *COL11A2* gene is only 28 kb in size. The exon numbers are greater than those found in the major fibrillar collagen genes because both the N-propeptides and the triple-helical domains are encoded by six to eight more exons.

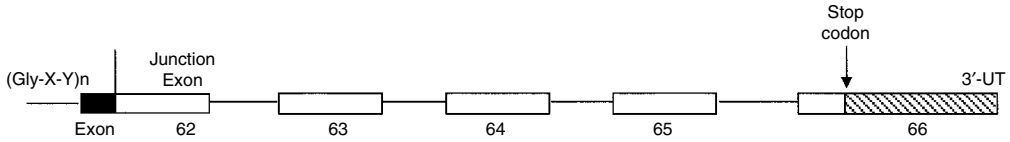
Propeptides

The N-propeptides of the α1(V) and α2(XI) collagen chains are encoded by 14 exons (Fig. 5), as compared with 5-8 exons for the α chains of the major fibrillar collagens. This reflects



Gene	Exon 1 (bp)	Exon 2 (bp)	Exon 3 (bp)	Exon 4 (bp)	Exon 5 (bp)	Exon 6 (bp)	Exon 7 (bp)	Exon 8 (bp)	Exon 9 (bp)	Exon 10 (bp)	Exon 11 (bp)	Exon 12 (bp)	Exon 13 (bp)	Exon 14 (bp)
α1(V)	282 + 109	168	214	163	132	138	240	168	57	42	63	75	93	57
α2(V)	232 + 79	216												
α1(XI)	318 + 106	168	214											
α2(XI)	220 + 82	150	211	163	192	78	63	180	60	42	63	75	87	57

Figure 5. Prototypic structure of exons coding for the N-propeptides of the minor fibrillar collagens V and XI. Data are from references indicated in Table 1. 5′-UT: 5′-untranslated region; NC: noncollagenous domain; COL: collagenous domain; TN: thrombospondin N-terminal motif; VR: variable region; CR: constant region; SP: signal peptide.



Gene	Exon 62 (bp) (Gly-X-Y)n + Globular	Exon 63 (bp)	Exon 64 (bp)	Exon 65 (bp)	Exon 66 (bp) Globular + 3′-UT
α1(V)	18 + 238	113	69	234	144 + 602
α2(XI)	18 + 250	113	--	207	138 + 430

Figure 6. Prototypic structure of exons coding for the C-propeptides of the minor fibrillar procollagens V and XI. The structure at the top is α1(V) procollagen. Variations on this structure are shown below.

differences in the domain structure of the N-propeptides of the two groups of fibrillar collagens. In type V and XI collagens, exon 1 encodes 232–318 bp of the 5′-untranslated region plus 79–109 bp of the coding sequence. Exons 2–9 and the 5′-end of exon 10 code for a large globular domain at the amino terminus of the N-propeptide, NC3, which shares little homology with the cysteine-rich region of the major fibrillar collagens. Exons 10–12 code for an interrupted collagenous domain, and exons 12–14 code for a short non-collagenous region (NC2) preceding the triple-helix. The NC3 domain contains three parts: a basic subdomain, an acidic variable region, and a short constant region. The basic subdomain has been isolated as a proline and arginine rich peptide (PARP) from tissues and is homologous to the N-terminus of thrombospondin-1 [50]. Similar motifs are also found in several nonfibrillar collagens (see below), and the motif is referred to as TN (thrombospondin N-terminal motif) or PARP. The α2(V) chain does not contain the TN motif. In type V and XI genes, the exon sizes are conserved in all subdomains of the N-propeptides, except for the acidic variable regions (Fig. 5). Moreover, exons coding for the acidic regions of the two type XI chains are subjected to alternative splicing, thereby leading to further variations in the N-propeptide sequences [51–55].

The exon organization of the regions coding for the C-propeptides of type V and XI collagens is similar to that for the corresponding region of the major fibrillar collagen genes, but with some differences (Fig. 6). The C-propeptide

of the α1(V) chain is encoded by 5, not 4 exons, because the sequence equivalent to exon 50 of the major fibrillar collagen genes is split into two separate exons, i.e. exons 63 and 64. The gene for the α2(XI) chain lacks the second of the two exons, and also the penultimate exon is 27 bp shorter than the corresponding exon of the gene for the α1(V) chain. Thus, the C-propeptide of the α2(XI) chain is shorter by about 30 amino acids than the corresponding domains of the other fibrillar collagens.

Triple-Helical Domains

The triple-helical domains of the α1(V) and α2(XI) chains are encoded by 47 and 48 exons, respectively (Table 3). Like the major fibrillar collagen genes, most exons are 54 bp, 45 bp, or 108 bp in size, and begin with complete codons for glycine residues. The first and last exons are junction exons, which encode both the propeptides and the triple-helical domain. However, each of the four 99 bp exons found in the major fibrillar collagen genes is split into two exons of 45 and 54 bp. As a result, type V and XI collagen genes lack exons of 99 bp, and contain more exons of 54 and 45 bp. In addition, they do not have the 162-bp exon found in the major fibrillar collagen genes, but do contain exons of 36 and 90 bp that are not found in the major fibrillar collagen genes. The exon sizes of the *COL5A1* and *COL11A2* genes are identical except that Exon 48 in the *COL5A1* gene is split into two exons of 54 bp in the *COL11A2* gene.

GENES FOR THE NONFIBRILLAR COLLAGENS

Type IV

Type IV collagen is the major collagen of basement membranes [56] (see also Part I, this chapter). There are six genetically distinct α chains. The α1(IV) and α2(IV) chains are found in all basement membranes, whereas the α3(IV), α4(IV), α5(IV), and α6(IV) chains have a more restricted tissue localization. Mutations in the latter group are found in patients affected with Alport syndrome or leiomyomatosis associated with Alport syndrome [57] (see also Chapter 25, this volume). Type IV collagen chains are characterized by the presence of N- and C-terminal globular domains that apparently are not cleaved during the processing of the protein. Also, the central triple-helical domain of the protein is characterized by a series of noncollagenous sequences that interrupt the Gly–X–Y sequences required for folding into a triple-helix.

Type IV collagen genes are exceedingly large in size and appear to have large introns near their 5′-ends. For example, the *COL4A1* gene is larger than 160 kb, and the *COL4A5* and *COL4A6* genes are approximately 230–250 kb and 425 kb in size, respectively (Table 1). The first intron of the *COL4A1* gene is more than 30 kb long and the second intron of the *COL4A6* gene is as much as 340 kb in size [58,59]. These large introns may contain regulatory elements controlling gene expression or may include another gene.

The primary structures of type IV collagen α chains suggest that they belong to two conserved subgroups, one consisting of the odd-numbered chains (the α1-like) and the other of the even-numbered chains (the α2-like). The gene structures support this classification and suggest that type IV collagen genes may have evolved through two separate events [59,60]. The first may have been a tandem duplication and later divergence of an ancestor gene to generate a primordial pair of α1 and α2 genes. The second event may have involved further duplication of the primordial gene pair onto two other chromosomes so that each odd-numbered gene is paired head-to-head with an even-numbered gene (Table 1).

The structure of the *COL4A1* gene [58] differs from the structures of the major fibrillar collagen genes in that only two of the exons are 54 bp, and only three are 45 bp (Fig. 7 and Table 4). The first 19 exons for the triple-helical domain

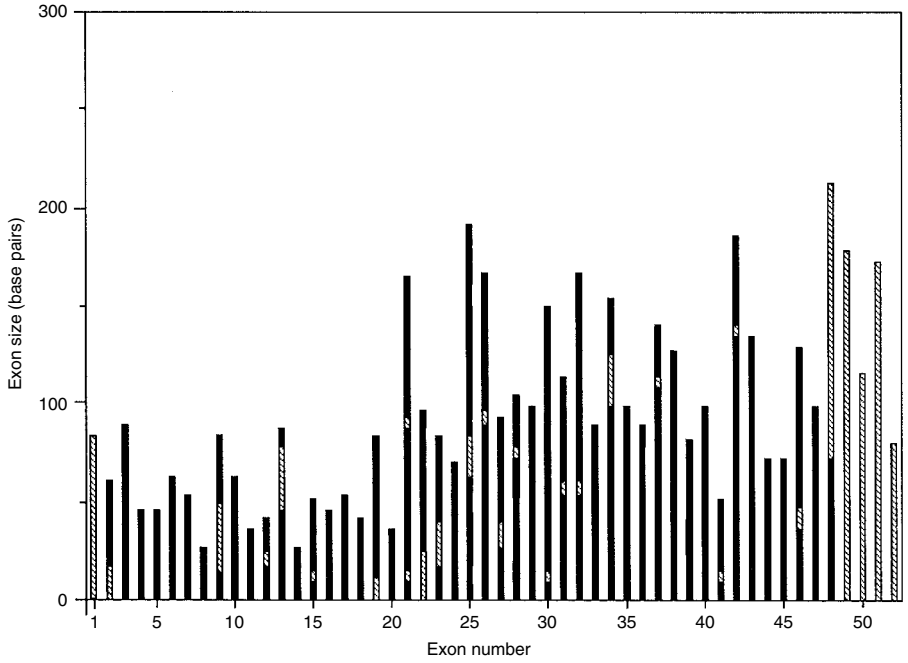


Figure 7. Sizes of exons in the gene for the α1(IV) chain of human type IV collagen. Data are from Soininen et al. [58]. Symbols: Solid bars, codons for Gly–X–Y sequences; hatched bars, codons for non-triple-helical sequences.

TABLE 4. Exons that Code for the α1 and α5 Chains of Type IV Collagen[1]

COL4A1		*COL4A5*		
Exon No.	Exon Size (bp)	Exon No.	Exon Size (bp)	Domain Encoded[2]
1	129 + 84	1	202 + 81	5′-UT + SP
2	60	2	60	NC2 + COL
3	90	3	90	COL
4	45	4	45	COL
5	45	5	45	COL
6	63	6	63	COL
7	54	7	54	COL
8	27	8	27	COL
9	84	9	81	COL
10	63	10	63	COL
11	36	11	36	COL
12	42	12	42	COL
13	87	13	93	COL
14	27	14	54	COL
15	51	15	57	COL
16	45	16	45	COL
17	54	17	54	COL
18	42	18	42	COL
19	85	19	133	COL
20	36			COL
21	165	20	174	COL
22	96	21	84	COL
23	84	22	93	COL
24	71	23	71	COL
25	192	24	192	COL
26	169	25	169	COL
27	93	26	93	COL
28	105	27	105	COL
29	98	28	98	COL
30	151	29	151	COL
31	114	30	114	COL
32	168	31	168	COL
33	90	32	90	COL
34	153	33	150	COL
35	99	34	99	COL
36	90	35	90	COL
37	140	36	140	COL
38	127	37	127	COL
39	81	38	81	COL
40	99	39	99	COL
41	51	40	51	COL
42	186	41	186	COL
43	134	42	134	COL
44	73	43	73	COL
45	72	44	72	COL
46	129	45	129	COL
47	99	46	99	COL
48	213	47	213	COL + NC1
49	178	48	178	NC1
50	115	49	115	NC1
51	173	50	173	NC1
52	79 + 1304	51	79 + 1116	NC1 + 3′-UT

[1]Data from Soininen et al. [58], and Zhou et al. [60].
[2]5′-UT: 5′-untranslated region; SP: signal peptide; COL: collagenous domain; NC: noncollagenous domain; 3′-UT: 3′-untranslated region. Exons that differ in size between genes are shaded.

begin with complete codons for glycine, but thereafter most of the exons begin with split codons for glycine in which the first G of the codon is in the preceding exon. Another feature is that although the gene for the α1(IV) chain has remarkable differences from the genes for fibrillar collagens, the number of exons is again 52.

The exon structure of the *COL4A5* gene is quite similar to that of the *COL4A1* gene, having 41 exons of identical sizes [60]. The split codons are also conserved. The gene has one less exon than the *COL4A1* gene because exons 19 and 20 that are separate in the *COL4A1* gene are fused (Table 4). The C-terminal noncollagenous region, NC1, of the *COL4A5* gene undergoes alternative splicing in human kidney and skin tissues [61]. Skipping of exon 50 introduces a stop codon at the first codon of exon 51, and the resultant α5(IV) chain lacks 84 amino acids in the NC1 domain. Exons coding for the NC1 domain of the *COL4A3* gene are also alternatively spliced from the mRNA. In this case, two exons are subjected to alternative splicing, yielding α3(IV) variants with NC1 domains of 232, 60, and 199 amino acids [62–64]. The multiple forms of NC1 domains in the α3(IV) and α5(IV) chains in the kidney may lead to autoimmune glomerular diseases.

The structures of the three even-numbered genes differ considerably from those of the odd-numbered genes [59,65–67]. The mouse α2(IV) gene, human α4(IV) gene, and human α6(IV) gene contain 47, 48, and 46 exons, respectively (Table 5). The NC1 domains of the α2-like genes are encoded by 3, not 5 exons. As with the α1-like genes, most exons in the second halves of the genes begin with split codons. Exons coding for the central portion of the triple-helical domains (exons 17–26) are most variable among the α2-like genes. The *COL4A4* gene has a small exon of only 9 bp (Exon 43), which is not found in the *Col4a2* and *COL4A6* genes.

The *COL4A4* and *COL4A6* genes each contain two promoters that are differentially utilized in tissues, resulting in α4(IV) chains with different 5′-untranslated sequences and α6(IV) chains with different signal peptides [17,18]. The alternative transcripts have different tissue distribution but the functional significance of this is not clear.

Type VI

Type VI collagen consists of three different α chains and it is ubiquitously distributed [68]. It appears to account for as much as 10–20% of the collagen synthesized by cultured skin fibroblasts. Mutations in type VI collagen genes cause Bethlem myopathy (MIM 158810) (see also Chapter 26, Part VI, this volume), a childhood onset muscular dystrophy with joint contractures [69]. The protein forms an unusual microfibrillar structure in which the monomers coil around each other to form dimers and tetramers. The monomer, consisting of α1(VI), α2(VI), and α3(VI) chains, has only a very short triple-helical domain of 335–336 amino acids, which is flanked by large N- and C-globular domains. The α1(VI) and α2(VI) chains, each of about 1000 amino acids, are highly homologous, whereas the α3(VI) chain is almost three times the size of the α1(VI) and α2(VI) chains because of much larger N- and C-globular domains.

The triple-helical domains of the α1(VI) and α2(VI) chains are each encoded by 19–20 exons that are generally multiples of 9 bp and begin with complete codons for glycine [70,71]. Some of the exons are 54 and 45 bp but most are 63 bp or other sizes (27,36, and 72 bp) that do not reflect the 54 bp unit (Table 6). The exon size patterns for the triple-helical domains of the two genes are almost identical, except that exons 17 and 18 in *COL6A1* are fused in the *COL6A2* gene. With only minor differences, exons coding for the triple-helical domain of the α3(VI) chain are similar in size to those of the *COL6A1* and *COL6A2* genes [72].

By contrast, there is less conservation of the exon structures of the globular domains, which primarily consist of motifs similar to the A domains of von Willebrand factor (vWF A) [73–77]. The vWF A motifs in the N- and C-globular domains of the three chains are encoded by one to four exons. The α3(VI) chain contains a total of 10 consecutive vWF A motifs, N10-N1, in the N-terminal globular domain. Except for N1, which is encoded by two exons, the other nine vWF A motifs are each encoded by a single exon [74]. Because of alternative splicing of exons coding for domains N10, N9, N7, and part of N3, the α3(VI) chains contain varying numbers of vWF A domains [74,76]. The alternative splicing pattern differs between normal and tumor cells [74]. Except for the exon coding for N3, the corresponding exons in the chick α3(VI) collagen gene are also alternatively spliced and the multiple transcripts are differentially expressed in embryonic chicken tissues [78,79]. Alternative splicing also occurs with the *COL6A2* gene, in which the last two exons are alternatively spliced to vary the C-terminal amino acid sequence [73]. The gene also has two promoters, producing mRNAs with different 5′-untranslated regions [75].

Type VII

Type VII collagen is the major component of the anchoring fibrils that connect epithelia to underlying stroma [80] (see also Chapter 15, this volume). It is a homotrimer of α1(VII) chains with a long, interrupted triple-helical domain flanked by non-collagenous domains at the N- and C-termini.

The *COL7A1* gene contains 118 exons and is apparently the most complex gene described to date [81,82]. Yet its size of 31 kb is very compact because of small introns with an average length of 188 bp. The triple-helical domain with 19 interruptions in the Gly–X–Y sequences is encoded by 81 exons (Table 7), the last of which is a junction exon encoding also the beginning of the C-terminal globular domain, NC2. All exons coding for uninterrupted Gly–X–Y sequences start with complete codons for glycine and are multiples of 9 bp in size. However, exon sizes found in fibrillar collagen genes are not predominant. Only 4 exons are 54 bp, 12 exons 45 bp, and 1 exon 108 bp. The most predominant exon size is 36 bp (a total of 25 exons). In addition, there are 8 exons of 63 bp, 5 exons of 72 bp, 6 exons of 81 bp, and 3 exons of 27 bp. The largest interruption in the Gly–X–Y sequence is 39 amino acids and encoded by three exons (exons 70–72).

The N-globular domain, NC1, primarily contains two types of conserved protein motif, i.e. nine fibronectin type III (FN III) repeats and two von Willebrand factor A (vWF A) motifs. The FN III repeats are each encoded by two exons as are those in the fibronectin gene, and the two vWF A motifs are encoded by four and three exons, respectively. A proline- and cysteine-rich domain unique to type VII collagen is encoded by two exons.

The C-terminal globular domain, NC2, is encoded by six exons in addition to the junction exon. Within this region, a Kunitz serine protease inhibitor motif is encoded by the penultimate exon, and the acidic region is encoded by the four preceding exons plus the junction exon. The majority of exons in the NC1 and NC2 domains begin with split codons.

TABLE 5. Exons that Code for the Mouse α2 Chain, and the Human α4 and α6 Chains of Type IV Collagen[1]

Col4a2		*COL4A4*		*COL4A6*		
Exon No.	Exon Size (bp)	Exon No.	Exon Size (bp)	Exon No.	Exon Size (bp)	Domain Encoded[2]
1	91 +	1	178	1A	? + 14	5′-UT + SP
2	45 + 44	2[3]	172	1B[3]	? + 11	5′-UT + SP
3	55	3	43	2	52	SP
4	81	4	78	3	81	COL
5	135	5	135	4	135	COL
6	45	6	45	5	45	COL
7	117	7	117	6	117	COL
8	72	8	69	7	69	COL
9	36	9	36	8	36	COL
10	63	10	63	9	63	COL
11	78	11	36	10	36	COL
		12	42	11	42	COL
12	93	13	81	12	93	COL
13	36	14	54	13	54	COL
14	51	15	60	14	69	COL
15	45	16	45	15	45	COL
16	54	17	54	16	54	COL
17	67	18	70	17	70	COL
18	111	19	105	18	108	COL
19	150	20	165	19	141	COL
20	92	21	90	20	105	COL
21	155	22	164	21	161	COL
22	73	23	73	22	180	COL
23	107	24	107	23	184	COL
24	202	25	184	24	72	COL
25	60	26	69			COL
26	57					COL
27	108	27	108	25	108	COL
28	222	28	219	26	222	COL
29	162	29	162	27	162	COL
30	171	30	171	28	171	COL
31	144	31	144	29	144	COL
32	123	32	108	30	126	COL
33	182	33	182	31	182	COL
34	64	34	64	32	64	COL
35	75	35	75	33	75	COL
36	108	36	108	34	108	COL
37	108	37	108	35	108	COL
38	72	38	72	36	72	COL
39	126	39	129	37	126	COL
40	117	40	111	38	117	COL
41	162	41	156	39	162	COL
42	99	42	108	40	99	COL
		43	9			COL
43	147	44	126	41	147	COL
44	117	45	117	42	117	COL
45	192	46	189	43	192	COL + NC1
46	287	47	287	44	287	NC1
47	255+	48	264+	45	258+	NC1 + 3′-UT

[1]Data from Buttice et al. [65], Boye et al. [66], and Zhang et al. [59].

[2]5′-UT: 5′-untranslated region; SP: signal peptide; COL: collagenous domain; NC: noncollagenous domain; 3′-UT: 3′-untranslated region.

[3]Alternative promoter.

Exons that differ in size between genes are shaded.

Types VIII and X

Type VIII collagen, expressed by corneal and vascular endothelial cells, and type X collagen, found in the zone of hypertrophic cells of cartilage, share similar protein and gene structures. Genes for type VIII and X collagens are unusual in the collagen family in that they contain only 3–4 exons (Table 8) and most of the coding sequences, including all the Gly–X–Y codons and codons for the globular C-terminus, are found in the large exon at the 3′-end of the gene [83–88].

TABLE 6. Exons that Code for the α1 and α2 Chains of Type VI Collagen[1]

COL6A1			*COL6A2*		
Exon No.	Exon Size (bp)	Domain Encoded[3]	Exon No.	Exon Size (bp)	Domain Encoded[3]
			1[4]	41–63	5′-UT
			2[4]	115–397	5′-UT
1	177–194	5′-UT + SP	3	142	5′-UT + SP
2	130	N1			
3	201	N1			
4	160	N1	4	596	N1
5	135	N1			
6	21	LK			
7	21	LK	5	21	LK
8[2]	9 + 36	LK + COL	6[2]	30 + 36	LK + COL
9	54	COL	7	54	COL
10	45	COL	8	45	COL
11	27	COL	9	27	COL
12	27	COL	10	27	COL
13	45	COL	11	45	COL
14	54	COL	12	54	COL
15	63	COL	13	63	COL
16	63	COL	14	63	COL
17	54	COL	15	90	COL
18	36	COL			COL
19	63	COL	16	63	COL
20	63	COL	17	63	COL
21	63	COL	18	63	COL
22	63	COL	19	63	COL
23	51	COL*	20	51	COL*
24	36	COL	21	36	COL
25	63	COL	22	63	COL
26	66	COL*	23	63	COL*
27	36	COL	24	36	COL
28	37	LK	25	46	LK
29	9	LK			
30	134	C1	26	153	C1
31	110	C1	27	453	C1
32	184	C1			
33	184	C1			
34	30	LK	28	39	LK
35	686	C2 + 3′-UT	29[5]	900	C2a/C2a′ + 3′-UT
36	917	3′-UT	30[5]	887	C2 + 3′ -UT

[1]Data are from Trikka et al. [77], and Saitta et al. [71,73,75].

[2]Junction exon.

[3]COL: collagenous domain; N1, C1-C2: vWF (von Willebrand factor) A motifs in the N- and C-globular domains; C2a, C2a′: alternative C2 domains; SP: signal peptide; LK: linker region; 5′-UT: 5′-untranslated region; 3′-UT: 3′-untranslated region.

[4]Alternative promoter.

[5]Alternatively spliced exon.

*Contains interruptions of Gly–X–Y sequence.

Exons that differ in size between genes are shaded.

Types IX, XII, XIV, XVI, and XIX

Collagen types IX, XII, XIV, XVI, and XIX belong to the FACIT (fibril-associated collagens with interrupted triple helices) subgroup of collagens [89]. They comprise several collagenous domains interspersed with noncollagenous domains and have a characteristic C-terminal collagenous domain, COL1. The COL1 domains are of similar size and contain two Gly–X–Y imperfections. Two cysteine residues separated by three other amino acids are found at the end of each COL1 domain. Another common feature is the presence of a TN (thrombospondin) motif in one of the noncollagenous domains.

Type IX

Type IX collagen is found predominantly in cartilage, and is also found in ocular tissue [90]. One of the three different chains contains a site to which a large glycosaminoglycan is attached, and forms a covalent crosslink with fibrils of type II collagen. Each of the three chains contains three collagenous domains, COL1–COL3 numbered from the C-terminus, which are separated by noncollagenous domains, NC1–NC4.

TABLE 7. Exons that Code for the Human α1(VII) Collagen Gene[1]

Exon No.	Exon Size (bp)	Domain Encoded[2]	Exon No.	Exon Size (bp)	Domain Encoded[2]
1	177	5′-UT + SP	60	36	COL
2	181	vWF A-1	61	81	COL
3	160	vWF A-1	62	36	COL
4	94	vWF A-1	63	63	COL
5	162	vWF A-1	64	45	COL
6	164	FN III-1	65	36	COL
7	130	FN III-1	66	36	COL
8	117	FN III-2	67	96	COL
9	147	FN III-2	68	36	COL
10	117	FN III-3	69	36	COL
11	150	FN III-3	70	48	NC
12	129	FN III-4	71	36	NC
13	144	FN III-4	72	123	NC
14	126	FN III-5	73	201	COL
15	144	FN III-5	74	36	COL
16	120	FN III-6	75	63	COL
17	144	FN III-6	76	69	COL
18	126	FN III-7	77	45	COL
19	147	FN III-7	78	63	COL
20	123	FN III-8	79	45	COL
21	147	FN III-8	80	36	COL
22	135	FN III-9	81	36	COL
23	147	FN III-9	82	45	COL
24	137	vWF A-2	83	33	COL
25	127	vWF A-2	84	63	COL
26	147	vWF A-2	85	36	COL
27	173	CP	86	81	COL
28	36	CP	87	69	COL
29	27	COL	88	36	COL
30	45	COL	89	42	COL
31	63	COL	90	45	COL
32	81	COL	91	45	COL
33	36	COL	92	36	COL
34	36	COL	93	60	COL
35	72	COL	94	108	COL
36	78	COL	95	72	COL
37	27	COL	96	36	COL
38	54	COL	97	60	COL
39	63	COL	98	45	COL
40	60	COL	99	36	COL
41	36	COL	100	36	COL
42	45	COL	101	57	COL
43	36	COL	102	72	COL
44	45	COL	103	72	COL
45	36	COL	104	36	COL
46	36	COL	105	81	COL
47	33	COL	106	54	COL
48	54	COL	107	54	COL
49	60	COL	108	63	COL
50	36	COL	109	63	COL
51	81	COL	110	117	COL
52	36	COL	111	78	COL
53	45	COL	112	54	COL + NC2
54	72	COL	113	49	NC2 (acidic)
55	45	COL	114	33	NC2 (acidic)
56	27	COL	115	87	NC2 (acidic)
57	30	COL	116	93	NC2 (acidic)
58	81	COL	117	198	NC2 (KM)
59	36	COL	118	350	NC2 + 3′-UT

NC1

[1]Data from Christiano et al. [81].

[2]NC: noncollagenous domain; COL: collagenous domain; vWF A: von Willebrand factor type A-like motif; CP: cysteine- and proline-rich domain; FN III: fibronectin type III-like motifs; KM: Kunitz serine protease inhibitor motif.

Analysis of mRNA transcripts of the α1(IX) gene indicates that it is transcribed from two different start sites in cartilage and cornea of chick embryos. Transcription in the cornea begins from a site that is 20 kb downstream from the promoter used in cartilage [91]. Because transcription begins from the second downstream site, the protein in cornea lacks the large globular domain of 266 amino acids comprising the N-terminal NC4 domain, which contains the TN motif, found in the cartilage protein. The expression of the two different forms of the α1(IX) chain is regulated

TABLE 8. Exons that Code for Rabbit Collagen VIII and Human Collagen X[1]

COL8A1[2]		*COL10A1*		
Exon No.	Exon Size (bp)	Exon No.	Exon Size (bp)	Domain Encoded[3]
1	69			5′-UT
2	120	1	81	5′-UT
3	331 (331)	2	169	5′-UT + NC2
4	2278 (>1907)	3	2940	NC2 + COL + NC1 + 3′-UT

[1]Data for the rabbit α1(VIII) gene are from Yamaguchi et al. [86] and data for the human α1(X) gene are from Reichenberger et al. [85]. Exons for the human α1(VIII) collagen gene that have been defined [155] are shown in parentheses.

[2]Rabbit α1(VIII) collagen gene.

[3]UT: untranslated region; NC: noncollagenous domain; COL: collagenous domain.

during chondrogenesis [92–94]. The downstream start site is located within intron 6 of human, mouse, and chick α1(IX) collagen [91,95,96]. Because the promoter is located only a short distance upstream from Exon 7, it is spliced directly to Exon 8. The NC4 domains of the α2(IX) and α3(IX) chains are similar to that of the short form of the α1(IX) chain and contain only three amino acids.

The *COL9A1* gene is 90 kb in size and contains 38 exons (Table 9). By contrast, the *COL9A2* and *COL9A3* genes are only about 15 kb and 23 kb in size, respectively, and each consists of 32 exons [96,97]. The exon size patterns of the three genes are essentially the same except for an additional seven exons coding for the NC4 domain of the long form of the α1(IX) chain (Table 9). Exons coding for the NC3 domains and the junction exons in the beginning of the COL3 domains also differ in size among the genes. For example, exon 2 in the *COL9A3* gene encodes one less Gly–X–Y sequence than its counterpart in the other two genes, but the difference is compensated for by one additional Gly–X–Y coding sequence in exon 4 of the *COL9A3* gene.

The COL2 domains of the three chains contain no interruptions of the Gly–X–Y sequences. Eleven of the 17 exons coding for each COL2 domain are 54 bp long. Other exons are 72, 45, and 36 bp in size, i.e. multiples of 9 bp. The last two exons in the COL2 domain are unusual in that they are separated by introns between the X and Y codons. In the COL3 domain, three exons are 54 bp, and other exons are 36 bp, 63 bp, and 24 bp in size. Exons in the COL1 domain also are unusual. They do not show the 9-bp size pattern and the last two exons begin with split codons for glycines.

Types XII and XIV

Collagen types XII and XIV are highly similar in primary structure and both are found in small amounts in association with type I collagen [98,99]. They are homotrimers of α1(XII) or α1(XIV) chains with two collagenous domains, COL1 and COL2, and three noncollagenous domains, NC1–NC3 [30,100,101]. The N-terminal NC3 domains of both collagens are extremely large and are present in two vastly different forms. The long form of type XII collagen contains 18 FN III motifs and 4 vWF A domains, whereas the short form lacks 8 FN III repeats and two vWF A domains in the N-terminus [101,102]. These two forms, however, are not generated by alternative promoters like the long and short forms of the α1(IX) collagen transcripts. Instead, they are products of alternative splicing of internal exons [103]. The long NC3 form is found primarily in embryonic tissues, whereas the short form is the predominant species in adult tissues. Further variation in the coding sequence is found in the 3′-end of rat and mouse α1(XII) collagen genes [104]. Mutually exclusive use of the last two exons, each containing a polyadenylation site, results in the long and short forms of the C-terminal NC1 domain. The long form, containing an extended acidic region followed by a basic region, is found primarily in ligament and tendon, whereas the short form has a broader tissue distribution.

The structures of the exons encoding NC1, COL1, and part of the NC2 domain of the chick type XII gene that have been analyzed [105] suggest that the gene is similar to the type IX collagen genes (Table 9).

Types XVI and XIX

Collagen types XVI and XIX contain more collagenous domains (a total of 5–10) than other members of the FACIT group and share higher sequence similarity with each other than with other FACIT members [106–108]. The gene for type XIX collagen, *COL19A1*, contains 51 exons that span more than 250 kb of genomic DNA [109]. The gene structure shares some similarity with that of the type IX collagen genes. The COL1 domain is encoded by exons that begin with split codons and are not integers of 9 bp (Table 10). Exons coding for the rest of the collagenous domains start with complete codons for glycine and essentially all are multiples of 9 bp in size. The most predominant exon size is 54 bp (a total of 14). Seven exons are 45 bp, five are 36 bp, four are 63 bp, and one each is 27 and 90 bp. Moreover, exons coding for the NC6 domain, which contains a TN motif, are similar in size to those encoding the NC4 domain of the *COL9A1* gene, which also contains a TN motif.

The gene structure for the TN motif in the NC11 domain of the mouse α1(XVI) collagen chain resembles that for the TN motif in the NC6 domain of *COL19A1* [110]. Exons coding for the collagenous domains that have been defined are 45 bp, 36 bp, and 54 bp in size and begin with intact codons for glycine. The exon organization thus supports the evolutionary relationship among FACIT members.

Type XIII

Type XIII collagen is distributed in very small amounts in many tissues [111]. It is an integral membrane protein with a transmembrane domain near the N-terminus [112]. The α1(XIII) chain has three collagenous domains, COL1–COL3 numbered from the N-terminus, and four noncollagenous domains, NC1–NC4. A noteworthy feature of type XIII collagen is that at least 10 exons encoding not only the noncollagenous but also the collagenous domains are

TABLE 9. Exons that Code for Human Collagen IX and Chick Collagen XII[1]

COL9A1		*COL9A2*		*COL9A3*		*COL12A1*		
Exon No.	Exon Size (bp)	Exon No.	Exon Size (bp)	Exon No.	Exon Size (bp)	Exon No.	Exon Size (bp)	Domain Encoded[2]
1	145							5′-UT + SP
2	74							NC4
3	78							NC4
4	133							NC4
5	397							NC4
6	84							NC4
1*	? + 72	1	167	1	? + 78			5′-UT + SP
7	21							NC4
8	75	2	75	2	69			NC4 + COL3
9	36	3	36	3	36			COL3
10	63	4	63	4	72			COL3
11	54	5	54	5	54			COL3
12	36	6	36	6	36			COL3
13	24	7	24	7	24			COL3
14	54	8	54	8	54			COL3
15	54	9	54	9	54			COL3
16	33	10	48	10	42			COL3 + NC3
17	57	11	57	11	57			NC3 + COL3
18	54	12	54	12	54			COL2
19	54	13	54	13	54			COL2
20	54	14	54	14	54			COL2
21	54	15	54	15	54			COL2
22	54	16	54	16	54			COL2
23	54	17	54	17	54			COL2
24	54	18	54	18	54			COL2
25	54	19	54	19	54			COL2
26	45	20	45	20	45			COL2
27	54	21	54	21	54			COL2
28	54	22	54	22	54			COL2
29	54	23	54	23	54			COL2
30	72	24	72	24	72			COL2
31	36	25	36	25	36			COL2
32	45	26	45	26	45	7[3]	54	COL2
33	33	27	33	27	33	6[3]	72	COL2
34	147	28	147	28	147	5[3]	36	COL2
35	55	29	55	29	55	4[3]	67	COL2 + NC2
36	189	30	189	30	183	3[3]	189	NC2 + COL1
37	78	31	78	31	78	2[3]	69	COL1
38	560	32	200+	32	191+	1[3]	171	COL1 + NC1 + 3′-UT

[1]Data are from Pihlajamaa et al. [96], Paassilta et al. [97], and Ninomiya et al. [105].

[2]UT: untranslated region; SP: signal peptide; NC: noncollagenous domain; COL collagenous domain.

[3]Exon numbered from the 3′-end of the gene.

*Alternative promoter.

Exons that differ in size between the type IX collagen genes are shaded.

alternatively spliced from the mRNA [113–116]. Therefore, the protein synthesized from the gene must vary considerably in structure.

The *COL13A1* gene is more than 130 kb in size and contains 42 exons [117,118]. The first exon encodes the 5′-untranslated region, a short cytoplasmic domain, and the transmembrane domain (Table 11). The last exon starts with the translational stop codon and contains only the 3′-untranslated sequence. Exons coding for uninterrupted collagenous sequences start with complete codons for glycine, except for exon 3, which starts with a split codon for glycine. Exon 3 is 8 bp long (Table 11) and is the shortest exon found in collagen genes known to date. The most frequent exon size is 27 bp (a total of 10 exons). Four exons are 54 bp, four are 45 bp, and the rest are 36, 63, 81, and 153 bp in size.

Four exons in the COL1 domain, two exons in the NC2 domain, and four exons in the COL3 domain are alternatively spliced in human tissues [118]. Exons that are alternatively spliced in mouse and human tissues are not always the same [116]. The alternatively spliced exons can be present or absent in any combination, but there are some common splice variants. The alternative splicing of exons 14 and 15, which code for the NC2 domain, shows tissue-specific differences that are conserved in mouse and human tissues.

TABLE 10. Exons that Code for Human Collagen XIX[1]

Exon No	Exon Size (bp)	Domain Encoded[2]	Exon No.	Exon Size (bp)	Domain Encoded[2]
1	85	5′-UT	27	78	COL4
2	123	5′-UT + SP + NC6	28	36	COL4
3	75	NC6	29	54	COL4
4	100	NC6	30	54	COL4
5	124	NC6	31	42	NC4
6	276	NC6	32	105	NC4 + COL3
7	81	NC6	33	54	COL3
8	126	NC6	34	54	COL3
9	63	COL5	35	54	COL3
10	45	COL5	36	54	COL3
11	45	COL5	37	45	COL3
12	54	COL5	38	51	COL3 + NC3
13	54	COL5	39	45	NC3 + COL2
14	36	COL5	40	27	COL2
15	54	COL5	41	54	COL2
16	54	COL5	42	45	COL2
17	63	COL5 + NC5	43	45	COL2
18	42	NC5 + COL4	44	63	COL2
19	63	COL4	45	36	COL2
20	36	COL4	46	45	COL2
21	36	COL4	47	171	COL2
22	54	COL4	48	67	NC2
23	54	COL4	49	129	NC2 + COL1
24	54	COL4	50	78	COL1
25	90	COL4	51	5300	COL1 + NC1 + 3′-UT
26	45	COL4			

[1]Data are from Khaleduzzaman et al. [109].
[2]UT:untranslated region; SP: signal peptide; NC: noncollagenous region; COL: collagenous region.

TABLE 11. Exons that Code for Human Collagen XIII[1]

Exon No.	Exon Size (bp)	Domain Encoded[2]	Exon No.	Exon Size (bp)	Domain Encoded[2]
1	1088	5′-UT + NC1	22	54	COL2
2	70	NC1 + COL1	23	87	COL2
3	8	COL1	24	54	COL2
4[3]	27	COL1	25	45	COL2
5[3]	36	COL1	26	69	COL2 + NC3
6[3]	27	COL1	27	24	NC3
7[3]	51	COL1	28	63	COL3
8	36	COL1	29	45	COL3
9	27	COL1	30	153	COL3
10	27	COL1	31	99*	COL3
11	27	COL1	32[3]	45	COL3
12	27	COL1	33[3]	42	COL3
13	27	COL1 + NC2	34	27	COL3
14[3]	57	NC2	35	81	COL3
15[3]	66	NC2	36[3]	36	COL3
16	108	NC2 + COL2	37	54	COL3
17	27	COL2	38	54	COL3
18	36	COL2	39	36	COL3
19	45	COL2	40[3]	87	COL3 + NC4
20	33	COL2	41	39	NC4
21	27	COL2	42	243,317[4]	3′-UT

[1]Data are from Tikka et al. and Kvist et al. [117,118].
[2]UT: untranslated region; NC: noncollagenous domain; COL: collagenous domain.
[3]Alternatively spliced exon.
[4]Assuming the end of exon is 30 bp from the first and second polyadenylation signals located at 273 bp and 347 bp from the beginning of the exon, respectively.
*Alternative exon in the mouse gene, this exon has not been defined in the human gene.

Types XV and XVIII

Collagen types XV and XVIII are expressed in the basement membrane zones of many organs [119,120]. Both contain large N-terminal and C-terminal noncollagenous domains connected by a central triple-helical segment, which consists of 9–10 collagenous domains interrupted by short noncollagenous sequences [21,121,122]. The N-terminal noncollagenous domains of both collagens each contain a TN motif, and seven of their collagenous domains share sequence similarity. The C-terminal noncollagenous domains of the two collagens are highly conserved with 55% identity in nucleotide sequence. Of interest is that a fragment of the C-terminal noncollagenous domain of type XVIII collagen, endostatin, has been shown to be an angiogenesis inhibitor [123].

Human α1(XV) and mouse α1(XVIII) collagen genes are similar in size and exon-intron organization [124,125]. The *COL15A1* gene is 145 kb long and contains 42 exons and the *Col18a1* gene is 102 kb long with 43 exons (Table 12). The two genes have 25 exons of nearly identical sizes, and the similarity even extends to the size of some introns. For

TABLE 12. Exons that Code for Human α1(XV) Collagen and Mouse α1(XVIII) Collagen[1]

COL15A1			*Col18a1*		
Exon No.	Exon Size (bp)	Domain Encoded[2]	Exon No.	Exon Size (bp)	Domain Encoded[2]
1	120	5′-UT + SP + NC1	1	79	5′-UT + SP + NC1
2	89	NC1	2	71	NC1
			3[3]	1770	5′-UT + SP + NC1′
3	548	NC1 (TN)	4	551	NC1 (TN)
4	75	NC1	5	87	NC1
5	81	NC1	6	54	NC1
6	148	NC1	7	130	NC1
7	113	NC1	8	77	NC1
8	135	NC1	9	187	NC1 + COL1 + NC2 + COL2
9	153	NC1	10[4]	26	COL2
10	150	NC1	11[4]	63	COL2
11	144	NC1	12	78	COL2
12	54	NC1 + COL1	13	54	COL2 + NC3
13	60	COL1 + NC2	14	159	NC3 + COL3
14	93	NC2 + COL2	15[4]	63	COL3
15	126	COL2	16[4]	27	COL3
16[4]	63	COL2	17	129	COL3 + NC4
17[4]	36	COL2 + NC3	18	63	NC4 + COL4
18	141	COL2 + NC3	19[4]	27	COL4
19	69	NC3 + COL3	20[4]	36	COL4
20[4]	36	COL3	21[4]	72	COL4
21	84	COL3 + NC4	22[4]	36	COL4
22	66	NC4 + COL4	23	90	COL4 + NC5
23[4]	36	COL4	24	30	NC5 + COL5
24[4]	63	COL4	25[4]	27	COL5
25	69	COL4 + NC5 + COL5	26[4]	63	COL5
26[4]	36	COL5	27	75	COL5 + NC6 + COL6
27[4]	54	COL5	28[4]	27	COL6
28	60	COL5	29[4]	54	COL6
29	69	COL5 + NC6	30	75	COL6
30	42	NC6	31	69	COL6 + NC7
31	46	NC6 + COL6	32	43	NC7 + COL7
32[4]	81	COL6	33[4]	63	COL7
33	50	COL6 + NC7 + COL7	34	44	COL7 + NC8
34	111	COL7 + NC8 + COL8	35	171	NC8 + COL8 + NC8 + COL9
35	145	COL8 + NC9	36	145	COL9 + NC10
36	65	NC9 + COL9 + NC10	37	74	NC10 + COL10 + NC11
37	129	NC10	38	132	NC11
38	60	NC10	39	33	NC11
39	60	NC10	40	249	NC11
40	186	NC10	41	198	NC11
41	116	NC10	42	116	NC11
42	1119	NC10	43	370	NC11 + 3′-UT

[1]Data are from Hägg et al. [124], and Rehn et al. [125].

[2]UT: untranslated region; SP: signal peptide; COL: collagenous domain; NC: noncollagenous domain; NC1′, the alternative N-terminal domain that contains sequence homologous to frizzled proteins; TN: thrombospondin N-terminal motif.

[3]Alternative promoter

[4]Collagenous domain without Gly–X–Y imperfection.

example, the first intron in both genes is very short (71 and 89 bp), whereas the second intron is exceedingly long (50 and 55 kb). The *Col18a1* gene contains one more exon (exon 3) than the *COL15A1* gene. Exon 3, located more than 50 kb downstream from exon 1, corresponds to an alternative promoter and also contains an internal splice donor site. Transcription from the alternative promoter together with the utilization of alternative splice donor sites results in two different N-terminal sequences, a short form with a 239-residue noncollagenous sequence and a long form with an additional 247 residues, which contains a cysteine-rich sequence homologous to that found in frizzled proteins [119,126]. Transcription from the upstream promoter gives rise to a third kind of α1(XVIII) chain with a different signal peptide and N-terminal noncollagenous sequence.

The central collagenous regions of the *COL15A1* and *Col18a1* genes are encoded by 25 and 29 exons, respectively. Eight exons in the *COL15A1* gene and 13 exons in the *Col18a1* gene encode uninterrupted collagenous domains, of which only one in each gene is 54 bp long. The other exons are 27, 36, 63, 72, and 81 bp in size, all being integers of 9 bp. However, the two genes do have exons of different sizes. For example, the *Col18a1* gene has four 27-bp exons, whereas the *COL15A1* gene has no 27-bp exons. In addition, the *COL15A1* gene contains an 81-bp exon not found in the *Col18a1* gene, whereas the *Col18a1* gene has a 72-bp exon not found in the *COL15A1* gene. In both genes, split codons are found in two consecutive exons that code for the collagenous domains.

The TN motif in the N-terminal noncollagenous domain of each gene is encoded by a large exon of about 550 bp that starts with a split codon. This is in sharp contrast to the exon structures of TN motifs in other collagen genes, which are encoded by multiple exons. The last three exons encoding the C-terminal NC1 domain are highly conserved between type XV and XVIII collagen genes, consistent with the high sequence similarity between the NC1 domains of the two collagen types.

Type XVII

Type XVII collagen, a component of hemidesmosomes in the skin basement membrane zone, is another collagen type with a transmembrane domain [127,128]. The α1(XVII) collagen chain consists of an intracellular noncollagenous domain at the N-terminus, a transmembrane segment, and an extracellular collagenous domain [129].

The *COL17A1* gene is approximately 52 kb long and contains 56 exons. The translation start site is located in exon 2 [130]. The intracellular domain is encoded by exons 2–17, with exon 17 also coding for the transmembrane segment (Table 13). Exons 18–56 code for the extracellular domain, which comprises 15 uninterrupted collagenous domains separated by short noncollagenous sequences.

TABLE 13. Exons that Code for Human α1(XVII) Collagen[1]

Exon No.	Exon Size (bp)	Domain Encoded[2]	Exon No.	Exon Size (bp)	Domain Encoded[2]
1	94	5′-UT	29	63	COL
2	63	5′-UT + ICD	30	36	COL
3	45	ICD	31	72	COL
4	105	ICD	32	27	COL
5	129	ICD	33	36	COL
6	48	ICD	34	36	COL
7	36	ICD	35	81	COL
8	48	ICD	36	36	COL
9	144	ICD	37	54	COL
10	159	ICD	38	42	COL
11	72	ICD	39	54	COL
12	72	ICD	40	60	COL
13	69	ICD	41	27	COL
14	162	ICD	42	33	COL
15	81	ICD	43	75	COL
16	45	ICD	44	51	COL
17	198	ICD + TM	45	123	COL
18	222	NC	46	138	COL
19	30	COL	47	69	NC
20	27	COL	48	141	COL
21	27	COL	49	90	COL
22	63	COL	50	111	COL
23	105	COL	51	147	COL
24	63	COL	52	390	COL
25	36	COL	53	138	COL
26	54	COL	54	63	COL
27	36	COL	55	81	COL
28	36	COL	56	126	COL + NC

[1] Data are from Gatalica et al. [130].

[2] Abbreviations: UT: untranslated region; ICD: intracellular domain; TM: transmembrane domain; NC: noncollagenous domain; COL: collagenous domain.

Exons 18 and 47 encode noncollagenous sequences only, and exon 56 also contains a noncollagenous sequence at the C-terminus of the chain. The largest collagenous domain of 242 amino acids is encoded by exons 19–34. The remaining collagenous domains are relatively short, ranging from 15 to 45 amino acids. A total of 17 exons encode only uninterrupted Gly–X–Y sequences, of which 15 exons are multiples of 9 bp in size. The exon sizes do not show the 54 bp exon pattern, the most frequent size being 36 bp. The most unusual feature is that all exons encoding collagenous sequences begin with a phase I split codon, in which the intron is found between the first and second base of the codon.

CONCLUDING REMARKS

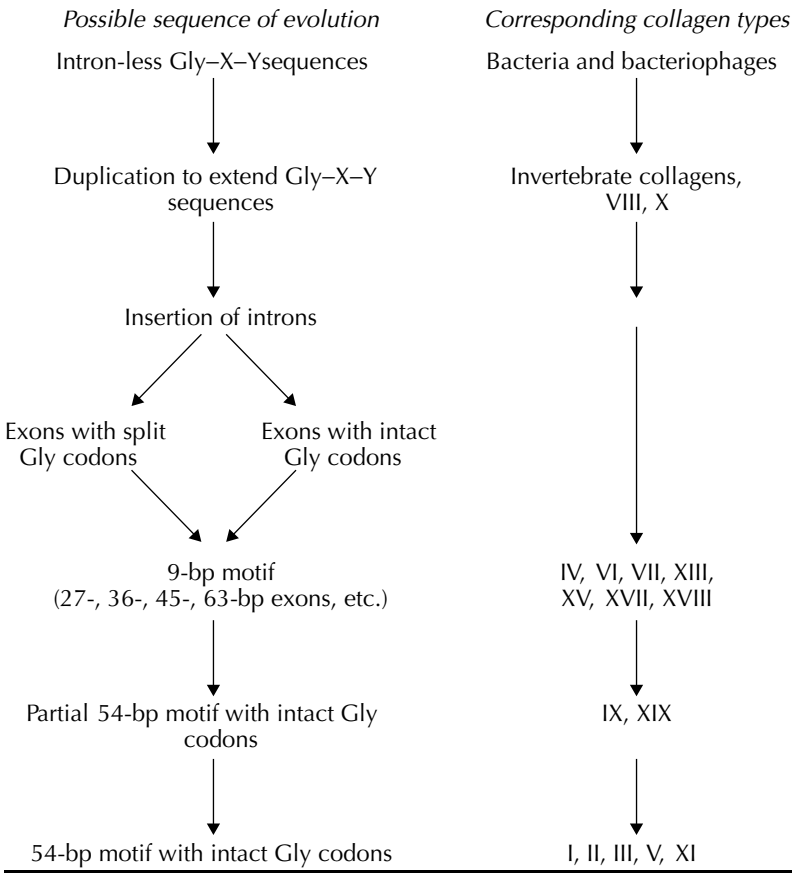
The unusual exon-intron structures of the genes for collagens have stimulated speculation as to how the genes evolved (Table 14). As pointed out by Engel [131] and Engel and Bächinger [131a], short sequences coding for Gly–X–Y tripeptides characteristic of collagens are found in bacteria and bacteriophages, organisms whose genomes do not contain introns. Therefore, one assumption as to how the genes evolved is to say that codons for repetitive Gly–X–Y tripeptides were first duplicated to extend and then separated by the insertion of introns. The introduction of introns occurred by two major mechanisms. One provided exons with split codons for glycine. The other mechanism provided exons with intact codons for glycine. Because the exons began either with intact or split glycine codons, a 9-bp motif appeared to develop, with exons of 27-, 36-, 45-, 54-, and 63-bp exon sizes. The exons gradually evolved toward more uniform structures in which a 54-bp motif was established and in which the exons began with intact glycine codons.

ELECTRONIC DATABASES

DNA sequences of many human collagen genes have been completely determined and are available in the public database <http://www.ncbi.nlm.nih.gov/Entrez/>. These include *COL1A1* (Accession no. AF017178), *COL1A2* (AF004877), *COL2A1* (L10347, AC004801), *COL4A5-COL4A6* (AL034369, AL109943, AL031177), *COL6A1–COL6A2* (AP001759, AJ011932), *COL7A1* (L23982, AC005903), *COL8A1* (AC024938), *COL9A2* (AF019406), *COL9A3* (AF026801), *COL10A1* (AL121963), *COL11A2* (U32169, AL031228), *COL12A1* (AL096771, AL080250), and *COL18A1* (AL163302).

For other collagen genes, only partial sequences or sequences of the complete exon/intron boundaries are available. These include *COL4A1* (M36963, M26576–26550), *COL4A3* (AF218541), *COL4A4* (Y17396–Y17443), *COL9A1* (AF036110–AF036130), *COL13A1* (M68974–M69010), *COL15A1* (AF052956–AF052975), *COL17A1* (U76564–U76604), *COL19A1* (AB004584–AB004688, AB004634).

TABLE 14. Possible Scheme for the Evolution of Exons Coding for Collagens



Partial sequences of many collagen genes can also be found in the "htgs" database of unfinished Human Genome sequences, which consist of unordered DNA pieces. These include *COL3A1* (AC066694), *COL4A1* (AC016380), *COL4A2* (AL159153), *COL4A4* (AC073149), *COL5A1* (AP001160), *COL5A3* (AC008742), *COL6A3* (AC025933, AC025895, AC025878), *COL8A1* (AC024938), *COL8A2* (AC012141), *COL9A1* (AC067861), *COL15A1* (AL354923), *COL16A1* (AL355514), *COL17A1* (AL360170).

RECENT DEVELOPMENTS

Since the completion of this chapter, the draft human genome sequences have appeared (see *http://www.ncbi.nlm.nih.gov/*, *http://genome.cse.ucsc.edu*, and *http://www.ensembl.org*). Many of the human collagen genes have been completely sequenced and hence their precise sizes and exon-intron structures are now known. However, there are still gaps in many collagen gene loci.

Two new collagen types have been identified. The complete cDNA sequences of the chick α1(XX) and human α1(XXI) chains have shown that they both belong to the FACIT collagen subfamily [176,177]. The *COL21A1* gene is located on human chromosome 6p11-12. Complete cDNA or genomic sequences of four additional collagen chains have been characterized [178] (and Manuel Koch, personal communication). The collagen gene family thus consists of at least 25 types.

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Chapter 3

Elastin and the Microfibrillar Apparatus

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INTRODUCTION

The physiologic function of many tissues requires that they possess elastic properties. Thus, the vertebrate body is encased in a deformable skin, and during systole the work of the heart is absorbed by expansion of the great vessels, which then elastically recoil during diastole, maintaining the blood pressure and assuring continuous perfusion of the tissues. These elastic properties are largely due to the presence of elastic fibers in the extracellular matrix. Although the elastic fibers may quantitatively comprise only a relatively small but important proportion of the total weight of some tissues, such as the skin (2–4%), in others, such as the large arteries and certain specialized ligaments, they are major components and may comprise greater than 50% of the dry weight. In the light microscope, the highly refractive elastic fibers are difficult to identify unequivocally with conventional stains such as hematoxylin/eosin and must be identified by characteristic staining reactions, such as those with orcein, resorcin-fuchsin, tetraphenyl porphinate, or other multicomponent stains [1,2]. Although these stains are relatively specific for elastic fibers, they do not provide unequivocal identification, and immunolocalization and ultrastructural analysis may be necessary. Electron microscopic examination of elastic fibers has revealed that they are comprised of two morphologically distinguishable components [3–5] (Fig. 1). The amorphous component, so named because it usually does not possess any apparent repeating structure or banding pattern, is quantitatively the larger portion, composing upward of 90% of the mature fiber. The microfibrillar component, as its name implies, is found as small fibrils approximately 10–12 nm in diameter, located primarily around the periphery of the amorphous component but, to some extent, interspersed within it. Although the exact composition of the microfibrils remains to be defined, they contain several glycoproteins [6], including fibrillin [7]. The name "elastin" has been reserved for the protein that makes up the amorphous portion of the elastic fiber and is primarily responsible for its elastic properties.

Early work on the histochemistry and chemical and physical properties of elastin has been reviewed by Partridge [8]. The present chapter focuses on the proteins and encoding genes of the elastic fiber.

CHARACTERIZATION OF ELASTIN

Isolation and Purification of Elastin

Because of the extensive cross-linking of elastin and its resultant great insolubility under all conditions in which there is no appreciable cleavage of peptide bonds, early purification methods employed relatively nonspecific and fairly harsh conditions, such as extraction of tissues with 0.1 M NaOH at 98 °C for 30 to 60 minutes [9], or repeated autoclaving in water until no further protein was solubilized. The resulting insoluble residue of these procedures was operationally taken to be elastin. However, the vigorous extraction procedures may result in significant peptide bond cleavage in the elastin, as well as destruction of the microfibrillar component. Milder methods have been developed in which the elastin polypeptide chains are recovered largely intact and which yield a protein fraction derived at least partially from the microfibrillar component. In one procedure that has been used and frequently adapted, the tissue is first extracted with 5 M guanidine, which removes the soluble collagen, glycoproteins, and proteoglycans, followed by digestion of the remaining contaminating insoluble collagen with highly purified bacterial collagenase [10]. At this stage, the elastic fibers contain amorphous and microfibrillar components, while no collagen or other proteins can be visualized in the electron microscope. The peripheral microfibrils are then solubilized by a second extraction with 5 M guanidine containing β-mercaptoethanol, leaving an insoluble elastin residue. The soluble proteins in this fraction are rich in acidic and other hydrophilic amino acids as well as being unusually rich in cysteine. Electrophoretic analyses have demonstrated that this fraction is a complex mixture of proteins and glycoproteins, some of which are derived from the microfibrillar component (see below) [6,11].

Although the amino acid composition of insoluble elastin prepared from several tissues by guanidine extraction is similar to that of elastin prepared by hot alkali extraction, this milder procedure may not be adequate for removal of all contaminating proteins and glycoproteins from tissues containing smaller proportions of elastin, and additional steps have been added in other purification schemes [12]. These have included extraction with detergent solutions, digestion with other proteolytic enzymes, such as trypsin, against which elastin is resistant, and reaction with cyanogen

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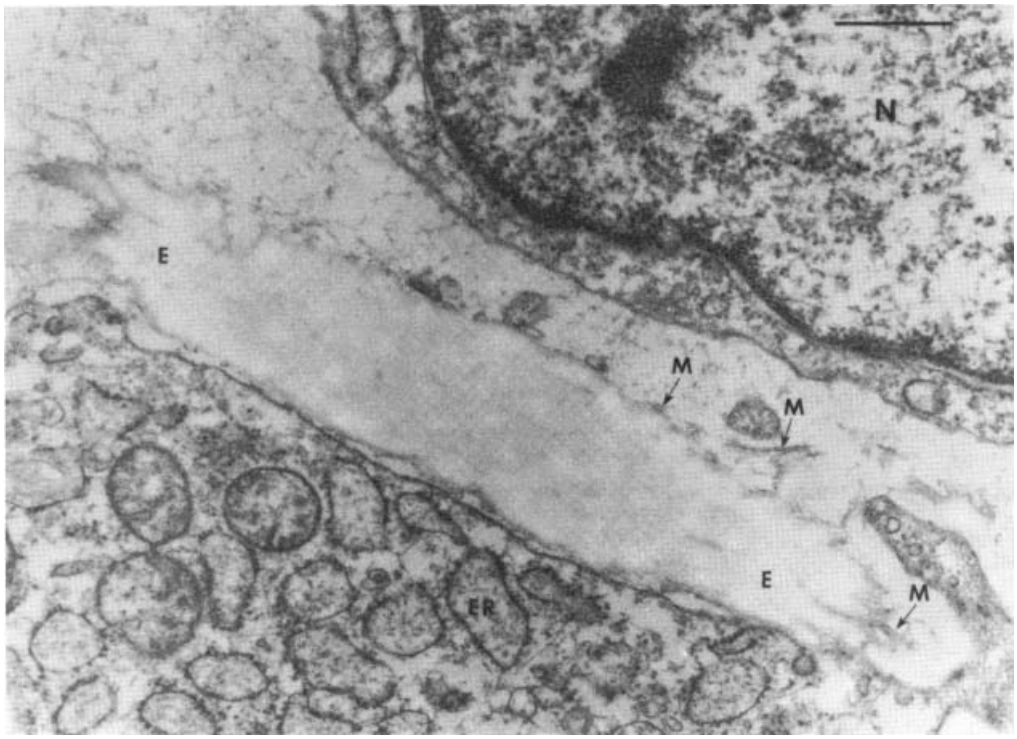

Figure 1. Electron micrograph of 15-day chick embryo aorta. A forming elastic fiber is situated between two cells; the nucleus (N) of one and the endoplasmic reticulum (ER) of the other are marked. Note the microfibrils (M) on the surface of the amorphous elastin (E). Some microfibrils are interspersed within the amorphous elastin. Bar = 0.5μm. Uranyl acetate and lead citrate; x45,000. (Reproduced from [147], with permission of the publisher.)

bromide in formic acid because it is generally agreed that most elastins contain no methionine (for exceptions, see Table 1). Depending on the starting tissue, one or another of these modified procedures should be suitable for preparing acceptable elastin comparable in purity to that prepared by the classical hot alkali treatment.

Occurrence of Elastin

Early work on the analysis and characterization of elastin was carried out largely on samples isolated from ligamentum nuchae or the aorta of mammals [8]. Consistent with its unique physical properties, the amino acid composition of elastin is peculiar, consisting of approximately 33% glycine, 10–13% proline, and more than 40% other hydrophobic amino acids. There are very small amounts of hydrophilic or charged amino acids. The amino acid composition is sufficiently similar to that of collagen to have suggested that it may be an unusual form of collagen. Although thorough analyses have clearly shown that this is not the case, there is limited evidence for a distant homologous relationship at the amino acid sequence level [13].

A very extensive survey of the occurrence and amino acid composition of elastin throughout the animal kingdom was carried out by Sage and Gray [14]. Analyses were performed on samples, mostly from the aorta and related vessels, from representative species of all vertebrate phyla and a number of invertebrate phyla. The elastin was purified by a relatively nondegradative procedure involving defatting of the tissue, extraction with a concentrated guanidine solution containing β-mercaptoethanol, and, finally, autoclaving. Where they could be compared, the resulting preparations had amino acid compositions very similar to that of elastin prepared using alkali extraction, cleavage with cyanogen bromide, or digestion with proteolytic enzymes. The results appeared very clear-cut with respect to the distribution of elastin within the vertebrate phyla. Elastin was found in every vertebrate species examined except for those in the *Agnatha* (jawless fish or cyclostomes). Later studies on the lamprey identified a novel protein, lamprin, that shares some chemical and physiologic features with elastin but is clearly distinct from it [15,16]. Although the amino acid compositions of all elastins have similar general characteristics in that they contain desmosines (cross-links characteristic of elastin) and are rich in glycine, proline, and hydrophobic amino acids and poor in hydrophilic ones, there is significant variation in composition among species within a phylum and considerable variation among phyla. Representative compositions are given in Table 1, which also contains some analyses of Starcher and Galione [12], who used a modified isolation method including digestion with CNBr. In contrast to previous results obtained solely with mammalian and avian elastins, in which histidine, methionine, and cysteine were found to be absent, these amino acids were found in the elastins of many reptiles, amphibians, and fish. Consideration of the changes in composition during evolution suggests that the earliest elastin was similar in amino acid composition and cross-linking to that of mammalian elastin, although there has been a progressive increase in hydrophobicity with evolutionary time. This trend toward greater hydrophobicity may be related to a parallel change in systolic blood pressure, which also increases from a low of 30 mm Hg in fish and

TABLE 1. Amino Acid Composition of Elastin from Aortas of Various Species[1]

Amino Acid	Species						
	Pig	Dog	Human	Chicken	Turtle	Yellow Fin Tuna	African Lungfish
Lys	5.2	5.2	4	2	6.8	15	12
His	1.0	1.9	0.5	0.7	3.6	2.6	5.3
Arg	7.9	9.1	9	5.0	7.6	16	15
Asx	6.4	7.6	6	7	3.4	26	14
Thr	15	24	12	9	18	63	33
Ser	12	16	8	6	11	34	23
Glx	19	22	18	13	24	43	35
Hyp	8.7	11	10	26	16	5.5	12
Pro	113	107	131	131	130	101	112
Gly	313	314	295	338	319	391	351
Ala	244	249	233	179	184	103	105
Val	128	99	143	173	151	64	110
Ile	18	28	23	20	17	19	43
Leu	54	46	58	56	58	45	80
Tyr	19	29	23	13	34	37	27
Phe	33	25	22	19	13	28	18
Cys	<1	2.3			<0.9	<1	<0.8
Met	<1	2.1			2.0	7.1	3.5
Ide	1.9	1.8	2.2	1.2	1.5	0.3	0.7
Des	1.3	1.3	2.8	1.4	1.2	0.3	0.5

[1]Taken from Sage and Gray [14] except for human and chicken data, which were taken from Starcher and Galione [12]. Values are expressed as residues/1,000.

amphibians to 120–150 mm Hg in mammals and birds. Hydrophobic effects may play a major role in the mechanism of elastin fiber formation and rubber-like elasticity.

Sage and Gray [14] did not find elastin in any invertebrate species, either by chemical or histologic methods, and this suggests that the earlier histologic identifications of elastin in invertebrates, based on characteristic staining reactions, were probably in error. It is true that other rubber-like proteins such as resilin [17], abductin [18], and an octopus elastomer [19] occur in the invertebrates, but they are not related to elastin because they do not contain characteristic desmosine cross-links and have distinctly different amino acid compositions.

Identification of Tropoelastin and the Primary Structure of Elastin

Although elastin can be partially solubilized by nonspecific hydrolysis with weak acids or alkali, the resulting peptides (called α-elastin when oxalic acid is used) are very heterogeneous and difficult to resolve because of their similarity in amino acid composition and chemical properties. This greatly limited sequence analysis to a few dipeptides, such as Ala–Leu and Leu–Ala [20]. The major significant achievements before the late 1960s were the elucidation of the structure of the desmosine cross-links by Partridge and his colleagues [21] and the demonstration that these were derived from lysine residues [22,23].

A major advance in elastin chemistry came about with the isolation of a soluble protein clearly related to elastin. It had been observed in nutritional studies involving trace metals that animals on a copper-deficient diet suffered aneurysms of the aorta and other defects that could be attributed to a decreased content of the amorphous component in their elastic fibers. This led to the isolation of a soluble polypeptide from the aorta of copper-deficient pigs [24]. The relationship of this protein, now called tropoelastin, to insoluble elastin was solidified by the work of Sandberg et al. [25], who showed that its amino acid composition was very similar to that of insoluble elastin except for the absence of cross-links and a corresponding increase in lysine residues. The total lysine content is 38 residues/mol in tropoelastin compared with about 6 residues/mol in mature elastin. Peptide maps obtained by pancreatic elastase digestion demonstrated that although insoluble elastin and the soluble protein tropoelastin share a number of peptides, there are peptides that are unique to each because of the retention in tropoelastin of many lysine residues. Tropoelastin has been isolated from copper-deficient chicks and calves and from lathyritic animals [26–29] (lathyrism being induced by feeding animals β-aminopropionitrile, an inhibitor of lysyl oxidase, the enzyme responsible for initiating cross-link formation). In addition to their similarity in amino acid composition, the tropoelastins from all species share a number of features, including a molecular weight of 72,000–74,000, unusually high solubility in concentrated solutions of short-chain alcohols, and a negative temperature coefficient of solubility in salt solutions [30,31]. This last property leads to the phenomenon of coacervation, or phase separation, of tropoelastin from a cold solution when the temperature is increased to greater than 25 °C.

The peptides resulting from tryptic digestion of porcine tropoelastin have been fractionated by ion-exchange chromatography and sequenced by automated Edman degradation [32,33]. Two classes of tryptic peptides were found: (i) small ones, rich in alanine, which are derived from regions that will form the cross-links; and (ii) larger peptides rich

in hydrophobic residues, which are derived from the regions responsible for the elastic behavior. Two of the small peptides, Ala–Ala–Ala–Lys and Ala–Ala–Lys, are repeated six times per mole of tropoelastin, while several others are repeated twice. As shown later by sequencing of cDNA (see below), these small peptides are spaced throughout the tropoelastin, being separated by the larger hydrophobic segments. Within some of the larger hydrophobic peptides, smaller limited repeats are discernible. Such repeating sequences have raised the possibility of a secondary helical structure, the β-spiral, peculiar to elastin in a portion of the molecule [34], but considerable evidence supports the concept that elastin is largely a random coil [35–37]. The sequences of the larger peptides derived from porcine tropoelastin show that although glycine constitutes approximately one-third of the residues in elastin, it is not found regularly as every third amino acid and there are numerous occurrences of glycine residues being adjacent to one another, unlike in the sequences found in collagen. However, chick tropoelastin does contain a collagen-like stretch of 17 Gly–X–Y triplets, of which most are Gly–Val–Pro [13]. Presumably, this region cannot form a collagen triple helix because proline is usually in the third rather than second position. However, this finding raises the possibility that the collagens and elastin do have a distant homologous phylogenetic relationship. Nevertheless, the apparent marked difference between chick and porcine tropoelastin, as well as differences in the amino acid composition of many other elastins (Table 1), suggests that exact interspecies homology is low. Presumably, such variation may occur because there are many ways to form a random coil, probably the major factor in the properties of elastin, and selective constraints are less than for ordered proteins. As noted, there is a preferential occurrence of prolyl residues on the amino side of glycine, with proline found only rarely on its carboxyl side.

BIOSYNTHESIS OF ELASTIN

The isolation and characterization of tropoelastin from copper-deficient animals suggested that this 72 kDa polypeptide was a soluble intermediate in the biosynthesis of insoluble elastin. To delineate the role of tropoelastin in the biosynthetic pathway, tissues and cells that rapidly synthesize elastin were incubated with radioactive amino acids, and the soluble labeled proteins were extracted and characterized [38,39]. Tropoelastin was identified by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, by its solubility in aqueous alcohol solutions, by its labeling pattern with various amino acids, by its resistance to cyanogen bromide degradation, and by immunoprecipitation with affinity-purified antibody to insoluble elastin. Additional evidence for the legitimate role of tropoelastin as a biosynthetic intermediate came from the observation that when isolated [^{14}C]lysine-labeled porcine tropoelastin was incubated with fresh preparations of normal newborn pig aorta, labeled desmosines, as well as lysinonorleucine and merodesmosine, could be recovered [40]. It was found that the intracellular pathway appears to be the classical one (i.e., synthesis on membrane-bound polysomes, transport to the Golgi apparatus, and packaging into secretory vesicles) followed by a number of secreted proteins [41], and approximately 20 minutes were required for the synthesis of the tropoelastin molecule and its secretion into the extracellular matrix [42]. Agents such as brefeldin A, monensin, and colchicine, which disrupt the pathway at various points, inhibit secretion significantly [39]. Furthermore, when elastogenic cells were treated with bafilomycin A1, a vacuolar H^+/ATPase inhibitor that prevents acidification of the *trans*-Golgi network and endosomal compartments without disrupting intracellular organelle formation, tropoelastin secretion was diminished and intracellular accumulation of tropoelastin was detected in the *trans*-Golgi network and small secretory vesicles [43]. These results suggest that, after exiting the Golgi, tropoelastin may be targeted to an acidic compartment prior to transport to the cell surface.

Although some of the prolyl residues in tropoelastin are hydroxylated posttranslationally, unlike the case of collagen, in which hydroxyproline stabilizes the triple helix and inhibition of hydroxylation inhibits secretion [44–46], inhibition of hydroxylation of proline in tropoelastin does not inhibit the rate of tropoelastin secretion, and the function of hydroxyproline, if any, in tropoelastin is unknown [39,47]. It has been suggested that ascorbic acid, a cofactor in the hydroxylation reaction, although not altering the synthesis of tropoelastin, markedly inhibits the accumulation of insoluble elastin [48]. It is conceivable that ascorbic acid mediates an increase in the degree of prolyl hydroxylation of tropoelastin, which interferes with fibrillogenesis and/or stabilization of elastic fibers. It is interesting that elastin isolated from aortas of Marfan syndrome patients shows considerably increased levels of hydroxyproline. It has been suggested that this may explain, at least in part, some of the compromised tensile strength properties of the aortas of Marfan patients [49], but the relation of this finding to mutations in fibrillin (see below) is not clear. Also, unlike the fibrillar collagens, tropoelastin is incorporated without proteolytic cleavage into the insoluble fiber [50].

The control of elastin synthesis has been investigated in a number of developing embryonic systems, including chick and sheep aorta and sheep nuchal ligament. In these systems, measurements of functional elastin mRNA by *in vitro* translation have demonstrated a strong correlation between messenger levels and the rate of elastin synthesis in the tissue, suggesting that the rate of synthesis is controlled by the mRNA level [51–54]. Northern hybridization experiments using cloned elastin cDNA as probe demonstrated that the functional mRNA levels are a reflection of the actual levels and confirmed the conclusion that the rate of elastin synthesis is largely controlled by the level of elastin mRNA [55,56]. In these Northern hybridization experiments, the probes hybridized to a 3.5 kb message, consistent with there being 2.5 kb of coding sequence, corresponding to about 800 amino acids, and an approximately 1.0 kb 3′ untranslated segment (see below). These results, considered collectively, strongly support the proposition that tropoelastin is the primary elastin gene product.

Cross-Linking

A critical feature of the elastic fiber, crucial to its proper function, is the extensive extracellular cross-linking of tropoelastin. This cross-linking is mediated by the enzyme protein-lysine 6-oxidase (lysyl oxidase; EC 1.4.3.13), which oxidizes selective lysine residues in peptide linkage to α-aminoadipic δ-semialdehyde (trivial name allysine). This is the same enzyme involved in collagen cross-linking, and its isolation, purification, assay, and properties are discussed in detail by Kagan [57]. Experiments using highly purified preparations of lysyl oxidase and pure collagen or elastin have demonstrated that the enzyme functions better on insoluble forms of the substrates. Thus, preincubation of soluble collagen to allow aggregation, or of tropoelastin to allow coacervation, results in increased oxidation by a

given amount of enzyme. This observation is consistent with the finding that a substantial amount of lysyl oxidase is associated with insoluble fibers (possibly with microfibrils) in the connective tissue matrix. The enzyme-substrate complex can be visualized using fluorescently tagged antibody against lysyl oxidase, and the enzyme can be extracted using high concentrations of urea, with retention of enzymatic activity upon removal of the urea by dialysis. When fibroblasts are grown in culture under conditions in which the majority of the collagen is secreted into the medium, lysyl oxidase also is found largely in the incubation medium, comparatively little being associated with the cell layer. These findings suggest that the enzyme acts in the extracellular space.

The course of cross-link formation in elastin is similar to that in the collagens, but hydroxylysine and histidine residues are not involved because there are none in mammalian and avian elastins, and some of the final products of the series of aldol, and aldimine condensations, the desmosines, are absent from the collagens. There are two bifunctional cross-links: dehydrolysinonorleucine, formed from one residue of allysine and one of lysine, and allysine aldol, formed from two residues of allysine. Their structures and those of the desmosines are shown in Figure 2 (for a review of cross-linking in collagen and elastin, see [58]). Dehydrolysinonorleucine also occurs as the reduced, and thus stabilized, secondary amine lysinonorleucine. Allysine aldol, shown as the dehydrated aldol, may be hydrated *in vivo*. A trifunctional cross-link, dehydromerodesmosine, which is also shown in Figure 2, may also be present in the reduced form *in vivo*. Although desmosine and isodesmosine are tetrafunctional, it is likely that each cross-link normally joins only two chains [59]. The lysine residues that serve as cross-links in elastin occur as pairs, mainly in polyalanine sequences, and, in contrast to the variability seen in the hydrophobic domains, there is strict conservation, especially in the numbers of residues between lysines. The conformation of the alanine-rich cross-linking domains is essentially α-helical [60], and the lysine residues are always separated by two or three alanines. In an α-helix, each residue is related to the next by a translation of 1.5 Å along the helix axis and a rotation of 100°, forming a rod-like structure with side chains extended outward in a helical array.

Thus, the side chains of amino acids separated by two or three residues in the linear sequence are spatially close to one another on the same side of the helix, whereas side chains of amino acids separated by one or four residues are situated on opposite sides of the helix and are unlikely to make contact (Fig. 3). These positional considerations have suggested that a critical step in the cross-linking pathway is the formation of a bifunctional "within chain" cross-link intermediate, which then condenses with another bifunctional intermediate on a second chain to form the tetrafunctional desmosine cross-links [61]. Alternatively, they could form by several routes by the repeated addition of single residues of lysine or allysine, with dehydromerodesmosine as an intermediate. Overall, desmosine formation requires an oxidation (loss of two protons). Perhaps there is a balance in the reduction of dehydrolysinonorleucine and dehydromerodesmosine.

Insofar as is known, all reactions subsequent to the oxidative deamination of lysine are spontaneous. Whether they occur essentially randomly between residues in the highly mobile elastin chains or in an ordered manner remains to be proven. In a very illuminating paper [62], Mecham and co-workers have presented strong chemical evidence that a major cross-linking site is formed through the association of sequences encoded by exons 10,19, and 25, and that three chains are joined by one desmosine and two lysinonorleucine cross-links. The data indicate that domains 19 and 25 join two chains by a desmosine cross-link, while domain 10 of a third chain bridges domains 19 and 25 through lysinonorleucine cross-links. It should be noted that domains 19 and 25 contain three lysine residues that can participate in cross-link formation. These findings, together with the high degree of sequence conservation for these three domains, suggest that these regions may have an important function in nucleating polymerization and alignment of tropoelastin monomers. Table 2 shows that there is an approximate balance of the various cross-links and cross-link precursors in mature elastin. About four of the lysine residues found in tropoelastin remain unaccounted for in elastin, either because of losses during analysis or because of their possible involvement in other unidentified derivatives (see below). The

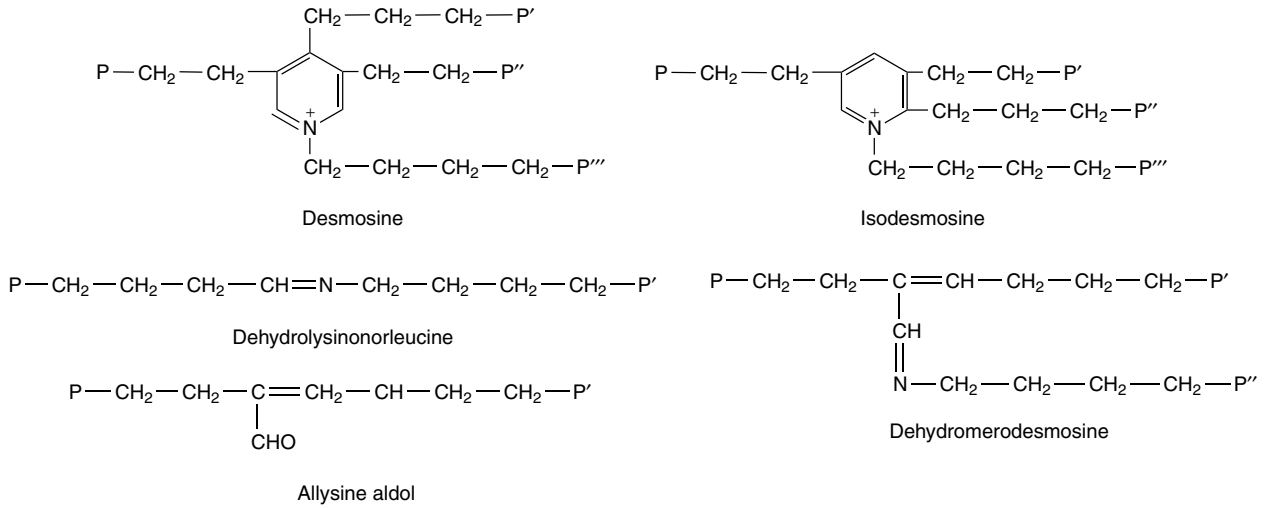

Figure 2. Structure of elastin cross-links.

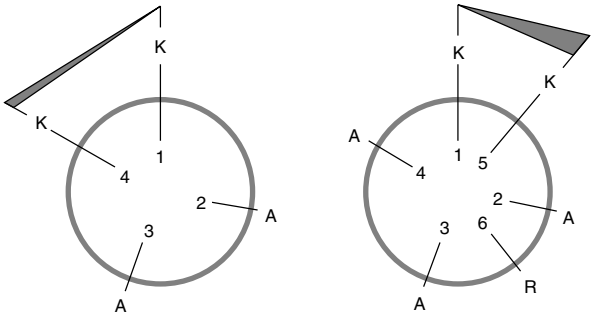

Figure 3. Cross-sectional view of the cross-linking regions of elastin containing lysine (K) and alanine (A) residues drawn as an α-helix. The polypeptide main chain forms the inner part of the helix, with side chains extending outward in a helical array. When the sequences found in elastin are substituted into the side chain positions, beginning with position 1, it can be seen that lysine side chains in the 4 or 5 position are close to the side chain of lysine 1 and able to form the ''within chain'' cross-link that is the precursor of the tetrafunctional desmosine cross-link. If lysines were to be in positions 3 or 6, they would be on the opposite side of the helix from lysine 1 and, hence, could not interact with it. Position 6 is frequently occupied by a phenylalanine or tyrosine residue.

TABLE 2. Numbers of Cross-link and Cross-link Precursors in Mature Elastin

Cross-link	Lysine Equivalents (Residues/mol Tropoelastin)
Desmosines	15
Merodesmosine	2
Lysinonorleucine	3
Allysine aldol	6
Allysine	2
Lysine	6
Total Residues	34

net result is a highly insoluble polymer in which some type of interchain lysine-derived cross-link occurs about every 65–70 residues as calculated from the cross-link content and mechanochemical properties [63], in good agreement with the sequence data discussed below.

BASIS OF ELASTIC PROPERTIES

Elastic recoil is a critical property of several tissues and organ systems. For example, during an average lifetime, the elastic fibers in the aortic arch undergo more than a billion stretch/relaxation cycles. Because the turnover of elastin appears to be quite low [8], individual fibers can conceivably last a lifetime. The rather amazing durability of the elastic fiber suggests that the elastomeric force results not from the stressing of chemical bonds, which could result in progressive deterioration, but rather results, upon stretching, from a decrease in the number of conformations accessible to the cross-linked polypeptide chains. The increase in the number of conformational states upon removal of the stretching force provides the free energy for elastic recoil. In other words, like a true rubber, the elasticity is due to an increase in entropy upon relaxation. This view has been essentially proven by a number of physical studies [64–66].

Structural Models

Determination of the structure of elastin, other than the primary amino acid sequence, has proven difficult. Elastic fibers yield only broad rings upon X-ray diffraction, indicating little short-range order [67]. Similarly, study of individual elastic fibers by polarized light microscopy has indicated that elastin is optically isotropic [63]. Nuclear magnetic resonance studies on elastin have clearly shown that the backbone chain of the protein is highly mobile, with individual residues in the chain able to rotate freely in three dimensions [35,37]. Although these observations have indicated that elastin exists predominantly in a kinetically free, largely random coil network, some observations suggest regions of local order in the molecule. For example, circular dichroism spectra indicate that the alanine-rich sequences in the cross-link regions are in an α-helix [60]. In addition, some electron microscopy studies have suggested the presence of a filamentous substructure. Whereas freeze-fracture electron microscopy of unstretched elastin revealed an isotropic structure, samples that had been stretched 150–200% appeared to contain ordered filaments approximately 13 nm in diameter [68]. Negative staining of insoluble elastin revealed a filamentous network, with the filaments having a diameter of 3–5 nm [69].

Urry and co-workers have carried out a variety of physical/chemical studies on α-elastin (peptides solubilized by hydrolysis of insoluble elastin with oxalic acid solutions) as well as on a number of synthetic polypeptides, some of which are found as limited repeating units in elastin, such as (Val–Pro–Gly–Val–Gly) and (Val–Ala–Pro–Gly–Val–Gly) [34,70]. Based on these studies and the observation that under certain circumstances elastin polypeptides can be found in thin filaments, Urry has proposed that a significant proportion of the hydrophobic segments of elastin are found in a structure designated a β-spiral, a loose water-containing helical structure in which β-turns act as spacers between suspended segments of the helix.

From these rather different views of the elastin molecule — one in which the polypeptide chain is largely a random coil and the second in which it is a β-spiral — two alternative structural models have been developed to explain the elastic force

In the first model, elastin is a network of largely random chains within the elastic fibers, which behave according to the classical theory of rubber elasticity [36,64–66]. In this model, the collection of chains has a random distribution of end-to-end chain lengths and displacement from this position of highest entropy provides the source of the elasticity.

In contrast, in the second model, Urry [34,70] has proposed that the entropic elasticity derives from the β-spiral structure, with essentially fixed end-to-end chain lengths. The peptide segments suspended between the β-turns are free to undergo large-amplitude, low-frequency rocking motions called librations. Upon stretching, there is a decrease in amplitude of the librations, which results in a large decrease in the entropy of the segment, and this provides the driving elastomeric force for return to the relaxed state.

It should be emphasized that while these models differ in their molecular conformational details, both rely on an entropy-driven mechanism to provide the elastomeric force. Further studies are required to prove whether either of these models is uniquely valid or whether the structure of elastin incorporates some features of each. Figure 4 presents a diagrammatic representation of cross-linked elastin in the

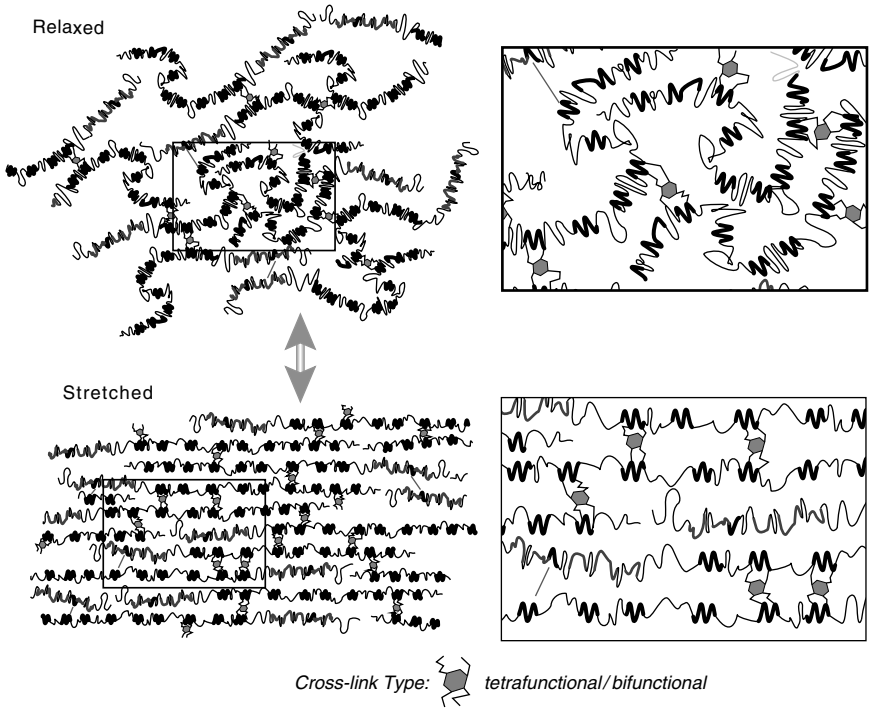

Figure 4. Diagrammatic representation of cross-linked elastin in relaxed and stretched states. The potential cross-linking domains, some of which are probably in an α-helical conformation, are in bold. For clarity of presentation, only some of the tetrafunctional desmosine or bifunctional cross-links are shown. The hydrophobic domains in the relaxed state are probably largely random coils, although limited portions may exist in a β-spiral conformation (see the text). In the stretched state, the imposed force brings the chains into relative alignment and limits their conformational freedom.

relaxed and stretched states. The figure emphasizes the limitation of motion and relative alignment of the chains imposed by the stretching force. Upon release of the force, the chains return to a more random state.

MOLECULAR BIOLOGY OF THE ELASTIN GENE (MIM 130160)

Analysis of Elastin cDNA

Analysis of cDNAs from a number of species, including man, have demonstrated that tropoelastin consists predominantly of alternating hydrophobic and paired lysine-rich domains, as indicated by open and filled boxes in Figure 5 [71–76]. The segregation of domains is conveniently visualized when the amino acid sequences are analyzed for the distribution of hydrophobic and hydrophilic segments. A graphic display of such an analysis (Fig. 5) permits ready identification of the lysine-rich potential cross-linking sequences, which project as relatively hydrophilic regions. Thus, the domain structure of the protein is a reflection of the gene in which functional domains are usually segregated into separate exons. In general, there is good agreement at the nucleotide and encoded amino acid sequence levels among the mammalian elastins, which differ, however, in multiple segments from those of the chicken. Among mammalian elastins, most amino acid substitutions are of a conservative nature, but some significant differences do exist. For example, near the center of bovine and porcine tropoelastins, a pentapeptide, GVGVP, is repeated 11 times, but this repeat segment is considerably different and more irregular in human tropoelastin and is replaced in rat tropoelastin by GVGIP [73–76]. Similarly, in human tropoelastin a hexapeptide, GVGVAP, is repeated seven times, but only five times, with conservative substitutions, in bovine tropoelastin, and it is absent altogether from rat, tropoelastin [74]. However, in the rat, several expansions of hydrophobic segments have occurred, significantly increasing the overall size of rat tropoelastin (cf. the sizes of rat and human exons 7, 22, and 30 in Fig. 6). In contrast, the length of cross-linking segments is highly conserved, indicative of a strong functional requirement (Fig. 6). Four exons (nos. 6,10,12, and 35) have a dual hydrophobic and cross-linking character, which suggests that they may play a critical role in molecular assembly of the cross-linked fiber. The chicken sequence is quite homologous to the mammalian sequences for the first 302 and last 57 residues [72]. In the central portion, although some segments are homologous, major differences do exist, which appear to be due to insertion, duplication, and deletion events. The most striking of these differences is the occurrence in chicken tropoelastin of the repeating tripeptide $(GVP)_{12}$ (residues 374–409), which is not found in the mammalian elastins. The presence of this repeating tripeptide suggests that elastin may have a distant evolutionary relationship to collagen. All in all, these observed variations suggest that a particular number of amino acids and a precise sequence in a given hydrophobic region are not critical to the adequate functioning of the molecule, although there appears to be a strong tendency to conserve the size of the total polypeptide chain to 750–800 residues.

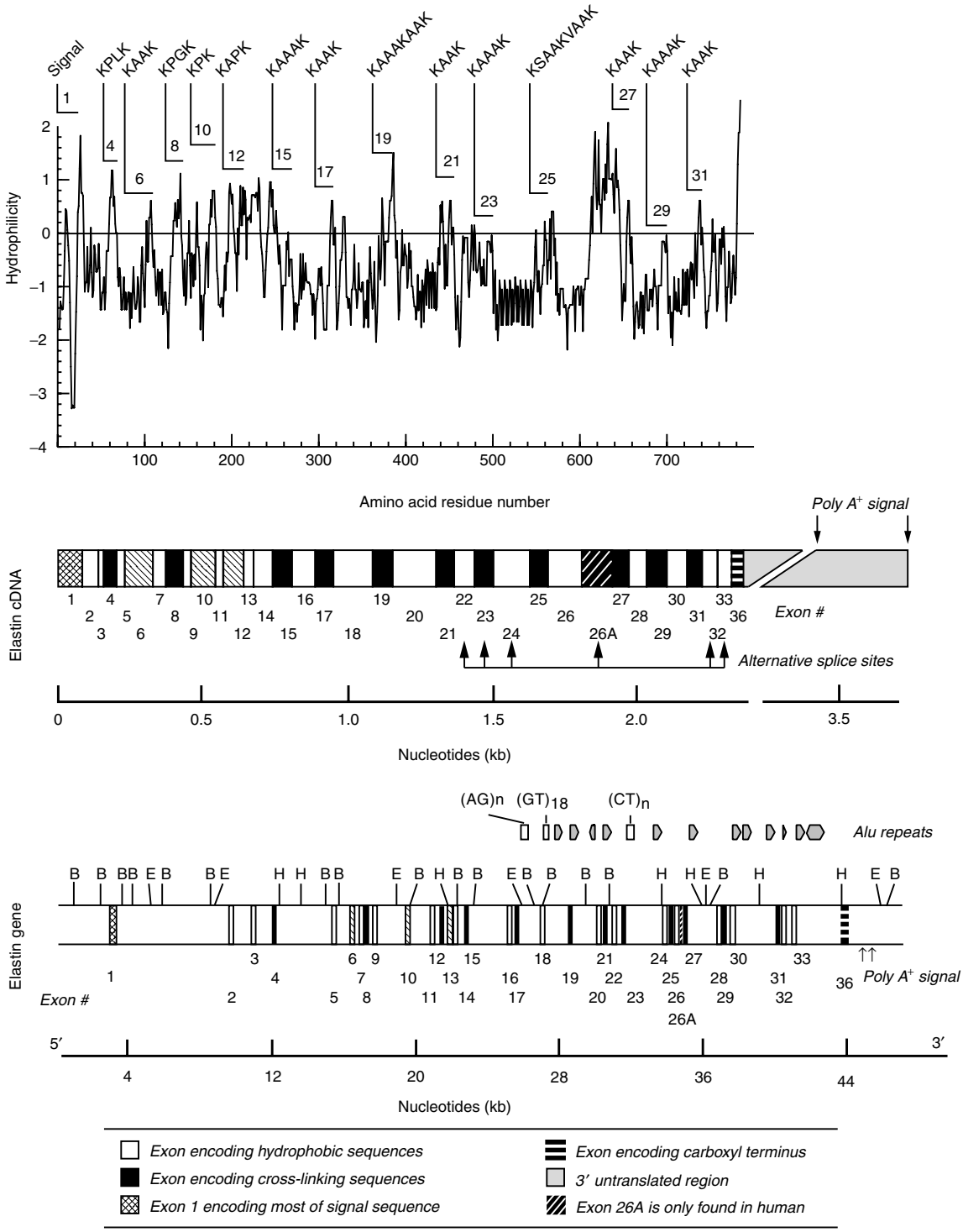

Figure 5. Diagrammatic representation of human elastin gene and cDNA and hydrophilicity/hydropathy analysis of tropoelastin by the method of Kyte and Doolittle [146]. The cDNA is divided into exons, which are numbered starting at the 5′ end and correspond to the gene diagram. To preserve a numbering system consistent with homologous sequences in the bovine and rat genes, the most 3′ exon has been designated 36 in the human gene. Exons 34 and 35, found in other species analyzed, are absent from the human gene. Restriction sites indicated in the gene are: B, *Bam*HI; E, *Eco*RI; H, *Hind*III. In the hydropathy diagram, the hydrophilic peaks correspond for the most part to potential cross-linking domains, and the numbers above these peaks identify exons. In the single-letter amino acid code, K corresponds to lysine, P to proline, L to leucine, A to alanine, G to glycine, S to serine, and V to valine. Most of the signal sequence is encoded by exon 1.

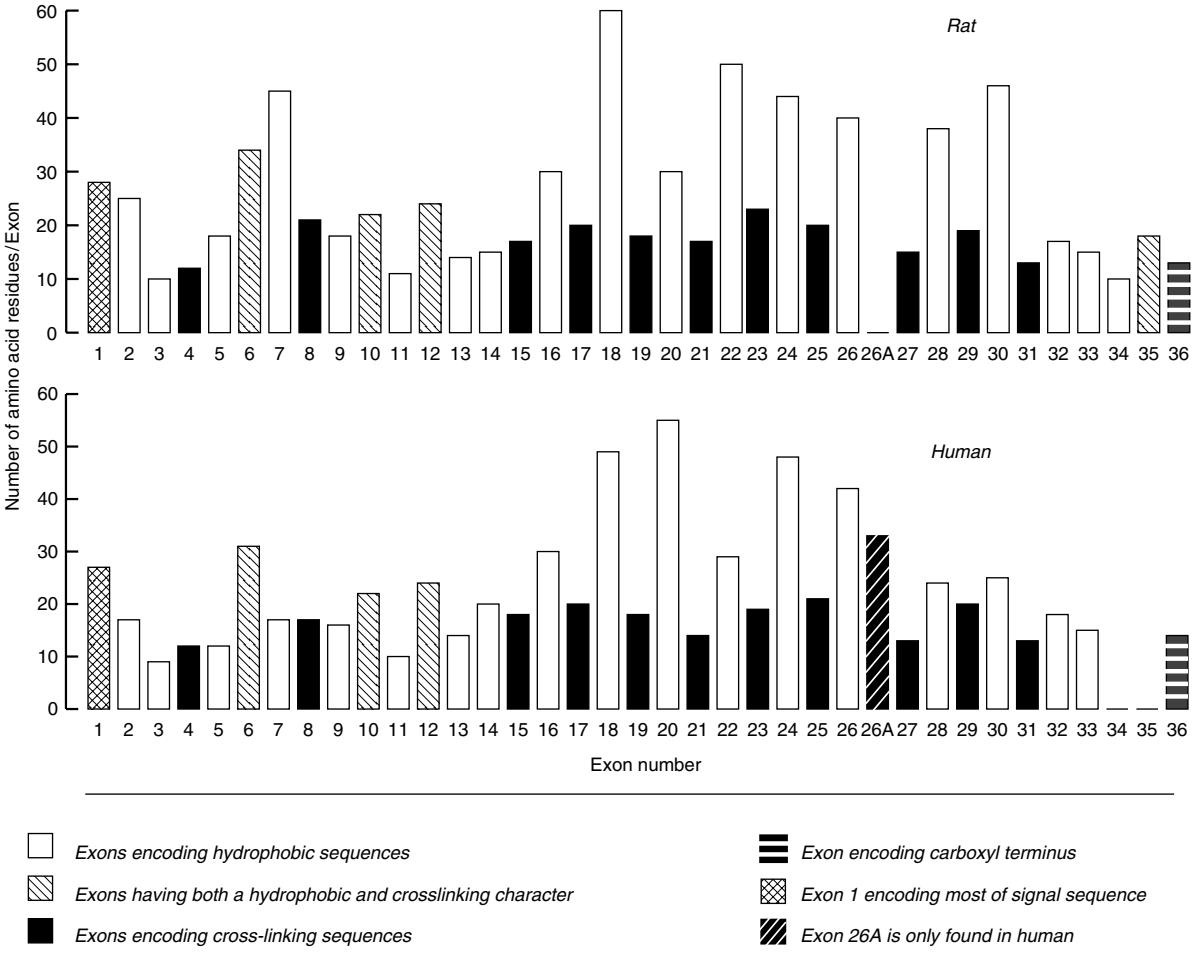

Figure 6. Comparison of exon sizes in elastin genes. Sizes for the entire human gene were determined by cDNA and genomic sequence analysis. The rat exon sizes were estimated by comparison with the human and bovine genes and by using the hydropathy analyses illustrated in Fig. 5, making the assumption that sequences encoding hydrophobic and cross-linking domains are segregated in separate exons.

In the cross-link domains, the lysines usually occur in pairs. This arrangement supports the suggestion that a given desmosine/isodesmosine serves to join only two tropoelastin molecules rather than the four that are theoretically possible. It is also apparent that these potential cross-linking sequences are not uniformly distributed and occur at shorter intervals in the first 200 residues, resulting in an asymmetry of the molecule. In addition, the potential cross-linking sequences in the first 200 residues frequently contain a proline or other residue between the lysine residues instead of the usual alanine. The conformation of tropoelastin in the cross-linking segments containing alanine residues is largely α-helical, which is undoubtedly important in the alignment and condensation of the lysine residues in desmosine formation. In contrast, the presence of proline residues clearly disrupts α-helix formation, and these segments may participate in cross-links other than the desmosines, such as the difunctional cross-link lysinonorleucine. The 200-residue amino-terminal segment of tropoelastin ends in a tyrosine-rich region, the function of which is unknown, but it may be involved in interaction with other matrix macromolecules or with the cell surface, mediating alignment of the molecules within the fiber. In two instances (exons 19 and 25), three lysines are found near one another, and they may have a critical role in cross-linking the tropoelastin, as discussed above.

There is rather extensive (80%) nucleotide homology in the 3′ untranslated region (Fig. 7), suggesting that this region may have a function either in stabilizing the mature mRNA or even in modulating translation. Additionally, all the cDNAs encode an unusual, highly conserved carboxyl terminal segment, GGACLGLACGRKRK, the highly basic character of which suggests that it may interact strongly with acidic microfibrillar proteins (see below).

Analysis of the Elastin Gene

The elastin gene exists as a single copy, and the human gene has been localized to chromosome7q11.1-q21.1 [77]. Analysis of the entire bovine and human genes has demonstrated that rather small exons (27–186 bp) are interspersed between large introns, resulting in a low coding ratio of about 1:20 [73–75,78] (Fig. 5). Another important characteristic is that hydrophobic and cross-link domains of

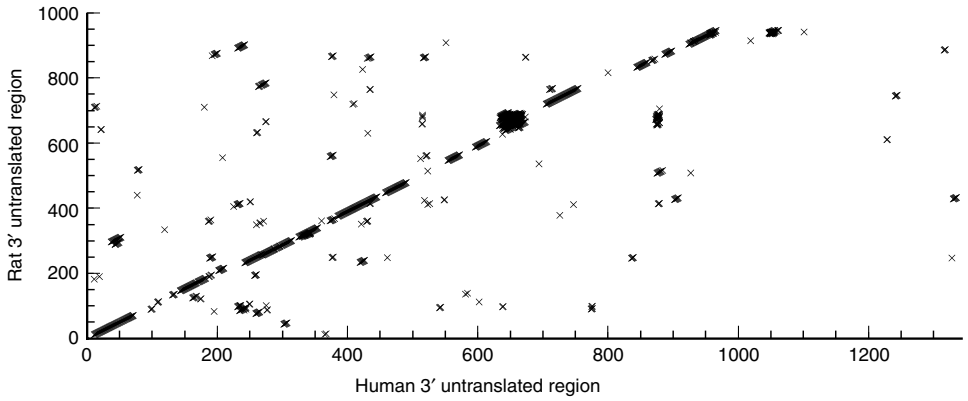

Figure 7. Comparison of the 3′ untranslated region of rat and human elastin cDNAs. The sequences were compared using the Wisconsin Sequence Analysis Package Program Compare, Version 8, Genetics Computer Group. Note the striking regions of homology scored on the diagonal.

the protein are encoded by separate exons, so that the domain structure of the protein is a reflection of the exon organization of the gene. Although all the exons are multiples of three nucleotides and glycine is usually found at the exon/intron junctions, the exons do not exhibit any regularity in size as is found in the fibrillar collagen genes. All exon/intron borders have the same phasing. Thus, the second and third nucleotides of a codon are included in the 5′ exon border, while the first nucleotide of a codon is found at the 3′ border of the previous exon. This consistent structure permits extensive alternative splicing of the primary transcript in a cassette-like fashion while maintaining the reading frame. Sequences homologous to exons 34 and 35 in other species are not found in the human gene. It is not presently known whether this difference has any functional significance.

The introns contain an unusual abundance of *Alu* repeat sequences clustered in introns toward the 3′ end of the gene (Fig. 5). In the human genome, *Alu* repeat sequences occur about once every 4 kb of genomic DNA, but in the elastin gene these sequences occur at about four times that frequency. In addition to *Alu* repeats, rather long stretches of alternating purines or pyrimidines occur. The function, if any, of these repetitive elements remains to be determined, but they may facilitate recombinational events. In other human genes, deletions apparently mediated by recombination between repetitive sequences have occurred, resulting in hereditary diseases, and evidence for genomic instability in regions of human DNA enriched in *Alu* repeat sequences has been presented [79–82], but no mutations in the human elastin gene, possibly mediated by similar mechanisms, have been found. However, disruption of the elastin gene has been shown to be the cause of supravalvular aortic stenosis, either as an isolated finding or as part of the Williams syndrome, and some of these cases may involve such recombinations (see Chapter 12, this volume). Several informative polymorphisms have been identified by PCR and restriction endonuclease analysis in the human elastin gene [83].

Alternative Splicing of Elastin mRNA

Analysis of bovine, human, and rat elastin cDNAs has clearly demonstrated alternative splicing of the primary transcripts [73–76,84]. In most cases, splicing occurs in a cassette-like fashion in which an exon is either included or deleted, but occasionally a splicing event may divide an exon, as in the case of exons 24 and 26. Both hydrophobic and cross-link domains are affected, so that two cross-link domains may be brought into apposition (deletion of exon 22) or the interval between cross-link domains may be increased (deletion of exon 23). It is not known whether these variations have any functional significance, although clearly a tighter or looser fiber network could be produced. S1 nuclease mapping experiments using elastin mRNA isolated from developing bovine and rat tissues have demonstrated that alternative splicing of some exons occurs frequently, but in the majority of cases it is infrequent [75,77]. These experiments indicate that splicing may be developmentally controlled and tissue-specific. They also suggest that the splicing pattern in the adult may differ significantly from that occurring during development. Translation of the alternatively spliced transcripts would result in significant primary sequence variation among individual tropoelastin molecules, and it is likely that such differences explain the finding of variant isoforms of tropoelastin in several species [85,86]. It remains to be determined whether variation in splicing occurs in disease situations. The possible variable expression of exon 26A in human elastin is particularly intriguing, because this segment, which is highly hydrophilic and atypical in amino acid sequence for elastin, appears rarely to be expressed under normal circumstances. Inclusion of this sequence might substantially alter the properties of the elastic fiber.

Regulation of Elastin Gene Expression

Sequence Analysis of the Promoter and Transcription Initiation

The elastin promoter does not appear to contain a functional canonical TATA sequence, and it is likely that the CAAT sequences are not functional (Fig. 8). The proximal promoter region is G + C-rich (66%), with a high frequency of CpG dinucleotides [75,78]. These features have previously been associated with the promoters of so-called "housekeeping genes," but more tissue-specific genes are being found to possess them as well, so that the distinction between these classes is now breaking down [87]. As with many genes, the elastin promoter contains a remarkable constellation of potential binding sites for transcription regulatory factors indicative of complex regulation (Fig. 8). These binding sites include multiple SP1 and AP-2 binding

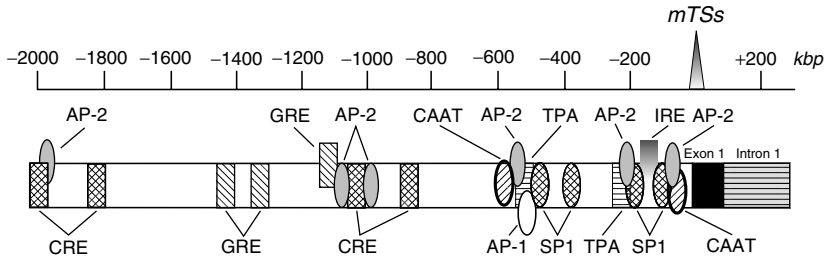

Figure 8. Schematic representation of the human elastin gene promoter. CRE, cAMP responsive element; GRE, glucocorticoid responsive element; IRE, insulin-like growth factor responsive element; TPA, 12-O-tetradecanoylphorbol-13 acetate; AP-1, activator protein responsive element–1; AP-2, activator protein responsive element–2; CAAT, CAAT–box; SP1, a GC–rich-binding zinc finger protein responsive element; mTSs, multiple transcription start sites.

sites, glucocorticoid-responsive elements (GRE), and TPA- and cyclic AMP (CRE)-responsive elements. The absence of a TATA box in the putative promoter region suggested that there may be multiple sites of transcription initiation, and S1 protection and primer extension analyses have shown this to be so [78]. It remains to be determined whether the multiple initiation has any physiologic significance and whether there is any relationship between the position of transcript initiation and the pattern of alternative splicing.

Functional Analysis of the Human Elastin Promoter

Transient transfection analyses using promoter/reporter gene constructs have demonstrated the presence of multiple positive and negative regulatory elements within 5.2 kbp of the elastin promoter and indicated that the core promoter necessary for basal expression is contained within the region −128 to −1 [88]. The positive regulatory and core promoter activities may be explained, at least in part, by the presence of multiple SP1 and AP-2 binding sites within these regions, which may act as general enhancer elements, and DNAse footprinting experiments have indicated that SP1 and AP-2 sites interact with their respective *trans* factors. This notion is supported by the observation that deletion of the segment −134 to −87 containing three putative SP1 binding sites reduced the activity to 10–20% of the reference −475 to −1 construct.

Significantly, when tissues of transgenic mice utilizing 5.2 kbp of the human elastin promoter were analyzed, there was reasonable correspondence between expression of the endogenous gene and the transgene within the tissues [89]. Nevertheless, because of some inconsistencies between the expression of transfected genes and the endogenous gene, collectively these observations suggest that not all elements for tissue- and development-specific expression of the elastin gene reside within the 5.2 kb promoter region tested. It has been observed in a number of genes, including three collagen genes, that the first intron contains segments that act as enhancer elements of promoter activity. Comparison of the intron sequences of the bovine and human elastin genes revealed regions of strong homology only in the first intron, which may contain regulatory elements.

Modulation of Expression by Hormones, Growth Factors, and Cytokines

Transcriptional Regulation

The elastin promoter contains three putative glucocorticoid response elements, which may be functionally active because glucocorticoids have been shown to increase elastin expression in the developing chick aorta, fetal rat lung, and cultured fetal bovine nuchal ligament fibroblasts [90–92]. However, it is clear that glucocorticoid effects on elastin production are context-dependent and that the hormones can either up- or down-regulate elastin production depending on the developmental stage and tissue. For example, glucocorticoids have been reported to down-regulate elastin synthesis in normal cultured human fibroblasts but not in those from keloids [93]. Direct proof that any of these effects are regulated at the transcriptional level is still lacking, and glucocorticoids may act through modulation of production of one or more cytokines.

Insulin-like growth factor-I (IGF-I) has been shown to significantly enhance elastin gene expression in rat neonatal aortic smooth muscle cells both *in vitro* and *in vivo* [94]. Transient transfection experiments with elastin promoter constructs demonstrated that the IGF-I was acting at the transcriptional level and that the responsive element was located between bases −195 and −136 [95]. Gel shift and DNA footprinting analyses suggested that IGF-I acts by releasing a transcriptional repressor, possibly SP3, which binds to an element located between −137 and −127 [96]. This sequence has homology to a retinoblastoma control element. In contrast, recombinant tumor necrosis factor-α (TNF-α) markedly suppressed elastin mRNA levels in cultured human skin fibroblasts and rat aortic smooth muscle cells and also suppressed the expression of elastin promoter constructs in transiently transfected cells, again indicating regulation at the transcriptional level [97]. Detailed analysis of the mechanisms involved strongly suggested that the down-regulatory effect of TNF-α was mediated through *jun/fos* binding to an AP-1 site located at −223 to −229 in the elastin promoter. Basic fibroblast growth factor has also been shown to reduce significantly elastin mRNA and protein levels, and the effect appears to be achieved at the transcriptional level [98]. Conflicting evidence has been presented on the effects of interleukin-1β. One group reported that the cytokine inhibited elastin synthesis and decreased elastin mRNA steady-state levels in cultures of a particular subtype of neonatal rat lung fibroblast [99], whereas another group reported that the cytokine increased elastin gene expression in cultured human skin fibroblasts, apparently at the transcriptional level [100].

Post-Transcriptional Regulation

Although it is quite possible that general transcription factors interact with other factors yet to be identified to achieve regulation of elastin expression, other mechanisms

must be considered. Presently, there is no evidence for translational control of elastin mRNA, but strong evidence for either up- or down-regulation of tropoelastin expression through alteration of elastin mRNA stability by several modulators has been presented. Elastin production, as determined by ELISA, was increased approximately threefold when porcine smooth muscle cells were incubated with TGF-β1 [101]. Although the human promoter may respond to TGF-β [102], the major effect of TGF-β is to increase elastin production by message stabilization [103,104]. Treatment of cultured human fetal lung fibroblasts with TGF-β1 produced a more than tenfold increase in elastin expression with no alteration in transcription but with increased message stability. A phosphatidylcholine-specific phospholipase C, a protein kinase C, and a *ras* -superfamily member are involved in mediating elastin message stabilization [104,105]. When cultures of elastogenic fetal bovine chondrocytes were exposed to 10^{-7} M 12-O-tetradecanoylphorbol-13-acetate (TPA), tropoelastin mRNA levels decreased greater than tenfold, and this was paralleled by a decline in the production of tropoelastin [106]. As determined by nuclear-runoff assay and transient transfection with a human gene promoter-CAT construct, tropoelastin transcription was unaffected by exposure to TPA. The half-life of tropoelastin mRNA in control cells was estimated to be about 20 hours, but exposure to TPA reduced this to 2.2 hours. Similarly, 10^{-7} M 1,25-dihydroxyvitamin D_3, a rather high unphysiologic dose, produced a marked decrease in steady-state and functional levels of tropoelastin mRNA, but transcription was not affected [107]. Collectively, these data indicate that tropoelastin expression can be controlled by post-transcriptional mechanisms.

Potential Morphogenetic Role of Elastin

The potential morphogenetic role of elastin may be critical in developing strategies to treat complex destructive diseases affecting the lung. Masaro and Masaro [108] reported that treatment with all *trans*-retinoic acid reversed the destructive effects of pancreatic elastase administration in a rat emphysema model. It had previously been shown that retinoic acid could increase elastin production in neonatal rat lung fibroblast cultures [109].

MICROFIBRILS

On electron microscopic examination, the microfibrils are seen to consist of apparently tubular structures, about 10–12 nm in diameter (Fig. 1), which possess different staining properties and susceptibilities to enzymatic digestion from amorphous elastin [6]. At high resolution, the microfibrils appear in cross-section as an outer electron-dense shell surrounding an inner lucid core and in longitudinal section as a beaded chain, suggesting that they may be composed of more than one protein. Apparently similar microfibrils are present in many tissues, including those that contain abundant elastin as well as those in which there is no visible or immunoreactive elastin, such as the ocular ciliary zonule and the periodontal ligament. In the dermis, the microfibrillary bundles that connect the deep dermal elastic plexus with the dermo-epidermal junction region are seen to consist of microfibrils indistinguishable from those associated with elastic fibers. In their superficial distribution, no immunoreactive elastin is associated with them, but as they traverse the dermis they are associated with an increasing amount of amorphous elastin.

Because of their insolubility and apparent complexity, chemical characterization of the microfibrils progressed slowly until the late 1980s. The problem of identifying structurally important components was compounded by observations localizing small amounts of several proteins, including amyloid P and decay-accelerating factor, to the elastic microfibrils by immunoelectron microscopy [110,111]. Although they may have some functional role, it is very unlikely that any one of them is a major structural component of the microfibrils. Nevertheless, within the last few years, great progress has been made in the convincing identification of certain proteins as structural components. These structural proteins can conveniently be divided into two groups based on their molecular size: those larger than 125 kDa and those smaller than 115 kDa. Table 3 lists these proteins and provides some distinguishing features, including their human chromosomal location. The larger-molecular-weight group consists of three gene families: fibrillins, latent TGF-β binding proteins (LTBPs), and fibulins. Significantly, these three families share protein domain motifs, as discussed below.

Fibrillins

The largest of these proteins and quantitatively the most important are the fibrillins, 350 kDa glycoproteins that form an integral part of the microfibril structure [7,112]. Electron microscopic images of monomeric fibrillin prepared from human fibroblast cultures reveal an extended flexible molecule approximately 148 nm long and 2.2 nm wide. Multiple fibrillin molecules probably align in a parallel head-to-tail fashion to form a major portion of the microfibrils. Molecular cloning studies have so far identified two distinct homologous human genes encoding fibrillin proteins, one (*FBN1*) located on chromosome 15q15–q21 and the other (*FBN2*) on chromosome 5q23–q31 [113,114]. Analysis of the deduced fibrillin amino acid sequences demonstrated that the proteins contain multiple repeats of a sequence motif previously observed in epidermal growth factor, having six conserved cysteines [115]. Many of these repeats contain consensus sequences that have been associated with calcium binding. A second motif containing eight cysteines, originally found in LTBPs [116], has also been identified in the fibrillins (Figure 9 compares the structures of the FBNs and LTBPs.) Of great interest is the report of a protein in jellyfish having extensive homology to mammalian fibrillin, which identifies fibrillin as a very ancient protein that evolved well before elastin [117].

Latent TGF-β-binding Proteins

Several members of the LTBP family have been cloned and shown to contain repeating domains similar to those found in FBN1 and FBN2 [116,118–122]. TGF-β is always secreted as a latent complex, and in some cases this complex is bound to an LTBP [119,121]. To date, four distinct LTBPs have been cloned and characterized, ranging in size from 125 kDa to 205 kDa. The function of LTBPs remains to be determined since it is clear that they are not necessary to maintain TGF-β in an inactive form and they do not appear to bind mature TGF-β. Although LTBPs may facilitate the secretion of TGF-β or binding of the inactive complex to the cell surface where activation takes place, they are also found as free proteins associated with components of the extracellular matrix. Immunohistological studies have localized both LTBP1 and LTBP2 to microfibrils in elastic fibers, strongly suggesting that one or more of the LTBPs may be a component of these fibrils [119,122]. Within the matrix, the LTBPs may act as a reservoir of TGF-β, which can be

TABLE 3. Microfibrillar Component Proteins

Microfibrillar Protein	Characteristic Features	Human Chromosomal Locus
Fibrillins	350 kDa	
FBN1 (MIM 134797)	Contain EGF and TGF-β- binding protein motifs.	15q15–q21
FBN2 (MIM 121050)		5q23–q31
Latent TGF-β-Binding Proteins (LTBP)	150–205 kDa	
LTBP1 (MIM 150390)	Contain EGF and TGF-β-binding protein motifs.	2p12–q22
LTBP2 (MIM 602091)	Secreted as a complex with latent TGF-β but also found as free proteins.	14q24
LTBP3 (MIM 602090)		11q12
LTBP4		19q13.1–q13.2
Fibulins	100–240 kDa	
FBLN1 (MIM 135820)	Contain EGF and anaphylatoxin motifs.	22q13.3
FBLN2 (MIM 135821)		3p24–p25
Microfibril–Associated Glycoproteins (MAGP)		
MAGP1 (MFAP2) (MIM 156790)	31 kDa; widely distributed in microfibrils.	1p36.1–p35
MAGP2 (MP25) (MIM 601103)	25 kDa	12p12.3–p13.1
Microfibril–Associated Proteins		
MFAP1 (MIM 600215)	Very acidic.	15q15–q21
MFAP3 (MIM 600491)		5q32–q33.2
MFAP4 (MIM 600596)	Frequently deleted in Smith–Magenis Syndrome[1]	17p11.2
MP70 (βig-h3), (MIM 601692)		5q31
Lysyl Oxidase (MIM 153455)	Probably not a structural component.	5q23–q31
Emilin (gp115) (MIM 130660)		Not known

[1]The Smith–Magenis syndrome (MIM 182290) is a multiple congenital anomaly/mental retardation syndrome in which patients manifest a complex phenotype that includes brachycephaly, midface hypoplasia, prognathism, a hoarse voice, growth and mental retardation, speech delay, hyperactivity, and self-destructive behavior. The clinical phenotype ranges from mild to severe.

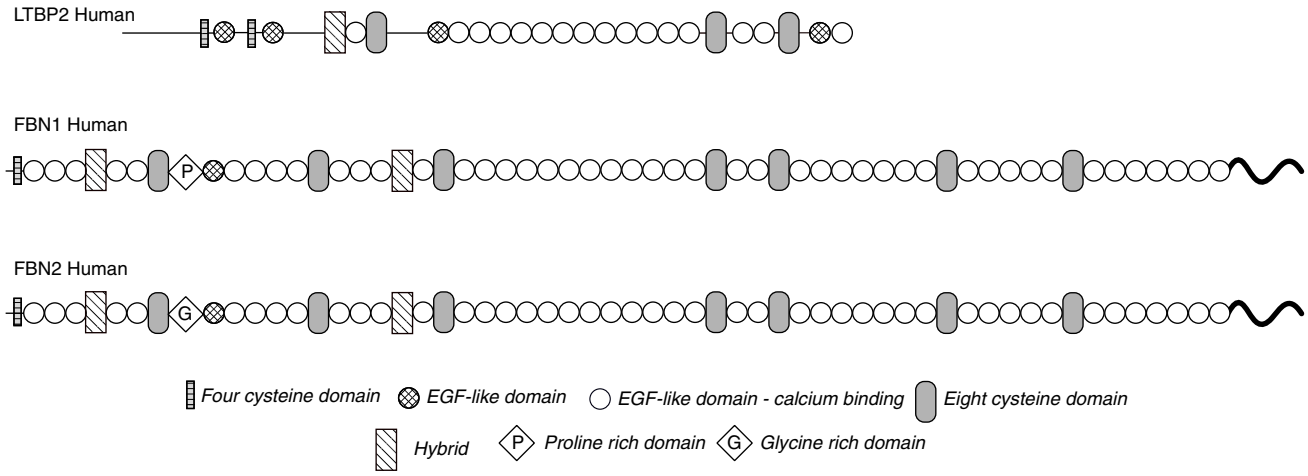

Figure 9. Schematic diagram of the domain structures of the *LTBP* and *FBN* gene families. Schematic representations of the domain structures of human *FBN1* (fibrillin gene found on human chromosome 15), *FBN2* (fibrillin gene found on human chromosome 5), and *LTBP2*. Phylogenetic analysis indicates that the two gene families are related.

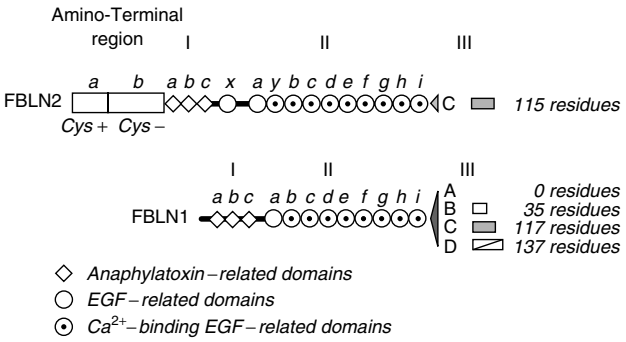

Figure 10. Domain models of fibulins (FBLN)1 and 2. The unique amino-terminal globular region in FBLN2 consists of a Cys-rich (a) and a Cys-free (b) segment. The carboxyl-terminal region III of FBLN1 exists in four alternatively spliced forms, whereas only a homolog of variant C has been detected in FBLN2.

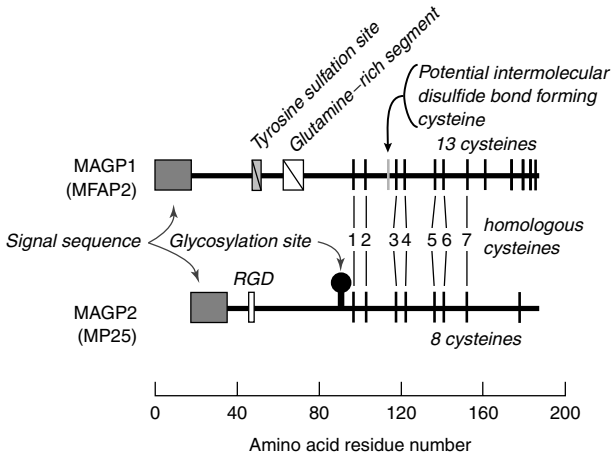

Figure 11. Schematic diagram of the structures of MAGP1, and MAGP2. The unpaired cysteine in MAGP1, which may be involved in intermolecular disulfide bonding, is indicated on the diagram. RGD (Arg–Gly–Asp) indicates a potential integrin receptor binding site.

quickly released and activated in response to various agents, including cytokines and proteases found in the inflammatory response.

Fibulins

The fibulins (FBLNs) are extracellular matrix and blood glycoproteins that can bind fibronectin and can self-associate [123,124]. Molecular cloning has identified two distinct fibulin genes (Table 3). The deduced amino acid sequences reveal that the molecules are modular, having several types of repeated cysteine-containing motifs, including EGF-like and anaphylatoxin-like domains. FBLN1 is alternatively spliced, and four forms have been identified, which differ from one another at their carboxyl termini (Fig. 10). The fibulins are expressed in all major tissues with the exception of the brain, and transcripts are found in fibroblasts and several epithelial, but not endothelial, cells. Fibulin protein is found widely distributed in connective tissues throughout the body. FBLN1 was found to be particularly evident in the basal lamina surrounding smooth muscle cells in blood vessels and in association with elastic fibers in the dermal layer of the skin. However, it did not appear to be associated with the microfibrils that contained fibrillin but was located primarily within the amorphous elastin rather than surrounding it [125].

Other Microfibrillar Proteins

A number of other proteins unrelated to the FBNs, LTBPs, or FBLNs appear to be microfibril constituents. When bovine nuchal ligament, a tissue rich in microfibrils, was extracted with reductive saline, five major bands having M_rs of 340, 78, 70, 31, and 25 kDa were identified upon gel electrophoresis [126]. Molecular cloning of the 25 kDa and 31 kDa components, which were designated microfibril-associated glycoproteins (MAGP), demonstrated that the two share limited regions of homology (Fig. 11) [127,128]. The deduced primary structure of the 31 kDa MAGP1 indicates that it contains two structurally distinct regions; the amino-terminal half of the protein is rich in glutamine, proline, and acidic amino acids, whereas the carboxyl-terminal half contains all 13 of the cysteine residues and most of the basic amino acids.

Further molecular studies have demonstrated that it is highly likely that the 70 kDa component is derived from the 78 kDa protein and, surprisingly, that they are identical to a protein, βig/h3, previously cloned from the lung adenocarcinoma cell line, A549 [129] (see also Chapter 26, Part I, this volume). The 340 kDa component is the bovine homolog of FBN1. The observation that these proteins can be extracted from tissues by several solvents, provided they contain a strong reducing agent, suggests that intermolecular disulfide bonding is an important feature of the association of the polypeptide chains in the fibrils. However, it is also possible that other types of bonds, including those formed by transglutamination, or even derived from oxidation of lysine residues, may be involved. Other candidate microfibril components include the enzyme lysyl oxidase [130], a 115 kDa protein called emilin [131], and several proteins designated microfibril-associated proteins (MFAP) [132–134]. One of the latter, MFAP1, is remarkable in that it is extremely acidic, with glutamic acid comprising 23% and aspartic acid 6% of the residues. The extremely acidic nature of the protein suggests that it may have an important function in the assembly of the very basic tropoelastin molecules.

FIBER ASSEMBLY

Information on fiber assembly is limited, but the notion that secreted tropoelastin molecules simply diffuse onto the surface of growing fibers where they bind and become cross-linked seems inadequate to explain the efficiency of the assembly process, the variable form of elastic fibers in different tissues, and the nonrandom nature of the cross-links [135]. This variability argues for a more directive role of the cell in fiber assembly. It is likely that assembly requires helper proteins both inside and outside the cell. Elastin fibrillogenesis takes place at sites close to the cell membrane, generally in infoldings of the cell surface [136]. Microfibrils are the first visible components of elastic fibers, forming a plexus upon which elastin appears as an amorphous material in discrete loci within each microfibrillar bundle. These amorphous areas gradually coalesce and generate the central core of elastin. The majority of microfibrils are progressively displaced to the outer aspect of the fiber, a position they retain in the mature tissue. The observation that microfibrillar aggregates take the form and orientation of presumptive

elastic fibers suggests that they direct the morphogenesis of elastic fibers by acting as a "scaffold" on which elastin is deposited. This appears to be true not only in developing tissues but also in autografts of regenerating skin [137]. Microfibrils may serve to align tropoelastin molecules in precise register so that cross-linking regions are juxtaposed prior to oxidation by lysyl oxidase.

How secretion of tropoelastin is targeted to sites of elastic fiber assembly remains unknown. The proper folding and trafficking of tropoelastin is thought to be mediated by intracellular chaperones, possibly BiP and FKBP65, which manifests prolyl *cis–trans* isomerase activity [138]. The relationship, if any, of these chaperones, which appear to be localized in early compartments of the secretory pathway, to the 67 kDa elastin-binding protein previously described [139] remains to be determined. Unequivocal identification of the binding protein has not been made, and candidates include the 67 kDa high-affinity laminin receptor, galactosyltransferase, 5′nucleotidase, and several others (for a review, see [140]). Hinek et al. [141] have suggested that the 67 kDa protein is an enzymatically inactive, alternatively spliced form of β-galactosidase, which binds tropoelastin in the endoplasmic reticulum and chaperones its secretion to assembly sites on the cell surface designated by cell-matrix receptors that interact with microfibrillar proteins [139]. At the plasma membrane, tropoelastin may remain bound to the 67 kDa protein until interaction with a microfibril induces the transfer of tropoelastin to the growing fiber. Biochemical studies have shown that the 67 kDa elastin-binding protein is a galactoside lectin and that its affinity for elastin is greatly diminished by interactions with carbohydrates. It is possible that galactosyl residues on microfibrillar glycoproteins provide specific signals for the release of tropoelastin at sites of fiber assembly. Supporting a matrix-assembly function for the 67 kDa protein are studies showing inhibition of elastin fiber assembly by the addition of lactose or galactose sugars to the culture medium of elastin-producing cells [142]. In the presence of lactose, the majority of newly synthesized tropoelastin is prematurely released directly into the medium from the cell layer before it can be transferred to acceptor sites on microfibrils. The 67 kDa membrane-associated protein that specifically binds tropoelastin also binds the hexapeptide Val–Gly–Val–Ala–Pro–Gly (found seven consecutive times in human elastin and five consecutive times in bovine elastin) [139]. The synthetic hexapeptide and tropoelastin are also chemotactic for ligament fibroblasts and monocytes [143]. The 67 kDa protein is associated with two other integral membrane proteins of 61 kDa and 55 kDa. Taken together, these observations suggest that the 67 kDa protein may be an elastin receptor, possibly transmitting a signal to the interior of the cell through association with the 61 kDa and 55 kDa proteins. Such a receptor could be involved in modulating the position of the cell surface where tropoelastin secretion is taking place and/or modulating the association of the cell surface with the growing elastic fiber, thereby affecting the form of the fiber.

Persuasive evidence exists that the carboxyl-terminal end of tropoelastin mediates interactions with microfibrils. This portion of the molecule contains two cysteine residues, which can form a disulfide bond that stabilizes a positively charged pocket suitable for binding acidic microfibrils [144]. This segment binds the 31 kDa MAGP1, and monospecific antibodies directed against either the carboxyl portion of tropoelastin or a domain near the amino terminus of MAGP1 block elastic fiber assembly [145].

RECENT DEVELOPMENTS

Elastin

Previous studies demonstrated that basic fibroblast growth factor (bFGF) decreases elastin gene transcription. Recent studies have provisionally identified the *cis* element and *trans*-acting factors responsible. The bFGF-responsive element has been localized to −564 to −558 of the elastin promoter. The data indicated that bFGF induced expression of the transcription factor Fra1, which binds to the element as a heterodimer with c-Jun to form an inhibitory complex [148]. In other work, Keeley and co-workers have identified a GA-rich sequence in the 3′-UTR of elastin mRNA which is highly conserved in several species, and which preferentially binds proteins found in tissues rapidly synthesizing elastin [149,150]. Since these proteins are particularly abundant when elastin mRNA is stable, they postulated that they may be involved in such stabilization. The GA-rich segment does not resemble other known *cis*-acting 3′-UTR sequences affecting mRNA stability and no other relevant occurrences of the sequence were identified in the data base.

Fibrillins

The genomic organization of the *FBN1* gene has been revised. Previously, the existence of three exons upstream of the exon containing the putative initiating methionine raised the possibility that alternative isoforms varying at their N-termini existed. New data that demonstrate extreme conservation between the human and porcine genes argue against this possibility and for a single translational start site [151].

Much recent work has centered on the interaction of the fibrillins with other components of the elastic fiber. The amino terminal domains of fibrillin-1 and fibrillin-2 have been shown to interact with tropoelastin in solid-phase binding assays [152]. A folded secondary structure of the fibrillin molecules was necessary for binding, which was mediated by ionic interactions involving the lysine side chains of tropoelastin. Mature elastin, the lysine side chains of which form crosslinks, did not bind. These results support the concept that fibrillins play an important role in elastic fiber assembly by binding tropoelastin and perhaps facilitating side chain alignment and crosslinking. Several studies have highlighted the importance of fibrillin/proteoglycan interactions for normal microfibril formation. Decorin interacts with MAGP-1 and fibrillin-1 individually and with both proteins to form a ternary complex [153]. Fibrillin-1 has also been shown specifically to bind heparin/heparan sulfate through both calcium-dependent and calcium independent sites [154]. Inhibition of sulfation or the attachment of glycosaminoglycans to core proteins can result in a significant reduction of the fibrillin network.

Latent TGF-β-binding Proteins

The ability of the members of the LTBP-fibrillin family to associate via their 8-Cys repeats with TGF-β isoforms has been analyzed [155]. TGF-β1 associates with the third 8-Cys repeat of LTBP-1 through the latency-associated peptide (LAP) by direct cysteine bridging. LTBP-1 and LTBP-3 efficiently bound all TGF-β isoforms, while LTBP-4 had a much weaker binding capacity. In contrast, LTBP-2, as well as fibrillin-1 and fibrillin-2, did not bind. A short, specific TGF-β-binding motif that contained a hydrophobic interaction surface was identified in the TGF-β-binding 8-Cys repeats.

Deletion of the *Ltbp2* gene in mice is embryonically lethal, death occurring between E3.5 and E6.5 [156]. Expression could not be detected in E6.5 embryos, but was detected in E3.5 blastocysts. These results imply that LTBP-2 performs a yet undiscovered function in early development, perhaps in implantation. The distribution of LTBP-1 has been examined in cultures of differentiating mouse embryonic stem (ES) cells [157]. During *in vitro* differentiation of embryoid bodies, extracellular fibers containing LTBP-1 colocalize with specific differentiating cell lineages, including endothelium. The results suggested that during differentiation of ES cells, LTBP-1 facilitates endothelial cell organization via a TGF-β-dependent mechanism.

Fibulins

Fibulin-1 and fibulin-2 have previously been identified as basement membrane and microfibrillar proteins with a broad binding repertoire for other extracellular ligands. Two novel fibulins, fibulin-3 and fibulin-4, have been cloned and shown to be most closely related to fibulin-1C [158]. They consist of a C-terminal globular domain III, also shared by the fibrillins, a central rod-like element composed of five calcium-binding epidermal growth factor-like (EG) modules and an N-terminal interrupted EG module which replaces the anaphylatoxin-like modules of the other fibulins. Both novel fibulins are expressed in several human tissues with particularly strong expression in the heart. Immunohistology of adult mouse tissues has shown that fibulin-3, fibulin-4, and fibulin-1 have overlapping but distinct extracellular tissue localizations, with particularly prominent staining of some large and small blood vessels.

EMILINs

The EMILINs (**e**lastin **m**icrofibril **i**nterphase **l**ocated prote**ins**) comprise a new extracellular glycoprotein family. First isolated from chicken aorta, they are now known to be widely distributed in association with elastin and localized at the interface between elastin and microfibrils [159]. Two human EMILINs have been cloned and are characterized by a C-terminal gC1q globular domain, a short collagenous sequence, a long coiled-coil region and a new cysteine-rich N-terminal domain also found in the protein multimerin. The gene for EMILIN-1 was mapped to chromosome 2p23 overlapping the promoter of the ketohexokinase gene [160]. The gC1q domain can form relatively stable homotrimers, which process is followed by the multimeric assembly of disulfide-bonded protomers.

Lysyl Oxidase

Several cDNAs and genes encoding lysyl oxidase-related proteins have been described. These lysyl oxidase-like proteins were identified by overall sequence homology and the presence of conserved domains, including the copper-binding site, and lysyl and tyrosyl residues known to be involved in the formation of the lysyl tyrosyl-quinone cofactor found in lysyl oxidase [161]. Presently, three human lysyl oxidase-related proteins have been identified. Multiple functions previously attributed to lysyl oxidase, such as tumor suppression, cell growth control and chemotaxis, possibly unrelated to amine oxidase activity, may be performed by these or other novel lysyl oxidase-like proteins.

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Chapter 4

Glycosylated Matrix Proteins

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INTRODUCTION

Connective tissues have many features in common, including resisting and dissipating mechanical load and providing shape, as well as acting to provide barriers regulating water flow and diffusibility of macromolecules. These common properties are reflected by their composition, many matrix constituents being shared among tissues, and others having similar structural and functional properties.

There are, however, connective tissues with rather distinct properties and, therefore, a partly different composition. One such example is bone, in which the major constituent, hydroxyapatite mineral, is important for tissue properties. At the same time, some of the features of bone, including mineralization and expression of select matrix molecules, are observed in pathology (e.g., in atherosclerotic lesions).

In view of the many common features among connective tissues, we have chosen to focus on cartilage and bone, covering many aspects of connective tissues. It is important to stress that the description is largely representative for other tissues.

Skeletal tissues play key roles in our ability to move and to maintain this function over many years. Rigid bones are thus used as levers, with mobility being contributed by the joints. Articular cartilage plays a fundamental role in providing low-friction movement as well as in absorbing and distributing mechanical load. The shape and organization of the skeletal elements are strictly directed toward achieving optimal function. In development, the shape of the skeleton is largely laid down as a cartilage scaffolding, providing the template for subsequent bone formation. In this process of endochondral bone formation, the cartilage undergoes distinct developmental changes and is eventually replaced by bone, except in the articulating cartilage of the joints.

Bone and cartilage are dynamic tissues, such that there is a continuous turnover in response to altered load and mechanical fatigue. A number of matrix proteins provide key elements in this process as building blocks that are assembled into the intricate organization of the tissue extracellular matrix. Others have roles in catalyzing the assembly process, and yet others send signals back to the cells about the properties of this matrix.

In recent years, an increasing number of cartilage and bone matrix proteins with functions in forming the tissue structure as well as in the remodeling process have been identified. Although our knowledge of specific functions of the particular proteins is somewhat limited, this information is being assembled and provides new insights into tissue properties.

Overall, we can view both cartilage and bone as composite materials. In cartilage, an organic matrix is made up of rope-like structures of collagen II fibers [1–3] that are linked together by a number of collagen-binding proteins. These latter also appear to provide linkage to other matrix constituents. In this fibrous network, large, extremely anionic proteoglycans provide a fixed charge density that maintains a high osmotic pressure, thereby promoting water retention [4–6]. The proteoglycans are also bound to other matrix constituents, including collagen, via a number of interactions.

In bone, the major organic component is collagen I, which forms the structural framework for the deposition of hydroxyapatite mineral, which is the predominant inorganic component. Together, these constituents form the rigid structure of bone. Other matrix constituents play important roles in regulating the deposition and organization of hydroxyapatite, such that there is always a thin layer of nonmineralized osteoid tissue.

An important feature in skeletal biology is a variable, pronounced mechanical load that will induce cellular responses [7–9]. When the load is abnormal, it may accelerate induction of material fatigue. An altered direction or magnitude of the load will result in a modified mechanical environment that induces remodeling of the tissue. In cartilage, this occurs with gradual modifications within the maintained scaffold of the tissue, where the general architecture remains intact, possibly because the collagen fibers play a fundamental role that should not be compromised. The collagen thus only turns over very slowly in comparison with, for example, the large proteoglycans [10]. In bone, on the other hand, remodeling occurs through the removal of the mineralized matrix by specialized cells, osteoclasts, creating a tissue defect that is then filled with new bone by another set of cells, osteoblasts. One consequence of these different modes of turnover is that cartilage changes its composition with aging and in disease, while bone maintains a much more conserved composition, although the total amount of tissue may change when whole tissue segments are replaced at appropriate locations. Thus, in pathological conditions, the most marked feature is an

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imbalance between the removal and building up of bone, with an overall loss of bone mass.

The increasing understanding of the roles of the many different matrix constituents involved in these processes provides an essential background for understanding the changes occurring in the tissues, with regard to both the formation of new tissue and the modifications of existing tissue.

Cartilage and bone, being rather different in structure and composition, are treated separately below, with the focus being on the noncollagenous proteins in their matrices.

CARTILAGE

The cartilage matrix, as illustrated schematically in Figure 1, consists of a fibrillar network based on collagen fibrils dispersed in an environment of highly negatively charged aggrecan molecules that form large supramolecular aggregates. A number of noncollagenous proteins, often substituted with oligosaccharides, polysaccharides, phosphate, and sulfate, contribute to the properties of the matrix by cross-bridging the various constituents, including the collagen fibrils. Also, among these molecules, some play apparent roles in catalyzing and regulating matrix assembly.

As depicted in Figure 1, the matrix can be divided into domains. The pericellular matrix contains molecules that provide feedback by interacting with cell surface ligands. The territorial domain contains a set of molecules, one of the roles of which is to protect cells and regulate early steps in matrix assembly. The interterritorial matrix, at a larger distance from cells, appears to play a primary role in contributing mechanical properties.

To understand the combined properties of the molecules in the assemblies that constitute the matrix, it is important to know the properties of the individual constituents. Following, is an account of some of the known key features of the various matrix constituents, excluding collagenous molecules, although information is provided on noncollagenous molecules that interact with collagens.

Aggrecan

Aggrecan, the large aggregating proteoglycan in cartilage ($M_r \sim 3 \times 10^6$), and versican (see below) in other tissues, play major roles in providing fixed charge groups that attract counter-ions. Thereby, an osmotic pressure is created in the cartilage, retaining water and restricting water flow [4,11–14]. Thus, the proteoglycan exerts a swelling pressure within the tissue, which is resisted by the collagen network [15]. One consequence of this striving to retain water is that deformation is counteracted and limited when the tissue is loaded. This is of essence for the function of aggrecan in taking up and distributing load over the cartilage tissue and further onto the underlying bone. In fact, the charge density of aggrecan is so extreme that it creates a very high swelling pressure in the tissue [4,13,14]. Over the years, considerable information on the detailed structure of aggrecan, its interactions, and its turnover has been gathered [6,16,17]. Thus, aggrecan contains several structural domains (Fig. 2). The core protein of $M_r \sim 210$ kDa contains an N-terminal domain, G1, that binds specifically ($k_d 10^{-8}$ M) [18] to hyaluronan via a decasaccharide sequence [18,19]. The binding of hyaluronan depends on an immunoglobulin fold, of which G1 contains two. Some information on the folding of these structures and their interaction with hyaluronan can probably be derived from data on TSG-6, another related hyaluronan-binding protein [20]. The binding of aggrecan to hyaluronan is such

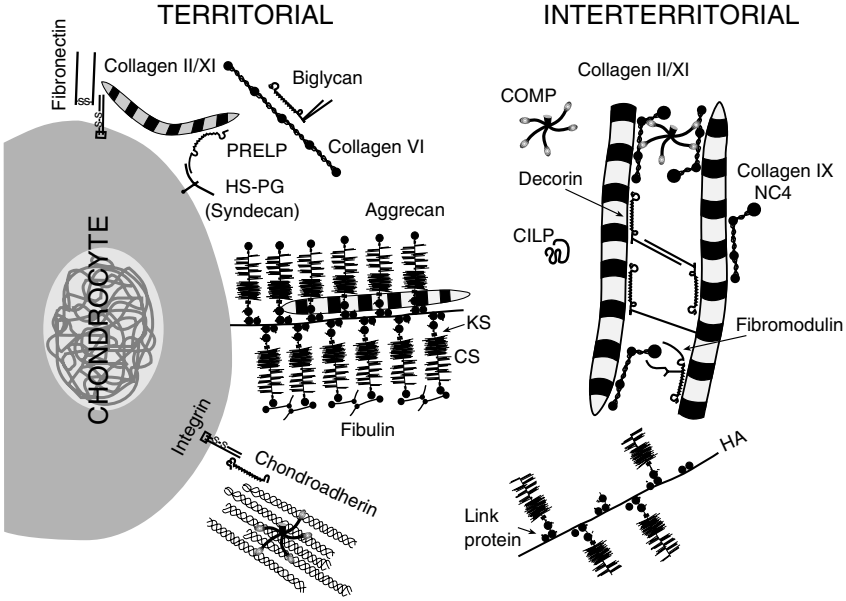

Figure 1. Schematic illustration of the organization of cartilage matrix and its molecular constituents. The composition and organization of the matrix is illustrated. Only those macromolecules are depicted for which there is available information on localization and/or interactions with other matrix constituents. The territorial matrix close to cells contains a number of matrix molecules that interact with cell-surface molecules, including receptors such as integrins. This compartment also includes aggrecan arranged in close association with cells and with collagen fibrils. The interterritorial matrix at a greater distance from cells contains interconnected collagen fibrils and interspersed aggrecan molecules having formed aggregates with hyaluronan. PRELP, proline- and arginine-rich end leucine-rich repeat protein, HS-PG, heparan sulfate proteoglycan; COMP, cartilage oligomeric matrix protein; CILP, cartilage intermediate layer protein; NC4, noncollagenous domain 4.

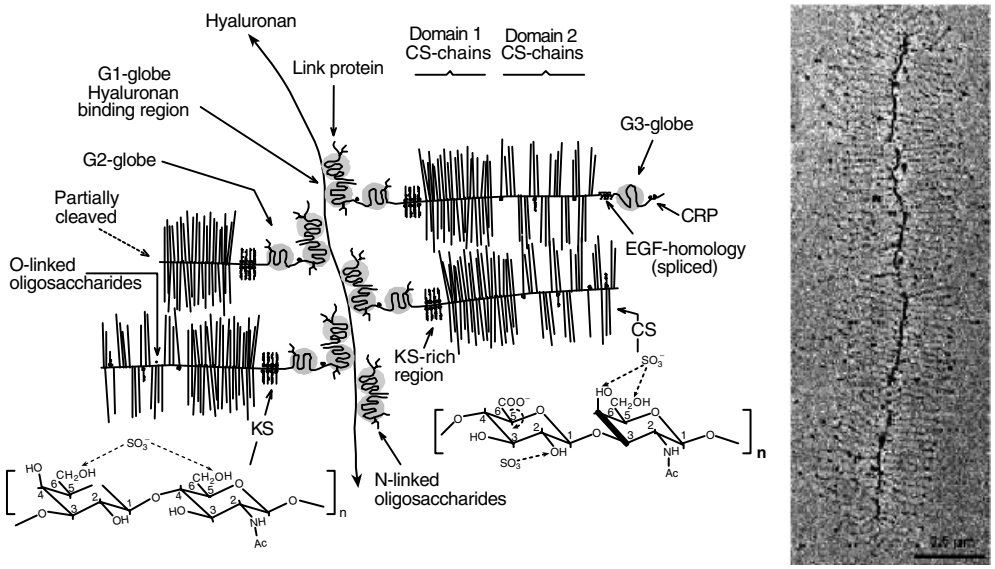

Figure 2. Schematic illustration of the aggrecan aggregate with hyaluronan. Several aggrecan molecules interact with hyaluronan, and the complex is stabilized by link protein. The aggrecan core protein contains glycosaminoglycan chains differently organized toward the N-terminus compared with toward the C-terminus. These glycosaminoglycan chains are composed of a number of repeat disaccharide units (illustrated) for CS approximately 40 and for KS 10–30. A rotary shadowing electron-microscopic picture of the aggregate illustrates the overall dimensions, where each side-chain filament represents an aggrecan molecule. The central core is hyaluronan with bound G1 and link protein. This electron-microscopic picture was kindly provided by Dr. Matthias Mörgelin, Lund University. CS, chondroitin sulfate; KS, keratan sulfate; CRP, complement regulatory-like protein; EGF, epidermal growth factor.

that more than one hundred aggrecan molecules may bind to a single, very long filament of hyaluronan, thereby forming complexes that may reach micrometers in dimension [17]. Such complexes are formed with hyaluronan bound at the cell surface [21], as depicted in Figure 1, as well as in various compartments in the matrix. This binding is stabilized by an additional protein, the link protein, that is homologous to the G1 domain and binds with the same specificity and affinity to hyaluronan [18] and the G1 domain [22,23]. This ternary complex is very stable and functionally not dissimilar to a covalent bond.

A second globular domain, G2, shows extensive homology to the G1 domain but lacks the ability to bind to hyaluronan [17,24,25]. The two domains are separated by a short, extended interglobular stretch of amino acids. This may be cleaved either between the amino acid sequences Pro–Glu–Asn and Phe–Phe–Gly (metalloproteinases) or between Glu–Gly–Glu and Ala–Arg–Gly (aggrecanase, ADAMTS) [26,27] in both normal and pathological turnover. Following G2, there is an extended domain carrying the majority of the some 30 relatively short keratan sulfate chains [28,29]. These chains contain a variable 5–20 disaccharide repeat of galactose and N-acetyl-glucosamine, the latter carrying a 6-O-sulfate group (Fig. 2). The polymer also contains extra sulfate groups at certain galactose residues, with the result that regions of higher charge are present [30]. The majority of the keratan sulfate chains are bound to a polypeptide motif of six amino acid residues in a continuous 23-fold repeat in bovine aggrecan [28,29]. The number of repeats in this domain varies among species [31].

The next major part of the core protein carries chondroitin sulfate chains attached in their reducing termini to serine residues via a xylose followed by a galactose-galactose-glucuronate sequence, which is followed by the repeat disaccharide shown in Figure 2 [32]. This is the domain carrying the majority of the charged groups and, indeed, one function of this part of the proteoglycan is to fix these charged groups in the matrix [4]. Thus, each of these chains contains, on average, some 40 to 50 repeat disaccharides of glucuronate and N-acetyl-galactosamine, the latter carrying an O-sulfate group at the 4 or 6 position [33,34], (Fig. 2). The sulfation pattern varies with age and in disease [35]. The distribution of the approximately 100 chondroitin sulfate chains is variable along this part of the core protein. Thus, two subdomains with somewhat different clusterings of these chains are observed [24,25,36], as indicated in Figure 2. Overall, however, the packing of the charged chondroitin sulfate is high.

There are additional carbohydrate substituents in the form of some 40 mucin type O-glycosidically linked oligosaccharides. These are linked to serine and threonine in the protein core via an N-acetylgalactosamine residue carrying a galactose-sialic acid disaccharide at the 3 position and an N-acetylglucosamine-galactose disaccharide in the 6 position [37]. This galactose is substituted with sialic acid in the oligosaccharide structures, while in keratan sulfate it is extended by a variable number of the repeat disaccharide shown in Figure 2. Interestingly, in fetal life there is little keratan sulfate in aggrecan, and all sites are occupied by the O-glycosidically linked oligosaccharides, totaling some 70 [38]. There appears to be a gradual replacement of these aggrecan molecules by those of the adult type with keratan sulfate through adolescence.

In the C-terminal part of the molecule, the globular G3 domain consists of a variably spliced EGF (epidermal growth factor) homology motif [39], a lectin homology motif, and a complement regulatory protein (CRP)-like

motif [25,36]. Data indicate that the lectin-like region may bind monosaccharides [40]. However, the best characterized interactions are from protein to protein, involving fibulins [41,42] as well as tenascin [43]. The interaction with fibulin involves several calcium-binding EGF repeats in the protein. Both the fibulins and the tenascins have the ability to self-interact to form multimers. Thus, this interaction of the C-terminal domain of the aggrecan may play an important role in linking cartilage molecules into a network, as illustrated in Figure 1. It is of interest to note that the fibulins are present in developing and growing young cartilage [44] but notably absent from adult normal cartilage [42]. Also, adult cartilage contains a much lower proportion of aggrecan containing the G3 domain, apparently as a result of progressive cleavage of the molecule, as indicated in Figure 2. We may therefore assume that this interaction of aggrecan with fibulins represents another molecular complex that primarily has a role in matrix assembly.

A very large complex of many aggrecan molecules bound to hyaluronan in cartilage is likely to have important functions in sequestering molecules in the matrix and in regulating the diffusion of nutrients and molecules participating in the assembly of the matrix, as indicated in Figure 1. It has actually been demonstrated that aggrecan, via its keratan sulfate-rich domain, appears to bind tightly to collagen *in vitro* ($k_d 10^{-8}$ M) [45]. In the tissue, collagen fibers in the pericellular and territorial domains are found located in the interior of the aggregate [45], as illustrated in Figure 1. The binding in the tissue should be much tighter, being multimeric as a result of the multimolecular assemblies in the aggregate and of collagen. It is particularly interesting that aggregates are already apparently formed at the cell surface, being bound to hyaluronan present there [21]. As a consequence, it is likely that the pericellular environment is organized in a way that may be important for promoting matrix assembly.

Aggrecan in Disease

A prominent feature of joint disease is that the levels of aggrecan in articular cartilage are considerably reduced. This happens as a result of the proteolytic degradation of aggrecan molecules, a process in which at least two distinct enzymes appear to be involved. The major activity seems to be that of the so-called aggrecanase, recently shown to be a member of the ADAMTS family of zinc-binding metalloproteinases [46–48]. Apparently, several of these enzymes [49] are responsible for both normal aggrecan turnover and increased turnover upon immobilization of the joint and, particularly, the increased release in many inflammatory processes, such as reactive arthritis [50]. Even though there may be extensive loss of aggrecan from the tissue in such conditions, the process can apparently be reversed since the outcome is usually complete restitution.

Other metalloproteinases that cause release of aggrecan result in a different pattern of cleavage. Thus, enzymes such as stromelysin (MMP-3) cleave between an asparagine residue and a phenylalanine residue in the interglobular domain between G1 and G2 (–Pro–Glu–Asn Phe–Phe–Gly–) [51,52]. Aggrecanase, on the other hand, cleaves between a glutamic acid and an alanine residue in this interglobular domain (–Glu–Gly–Glu Ala–Arg–Gly–) [26,49,51–53]. There are additional cleavage sites in the domain carrying the chondroitin sulfate chains [54]. The relative role of the activity of metalloproteinases such as stromelysin and aggrecanase is not clear, although it may be speculated that they may have different relative activities in different pathological joint conditions.

One consequence of the loss of aggrecan from the tissue, and thereby the loss of fixed charged groups, is that the osmotic environment is altered and the ability to retain water impaired. This causes a less efficient distribution of load over the tissue, and the process may become a vicious circle with impaired ability of the cells to accomplish repair.

In both osteoarthritis and rheumatoid arthritis, loss of aggrecan is a typical finding. However, very early in the process of osteoarthritis, the level of aggrecan appears quite conserved in cartilage. At the same time, the staining of fixed charged groups, by, for example, alcian blue, ruthenium red, and toluidine blue, may be impaired, possibly due to blocking of the polyanionic groups by other newly synthesized matrix proteins laid down in the matrix (P. Lorenzo, M. Bayliss, and D. Heinegård, unpublished).

Versican

Versican is a proteoglycan with a wide distribution among connective tissues, including blood vessels and cartilage. This proteoglycan is also present in brain. It has a number of features in common with aggrecan [55]. Versican contains an N-terminal hyaluronan-binding motif and a C-terminal globular domain homologous to corresponding domains in aggrecan. Its central extended domain carries the glycosaminoglycan chains [55–57], typically chondroitin sulfate. Versican, in contrast to aggrecan, contains no G2 globular domain, thus only having two globules.

Altogether, the two molecules have many properties in common. These include a capacity to bind tightly to hyaluronan in an interaction stabilized by link protein [56,57]. The C-terminal domain also shares many features of the homologous domain of aggrecan. The C-type lectin motif in this domain binds tightly to tenascin-R. This interaction is common for all four molecules of the hyaluronan-binding proteoglycans (i.e., aggrecan, versican, neurocan, and brevican) [43,58]. In contrast, only aggrecan and versican show a pronounced binding to fibulins 1 and 2 [41,42]. This binding depends on divalent cations and involves EGF domains in the fibulins. It is of particular interest that this interaction may play roles in matrix buildup by providing a bond between one set of molecular complexes in the form of versican aggregates to other putatively network-forming molecules such as fibulins and tenascins. The central domain where the glycosaminoglycan chains are attached is quite variable in that there are variants resulting from differential splicing. There are four such variants, V0, V1, V2, and V3 [59–61]. The number of glycosaminoglycan chains varies from 5 to 23 depending on which alternatively spliced exons are present. The V0 and V1 forms are present in most tissues, while the V2 forms show a more restricted distribution in brain compartments [62]. It appears that the V2 variant represents a regulating factor in axonal growth [63]. The V3 variant has so far only been observed at the mRNA level. The overall functional implications of this variability are currently not apparent. Although it can be speculated that versican may play a role in contributing a negative fixed charge density to tissues, it is not clear how similar this may be to the contribution of aggrecan to cartilage properties. Other possible roles for versican are in tissue assembly and in regulating cellular activities [64].

The Collagen Network

A major constituent of cartilage is the collagen fiber network, which consists predominantly of collagen II, with

a very minor proportion of collagen XI molecules [65] (see also Chapter 2, Part I, this volume). Some of these fibers are also associated with collagen IX at their surfaces, as indicated in Figure 1, to a large extent covalently cross-linked to the collagen II in the fibrils [66]. Collagen IX contains globular domains, in which the basic C4 domain extends out and away from the collagen fiber, free to interact with surrounding matrix constituents.

The collagen fibers are of variable dimensions, such that they are thinnest closer to cells and much more variable and of larger diameter further away in the interterritorial matrix [67]. The process of collagen fibril assembly is regulated by constituents produced by the cells, which also produce molecules that provide the completed fibril with a surface coat that enhances its ability to interact with other surrounding fibrils.

Following is an account of molecules with the ability to bind to the collagen fiber and to modulate fibril assembly.

Thrombospondins

There are currently five known members of the thrombospondin family (see also Chapter 5, this volume). In cartilage, COMP (cartilage oligomeric matrix protein), or thrombospondin-5, is particularly prominent, but thrombospondins −1 and −4 have also been identified in this tissue [68,69]. COMP is found primarily in cartilage and related structures such as meniscus and intervertebral disc. It is also present in structures with related morphology, such as pressure-loaded parts of tendons [70–74]. Very low levels (several orders of magnitude less) may be found in tissues such as synovial capsule [75]. The proteins of this family form two distinct subgroups, being either homotrimers or homopentamers.

COMP — cartilage oligomeric matrix protein. COMP is a classical example having five identical subunits, each with a molecular mass of about 87 kDa [76]. The subunits contain important elements found also in other connective tissue proteins, including calcium-binding modules [72]. The subunits are linked together close to their N-termini in a heptad-repeat structure [77], which is further stabilized by disulfide bonds. The portion of the molecule containing the heptad repeat has been expressed in recombinant form and its structure determined by X-ray crystallography [77]. Interestingly, it forms a central hydrophobic channel, where retinoids and 1,25-dihydroxyvitamin-D_3 appear to be possible ligands. In view of the close proximity of COMP to cells in growing cartilage [78], this may imply a function in the delivery of these hydrophobic factors to cells. The C-terminal part of each chain forms a globular structure, apparently in an overall design suitable for cross-bridging several other matrix components.

COMP shows a distinctly different distribution in adult cartilage from that in young immature cartilage, including the proliferative zone of the growth plate, in which it is particularly abundant. Thus, in young cartilage there is a pronounced pericellular distribution, whereas in older cartilage the protein appears to be restricted to the interterritorial matrix at a distance from the cells [78]. Whether this results from a difference in structure of the COMP molecule resulting in a different ability to interact, or whether it is dependent on changes in other matrix proteins, is not clear. There are indeed some changes in COMP structure with aging, albeit not in the protein itself. Thus, the oligosaccharide substituents differ between young and old cartilage. At all times, the middle of the three putative carbohydrate attachment sites is not occupied; the C-terminal site carries an N-glycosidically linked oligosaccharide, which is the same in the two age groups, while the N-terminal site shows differences. In the adult, at this site, there is only one type of oligosaccharide, which differs from the four different substituents observed in young tissue [76]. The implications of these differences are not yet clear.

Thrombospondin-1. Thrombospondin-1 is structurally related to COMP but shows considerable structural differences. It contains three subunits of approximately 130 kDa, which are considerably larger than those of the COMP subunit [79]. These chains are also bound in a heptad-repeat structure located at a similar distance from the C-terminus as in COMP, leaving a free, extended N-terminal domain that contributes heparin-binding properties to the molecule. Also, the chains of thrombospondin-1 have a globular domain at their C-termini.

It is likely that many of the functional properties of the thrombospondins are shared among the various members of the family. However, whereas, for example, thrombospondin-1 contains a heparin-binding domain and has well-documented cell-binding properties, this is not the case for COMP. Other functions are most likely shared and are discussed for COMP only.

Function of COMP. Details of the functional role of thrombospondins, such as COMP, in cartilage are not well-known. However, mutations affecting the calcium-binding motif, impairing calcium binding, result in severe growth disturbances; e.g., multiple epiphyseal dysplasia or pseudoachondroplasia [80] (see also Chapter 23, Part II, this volume). One may conclude that part of the phenotype depends on the deposition of the mutated COMP in the endoplasmic reticulum of the cells, thereby disturbing trafficking and secretion. This may occur through the intracellular formation of complexes between the mutated COMP and other molecules perhaps specific for the chondrocyte. One such molecule may be collagen IX, which has been demonstrated in intracellular deposits [81]. Indeed, more recent data show that collagen IX interacts with COMP at $k_d 10^{-9}$ M regardless of the presence of the mutation [82].

It has been reported that COMP can bind to cells [83], but details of this interaction have not been elucidated. It is not clear whether this is direct binding to a cell-surface receptor or whether it involves secondary interactions with other matrix constituents. There is no identifiable cell-binding motif in COMP that is common to all species. In some species, the protein contains an Arg–Gly–Asp (RGD) sequence implicated in integrin binding, but in others this is lacking. Also, the prevalence in proteins of this sequence is much more common than the ability of proteins to bind cells. An additional piece of information to consider is that, in normal adult articular cartilage, the protein is located at a distance from the cells [78], thereby automatically excluding direct interactions.

One clue to the function of COMP was obtained from studies with collagens I and II, to which the protein was demonstrated to bind with dissociation constants of the order of $k_d 10^{-9}$ M via the C-terminal globular domains present on each chain. This interaction is dependent on the presence of zinc ions, and calcium does not seem to promote the interaction. There are four apparently identical COMP-binding sites on the collagen molecules. However, a single COMP molecule cannot span two such sites in a single molecule. Therefore, it is likely that COMP will cross-bridge to neighboring collagen molecules, perhaps thereby

playing a role in fibril assembly and/or stability, as indicated in Figure 1. More recent data indicate that COMP may also bind to collagen IX [82], as indicated in Figure 1. In view of the presence of covalently cross-linked collagen IX on the surfaces of collagen II fibers [66], this would provide a site for interactions of COMP also with collagen fibrils. This may also provide an explanation for the fact that mutations in collagen IX result in a phenotype similar to that of pseudoachondroplasia or multiple epiphyseal dysplasia [84].

Matrilins

Matrilin-1, also named CMP (cartilage matrix protein) [85–87], is the first described member of a novel family of connective tissue proteins. Matrilins -1 and -3 are particularly prominent in cartilage [88]. Indeed, matrilin-1 is selectively found in some cartilages, including tracheal, nasal, ear, and epiphyseal cartilage, while it is absent from adult articular cartilage and intervertebral disc structures [89]. It is a trimer with three identical subunits, each with a molecular mass of around 50 kDa. The subunits are held together by a coiled-coil domain, and the interaction appears to be stabilized by disulfide bonds. A functional structure present in all matrilins is the von Willebrand factor A domain (vWF A). Matrilin-1 contains two such domains, while matrilin-3 contains only one [88,90,91] The vWF A domain would be expected to contribute collagen-binding properties to the matrilins. Indeed, it has been shown that matrilin-1 can bind to collagen and appears to colocalize with collagen in cartilage [92]. In addition to their collagen-binding properties, it appears that matrilins may self-assemble, forming their own network [93], probably through the vWF A domains. The much increased accumulation of the protein in cartilage with age [94] may involve the formation of large complexes and perhaps its own fibrillar structure.

The detailed functions of the several members of this family are, however, not clear, and their potential for variability is extended through their being able to form multimers with combinations of various subunits. Thus, a composite protein containing subunits of both matrilins -1 and -3 is present in cartilage [95,96]. As a consequence, there are several levels of fine-tuning of the functions of the matrilins.

LRR Leucine-Rich Repeat Proteins

LRR (leucine-rich repeat) proteins (Figure 3) constitute a family of proteins with a central domain containing a number of repeats of approximately 25 amino acids with leucine residues at conserved locations. In the extracellular matrix, there are nine known closely related such proteins with core proteins of molecular weights around 40 kDa. These are decorin [97], biglycan [98,99], asporin [100], fibromodulin [101], lumican [102], keratocan [103], PRELP [104], osteoadherin [105], and chondroadherin [106], containing 10–11 repeats. There are also three members with six repeats yielding core proteins of around 30 kDa: mimecan/osteoglycin [107], epiphycan/PG-Lb [108,109], and opticin [110]. Little is known of the functions of some of these (keratocan, mimecan, opticin, and epiphycan), and they are not further detailed here. From X-ray crystallographic studies of another LRR protein, ribonuclease inhibitor [111], some information on the structure of the repeats can be inferred [112], albeit that this protein has a larger number of somewhat longer repeats. Studies of the ribonuclease inhibitor showed each repeat to comprise a β-strand and a parallel α-helix in a hairpin structure aligned parallel to a common axis creating a horseshoe-shaped structure, the outer face of which is

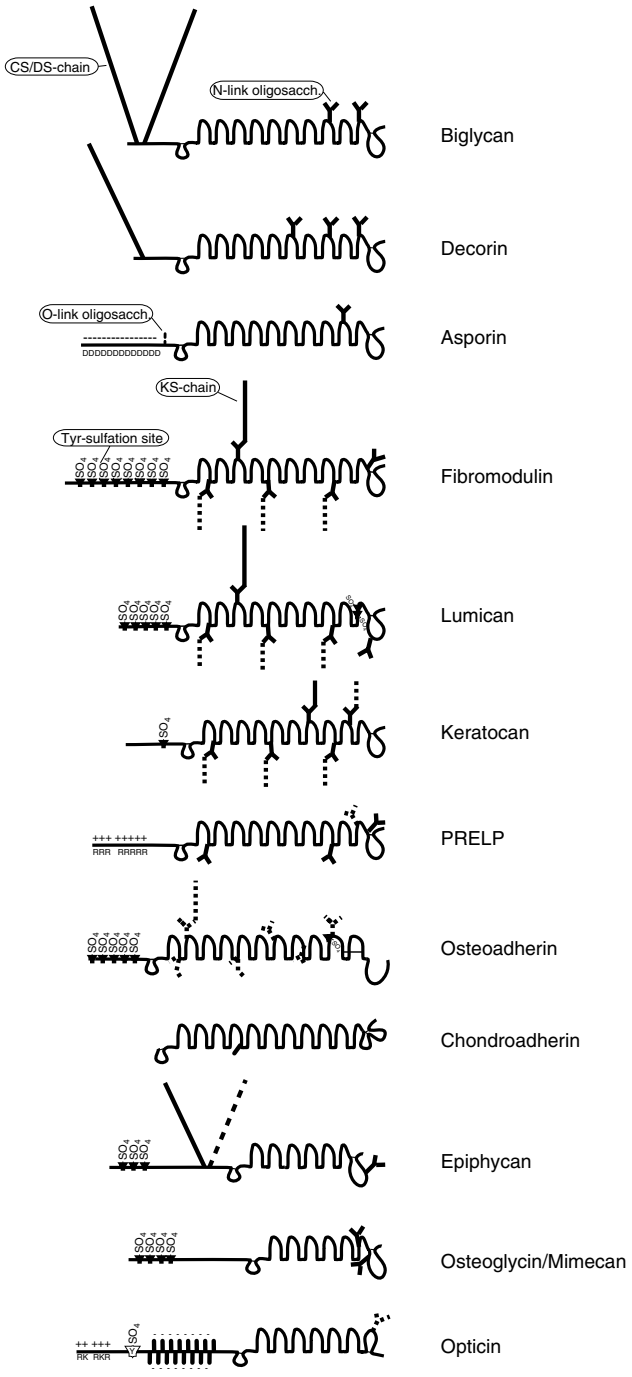

Figure 3. Extracellular matrix LRR proteins. The 10–11 leucine-rich repeats in the larger proteins and 6 such in the smaller ones are indicated and are surrounded by disulfide loop structures as outlined. Most of the proteins contain an N-terminal domain containing various substituents from long glycosaminoglycan chains to tyrosine sulfate-rich sequences. In the case of PRELP, there are no substituents on the N-terminal extension, but the protein has functionally active clusters of basic amino acids. Opticin contains an array of O-glycosidically linked oligosaccharides. There are N-glycosidically linked oligosaccharides attached at sites in the repeats, as indicated. In some cases, these are extended into keratan sulfate chains. Dashed lines indicate possible substituents. LRR, leucine-rich repeat; CS, chondroitin sulfate; DS, dermatan sulfate; KS, keratan sulfate.

formed by the α-helix, while the inner surface is made up of the β-sheet. This β-sheet is the primary structure interacting with other proteins. The repeat region is flanked by disulfide loops. The majority of LRR proteins have an N-terminal extension with distinct functional properties, as detailed in the sections that follow. In osteoadherin, there is also an extended C-terminal domain.

In general, LRR proteins can be divided into subfamilies on the basis of their gene structures and sequence homologies. Thus, decorin, biglycan, and asporin form one subfamily and fibromodulin, lumican, keratocan, osteoadherin, and PRELP another, while chondroadherin is distinct. The molecules with a lower number of repeats are again distinct and form a fourth subfamily. One central function of LRR proteins appears to be to interact with other matrix constituents, primarily collagens and, in some cases, cells.

Decorin and biglycan. Decorin and biglycan are encoded by genes containing eight exons. The biglycan gene maps to chromosome X (Xq28), while the decorin gene maps to chromosome 12 (12q23) in human [113] and chromosome 10 in mouse [114]. The proteins are rather ubiquitously distributed among tissues. The two proteins contain N-terminal extensions with either one (decorin) or two (biglycan) glycosaminoglycan chains (Fig. 3). The chains vary among tissues but are commonly dermatan sulfate containing two types of repeat disaccharide, one of which is iduronate-N-acetyl-galactosamine primarily sulfated at the 4 position. Some of the units, in addition, carry a sulfate at the iduronate. The other repeat is a glucuronate-N-acetyl-galactosamine with a sulfate in the 4 or 6 position. The two building blocks are then united in variably long stretches forming a rather complex extended structure with a large variability among tissues [115] as well as during growth and in pathology. Available data indicate that these chains provide functional properties, including the ability to self-interact [116] and to bind to other matrix constituents. In addition, the glycosaminoglycan chains of biglycan mediate binding to α-dystroglycan [117]. There are also N-glycosidically linked oligosaccharide substituents as indicated in Figure 3.

Both decorin and biglycan are synthesized with a propeptide of as yet unknown function. In tissue studies, it has been shown that the propeptide is sometimes cleaved off but that in other cases the proteoglycan in the tissue still contains the propeptide [118,119]. It has been proposed that the propeptide has a role in the processing of the proteoglycan [118].

Decorin has been shown to bind to cells, particularly colon carcinoma cells [114,120]. Cells with bound decorin show growth arrest [121], indicating that decorin may play a role in the regulation of cell behavior.

Decorin and biglycan share the feature of binding to collagen [122–124], as indicated in Figure 1. In the case of decorin, this has been shown to involve the repeat domain, where repeats 4 and 5, particularly, show a tight binding, with a dissociation constant in the nanomolar range [125,126]. However, other parts of the repeat domain are involved in strengthening the interaction. Decorin binding occurs at a site very close to the C-terminus of collagen I and also close to one of the intermolecular cross-linking sites of the collagen heterotrimers [127]. Decorin core protein [128], as well as its glycosaminoglycan chains [129,130], has been shown to occur bound to collagen fibers in the tissue. The proteoglycan or its core protein can modulate collagen fibril formation *in vitro*. Therefore, it may have a role in collagen fibril assembly and maintenance of the fibrillar network. This contention has been corroborated by results from decorin gene inactivation. Decorin knockout mice have an altered skin phenotype, the skin being fragile, with reduced tensile strength [131]. Collagen fibrils in both skin and tendon of these animals show increased size and irregular contours. This is in accord with the previously demonstrated inhibitory effect of decorin on collagen fibrillogenesis *in vitro* [122,132–134].

Decorin also appears to have the capacity to bind fibronectin [135], a prominent matrix protein in many tissues (see also Chapter 5, this volume), albeit a very minor constituent of articular cartilage. Binding to thrombospondin-1 has also been implied [136]. The role of these interactions is not clear, although there are indications that cell binding may thereby be modulated [136,137].

Biglycan does not appear to bind to collagen fibrils. Precipitation assays *in vitro* show that decorin is effectively precipitated by forming collagen fibers, whereas biglycan is not [125]. However, biglycan binds to collagen VI *in vitro* (as indicated in Fig. 1) using the same site as decorin [138]. The dissociation constant, k_d, is approximately 10^{-9} M, and the binding site is located close to the junction between the triple-helical and the nontriple-helical domains in the N-terminal part of collagen VI [138]. Whether biglycan has a role in regulating collagen VI beaded filament assembly is not known. Interestingly, the biglycan null mouse has particularly marked bone alterations [139], but it is not known whether alterations in the collagen VI network are a component of these bone changes.

More recently, an interaction of biglycan with α-dystroglycan has been demonstrated [117]. This interaction is mediated by the glycosaminoglycan chains of biglycan. Such interactions open up entirely new possibilities for associations between molecules in the extracellular matrix. In this particular case, the result may be an interaction between cells and their surrounding matrix.

Asporin. Asporin [100,140] is a novel member of the subgroup of LRR proteins comprising decorin and biglycan. Asporin is very closely related to decorin, showing the characteristic motif of the C–X3–C–X–C–X6–C sequence and the typical gene of 6 exons found in decorin and biglycan [141]. Asporin, however, is not a proteoglycan, containing no glycosaminoglycan chains. Although asporin contains a propeptide sequence, this differs considerably in its N-terminal region [100,140]. However, as in most of the LRR proteins, this domain is anionic, having a stretch of between 11 and 15 amino acids of consecutive aspartic acid residues. Asporin also differs in glycosylation in containing only one, albeit variable, N-glycosidically linked oligosaccharide. It also appears to contain one O-glycosidically linked oligosaccharide [100].

Asporin is found in cartilage and in tissues rich in smooth muscle cells such as uterus and aorta. Its role in the tissues is not clear, although preliminary data indicate that it may interact with collagen [100]. The protein migrates as a 39 kDa protein upon SDS-polyacrylamide gel electrophoresis [100].

Fibromodulin, lumican, and keratocan. These proteins, illustrated in Figure 3, are encoded by genes containing three exons, the major protein portion of each being encoded by the second exon [142]. They show a wide tissue distribution, although one somewhat more restricted than that of decorin and biglycan. The N-terminal extension of

bovine fibromodulin contains some eight sulfated tyrosine residues [143], as illustrated in Figure 3. Similarly, lumican has the typical motif for several tyrosine sulfate residues, albeit somewhat fewer than fibromodulin [144]. Keratocan contains even fewer putative tyrosine sulfation sites [103]. Fibromodulin and lumican have one or two keratan sulfate chains bound via typical N-glycosidic linkage to any of four of the putative sites for N-glycosylation [101,145–147] (Fig. 3). Because little is known about the functional properties of keratocan, this protein will not be further discussed. However, fibromodulin and lumican both bind to fibrillar collagen, as indicated in Figure 1, and inhibit fibril formation *in vitro* [124,133,134]. Fibromodulin has been demonstrated to occur bound to collagen fibers in cartilage and localized to the fiber gap region [67]. Interestingly, the quantity of protein bound varies with the thickness of the collagen fiber, such that thinner fibers have a higher density of bound fibromodulin molecules [67].

Despite the *in vitro* inhibition of collagen fibrillogenesis, fibromodulin null mice unexpectedly show a higher proportion of thin collagen fibrils in tendon [148]. There are also alterations of the collagen network in cartilage. Lumican protein is increased some fourfold in the fibromodulin knockout mouse. At the same time, lumican mRNA in tendon is less in the knockout mouse than in the wild type [148]. This indicates that the increased level of protein is due to decreased degradation rather than enhanced synthesis. An explanation was obtained in studies of the binding of lumican and fibromodulin to collagen, in which the two proteins bound to the same site, although the binding of fibromodulin was four to five times stronger [149]. The two molecules bind to forming collagen fibers with dissociation constants in the range of 1–10 nM. Thus, the picture emerges that, in collagen fibril assembly *in vivo*, early fibrils have bound lumican, which, at some stage, is replaced by fibromodulin that may then have a role in the further assembly of formed fibrils to result in thicker-diameter aggregates.

The lumican knockout mouse, on the other hand, shows thicker and irregularly shaped collagen fibers in skin [150] and other tissues, reminiscent of the decorin knockout mouse. It is not clear whether there are secondary changes in any of the related molecules in the lumican and decorin null mice.

PRELP (proline- and arginine-rich end leucine-rich repeat protein). PRELP was first isolated from articular cartilage [151]. It contains a leucine-rich domain similar to that of the other members of the family. It is closely related to fibromodulin and, most closely, to osteoadherin. Its structure, however, differs significantly in the N-terminal extension [104], indicated in Figure 3. This part of the protein contains clustered arginine and proline residues indicative of heparin-binding activity. In fact, it can be shown that the molecule binds to heparan sulfate and heparin via this domain with binding constants in the order of $k_d 10^{-8}$ M [152], as indicated in Figure 1. Indeed, cells attach to PRELP via heparan sulfate chains on their cell-surface proteoglycans, such as syndecan (Bengtsson, Heinegård, and Aspberg, unpublished results), as indicated in Figure 1. In view of the fact that PRELP can also bind collagen with a nanomolar dissociation constant (Bengtsson, Heinegård, and Aspberg, unpublished results), it is possible that the molecule can serve as an anchor from the matrix back to the cell. This is likely to create a different type of signal from that traditionally elicited by ligand binding to integrin receptors.

Chondroadherin. Chondroadherin, illustrated in Figure 3, differs from other members of the leucine-rich repeat region family in having no N-terminal extension and a short C-terminal extension [106]. The latter is cleaved off in a proportion of the molecules present in the tissue. As indicated previously, chondroadherin has a different gene organization, with four exons [153], and differs also at the protein level in having an extra disulfide bond in the C-terminal loop structure. There is no, or potentially only one, carbohydrate substituent in chondroadherin and no hexosamine. Thus, this is the only member of this family of proteins with no N-glycosidically linked oligosaccharide. However, the serine residue corresponding to position 123 in the human protein contains a substituent, indicated in Figure 3, that may contain a xylose residue [106,154].

Chondroadherin has been shown to bind cells via their $\alpha_2\beta_1$ integrins [155], as illustrated in Figure 1. Usually, cells spread when integrin binding occurs, but in the case of chondroadherin binding, cells remain rounded, although the synthesis of matrix constituents is altered [155] (Johnson, Olsson, and Heinegård, unpublished results). Integrin binding to chondroadherin does elicit signals, since tyrosine phosphorylation reactions are induced (Johnson, Olsson, and Heinegård, unpublished results). These effects, resulting from chondroadherin binding to cells via $\alpha_2\beta_1$ integrin receptors, are different from those elicited by collagen binding to the same integrin, which does result in cell spreading [155].

A further interaction of chondroadherin has been identified. Upon the induction of mild proteolysis by endogenous proteinases, complexes of collagen monomers with bound chondroadherin are released from cartilage explants [156]. The collagen appears to have been processed for assembly, because no propeptides are present. Two binding sites can be demonstrated, and an identical pattern is observed when recombinant chondroadherin is added back to collagen monomers. Thus, it seems that chondroadherin may have a role in modulating interactions at the cell surface and perhaps also in early collagen fibril assembly, as indicated in Figure 1.

Chondroadherin is particularly enriched in the lower proliferative, prehypertrophic area of cartilage growth plate, where the major mRNA message can also be detected [157]. This localization, to an area with decreasing cell proliferation, is consistent with a role of the protein in regulating cell growth.

Epiphycan and mimecan. These two proteins represent a distinct group within the LRR family. Each contains six leucine-rich repeats [107,108,158,159] and shows some 50% amino acid identity to the others. Their gene organization is different in that they comprise seven exons, the coding sequences being located in exons 5–7 [160,161]. Both proteins have an N-terminal extension with putative sites for tyrosine-O-sulfation.

Epiphycan appears as a chondroitin/dermatan sulfate proteoglycan in the growth plate. Mimecan, on the other hand, can occur as a keratan sulfate proteoglycan in cornea [107], while in other tissues the oligosaccharides do not become extended with polylactosamine sulfated disaccharides to form keratan sulfate. A fragment of mimecan, isolated as osteoglycin, lacks the N-terminal domain carrying the tyrosine sulfate but retains other structural features [107].

Opticin. This protein, originally identified in the eye, is present in the retina as well as in ligaments and skin [110]. The mature protein has an M_r of some 35 kDa. It has

six leucine-rich repeats in the central domain. There is an additional repeat in the C-terminal cysteine cluster. The N-terminal extension of the protein shows the unique presence of a number of O-glycosidically linked oligosaccharides [110] in a structure reminiscent of that in mucins, as indicated in Figure 3.

General comments on the LRR-protein family. With a number of extracellular matrix LRR proteins identified, some overall features can be deduced. There are four subfamilies, with decorin, biglycan, and asporin constituting class I; fibromodulin, lumican, keratocan, PRELP, and osteoadherin constituting class II; epiphycan, mimecan, and opticin constituting class III, and chondroadherin class IV. The genes of the LRR proteins appear to be arranged in clusters of four. Decorin, lumican, keratocan, and epiphycan (classes I, II, II, and III, respectively) map to chromosome 12q23. Asporin, osteoadherin, and mimecan (classes I, II, and III, respectively) are found on chromosome 9q32. *ECM2*, a gene encoding LRR protein containing an amino-terminal von Willebrand factor repeat, is located in the same region [162]. Fibromodulin, PRELP, and opticin (classes II, II, and III, respectively) are positioned on chromosome 1q32. Biglycan (class I) differs from the others in not being part of a cluster. The gene for this protein is found in isolation on the X chromosome. A picture is emerging showing that several duplications have occurred during evolution, with an ensuing clustering of the LRR genes. The biglycan gene may then have relocated to the X chromosome. Alternatively, there may be four more LRR protein genes remaining to be identified, one on chromosome 1 and three on the X chromosome.

Perlecan

This very large protein, having a core protein exceeding 400 kDa (467 kDa in man) [163–165], usually carries three heparan sulfate chains, sometimes fully or partially replaced by chondroitin sulfate chains, in its N-terminal domain [163,166], as well as at least one chain in its C-terminal portion [167]. It is built from a number of modules found in many other connective tissue proteins. These include LDL-receptor class A modules, a large number of Ig-like repeats, several laminin-1 EGF-like repeats, and laminin-1 globular homology 1 and 2 modules, as well as a number of EGF-like repeats [163–165].

As expected, perlecan interacts with a number of other proteins, including integrins, dystroglycan, collagens, and laminins. The proteoglycan also binds and sequesters growth factors, particularly from the FGF (fibroblast growth factor) family [165,168]. Perlecan is found particularly in basement membranes, which were the original sources of the isolated proteoglycan. Subsequently, it has been identified in cartilage [169–171], where it appears after collagen II during development.

Significant progress in understanding perlecan biology has been made in studies of mice with the core protein gene inactivated [168,172]. The majority of such mice die during embryonic development (stages E10–E12). The mice show cardiac defects [168], apparently resulting from a deficient basement membrane, although its formation appears normal. The mice also show defects in cephalic development. Those mice that survive embryonic development die at birth or during the neonatal period. They show extensive chondrodysplasia with abnormal endochondral ossification [168]. Although cartilage formation appears normal, the turnover of growth cartilage, in particular, shows major alterations. Thus, the collagen network is deficient, with a much decreased number of collagen fibrils [168]. At the same time, the synthesis of a number of matrix proteins, including collagen II, matrilin-3, and COMP, is increased 3–5-fold as demonstrated by mRNA Northern blot assay.

It was suggested that one role of perlecan is to neutralize proteinases that function in normal cartilage turnover, particularly that of the growth plate [168]. Alternatively, the phenotype observed in the perlecan knockout mouse may have resulted from a perturbed homeostasis of growth factors [173] in the absence of perlecan.

CILP (Cartilage Intermediate Layer Protein)

CILP (cartilage intermediate layer protein) was initially isolated from articular cartilage but is present also in other cartilages, although it is restricted to this type of tissue [174]. Cloning of cDNA and the consequent deduced amino acid sequence indicated that the gene codes for a larger precursor protein that actually contains two different proteins [175]. The N-terminal part contains the approximately 82 kDa CILP protein and the C-terminal part another protein that, in terms of sequence homology, corresponds to a previously described nucleotide pyrophosphohydrolase (NTPPHase) [176]. The precursor protein is cleaved by a furin type proteinase at the time of secretion such that the two proteins are only found separately in the extracellular environment [175].

There are few examples of one gene encoding two different proteins. The CILP gene is organized in nine exons [177]. Surprisingly, the C-terminal portion of CILP and the entire NTPPHase are encoded by exon 9, such that the border between the two proteins occurs within this exon.

CILP, although markedly up-regulated in early osteoarthritis (Lorenzo, Bayliss, and Heinegård, unpublished results), has no known function, and its sequence represents an entirely novel protein [175]. CILP is not distributed evenly throughout articular cartilage but is particularly enriched in the middle to lower portions of the tissue [174]. This suggests some special functional requirement of this part of the tissue in which CILP is found. The enzyme NTPPHase may play a role in generating pyrophosphate, which, in turn, may be involved in the formation of calcium pyrophosphate precipitates and/or inhibit hydroxyapatite crystal growth [178]. Thus, the up-regulation of this molecule in osteoarthritis may be particularly significant in the regulation of mineral homeostasis and, perhaps, the formation of crystal deposits [179].

Fibronectin

Fibronectin (see also Chapter 5, this volume) is not a cartilage-specific component, although a specific splice variant is present in this tissue [180,181]. It promotes cell binding via the $\alpha_5\beta_1$ integrin receptor and also via a number of other integrins. Fibronectin contains the classical cell-binding sequence identified as arginine-glycine-aspartic acid (RGD) by Pierschbacher and Ruoslahti [182]. Indeed, cell binding to immobilized fibronectin can be inhibited by the addition of RGD-containing short peptides, which are able to saturate the integrins while being unable to form bridging structures. Fibronectin contains domains that promote binding to other matrix components as well as an additional cell-binding sequence, LDV (Leu–Asp–Val) [183]. Thus, its heparin-binding domains appear to play a role in concert with integrin binding in promoting cell division and growth [184]. Collagen binding provides a means of anchorage to the matrix [185]. In addition, fibronectin has the ability to self-interact to form its own fibrillar network [186–188]. A role for fibronectin in joint disease, such as osteoarthritis,

is indicated by its up-regulation in this condition [189], whereby it may play a role in regulating cellular responses to events in the matrix.

Interestingly, fragments of fibronectin containing only the heparin-binding sequence have been shown to modify cell behavior [190]. Thus, when such fragments are injected into joints, they may induce catabolic events [191,192]. It is possible that one mechanism involved in cartilage breakdown in joint disease is the prior formation of fibronectin fragments.

Other Proteins in Cartilage

There are a number of other proteins that have been studied in cartilage. These include a 39 kDa protein (GP-39, YKL-40), which is found predominantly in the more superficial parts of articular cartilage [193,194], although it is also found in other tissues, including the intestine, liver, and synovial capsule [195,196]

A further protein, containing γ-carboxyglutamic acid, is matrix Gla protein [197–199], which has a ubiquitous distribution in a number of tissues, including blood vessels and cartilage. This low-molecular-mass protein appears to play a role in the regulation of calcification. Upon gene inactivation in mice, the prominent phenotype is of excessive, lethal calcification of blood vessels within the first months of life of the animals [200].

A novel proteoglycan, containing both keratan sulfate and chondroitin sulfate chains, is present primarily in the superficial part of the articular cartilage and is also produced in the synovial capsule [201,202]. Little is known of its functional properties.

BONE

Throughout life, a hallmark of bone is a dynamic remodeling process, outlined in Figure 4 (see also Chapter 1, Part III, this volume). In this process, bone tissue that has been fatigued is removed and replaced by newly formed bone. The process also serves to modify the architecture of the bone in its adaptation to altered load. In certain disease states and in post-menopausal women, the intricate balance between the degradation and removal of bone tissue on the one hand and the synthesis and formation of new tissue on the other is disturbed, such that over each remodeling cycle there is a net loss of bone. This eventually leads to mechanical failure in osteoporosis. To understand these processes to develop novel therapeutic interventions, it is essential to learn not only about tissue breakdown but also about factors involved in the recruitment of bone-forming cells and the formation of new tissue. As indicated schematically in Figure 4, the breakdown process is accomplished by osteoclasts and the synthesis by osteoblasts.

The Bone Breakdown Process

The overall breakdown process is initiated by stimuli from osteocytes and/or bone lining cells that, when sensing a particular altered mechanical stimulus, appear to produce proteinases that initially degrade the thin osteoid layer covering bone. Among other factors, they also produce osteopontin, which binds particularly to the mineral phase, as outlined schematically in Figure 4. Thus, the bone surface covered with the cell-binding osteopontin will be exposed and participate in recruiting osteoclastic precursor cells derived from the monocyte lineage. Binding to osteopontin via their $\alpha_v\beta_3$ integrin receptors will transmit signals to the cells that, in concert with growth factors, particularly the RANK (receptor activator of c-jun N-terminal kinase)-ligand, TNF (tumor necrosis factor)-α [203–205], and M-CSF (macrophage colony stimulating factor) [206,207], are likely to affect the differentiation of the precursor cells into mature osteoclasts. Growth factors produced by the osteoblasts/osteocytes also participate in the process. Cells developing into mature osteoclasts progressively form a ruffled border region, beneath which are secreted protons to dissolve the mineral, proteolytic enzymes to dissolve the organic matrix, and enzymes that accomplish dephosphorylation of phosphoproteins, such as osteopontin and BSP (bone sialoprotein) [208]. An essential feature of the resorption process is that the osteoclast ruffled border region is sealed off from the surroundings, forming an extracellular, lysosome-like compartment. This is achieved by the clear zone rim of the osteoclast contact area [207, 209], where the cells attach to the bone surface, as is illustrated in Figure 4. Thereby, an enclosed domain is formed, beneath which enzymes and protons can be retained in sufficiently high concentrations for efficient activity.

In this process, the osteoclast forms a resorption pit, and when it leaves the area a novel bone surface will be exposed. This appears to serve as a substratum for the recruitment of cells that will accomplish the formation of new bone to replace that removed. The cells recruited form new bone tissue initially in the form of nonmineralized osteoid, which subsequently becomes mineralized in a series of tightly regulated processes. Bone contains a number of matrix proteins, which appear to play important roles in different phases of the remodeling process. An account of some of these proteins and their known functions follows.

Osteopontin

Osteoclasts bind to the mineralized matrix of bone via their $\alpha_v\beta_3$ integrin receptors [210,211]. Thus, it has been shown that competitors for integrin interaction, including short RGD (arginine-glycine-aspartic acid)-containing peptides, can inhibit bone resorption *in vitro* [212,213]. As illustrated in Figure 4, a multitude of data indicate that the ligand for the integrin involved in osteoclast binding is the phosphorylated glycoprotein osteopontin [214], which contains an active RGD cell-binding sequence [215]. Several clustered sequences of phosphorylated serine residues [216] in combination with stretches of polyaspartic acid are likely to provide for binding of the protein to the hydroxyapatite mineral [217,218]. Thus, the protein is, *de facto*, bifunctional and should have the potential to attach cells to the mineral phase.

The synthesis of osteopontin is up-regulated by 1,25-dihydroxyvitamin-D_3 [219]. Transforming growth factor (TGF)-β also up-regulates its production, while glucocorticoids have a down-regulatory effect. In bone, osteopontin is primarily produced by osteoblasts, which are often seen surrounding osteoclasts in areas of bone resorption [220]. It can bind to isolated osteoclasts *in vitro* via their $\alpha_v\beta_3$ integrin receptors [221,222]. Direct evidence for a role for osteopontin in the bone resorption process was obtained in immunolocalization studies using immunogold electron microscopy of areas in bone displaying osteoclasts. Osteopontin appeared to be localized almost exclusively at the clear zone of the cells, representing the attachment areas, while none was detected in the ruffled border regions [211,222,223]. Other areas of bone more distant from osteoclasts showed only small amounts of osteopontin. Furthermore, the $\alpha_v\beta_3$ integrin cell surface receptor was found to be enriched several-fold in the clear zone area of the osteoclast, with little staining in the ruffled border resorption area

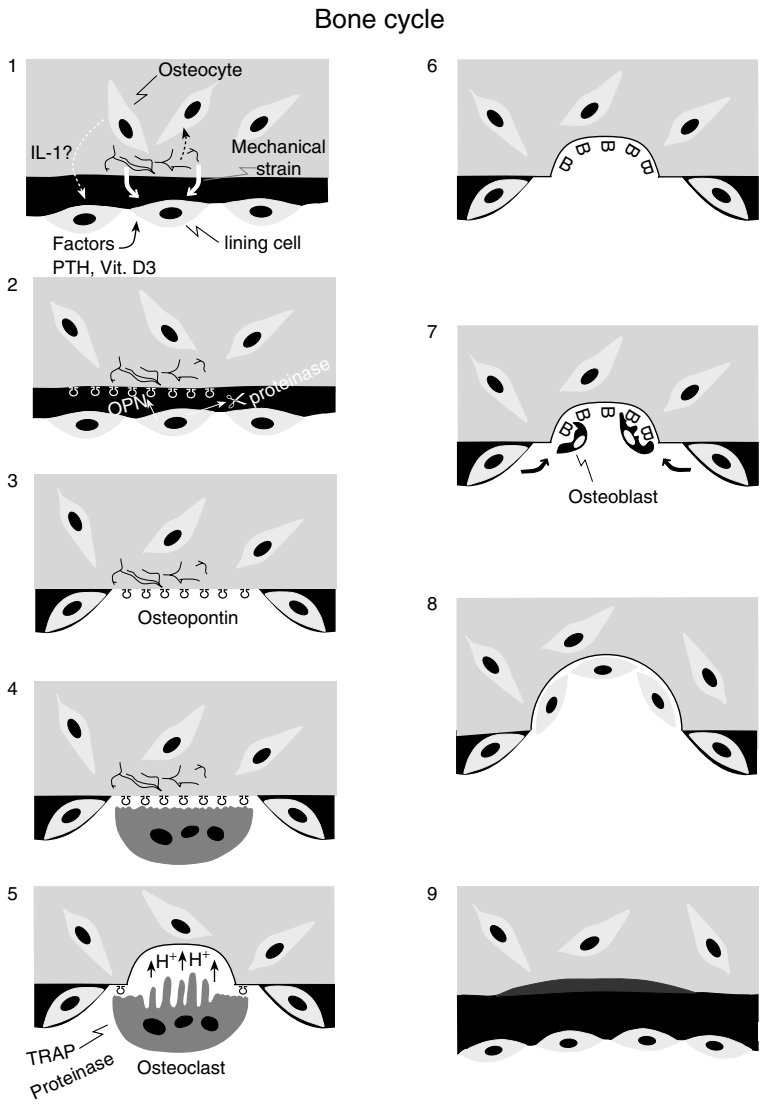

Figure 4. Schematic illustration of the bone remodeling cycle and molecular constituents involved. The catabolic phase includes the triggering of lining cells/osteoblasts/osteocytes to remove the thin layer of osteoid and to condition the bone matrix to recruit osteoclast precursor cells. This activity includes the deposition of the cell-binding matrix protein, osteopontin. The osteoclastic precursor cells develop into resorbing osteoclasts, each with a central ruffled border region dissolving mineral as well as digesting the organic matrix, and a peripheral clear zone area where attachment and sealing occurs. The resorption pits resulting from osteoclastic activity recruit novel osteoblasts essential for new bone tissue formation. IL-1, interleukin-1; PTH, parathyroid hormone; OPN, osteopontin.

of the membrane [210,211,222–225], as outlined schematically in Figure 4 (section 5). Osteopontin production in bone appears to occur exclusively in osteoblasts, in which both mRNA and protein can be detected [220,226], while osteoclasts residing in the bone are devoid of immunoreactivity at the electron microscope level [223]. Thus, it appears that the mature osteoclast is bound via its $\alpha_v\beta_3$-integrin receptor to osteopontin at the mineralized bone surface. Verification that early events in osteoclast binding also involve osteopontin was obtained in studies of the mutant *ia/ia* rat [223], which develops osteopetrosis. This appears to be due to a failure in osteoclast maturation, in which the cells are primarily arrested at the stage at which they have a large clear zone but no significant ruffled border domain. The mechanism may involve the intracellular retention of secretory vesicles that do not fuse with the plasma membrane. Accordingly, there is no secretion of proteinases and other enzymes necessary to accomplish bone resorption. Interestingly, none of these vesicles contain osteopontin [223], indicating that osteoclasts do not produce the protein. Furthermore, osteopontin is localized facing the whole clear zone area, and there is very pronounced enrichment of the $\alpha_v\beta_3$ integrin receptor along the corresponding region of the cell membrane, as indicated in Figure 4 (section 4). It is likely that osteoclastic precursor cells bind to osteopontin and that one of the signals inducing their differentiation into mature, resorbing osteoclasts is triggered by this interaction. Further work is necessary to substantiate this hypothesis, but additional support for a role

of osteopontin in bone resorption has been obtained in gene inactivation studies. Thus, the osteopontin null mouse shows a developing osteopetrosis, although this is slow and only readily apparent in old animals (Franzén, Hultenby, Reinholt, and Heinegård, unpublished results). This indicates an altered, defective osteoclast anchorage due to the lack of osteopontin. Further evidence for a key role of osteopontin in bone resorption is that the accelerated bone resorption that follows ovariectomy does not occur in mice with an inactivated osteopontin gene [227].

An additional potential role of osteopontin in bone mineralization is possible because it is found highly enriched at the mineralization front [226]. In support of this supposition, it has been shown that osteopontin can inhibit mineral growth *in vitro* [217,218].

Osteopontin is also produced by numerous other cells, including smooth muscle cells in atherosclerotic lesions, kidney tubular epithelial cells, macrophages in placenta, a number of cancer cells, and even microglial cells in the brain [228]. Some of its functions in these situations may be related to cell activation and migration in defense reactions and wound healing.

BSP — Bone Sialoprotein

BSP (bone sialoprotein) is restricted to bone, possibly having a role in the buildup phase of the tissue. Thus, it is particularly enriched in areas where new bone is formed by osteoblasts, and an example is its high abundance at the interface between bone and cartilage in the growth plate [78,220,229]. BSP is clearly an osteoblast product, as shown by both immunolocalization and *in situ* hybridization [78,220]. It is also found at sites of new osteoid production. Interestingly, BSP contains a cell-binding sequence that, albeit containing RGD and primarily binding to the $\alpha_v\beta_3$ integrin [230–232], can also bind to other integrins. BSP can bind to the mineral phase of bone, possibly via its multiple phosphorylated serine residues, as well as a tyrosine sulfated domain, around the cell-binding sequence in the C-terminal region of the molecule [233]. A high degree of substitution, particularly by O-glycosidically linked oligosaccharides, provides additional negative charge. Further, extended, long polyglutamic acid sequences contribute charge and possibly mineral binding. The finding that many of the sites exist with or without substituent (Zaia, Boynton, Heinegård, and Barry, unpublished results) indicates a possible modulation of functionality of the protein.

In vitro BSP can bind to osteoblasts as well as osteoclasts [221]. *In vivo*, however, it shows no significant presence at sites around osteoclasts [220,234]. The production of BSP by osteoblasts is regulated in the opposite way from that of osteopontin, that is, it is up-regulated by glucocorticoids [235] but pronouncedly down-regulated by 1,25-dihydroxyvitamin-D_3 and TGF-β. It is attractive to hypothesize a role for BSP in osteoblast function and perhaps bone formation. It may also play a role in osteocyte function, because it can be demonstrated in bone at sites appearing to represent the extensions of osteocytes [220].

Osteoadherin

Osteoadherin (Fig. 3) is a member of the LRR protein family [105]. It has the capacity to bind to osteoblasts via their $\alpha_v\beta_3$-integrin receptors [236]. The protein was isolated originally from bovine bone, in which it occurs as a keratan sulfate proteoglycan [236], although this appears not to be the case in other species. Interestingly, the isolated protein occurs in apparently stoichiometric quantities in a disulfide-linked complex with osteonectin/SPARC [236]. The cell-binding activity of osteoadherin appears not to be influenced by the presence of osteonectin. The active site for cell binding has not been identified, but osteoadherin does not contain the traditional RGD amino acid sequence that is typically involved in $\alpha_v\beta_3$-integrin binding. The structural features of osteoadherin are slightly different from those of other LRR proteins in that there is a larger C-terminal extension [105]. Similarly to other members of the family, there is an N-terminal domain apparently containing tyrosine sulfate. There are also unique sulfated tyrosine residues in the more C-terminal part (Önnerfjord, Gantcheva, and Heinegård, unpublished results), as illustrated in Figure 3.

The functional role of osteoadherin in bone is presently not clear, but its expression is particularly abundant along bone trabeculae [237] in a pattern not dissimilar to that of BSP. It is therefore possible that it is also involved in the building up of bone by osteoblasts.

Osteonectin/SPARC

This approximately 40 kDa protein appears to have roles in regulating cellular activities. It is particularly abundant in bone, in which the protein was first detected [238], but is expressed and present in many other tissues, including basement membranes, in which it has been referred to as BM-40 [239,240]. It contains several EF-hands mediating calcium binding [239]. *In vitro* studies show osteonectin to have particular roles in modulating cell division and cell migration [241–243], although these effects appear not to involve direct cell binding. However, the protein has been shown to interact with collagen [244], and the fact that the cell-binding protein osteoadherin is isolated as a complex with osteonectin may provide clues for understanding the effect of osteonectin on cells. Inactivation of the osteonectin gene results in particular effects on eye development and leads to the development of cataracts [242].

Osteocalcin

Osteocalcin is an abundant low-molecular-mass bone protein of less than 10 kDa [245,246]. It is unique in containing carboxyglutamic acid residues, more commonly found in blood coagulation factors. This represents a post-translational modification of three of the glutamic acid residues and provides extra charge and enhanced calcium binding. The role of the protein in bone is not quite clear, but its synthesis is regulated by factors that influence bone turnover, such as 1,25-dihydroxyvitamin-D_3 [247]. Upon gene inactivation, mice display increased bone formation [248,249], indicating a role for the protein in regulating osteoblastic functions.

Other Bone Proteins

There are numerous other proteins in bone, including thrombospondin-1 [83,250]. This protein can bind cells and influences cellular activities [251–253]. It also plays a role in angiogenesis, much like another member of the family, thrombospondin-2 [254]. Indeed, animals with an inactivated thrombospondin-2 gene show alterations in bone mineral density [255], perhaps an effect of altered blood vessel formation.

Bone, in addition, contains abundant proteins not synthesized in the tissue. Thus, α_2 HS glycoprotein is very abundant, particularly in growing bone [256–258]. Its exact role in the tissue is not clear.

CONCLUDING REMARKS

Our increasing understanding of the molecular constituents of bone and cartilage provides important clues to the organization of the tissues and to understanding the processes of remodeling. This involves the breakdown and rebuilding of the tissues and is a key element in normal homeostasis and in dealing with altered mechanical requirements. Such knowledge is of paramount relevance in understanding alterations in disease states, including those with a genetic background caused by mutations in particular genes. Conversely, the study of altered phenotypes and tissue properties following such gene mutations will provide additional information about the specific functions of a given protein. Seen against the background of a rapidly increasing incidence of diseases affecting bone and cartilage, including osteoarthritis, osteoporosis, and rheumatoid arthritis, we need to expand our knowledge of molecular mechanisms to enhance the potential for developing new means of specific therapeutic intervention.

RECENT DEVELOPMENTS

Mutations in the perlecan gene (*HSPG2*) have recently been identified in several different human chondrodysplasias [259,260] with phenotypes similar to that observed in mouse gene targeting experiments [261,262].

We have previously shown that the LRR protein PRELP binds heparin and heparan sulfate [263]. We have now found PRELP to be present at the basement membrane/stromal junction in many tissues and able to bind both perlecan in the basement membrane and collagen in the stroma, suggesting that this LRR molecule may be involved in basement membrane anchoring.

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Chapter 5

Adhesive Glycoproteins

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INTRODUCTION

The formation of tissues and organs in the early embryo, their development and growth, their destruction and regeneration in the adult, and their pathological alterations are the result of complex, tightly regulated patterns of cellular activities and interactions, including proliferation, migration, adhesion, differentiation, matrix production, and programmed cell death. In recent years, it has been firmly established that cell interactions with the extracellular matrix are often critical during these processes [1–4]. In searching for the cellular effectors in the matrix, numerous extracellular glycoproteins have been isolated and characterized, which have specific adhesive affinities on the one hand for the cell surface and on the other for other molecules of the matrix.

These glycoproteins are able to regulate cellular activities both directly, through interaction with cell-surface receptors, in particular integrins, and indirectly by structuring a defined three-dimensional extracellular matrix, which is required by most cells to express their tissue-specific features. The number of extracellular, adhesive glycoproteins seems boundless, and the literature on them has virtually exploded since the first edition of this volume. The expansion of our knowledge of matrix proteins is best illustrated by the laminin field, in which 12 or more* different laminins are known at present in contrast to three laminin isoforms known in the early 1990s.

The best-studied and longest-known matrix glycoprotein with adhesive properties is fibronectin, although collagen and its denatured form, gelatin, have been used for cell-adhesion studies for more than 30 years. Numerous large, extracellular glycoproteins have since been discovered, such as the laminins, tenascin/cytotactin, thrombospondin, vitronectin, osteopontin, chondroadherin, and others. Each has different patterns of affinities for both cells and other matrix molecules. It is unlikely that all relevant proteins have yet been found.

Much of our knowledge of the role of adhesive glycoproteins in modulating cellular functions is based on *in vitro* experiments using isolated, purified matrix components as substrates for cell biological studies. Most cells show multiple interactions with different adhesive components, and this is consistent with the presence on them of several types of matrix receptors. Depending on the experimental system, cells recognize various matrix components with a wide range of specificities and affinities, and the cellular response depends on the concentration, purity, and spatial arrangement of the proteins offered as substrates. Thus, the evaluation of the specificity and affinity of cell-protein interactions *in vitro* is subject to individual interpretation, and the classification of a glycoprotein as being "adhesive" or not has frequently been preliminary and controversial.

Collagens are not only the major structural glycoproteins in connective tissues, but they also exhibit multiple interactions with cells [2,5–8]. Among the 20 collagen types known in 2000, types I, II, IV, and VI have been the most intensively studied in terms of cell adhesion; we therefore focus on the adhesive aspects of these collagens, as the other collagens are considered in Chapter 2 of this volume.

In view of the space restrictions on this chapter, we have excluded matrix glycoproteins such as osteopontin, osteocalcin, matrilin, and others, which are dealt with in Chapter 4, although they do display adhesive properties. Similarly, a number of small proteoglycans such as decorin, biglycan, fibromodulin, lumican, and others, undergo specific interactions with other matrix proteins and cell surfaces and could be included in this chapter, but they also are dealt with in Chapter 4, together with the large proteoglycans. Fibrinogen and von Willebrand factor are considered to be major adhesive glycoproteins, but because they are not integral constituents of the extracellular matrix, they are also excluded.

Common Features of Adhesive Glycoproteins

Three criteria may be used to identify adhesive glycoproteins as a distinct group of matrix proteins. (1) The typical adhesive glycoprotein is an extracellular, multidomain, and multifunctional protein bearing various binding regions for both cells and other matrix components. In most cases, the domains can be isolated by limited proteolysis; they are independently active and retain their affinities to other macromolecules or cells, independently of the rest of the molecule (Fig. 1). They often coincide with distinct structural elements common to several adhesive glycoproteins, such as fibronectin type III homologies, EGF (epidermal

*At the time of going to press, this number had increased to 15.

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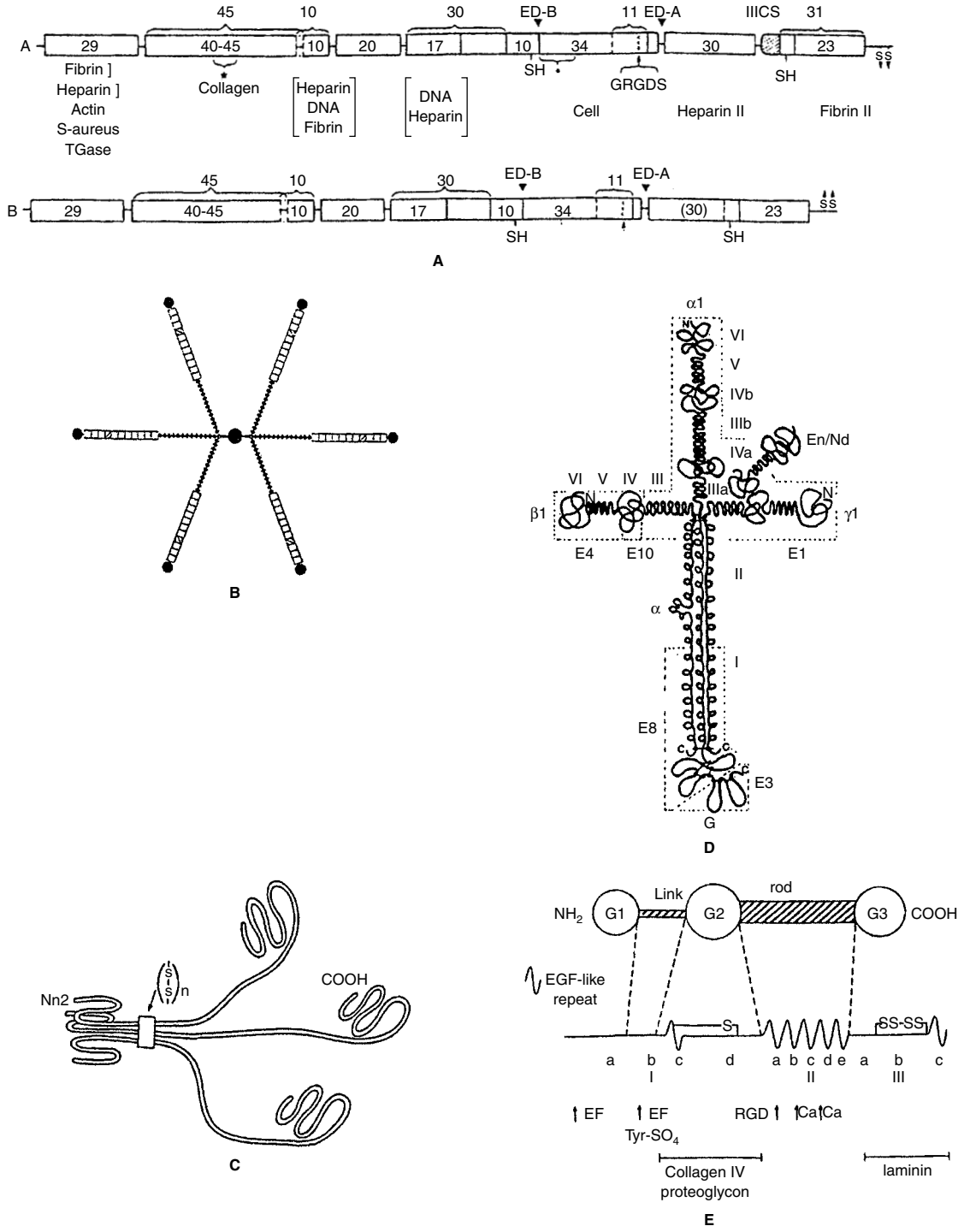

Figure 1. Structural Models of Adhesive Glycoproteins. **A:** Alignment of functional domains of the fibronectin A and B chains and their interactions with other macromolecules. Numbers indicate the molecular weights of the domains in kDa. (Reproduced from [54] with permission.) **B:** Model of tenascin. In each half, three subunits assemble to triplets to meet in the center globule. See Figure 3 for structural details. Reproduced from [187] with permission. **C:** Model of thrombospondin. (Reproduced from [28] with permission.) **D:** Laminin model. The Roman numerals indicate discrete structural domains (see also Fig. 3); α = α-helical domains; G =carboxyl-terminal globular domain. Elastase-derived (E) fragments are marked with dashed lines. Nidogen binding to the γ1 chain is indicated (En/Nd) (modified from [502]). Reproduced from Sasaki et al. [501] with permission. **E:** Model of nidogen (entactin). Correlation between the morphological domain structure of nidogen and subdomains predicted from the amino acid sequence and the localization of functional sites. Ib-d, IIa-e, and IIIa-c refer to these subdomains. S denotes extra disulfide bridges besides those in EGF-like repeats. EF and Ca indicate putative calcium-binding sites; Tyr-SO_4, tyrosine sulfation site; and RGD, a putative cell-binding sequence. Major binding domains for laminin-1, collagen IV, and proteoglycan are identified by horizontal bars (from [312]).

growth factor)-like repeats, VWA (von Willebrand domain A)-like domains, or the NH_2-terminal sequence of type I procollagen (Fig. 3). (2) Many glycoproteins contain short peptide sequences that are essential (but not always sufficient) for their adhesive properties, such as the amino acid sequence Arg–Gly–Asp (RGD) in fibronectin, vitronectin, and osteopontin, which functions as a major cell-recognition site [9,10]. The discovery of this versatile cell-binding site has opened new experimental pathways to elucidate specific molecular mechanisms of matrix effects on cells. (3) Their interaction with cells is mediated by specific transmembrane receptors, the integrins or transmembrane heparan sulfate proteoglycans (syndecans, glypicans, CD 44, and others). A selection of *in vitro* and *in situ* effects of RGD-containing peptides in cell biology illustrates the wide spectrum of activities associated with this sequence: RGD-containing peptides inhibit the adhesion to fibronectin, vitronectin, thrombospondin, and laminin fragments of numerous cells, including normal and transformed fibroblasts, epithelial cells, hematopoietic cells, and various embryonal cells [9,11]; they block the migration of neural crest cells *in vitro* and *in vivo* [7] and interfere with amphibian and *Drosophila* gastrulation [12–14] and the metastasis of B16 melanoma cells [15,16]. GRGDS (Gly–Arg–Gly–Asp–Ser) can serve as substrate for cell adhesion, is chemotactic for fibroblasts, mediates cell-cell adhesion, and triggers somite segmentation [3,17].

In this chapter, we try to summarize present knowledge about the major adhesive glycoproteins (Table 1), focusing on their common structural and functional features and on their interaction with cells. In view of the several thousand publications solely on fibronectin and laminin published to date, the citation of published work in this chapter has had to remain selective and incomplete. However, comprehensive overviews on fibronectin can be obtained from several review articles [17–20] and two excellent books [21,22]. A number of informative review articles are also available for the laminins [23–25], thrombospondins [26–28], tenascins [29–32], and vitronectin [33–35]. The adhesive properties of matrix proteins, their specificities, and their roles in cellular function cannot be understood, however, without reference to cellular matrix receptors, in particular the integrins and cell-surface heparan sulfate proteoglycans. For this reason, a short overview of these receptors stands at the end of the chapter, although it can only remain very general in light of the fact that over 12,500 articles on integrins have been published at the time of writing.

FIBRONECTIN

Fibronectin occurs both as a soluble, monomeric molecule with an M_r of 540 kDa in plasma and other body fluids and in polymeric, fibrillar form on the cell surface, in the interstitial space, and in thrombi. The independent discovery of fibronectin, the "prototype of a cell adhesion protein" [17], in the early 1970s in several laboratories as a result of completely diverse lines of investigation illustrates its multifunctional, complex nature. During a search for transformation-sensitive cell-surface proteins in fibroblasts, fibronectin was identified and extracted with urea from cell cultures [36–39]. By immunoelectronmicroscopy, the protein was also identified as an integral component of peri- and extra-cellular matrix fibers [40–41]. It was found to be homologous to a previously isolated plasma protein ("cold insoluble globulin" [42]) and was identified as the major cell-adhesion-promoting activity in plasma [43–45]. Another group discovered this same protein while searching for gelatin-binding activity in serum [46].

Structural and immunological comparison of the isolated molecules, and cleavage into smaller fragments retaining the originally addressed functions, demonstrated that the plasma and cell forms of fibronectin were very similar. Sequence analysis at both protein and cDNA levels revealed the existence of three dominant splice regions in the fibronectin gene, potentially giving rise to up to 20 different splice variants [47,48]. All fibronectin molecules are composed of two nearly identical subunits, with various binding regions arranged in a linear fashion (Figs. 1A, 2d, 3). At least two cell-binding domains [49–52] are identifiable in addition to a gelatin-binding domain [53,54], a self-assembly site involved in the formation of fibrils [55–57], three heparin-binding sites, and other domains binding to DNA, proteoglycans, fibrin, and hyaluronic acid [22,54,58].

The broad interest fibronectin is receiving in cell biology and pathology reflects its wide range of biological activities and its ability to regulate cellular activities *in vitro*. Fibronectin has been shown to promote migration and proliferation of embryonic and tumor cells *in vitro* and *in vivo* and to control cell differentiation, cell shape, and cytoskeletal organization. It is also involved in wound healing, hemostasis, metastasis, and control of tissue and organ development.

The key role of fibronectin in early embryonic development became strikingly evident after complete fibronectin gene inactivation by homologous recombination [59]. Mouse embryos deficient in both alleles of the fibronectin gene (fibronectin knockout mice) died at early stages. The fetuses lacked somites and notochord and showed an abnormal heart and vasculature and a deformed neural tube. This phenotype underlines the importance of fibronectin for the proliferation, differentiation, and migration of mesodermal cells.

Cellular Origin and Distribution

Hepatocytes secrete into human plasma a soluble form of fibronectin [19,60]. Following blood coagulation, about 150–250 μg/mL of fibronectin, which is partially degraded by proteases of the coagulation cascade, remains in serum. Soluble forms of fibronectin are also found in most other body fluids, such as synovial fluid [61], seminal plasma [62], saliva, and cerebrospinal fluid [21], but only in μg amounts per mL. In cultured fibroblasts from many species, including chick, rat, human, hamster, and mouse, fibronectin is secreted and can be identified on the cell surface or in the pericellular matrix by immunofluorescence and by surface iodination [39]. Most other mesenchymal cells, such as chondrocytes [63], myoblasts [64], and also epithelial cells [65,66], show pericellular labeling with anti-fibronectin in cell culture. Transformed cells produce less fibronectin and show less on their surfaces; however, exogenously added fibronectin binds to the cell surface and restores the normal fibroblastic phenotype [67,68]. This puzzling phenomenon may find its explanation in a phosphorylation of integrin receptors and a resulting change in the interaction with talin and the formation of adhesion plaques and stress fibers in transformed cells [69].

Extensive immunohistological surveys have located fibronectin in most embryonic mesenchymal tissues [70], and in all reticular and connective tissue fibers, for example, in liver, kidney, and lung [71,72]. It is also associated with basement membranes [73,74], although it is probably not an integral constituent of the basal lamina [75,76].

TABLE 1. Adhesive Glycoproteins

Name	Mol. Mass kDa	Subunits kDa	Cellular Receptors	Cell-Binding Motif	Cellular Origin, Tissue Distribution	Remarks
Fibronectin (FN)	500	A: 250 B: 250	α5β1, α3β1, αvβ1, αvβ3/β6 integrins α4β1 integrin syndecans	GRGD(S) REDV	Most mesenchymal and epithelial cells Plasma FN: hepatocytes	At least 8 splice variants monomer in plasma extracellular fibrils
Tenascin-C (TN-C)	~1200	190,200, or 220	α2β1 α9β1, αvβ3	RGD	Embryonic mesenchyme, tumors, glioma cells, brain, cartilage, skin	Synonyms: cytotactin, hexabrachion Transient in development and wound healing; tumor marker
Tenascin-R (TN-R)	960	160		RGD		Role in neuronal guidance
Tenascin-X (TN-X)	?				Embryonic tissue	
Thrombospondin TSP-1 TSP-2	 450 450		αvβ3 syndecans CD47/IAP, CD36	RGD WSXWSXW-XXCSVTGC	Platelet granules fibroblasts smooth muscle	Role in hemostasis, cell proliferation, collagen fibril assembly, neurite outgrowth
Vitronectin (VN)	70	70	αvβ1, αvβ3, αvβ5	GRGDS	Serum, liver	Binds PAI-1; thrombin-antithrombin complex, collagen; role in extracellular proteolysis
Laminins 1–12 (LN)	500-900	α, β, γ	α1, α2, α3, α9 − β1, α6β1, α6β4, α7β1	RGD (cryptic) ?	Epithelial, endothelial cells, embryonic fibroblasts, myoblasts, tumor cells	LN-1 (EHS-Lam) embryonic LN2/4 in muscle, LN5 skin BM, LN 8,10: endothelial cells
Nidogen/ Entactin	150	150			Embryonic mesenchyme, epithelia	Binds specifically to the γ1 chain of laminins
Collagen I Collagen II	290	$\alpha1(I)_2$ α2(I) $\alpha1(II)_3$	α1β1, α2β1, α3β1 syndecans α10β1	GFQGER in α1(I) chain	Fibroblasts, osteoblasts, almost all tissue cells Chondrocytes	Fibril- forming collagens
Collagen IV	450	$\alpha1(IV)_2$ α(IV)	α1β1, α2β1	(R, G, D) (nonlinear)	Epithelial cells, myoblasts, basement membranes, tumor cells	Forms cross-linked network
Collagen VI	400	α1, α2 α3		RGD	Fibroblasts, chondrocytes, ligaments	Microfibrillar collagen, highly disulfide cross-linked

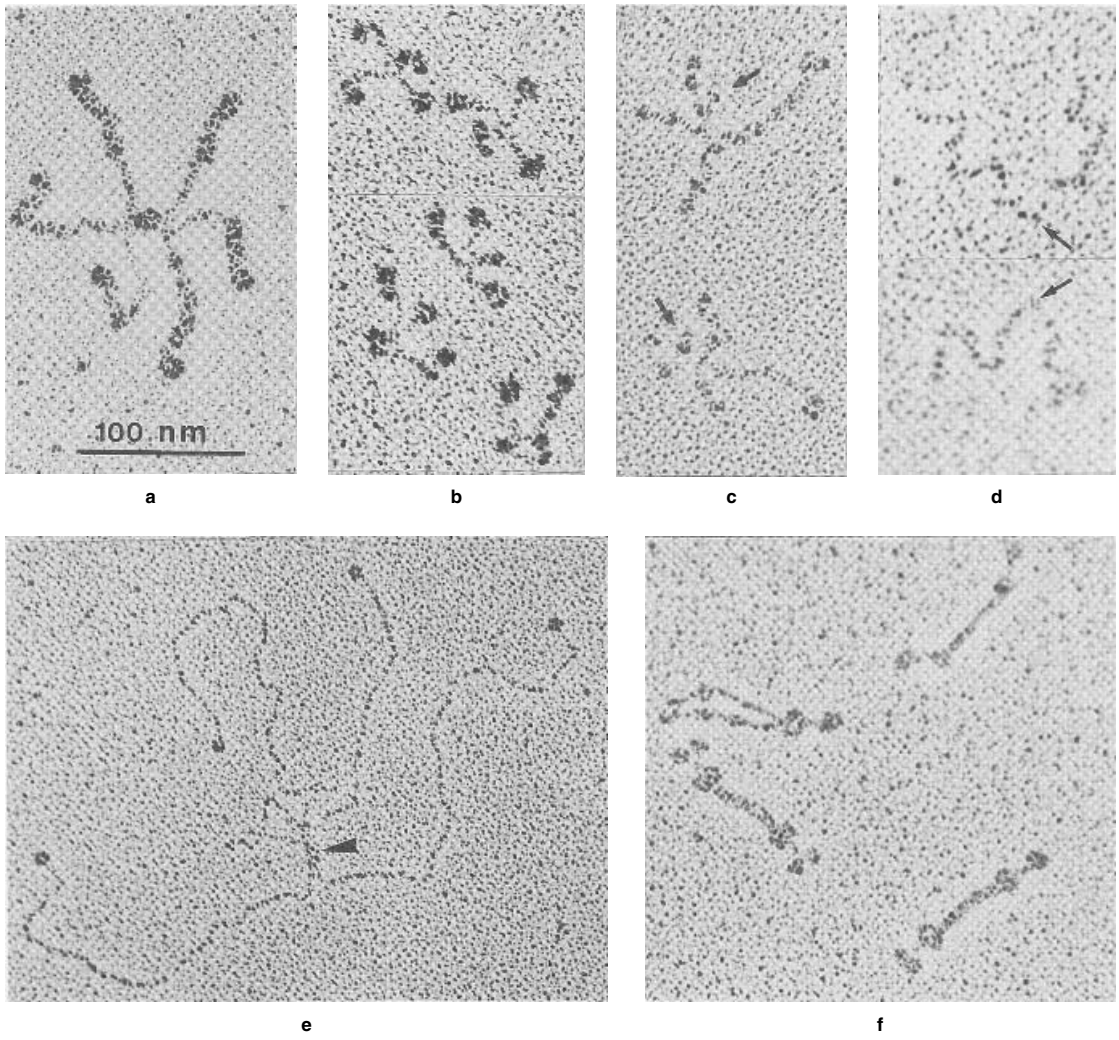

Figure 2. Ultrastructural images of adhesive glycoproteins revealed by rotary shadowing electron microscopy. (**a**) Tenascin-C from chick embryo fibroblast cultures, showing its six-armed ''hexabrachion'' structure (reproduced with permission from [217]). (**b**) Thrombospondin from human platelets (reproduced with permission from [28]). The bolas-like structure is caused by the globular domains at the carboxyl terminus of each of the three chains. The fourth globule consists of the associated three amino termini. (**c**) Laminin-1-nidogen complex from mouse EHS basement membrane tumor. Three short arms and one long arm comprise the laminin molecule. Nidogen (arrow) is bound to domain III of the B chains (see Fig. 1). Nidogen is indicated by an arrow. (**d**) Fibronectin from human plasma; two long, flexible arms are linked together at their carboxyl termini by a cystine bridge (arrows). (**e**) Human type IV procollagen tetramer from PYS (parietal yolk sac) cell cultures. Four molecules assemble through their aminotermini (''7S domains'', arrowhead). The globules represent the non-triple-helical carboxyl-terminal (NC1) domains. (**f**) Human type VI collagen tetramers from human placenta (for structural details, see Chapter 2, this volume). All figures are printed at the same magnification.

Primary Structure

Fibronectin consists of two polypeptide chains, each about 2500 amino acid residues long, that are covalently linked at the carboxyl terminus by cystine bridges (Figs. 1A, 3). The fibronectin gene exists in only a single copy, which is 50 kb long and contains 47 exons [77,78]. Therefore, both chains are either identical or may differ as a result of differential splicing. The entire amino acid sequence has been determined for human plasma fibronectin by cDNA sequencing [78,79] and for bovine plasma fibronectin by protein sequencing [80,81]; rat and chick fibronectin sequences are also available [21,82]. The two polypeptide chains are identical (with the exception of the short IIICS domain, which is lost in one chain of plasma fibronectin by differential splicing; see below) and consist of three types of structural repeat (''homologies''): nine type I repeats (each 45 amino acid residues long) at the amino end and three at the carboxyl terminus; two type II repeats (each of 60 amino acid residues) after the first six type I repeats; and 15–16 type III repeats (90 amino acid residues each) in the middle of the molecule (Fig. 3) (for reviews, see [22,83]). Each repeat is encoded by a single exon. Type I and II homologies, which are disulfide-bonded loops, have also been found in factor XII and plasminogen activator, in gelatinases, and in the seminal plasma proteins PSPA3 and PDC 109 [84], among others. Type I modules are linked together by β-strands with little flexibility and thus confer rigidity on this part of

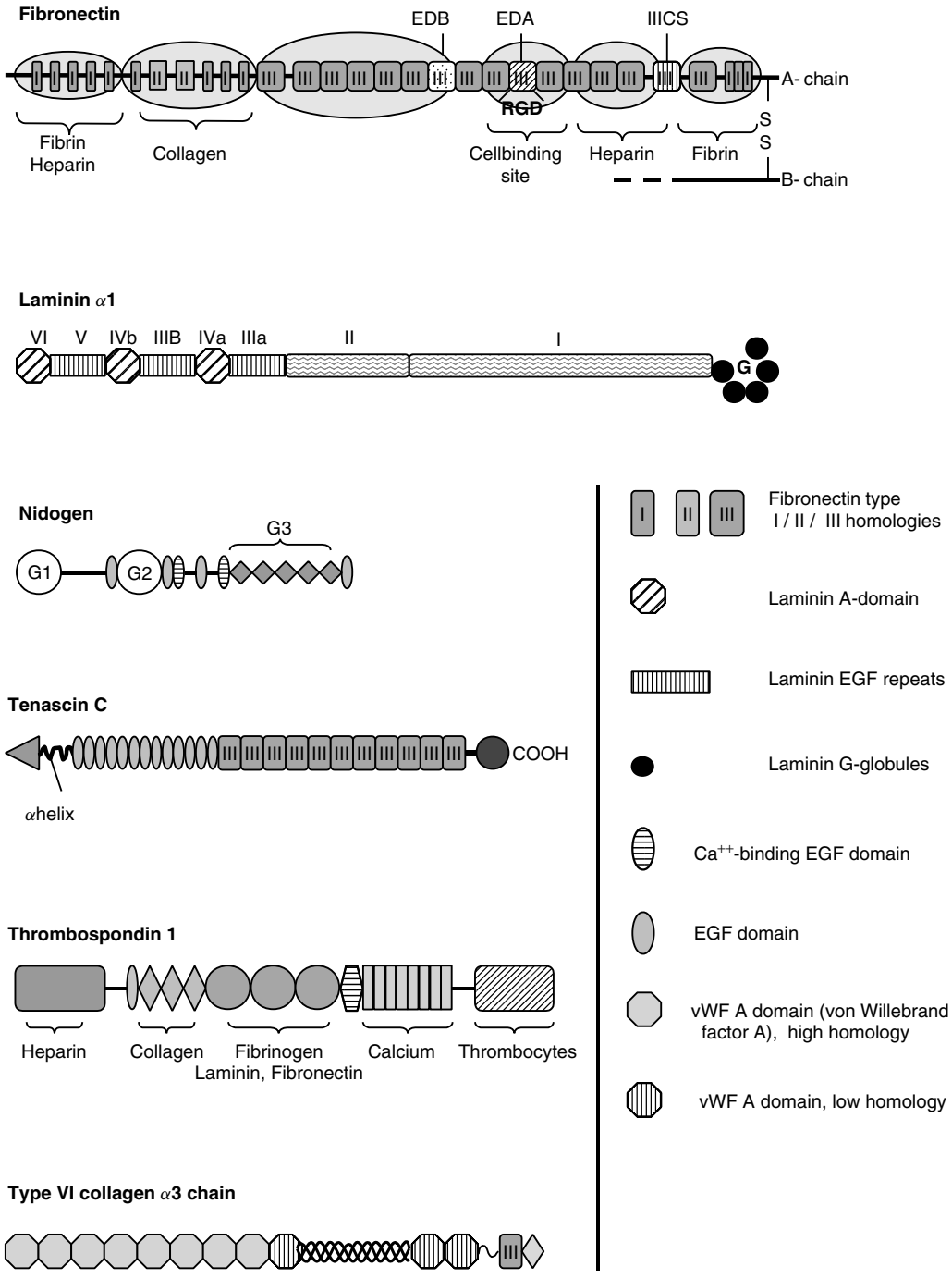

Figure 3. Modular Structure of Matrix Macromolecules. The general theme of adhesive matrix proteins appears to be a reiteration and amplification of structural motifs. A series of the most frequent recurrent motifs is shown. In **fibronectin**, three repeat types are found (12 class I; two class II; and 17 class III). Two of the type III repeats, EDA and EDB, can be differentially spliced, and in IIICS the splicing is complex (after [58] and [17]). Type III repeats are also found in tenascin-C (below) and other proteins. In **laminins**, the motifs are different; in the short arms of the molecules, globular domains (VI and IV) are separated by regions of cysteine-rich EGF-like repeats (V and III), while in the long arm, long regions of α-helix (II and I) are seen [132,501]**.** In **nidogen**, the EGF-like repeat is found again. Nidogen consists of three globular domains (G1-G3), with two of the globules (G2 and G3) connected by a rod-like segment containing two EGF-like and two Ca- binding motifs (after [360]). In **tenascin C**, each arm of the hexamer extends from a central N-terminal globule, followed by $13\frac{1}{2}$ EGF-like repeats and a number of fibronectin type III repeats which may be interrupted by fibronectin type I repeats depending on the splice variant. The arms end with a C-terminal globular domain (after [187]). The motifs in **thrombospondin** (here TSP-1) have not yet been found elsewhere, but the terminal globular domains are separated by seven distinct regions, three of which (circles) are reiterations (after [26,28]). **Collagen VI** has 17 von Willebrand factor (vWF A)-like repeats per trimer, eight strongly and three weakly homologous in the α3 chain and three weakly homologous in each of the α1 and α2 chains. Each chain also bears several RGD-sequences in the triple helical part [466].

the fibronectin molecule [85]. Type II homologies are also constituents of prothrombin [28] and tenascin (see below). Structural analysis of the type II-like domain of PDC 109 by nuclear magnetic resonance (NMR) revealed several loops forming a tight globular structure with a surface-exposed hydrophobic depression, a potential ligand-binding site 1 [84]. Type III homologies are found in 2% of all animal proteins, including, for example, tenascin, and collagens XII and XIV. They are composed of seven β-strands, forming two layers of β-sheets, which are held together by hydrophobic forces. The FN-III$_{10}$ module contains the cell-binding sequence, RGD, at the apex of a loop in a rather flexible conformation, allowing it to adopt more than one conformation when interacting with different integrin receptors.

The widespread occurrence of the conserved structural repeats in many extracellular macromolecules, and the fact that each repeat is encoded by one exon, indicates that fibronectin and many other extracellular matrix molecules have evolved by gene duplication and exon shuffling within ancestral precursors containing type I, II, and III repeats.

Carbohydrate, Sulfate, and Phosphate Groups

The fibronectin molecule contains 4–10% (wt/wt) carbohydrate, depending on its cellular origin. Fibronectin from transformed and embryonal cells contains more O- and N-linked carbohydrates than that from adult fibroblasts or plasma, which lacks the EIIICS segment with three carbohydrate binding sites in one chain. Placental and amniotic fluid variants also contain lactosaminoglycans in addition to the mannose- and sialic acid-containing N-linked sugars.

The carbohydrates protect fibronectin against proteolytic attack; whether they control the multiple interactions of fibronectin with cells and other macromolecules remains controversial. It has been reported that a secreted, nonglycosylated fibronectin, prepared in cell cultures in the presence of tunicamycin, was fully effective in mediating cell attachment and spreading [86]. Lack of carbohydrate may increase the interaction of fibronectin with gelatin and enhance its ability to promote the adhesion and spreading of fibroblasts [87].

Other post-translational modifications of fibronectin include phosphorylation of a serine residue at the carboxyl terminus and tyrosine sulfation in certain variants [88]. The biological function of these modifications is, however, unknown.

Splicing Variants

Variations in the solubility and subunit size of fibronectin from different sources had suggested heterogeneity among fibronectin molecules [89]. Sequencing of cDNA clones derived from different cells subsequently revealed the existence of at least ten different splicing variants, all derived from the same gene [17,47]. Two type III homologies, termed EIIIA and EIIIB (Fig. 3), may be completely spliced in or out; for example, they are both absent from plasma fibronectin but retained in fibronectin expressed in fibroblasts [90–92]. The EIIIB module is highly conserved among rat, human, and chicken; it is important for the conformation of fibronectin and contains the cryptic epitope responsible for fibril formation. Another domain, called V or IIICS (“connecting segment”), may also be partially spliced, giving rise to five splicing variants [91]. For example, in plasma fibronectin, the IIICS segment is partly spliced out by exon subdivision in one subunit [93]. The IIICS segment contains a second potential cell attachment site [51,94] and two binding sites for O- and one for N-linked oligosaccharides [81,92].

The role of some splice variants of fibronectin is still unclear. EIIIA expression is higher in transformed cells and tumors than in normal mammalian cells [95], but this is not observed in chick cells [69]. Using *in situ* hybridization and ribonuclease protection assays with probes specific for the spliced regions, fibronectin variants lacking either EIIIA or EIIIB sites were found to be distributed differently in the chick embryo [96]. In wound-healing experiments in adult rats, the fibronectin variant lacking both EIIIA and EIIIB, which is the major form in rat skin, was found in dermis and muscle, whereas the two segments were re-expressed in the fibronectin form found at the base of the wound [97].

Domain Structure and Molecular Interactions

Like most extracellular matrix molecules, the fibronectin molecule represents essentially a linear arrangement of modules that retain their structural and functional properties as isolated entities [98]: two or three fibrin-binding domains, a collagen-binding domain, two or three heparin-binding domains, at least two cell-binding domains, and a fibril-assembly domain (Fig. 3).

The multidomain structure of fibronectin has been discussed comprehensively in several review articles [17,22,58, 83,98]. Fibronectin binds not only to cells but also to a number of macromolecules, including denatured collagen I (gelatin) and, to a lesser extent, also native collagen [99], heparin [100], fibrin [101], thrombospondin [102], DNA [103], and actin [104]. The binding sites for these components are in separate domains; thus, fibronectin can undergo multiple simultaneous interactions with other molecules and with cells. Because the binding domains are generally more resistant to proteases than are their intervening bridges, they can be isolated as proteolytic fragments retaining full activity [50,98].

The amino-terminal 70 kDa domain is involved in matrix assembly and in fibronectin incorporation into the pericellular matrix of fibroblasts [105]. A 29 kDa fragment of this domain contains both a heparin- and a fibrin-binding site (see below); it is also involved in the binding of staphylococci to fibronectin [106–108], indicating that fibronectin might be a target for staphylococcal infection. In accordance with this notion, a fibronectin receptor has been identified in staphylococci and cloned [109,110].

Collagen-Binding Domain

The high affinity of fibronectin for denatured collagen (gelatin) ($k_d = 2–5 \times 10^{-9}$ M) [20] is unique and rather specific; thus, fibronectin can be purified from plasma by a single-step chromatography on gelatin-Sepharose to an extent of >90% purity [46,111]. Fibronectin also binds triple-helical collagen types I, II, III, IV, and V [99], but significantly only at 37 °C, when the collagen triple helix is partially denatured [112,113]. The general understanding is that fibronectin preferentially recognizes unfolded collagen structures. This is also supported by the fact that the fibronectin-binding sites in collagen types I and II are located in their single collagenase cleavage sites, where the triple helix is loosened [114]. The affinity of fibronectin for these sites may be sufficient to allow it to bind to collagens at 37 °C *in situ* thereby explaining the apparent codistribution of fibronectin and type I collagen in cell cultures and the electron-microscopical observation of fibronectin periodically decorating collagen type I fibrils [115].

The collagen-binding domain in fibronectin is located in a 40–45 kDa proteolytic fragment located next to the amino-terminal domain [53] (Fig. 1A). By preparing fusion proteins from overlapping cDNA clones from this region, the collagen-binding sequence has been restricted to 14 amino acid residues in the second type II repeat and the following type I repeat [79] (Fig. 3).

Fibrin-Binding Domains

Three binding sites for fibrin have been identified on each arm of fibronectin: one in the amino-terminal 29 kDa fragment [116], one close to the carboxyl terminus [118], and a third adjacent to the collagen-binding site, which is sensitive to trypsin and cathepsin D [117]. These interactions are of low affinity at 37°C but may be stabilized by covalent cross-linking through factor XIII (transglutaminase) [118]. In fibrinogen, the fibronectin-binding site is located in the carboxyl terminus of the α-chain.

Heparin-Binding Domains

The heparin-binding sites of fibronectin are most likely involved in the adhesion and spreading of certain cell types, such as 3T3 fibroblasts and melanoma cells. In these cells, but not in others, such as normal rat kidney (NRK) or NIL8 fibroblasts, the formation of focal adhesion plaques on fibronectin requires the presence of the heparin-binding domain in FN III repeats 12–14 [119,120] bound to syndecan [121–123]. One heparin-binding site is located in the amino-terminal domain; it is of low affinity and modulated by Ca^{2+}. The interaction is ionic and can be dissociated with 0.25 M NaCl. Heparin binding at the carboxyl-terminal site is stable up to 0.5 M NaCl and Ca^{2+}-independent [124].

Other glycosaminoglycans that bind to fibronectin are hyaluronan and chondroitin sulfate proteoglycan [125]. Hyaluronan binding is stable even at 1–2 M NaCl, suggesting a nonionic interaction [126].

Cell-Binding Domains

The search for the cell-binding site of fibronectin culminated in two exciting discoveries: the ubiquitous cell-binding motif, Arg–Gly–Asp (RGD), and the integrin superfamily of matrix receptors. Initially, the smallest fragment retaining cell-binding activity *in vitro* was a 75 kDa tryptic fragment [50]. For certain cells, such as NRK cells, activity could be retained in an even smaller 11.5 kDa pepsin fragment [127]. Sequencing of this peptide, and the production of progressively shorter, overlapping peptides for cell-adhesion experiments, finally narrowed the cell-binding activity down to the sequence Arg–Gly–Asp–Ser (RGDS) [50,128] in the 11th type III repeat (Fig. 3). The sequence motif, RGD, was later found in other extracellular cell-adhesive proteins, such as vitronectin (RGDV, Arg–Gly–Asp–Val) [129], fibrinogen [9], thrombospondin [130], collagen types I and VI [131], the α-chains of murine and human laminin [132], nidogen [133], von Willebrand factor, and others (for an overview, see [10]). The pentapeptide GRGDS supports cell adhesion when fixed to nonadhesive surfaces and strongly inhibits cell adhesion to fibronectin or vitronectin at concentrations between 0.1 and 0.5 μg/mL. Substitution of the aspartic acid residues in the peptide by glutamic acid (to give GRGES) completely abolishes its cell-adhesive activity, indicating high specificity of the cell-binding sequence [10].

An RGD-independent cell-binding site for melanoma cells has been located in the IIICS region of fibronectin [49]. Interestingly, adhesion of these cells is also inhibited by the GRGES sequence, which is completely inactive toward other cell types. Further analysis of this region has demonstrated two cell-binding sites, one in the amino-terminal part of the IIICS domain, which is recognized by melanoma and ganglion cells [134], and another near the carboxyl end of the IIICS domain, which contains an REDV sequence as the minimal cell-recognition signal.

Because the RGD sequence is not unique to fibronectin, the involvement of flanking sequences, distal adhesion elements, or conformational differences is postulated to explain the specificity of cell interactions with various glycoproteins. The elucidation of the exact mechanism of the molecular interactions among the various integrins and cell-binding structures of the glycoproteins is one of the most challenging tasks in current matrix research [135].

Fibronectin Monomers and Fibril Assembly

Although the fibronectin monomer as isolated from plasma appears as a thread-like molecule with the two chains unfolded by rotary shadowing (Fig. 2d), it is likely that the chains fold back in aqueous solution into a more globular molecule because of an alternating positive and negative charge distribution [82]. The assembly of monomers into fibronectin fibrils, such as observed on cell surfaces and in the interstitial space, requires the unfolding and activation of a cryptic site in the first type III repeat ($FN\text{-}III_1$) [55], which is then able to interact with another site in the fibronectin molecule to form fibrils [56,57] or a functionally distinct form, so-called "superfibronectin" [136]. Fibronectin assembly on cell surfaces is a multistep event and requires β1 or β3 integrins, while collagen and fibrinogen fragments may contribute to the unfolding process. Not all cells that synthesize fibronectin assemble fibronectin fibrils on their surfaces (e.g., macrophages [137], activated neutrophils [138], and activated T-lymphocytes [139]). This demonstrates that fibronectin fibril assembly is a cell-specific event involving distinct cell-surface molecules. Blocking antibodies against the β1 integrin subunit can prevent fibronectin assembly on the cell surface [140], which can be restored by the overexpression of β1 integrin subunits in β1 integrin-deficient cells [141]. Also, $\alpha v\beta_3$ integrin is able to induce fibronectin fibril formation [141], while other fibronectin receptors such as α4β1 or αvβ1 are unable to do so. Non-integrin membrane proteins such as syndecans or collagens [114] or a GGKD (Gly–Gly–Lys–Asp) membrane protein [142] may also promote fibril formation.

Biological Activities of Fibronectin

Cell Adhesion and Spreading In Vitro

The list of primary cells, cell lines, and tumor cells that have been shown to adhere to fibronectin *in vitro* [3,21] includes almost all embryonal and adult mesenchymal and epithelial cells and connective tissue cells; in addition, circulating lymphocytes and activated macrophages can be induced to become adherent to fibronectin.

Initial adhesion to fibronectin *in vitro* is inhibited by RGD peptides [50,128,143] and is usually followed by spreading and the formation of focal adhesion plaques, which involve the heparan sulfate-binding region. Immunofluorescence and electron-microscopical studies have suggested transmembrane linkages between extracellular fibronectin and intracellular actin filaments [144–146], which, via the mediators vinculin, talin, and fibulin, can bind to the cytoplasmic domain of the β-subunit of the α5β1 integrin fibronectin receptor [147–150].

Cell Proliferation and Migration

Fibronectin and vitronectin provide the major substrate in serum-containing cell culture, allowing the attachment and proliferation of anchorage-dependent cells. Fibronectin also stimulates the proliferation of myoblasts [151], keratinocytes [152], and hepatocytes [153] and allows the migration of neural crest cells [4] and melanoma and other carcinoma cells *in vitro* [15,16]. Other cells, such as myoblasts, are arrested by fibronectin and do not migrate [154,155]. The fact that cells adhere strongly to fibronectin, yet detach during mitosis and migration, illustrates the reversible character of fibronectin-cell interactions and reflects the degree of complexity of such interactions in tissues and organs.

Cell Shape and Differentiation

Cell spreading on fibronectin can induce the depolarization of epithelial cells and suppress the expression of differentiation markers, whereas laminin has the opposite effects (e.g., supporting the polarization of epithelial cells [156]). Spreading on fibronectin is also an early step in the dedifferentiation of chondrocytes [157,158] and myoblasts [159]. Moreover, fibronectin inhibits or delays several aspects of differentiation *in vitro*, such as the differentiation of keratinocytes [152], the formation of cartilage nodules in chondrocyte cultures, and the chondrogenic differentiation of limb bud mesenchyme [160]. On the other hand, transient expression of fibronectin during the condensation phase of limb bud mesenchyme [161–163] does suggest a role in chondrogenic differentiation *in situ*.

Role of Fibronectin In Vivo

The multiple effects of fibronectin on cells *in vitro* have been interpreted in terms of their possible physiological roles in plasma and the extracellular matrix. In plasma, fibronectin is assumed to have an opsonic effect on tissue-degradation products because of its affinity for denatured collagen, actin, DNA, and proteoglycans. As a major fibrillar component of blood clots, it certainly plays a central role in hemostasis and wound healing [164].

The location of fibronectin along migratory pathways in embryonic tissues indicates a controlling function in cell migration and proliferation [165–168]. Immunohistological studies suggest that neural crest cells can migrate along fibronectin-rich pathways in the early embryo [165,169]. Similarly, latex particles coated with the cell-binding domain of fibronectin are arrested in the chick embryo after microinjection, probably by binding to matrix receptors [170], while beads coated with the collagen- or heparin-binding domain of fibronectin are translocated ventrally. This illustrates the delicate balance between adhesion- and migration-stimulating activities of the extracellular matrix, which are influenced not only by the adhesive glycoproteins but also by the expression of matrix receptors at different sites and stages in development [17].

Another major function of fibronectin may be in the homing of lymphocytic cells. Whereas circulating lymphocytes do not bind fibronectin, activated T cells develop fibronectin receptors (VLA, very late antigens) and adhere to fibronectin and other matrix proteins, which may be the general mechanism by which inflammatory cells adhere to tissues during inflammation [18,171].

Until it is possible to probe at the immunocytochemical level for the specific signals generated by cell-fibronectin interactions, it will be difficult to identify particular functions of fibronectin in developing or adult tissues. The complexity of the analysis of such cell-fibronectin interactions is further illustrated by the fact that fibronectin is often interwoven with, or close to, other adhesive glycoproteins, such as collagens and laminins, which can have the opposite effect on cells *in vitro*. Antibody-blocking experiments *in situ* are subject to multiple artifacts, but useful information might be obtained from mutants with defects in fibronectin or its receptors should they be found.

TENASCIN

The tenascins are a family of large and complex extracellular glycoproteins ($M_r > 10^6$) with a unique molecular shape (Fig. 1B) and widespread tissue distribution. At present, five or six different tenascins are known (Tenascin-C, -R, -X, -Y, and -W), some of which occur as different splice variants. The six-armed tenascin-C (hexabrachion) consists of two three-armed units that are linked together at their amino ends by a central globule; each arm carries a further globule at its carboxyl-terminal end (Fig. 2a) [29–32,172,173].

Tenascin-C was independently discovered under different names in several laboratories. From glioma tumors, a "glioma mesenchymal extracellular matrix protein" [174] was extracted, which was later shown to be identical or homologous to the "myotendinous antigen M1" [175] and the "hexabrachion" molecule, a large, six-armed glycoprotein (Fig. 2a) coextracted with fibronectin from fibroblast cultures [176], "cytotactin" isolated from fetal chicken brains [177], and "J1 glycoprotein" involved in neuronal cell adhesion and neural development [178]. Tenascin-R (TN-R), a smaller molecule than tenascin-C (TN-C), has been originally discovered as "restrictin" in chicken brain [179] and as J1-160/180 (janusin) in rat brain [180]. TN-R is largely restricted to the central nervous system and the retina, where it plays a major role in neuronal differentiation and axonal growth (see below) [30].

Tenascin-X (TN-X), the largest of the five tenascins, is ubiquitously expressed in human fetal tissues, particularly blood vessels and skeletal, cardiac, and smooth muscle, but is absent from brain and testis [181]. Its distribution is nearly complementary to that of tenascin-C [182]. Tenascin-Y (TN-Y) has been found in chicken heart and skeletal muscle and, like tenascin-X, is structurally homologous to TN-C [29]; tenascin-W has been discovered in zebrafish [183].

Tenascin Structure

Tenascins are largely a composite of structural domains found in other molecules, with only short, tenascin-specific sequences (Fig. 3) [184–187]: the amino-terminal globular domain of each of the arms of TN-C is part of the central globule; it is followed by a short stretch of four α-helical heptad repeats, which allow the assembly of three arms into a triple-stranded coiled-coil structure in the connecting region [184,187] (Fig.1B). TN-C and TN-R, but not TN-X, contain a single cysteine residue at their amino terminus, which can connect two three-armed trimers to form the hexabrachion; yet, TN-R has been isolated so far only as a single trimer [188]. The α-helical part is followed by $13\frac{1}{2}$ EGF-like repeats in TN-C, four in TN-R, and 17 EGF repeats in TN-X. The outer, thicker arm of TN-C, representing two-thirds of each chain, consists of 8–16 fibronectin type III homologies (TNfnIII) containing numerous N-glycosylation sites [189]. Upon SDS-polyacrylamide gel electrophoresis, tenascin-C separates into three subunits of M_r 190 kDa, 200 kDa, and 230 kDa that are structurally related but differ in stretches of up to 273 amino acid residues due

to differential splicing of the same gene product (see below). Eight splice variants of TN-C have been found so far, all of which contain FN III repeats 1–8, which may be interrupted by additional other FN repeats [31] (Fig. 3). In TN-C, an RGD sequence has been located ten residues from the end of the third FN III domain [185,190]. This RGD sequence is involved in αv integrin-mediated cell adhesion of TN-C [191–193]; another cell-adhesive domain is located in the C-terminal globule [194] (see below) and shows 40% homology to the β- and γ-chains of fibrinogen.

Tenascin Distribution in Tissues

Tenascin-C shows two patterns of distribution: a transient appearance in certain embryonic tissues, in wound healing, and in tumors, and a permanent expression in some adult tissues, such as the myotendinous junction, nervous tissue, and cartilage [195]. During chick embryogenesis, TN-C appears first with gastrulation and subsequently in association with the basement membrane of the neural tube and in myotendinous junctions, smooth muscle, kidney, lung, and other tissues in the developing chick embryo [196,197]. Typical is the appearance of TN-C in certain cell-migration pathways [198,199].

The presence of TN-C is most prominent in the extracellular matrix of nervous tissues [174,197]. It is synthesized by astrocytes [174] and has been located in glial fibers in the neural tube and in the retina [198]. A characteristic feature of TN-C is its temporary and strictly spatially restricted appearance in certain embryonic mesenchymes. A strong expression of TN-C is found in maxillary mesenchyme during tooth development, but complete turnover follows differentiation of mesenchyme to odontoblasts. Generally, TN-C is pronounced in embryonic mesenchyme adjacent to epithelia, for example, in mesenchyme surrounding fetal glands and hair follicles, the developing kidney [201], and gut [202]. In the developing kidney, TN-C appears in the mesenchyme shortly after its interaction with epithelium [201], suggesting the induction of TN-C synthesis by the epithelium. In fact, an epithelial cell line (MDCK, Madin-Darby canine kidney) is able to stimulate significantly TN-C synthesis in melanoma cells, and mouse mammary epithelium induces tenascin-C synthesis in the adjacent stroma [203]. During limb bud chondrogenesis, TN-C is pronounced in the cartilage blastema but not in the surrounding mesenchyme [204,205]. It is also present in the perichondrium and in osteoprogenitor cells in endochondral bone formation, but is absent from bone matrix [204,205]. The localization of tenascin in bone marrow [206,207] and thymus [208] indicates a role for tenascin in hematopoiesis [209]. Adult tissues that have been shown to contain TN-C include brain [177,210], gizzard, skin [172,197], and cartilage [205,211,212].

There is considerable interest in the selective accumulation of tenascins in human tumors such as gliomas [174] and mammary tumors [172,213]. Tenascin is associated not only with malignant tumors, in particular breast, lung, and cervical carcinomas [214], but also with benign tumors, fibrocystic diseases, or neuroblastomas [213]. The majority of the tenascin in tumors is synthesized by stromal cells from the host tissue, but carcinoma cells may also be induced to produce tenascin. The role of tenascin in tumorigenesis is not yet clear. By cross-breeding TN-C-deficient mice with a strain that develops spontaneous mammary tumors, Talts et al. [215] demonstrated that the absence of TN-C had no effect on the number and size of tumors, their rate of proliferation, or apoptosis. Tumor organization, however, was different in the absence of tenascin, showing smaller tumor cell clusters and more monocytes in the stromal compartments. Similarly, tenascin is up-regulated in fibrotic tissues, such as the fibrotic liver, or in the heart muscle of myocarditis patients [216].

Adhesive and Antiadhesive Activities of Tenascin

Tenascins are involved in a variety of cellular interactions and activities that may, in part, appear conflicting, thus illustrating the complexity of tenascin function. Primary chick embryo and human fibroblasts, tumor cells, and endothelial cells attach to TN-C but do not spread [190,217]. TN-C also inhibits the adhesion of chick fibroblasts to fibronectin, and this inhibitory activity can be neutralized by a monoclonal antibody against the C-terminal part of the TNfnIII homologies [217]. The complex role of tenascin in regulating cell adhesion is best illustrated by its apparently "double-faced" (tenascin-R is also called "janusin" [239]) behavior toward nerve cells. TN-C promotes neurite outgrowth on polylysine coated culture substrates [173] but inhibits β1-integrin-mediated cell adhesion and neurite outgrowth on fibronectin [180,218]. Based on its localization, TN-C was originally thought to form a barrier to advancing neurites, but more recent evidence has indicated that it may guide axonal growth in both a permissive and a repellent way [219,220]. In fact, it has been shown that neurite outgrowth and neurite guidance are facilitated by distinct sequences within the alternatively spliced FN III repeats A–D of TN-C [221]. RGD- and αv-integrin-dependent adhesive activities of TN-C are located in the third FN III repeat [191–193], the same site that is responsible for glioma cell adhesion and migration on TN-C [222]. This is consistent with the observation that the migration of human glioma cells on tenascin is inhibited by function-blocking anti-β1, α2, and αv antibodies [223,224]. The β1-integrin-dependent cell adhesion and neurite outgrowth on fibronectin involves a disialoganglioside-mediated signaling mechanism [225].

Endothelial cell attachment and spreading on human tenascin is also mediated by α2β1 and αvβ3 integrins [226]. The RGD site is, however, cryptic and not accessible in the native, intact tenascin molecule due to sterical hindrance [193]. In addition to this attachment site, an RGD-independent adhesive epitope is located in the same FN III_3 repeat, which is, however, α9β1-integrin-dependent [194].

The antiadhesive effect of tenascin is probably the result of two independent mechanisms. One may be sterical hindrance of cell-adhesion receptors for other matrix components by the large, umbrella-like structure of the tenascin molecule, and the other may be an active process of cell rounding and detachment, mediated by integrin recognition of tenascin.

Tenascin also binds to cell surfaces and thus can agglutinate cells [227]. Previously, the agglutinating activity was believed to be associated with fibronectin; it is now known, however, to have originated in tenascin that had contaminated cellular fibronectin preparations.

In contrast to fibronectin, tenascin undergoes only a few, selective interactions with other macromolecules. It binds to heparin through its third TNfnIII repeat [228,229] and to fibronectin *in vitro*, yet with low affinity. More marked is its binding to large, aggregating chondroitin sulfate proteoglycans [230], such as versican and phosphacan, which copurify with cytotactin from embryonic chick brain [210,231].

Tenascin-C-Deficient Mice Develop Normally

Most surprisingly and, in part, disappointingly, the generation of a TN-C-deficient mouse by homologous recombination initially did not support such a significant role for TN-C in cell and tissue function as had been predicted from *in vitro* studies [232,233]. The mice were viable and fertile, and all tissues, including central nervous system, brain, lung, and thymus, developed apparently normally. Also, wound healing was not impaired [233]. Initial suggestions that a truncated form of TN-C was still expressed in the nervous system of TN-C knockout mice [234] have not been confirmed [235]. Substitution of TN-C functions by TN-R, TN-X, or TN-Y is highly unlikely in view of their completely different tissue distributions. More recently, however, careful investigation of the TN-C knockout mouse has revealed alterations in the structure of neuromuscular junctions and peripheral nerves [236,237] and suppression of hematopoietic activity [209]. Furthermore, corneal wound healing does seem affected [238]. Spontaneous carcinomas developing in TN-C-deficient mice developed to a different size and morphology from those in normal mice and were infiltrated by an excessive number of macrophages and monocytes [215]. Thus, while distinct functions of TN-C found at the cellular level *in vitro* may be redundant, a major role of tenascin may relate to the functionality of the entire organism, such as by supporting reparative and remodeling activities in response to stress and injury.

Tenascin-R — a Regulator of Neuronal Growth in the CNS

Tenascin-R (TN-R), previously discovered as restrictin [179,180,188] or janusin [239], is largely restricted to the central nervous system, where it seems to play a complex role in the guidance of axonal growth and neuronal differentiation [30,180]. TN-R is synthesized by oligodendrocytes during myelination [225] and other neuronal cells such as motoneurons in the spinal cord and horizontal cells in the retina; it concentrates around the nodes of Ranvier [240] and perineuronal nets.

From *in vitro* studies, a variety of functions have been ascribed to tenascin-R. It promotes the adhesion and differentiation of astrocytes and oligodendrocytes [180,241], and neurite outgrowth and neural polarization when offered as a uniform substrate [200], but repels growth cone progression and causes neurite defasciculation when offered as a substrate boundary with a neurite-outgrowth-promoting molecule [218]. The interactions of TN-R with neuronal cells are thus complex and may involve different cellular receptors. One of these is contactin F3/F11 (F3 in rodents; F11 in chicken), an adhesion molecule of the immunoglobulin superfamily, which is expressed predominantly by neurons [242]. TN-R and TN-C binding to the Ig-like domains of contactin causes clustering of the receptor and seems to induce tyrosine phosphorylation of *fyn*, an *src*-like kinase [243], suggesting that TN-R-elicited signals can be transmitted by the contactin molecule. Interestingly, the β2-subunit of the neuronal sodium channel contains an Ig motif that is structurally homologous to one of the six Ig motifs of contactin. In fact, TN-C and TN-R also bind to the purified sodium channel or a recombinantly produced β subunit, suggesting that tenascin may localize sodium channels in high density at axon initial segments and nodes of Ranvier [244]. This notion, however, was not confirmed by investigations into the TN-R-deficient transgenic mouse [240].

Cell-surface gangliosides also play a regulatory role in the adhesion and differentiation of oligodendrocytes. TN-R promotes the stable adhesion and differentiation of oligodendrocytes by a sulfatide-mediated autocrine mechanism; it is antiadhesive for ganglioside GD3-positive preoligodendrocytes and inhibits their integrin-dependent adhesion to fibronectin by a disialoganglioside-mediated signaling mechanism.

As a major constituent of the perineuronal matrix, TN-R is likely to interact with other matrix components. It codistributes with versican, a large hyaluronan-binding chondroitin sulfate proteoglycan in the cerebellum [245], and phosphacan, a nervous-tissue-specific chondroitin sulfate proteoglycan in retinal optic nerve and brain [246]. *In vitro*, TN-R binds to phosphacan with high affinity and can be isolated by affinity chromatography on the amino-terminal ECF domain of phosphacan [246,247].

The functions of TN-R suggested by these *in vitro* studies were partially confirmed by the analysis of a mouse deficient for TN-R in both alleles [240]. The TN-R-deficient mice were viable and fertile, similar to TN-C knockout mice [231,232], and the anatomy of all major brain areas and the myelin structure appeared normal, but a loss of immunoreactivity and a more diffuse distribution of phosphacan was observed [240]. Furthermore, measuring action potentials in optic nerves indicated a significant decrease in conduction velocity in the TN-R-deficient mice. The authors concluded from these and other observations that TN-R has an essential role in the formation of perineural nets and in the conduction velocity of optic nerves.

THROMBOSPONDIN

Thrombospondin-1 (TSP-1) was originally detected in platelets as the major high-molecular-weight glycoprotein (M_r 450,000) of α granules released after thrombin activation [248,249]. Like fibronectin, it is a normal constituent of both plasma and serum, and the extracellular matrix, but also plays a major role in hemostasis and angiogenesis as well as in matrix organization [26–28,250,251]. The three-armed molecule, with its remarkable, bolas-like shape (Fig.2b), is able to interact with a number of plasma proteins, such as fibrinogen, thrombin, and urokinase, and with matrix proteins, including fibronectin, collagens [252], and heparan sulfate proteoglycans. Furthermore, it regulates cellular activities such as proliferation and migration, not only by direct interaction with cell-surface receptors such as integrins, CD36, CD47, and syndecans. A new, exciting aspect of thrombospondin has arisen with the finding that a distinct peptide located in TSP-1 activates latent TGF-β [253] and TSP-1 thus may interfere with the regulation of collagen, fibronectin, and matrix metalloproteinase (MMP) synthesis.

Further proteins have subsequently been discovered that are similar to thrombospondin in protein structure and DNA sequence. Thrombospondin-2 (TSP-2), like TSP-1, is a three-armed molecule and shares several properties with it, such as inhibition of angiogenesis, impairment of wound healing [254], and control of collagen fibril arrangement [255]. TSP-3 [256–258], TSP-4 [259–262], and TSP-5 are five-armed molecules; TSP-5 is also called COMP (cartilage oligomeric matrix protein [263]) (see also Chapter 4, this volume), with predominant occurrence in cartilage. This chapter focuses predominantly on TSP-1 and TSP-2, which are, besides COMP/TSP-5, the most intensively studied members of the TSP family.

Thrombospondin Structure

Electron-microscopic investigation of TSP-1 by rotary shadowing (Fig. 2b) and sequence analysis revealed that the molecule is composed of three identical dumbbell-like subunits, each with a large globular domain at the carboxyl end and a small globular domain at the amino-terminal end (Figs. 1C, 3) [26,27]. The three arms are linked together by a central disulfide knot. Similarly to tenascin, each thrombospondin subunit of 1152 amino acid residues is composed of several structural domains (Fig. 3) and repeats that are post-translationally modified by glycosylation and β-hydroxylation of asparagine residues [26,250]. The middle part of each constituent subunit and part of the large C-terminal globule contain the binding sites for collagen, fibrinogen, laminin, and plasminogen. The globular domains at both ends of the subunits are unique sequences unrelated to other protein domains. The C-terminal 18 kDa fragment of each subunit contains the thrombospondin-specific sequence RYVVMWKIRVVM (Arg–Tyr–Val–Val–Met–Trp–Lys–Ile–Arg–Val–Val–Met), (4N1K motif), which plays a key role in TSP binding to cells. This interaction is mediated by the integrin-associated protein, CD47 [264,265].

The stalk region of the molecule begins at the amino end of each subunit, with a cysteine-containing sequence involved in trimer formation and three types of repeat domains (Fig. 3). The amino-terminal region of the 70 kDa domain contains a procollagen-like domain with 37% identity to the amino-terminal propeptide of type I collagen. It is followed by three type 1 repeats (''properdin repeats''), three type 2 repeats with EGF homology, and seven aspartic acid-rich type 3 repeats containing Ca^{++}-binding sites of the calmodulin/parvalbumin type. One TSP-1 molecule binds up to 35 Ca^{++} ions; the binding of Ca^{++} significantly affects the structure of TSP-1 and its resistance to proteases, as well as its function in tissues [266].

Both murine (*tsp-1*) and human (*TSP-1*) genes are about 20 kb in length, each containing 22 exons, and are transcribed and processed into mRNA species of 6 kb length. The human gene is located on human chromosome 15q15 and the murine gene on chromosome 2, band F. The promoter of the *TSP-1* gene contains multiple binding sites for transcription factors; for example, a serum response element. Splice variants of TSP-1 mRNA have been found in some tumor cell lines but not yet in tissues [267].

Cellular Origin and Tissue Distribution

After exposure to thrombin, platelets release thrombospondin from their α granules into the plasma (30–90 $\mu g/10^6$ platelets), some of which binds to the platelet surface. As a result of this release, the thrombospondin level increases several orders of magnitude, from 97–163 ng/mL in normal plasma to 15–17 μg/mL in serum after blood clotting; in blood clots it is uniformly incorporated into fibrin fibrils. Other major sources are human umbilical cord endothelial cells and aortic endothelial cells [268]. Thrombospondin synthesis *in vitro* has also been demonstrated in skin and lung fibroblasts [269,270], smooth muscle cells [271], keratinocytes [272], and monocytes and macrophages [273].

Thrombospondin-1 and thrombospondin-2 are developmentally regulated and expressed in a temporally and spatially restricted pattern in the developing organism [274]. By immunofluorescence staining, they have been located in a fibrillar distribution in vessel walls, skin, muscle, kidney, glandular epithelium, and embryonic basement membranes [275]; at the ultrastructural level, they appear as granular structures in the vicinity of cells. In cartilage, TSP-1, TSP-2, TSP-3, and TSP-5 (COMP) occur in different sites and at different developmental stages; TSP-1 is expressed in young proliferating cartilage, whereas TSP-3 occurs only in mature cartilage of the growth plate [258].

Cellular Interactions and Functions

Thrombospondin is a major regulator of hemostasis. It plays an important role in the second, irreversible phase of platelet aggregation by stabilizing the platelet-glycoprotein (GP) IIb-IIIa-fibrinogen complex [276,277], a reaction that can be inhibited by antithrombospondin antibodies [278]. Immunofluorescence analysis and cell-surface iodination have localized thrombospondin at the platelet surface [279]. A platelet glycoprotein of 88 kDa that has been identified as the thrombospondin receptor is probably identical to GP IV [280]. Binding of thrombospondin to platelets is inhibited by OK Mb, a monoclonal antibody against GP IV, but not by the tetrapeptide RGDS (Arg–Gly–Asp–Ser) [281]. The binding of thrombospondin to platelets increases dramatically in the presence of calcium; accordingly, cell adhesion to thrombospondin substrates is abolished by EGTA [130].

TSP-1 plays a key role in regulating angiogenesis by controlling the adhesion, proliferation, and migration of endothelial and smooth muscle cells [282–284]. TSP-2 has also been shown to inhibit angiogenesis [285]. The interaction of TSP-1 with endothelial cells is rather complex and dependent on its aggregation state: in insoluble, matrix-bound form, it promotes endothelial cell proliferation, whereas in soluble form it inhibits proliferation. It also inhibits chemotactic responses of endothelial cells [283] but supports chemotaxis of peripheral monocytes [286]. The importance of TSP-1 and TSP-2 in angiogenesis was confirmed by the results of studies on TSP-1-deficient mice, which showed delayed organization and prolonged neovascularization of skin wounds [287].

The adhesion of U937 monocytes, NRK cells, and endothelial and smooth muscle cells to thrombospondin substrates is inhibited by GRGDSP (Gly–Arg–Gly–Asp–Ser–Pro) and GRGDAC (Gly–Arg–Gly–Asp–Ala–Cys); in accordance with this finding, αvβ3-integrin-binding sites have been located on the TSP-1 molecule, indicating the involvement of integrins in TSP binding to these cells, although thrombospondin only supports their adhesion, not their spreading [130,288], and prevents the formation of focal adhesion plaques. In addition, thrombospondin promotes the RGD-independent haptotactic migration of smooth muscle cells [289] and human melanoma cells [290].

TSP-1 interacts also with cell-surface receptors other than integrins, particularly cell-surface heparan sulfate proteoglycans such as syndecans and CD36 [291], as well as CD47, an integrin-associated protein. By interaction with these receptors, thrombospondin is able to elicit various kinds of cellular signaling. It is able to regulate intracellular calcium levels in fibroblasts, a process involving the RGD motif in the type 3 repeats as well as the C-terminal globular domain [292]. Signaling via the 4N1K motif couples the CD47 transmembrane receptor to heterotrimeric G_i proteins [265].

Interactions with Other Molecules

Thrombospondin binds to plasma proteins involved in hemostasis and thrombosis, such as fibrinogen, fibrin,

plasminogen, and HRGP (hydroxyproline-rich glycoprotein), and to extracellular matrix components, including fibronectin, collagen, heparan sulfate proteoglycan, heparin, and laminin [250,293]. Among collagens, type V, which is generally the least interactive of all collagens studied, shows the highest affinity for thrombospondin [294]. Rotary shadowing analysis of the thrombospondin-collagen V complexes has shown that the type V collagen molecule binds to thrombospondin via the end of its triple helix [252]. Interestingly, the formation of collagen fibrils is significantly disturbed in TSP-1 and TSP-2 knockout mice [252,255].

The list of interactions of thrombospondin with structural molecules and enzymes involved in hemostasis and fibrinolysis is still growing. Elucidation of the molecular mechanisms of these interactions, and the affinities and molecular structures involved, might eventually help in understanding the complex role of this molecule.

VITRONECTIN

Vitronectin was identified as one of the major cell-attachment factors in serum. The 70 kDa protein has multiple functions as a regulator of proteolysis in blood and tissues and as a molecule protecting cells from cytolysis by complement factors. The various functions of this multidomain protein may be explained by its conformationally labile structure, which can be induced to take up different conformations [33–35,295,296].

Synthesis and Distribution

Vitronectin is found in serum concentrations of 300 μg/mL or more produced by hepatocytes [297], but it is also synthesized by other cell types, such as megakaryocytes [295] and neural crest cells [298]. Although vitronectin mRNA has been found in many embryonic murine tissues, the distribution of the protein in adult murine tissues is somewhat restricted to the extracellular matrix of vessel walls, skin, and bone [34].

As its name implies, vitronectin has an affinity for glass. It was first partially purified from serum over glass beads [299] and shown to promote the attachment and spreading of cells *in vitro*. Subsequently, it was used as an addition to serum-free media to promote cell attachment [297,300]. It can be isolated routinely in good yield from both serum and plasma using glass bead columns and anion-exchange chromatography [301].

The traditional tissue-culture growth-factor supplement, fetal calf serum, contains up to ten times as much vitronectin as fibronectin. Under normal culture conditions, it is probably the principal cell-attachment and spreading factor that cells use.

Structure

Vitronectin consists of four distinct domains; the amino-terminal domain, with 44 amino acids, is also called somatomedin B [302,303] and is found in serum as a separate entity after proteolytic processing. In the intact molecule, the somatomedin B domain is followed by a connecting segment, which is characterized by a cluster of negatively charged residues, including sulfated tyrosine residues. This domain carries an RGDV(F) sequence, 45 residues from the N-terminus, which represents the major cell-attachment site recognized by α3β1, αvβ3 $\alpha_{IIb}\beta3$ and αvβ5 integrins [129,304] and contains a cryptic collagen-binding site [293,305]. The C-terminal half of the molecule consists of two basic, hemopexin-like domains, which can interact with the acidic residues of the connecting segment of the same molecule, thus allowing the formation of intramolecular bonds or intermolecular bonds to form vitronectin polymers [306]. In an open conformation, these domains may also bind to heparin [303,307]. In addition, there is a glycosaminoglycan recognition site some 350 residues from the N-terminus, a site recognizing the thrombin-antithrombin complex [308], and a site permitting attachment to native collagens I and III [305].

Upon SDS-polyacrylamide gel electrophoresis, vitronectin shows two bands of 65 kDa and 75 kDa [297,309]. The 75 kDa form may be a precursor of the 65 kDa form produced by COOH-terminal proteolysis. Vitronectin has been cloned, and the cDNA codes for a protein of 52 kDa [308]; the difference in molecular weight from the above is due to N-linked sugars, the sequence having three consensus sites for N-glycosylation.

Functions

A specific feature of vitronectin is its ability to bind through its somatomedin B domain to PAI-1, the plasminogen activator inhibitor and member of the serpin (serine-protease inhibitor) family [302,310]. Furthermore, vitronectin can form complexes with thrombin and antithrombin and mediates endocytosis [293]. Thus, it is to be considered an important regulator of extracellular proteolysis, particularly of fibrinolysis in blood and tissues.

Because vitronectin can bind extracellular matrix components through its glycosaminoglycan and collagen recognition sites, and cells and platelets through its RGD sequence, it is tempting to infer that it has a role in thrombus building and removal following damage to the endothelium. Furthermore, vitronectin protects tissue cells from cytolysis by complement factors by blocking the membrane-binding site of the C5b-7 complex and preventing polymerization of complement factor C9 [311].

LAMININS

The laminins constitute a family of large adhesive glycoproteins, which occur predominantly in basement membranes and modulate diverse biological functions. They are heterotrimers composed of three chains, α, β, and γ (Fig. 1D), each chain being coded for by a separate gene [24,25,312,313]. To date, five α, three β, and three γ chains have been identified, which may combine to form at least 12 different isoforms [314–320] (Table 2). The genes coding for the individual laminin chains are grouped in relation to α (*LAMA*), β (*LAMB*), and γ (*LAMC*) chains, such that *LAMA1* codes for laminin α1, *LAMA2* codes for laminin α2, and so forth [25]. The heterotrimeric chain composition of 12 different laminin isoforms listed in Table 2 and illustrated in Figure 4 has been deduced from colocalization of chain-specific antibodies by immunohistochemistry and/or their ability to isolate or immunoprecipitate the heterotrimer. The existence of some laminin isoforms, however, such as laminins 3 and 9, is based entirely on colocalization of chain-specific antibodies, which is not sufficient to confirm the existence of such heterotrimer assemblies *in vivo*. The current data suggest that further novel laminin isoforms remain to be identified and characterized.

Primary Structure

Laminin-1, composed of α1, β1, and γ1 chains, was the first of the laminin isoforms to be identified. It was originally isolated from the culture medium of a murine embryonal carcinoma cell line, M1536-B3 [321], and subsequently in large

TABLE 2. The Laminin Family

Laminin Isoform	Chain Composition	Tissue Distribution
Laminin-1	α1β1γ1	developing epithelia
Laminin-2	α2β1γ1	muscle fiber, peripheral nerves
Laminin-3[1]	α1β2γ1	myotendinous junction?
Laminin-4	α2β2γ1	neuromuscular junction, glomerulus
Laminin-5	α3β3γ2	epidermis
Laminin-6	α3β1γ1	epidermis
Laminin-7	α3β2γ1	epidermis
Laminin-8	α4β1γ1	endothelium, smooth muscle, fat, peripheral nerves
Laminin-9	α4β2γ1	neuromuscular junction, glomerulus
Laminin-10	α5β1γ1	mature epithelium and endothelium
Laminin-11	α5β2γ1	neuromuscular junction, glomerulus
Laminin-12	α2β1γ3	surface of ciliated epithelia

[1] It is debatable whether laminin-3 exists *in vivo*; unpublished data have suggested the coexistence of laminin α1, β2, and γ1 chains at the myotendinous junction in human muscle (D. Gullberg, Uppsala, Sweden).

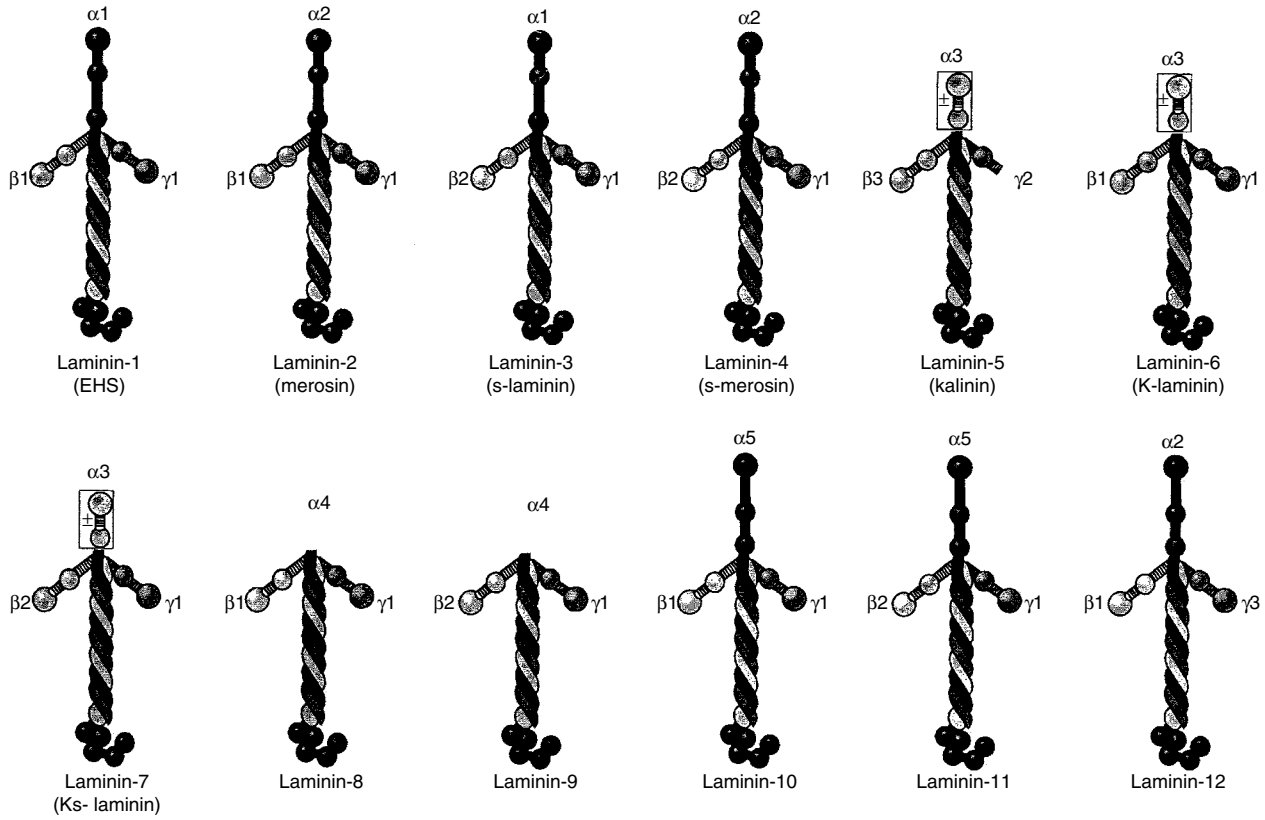

Figure 4. Laminin isoforms. To date, 12 different laminin isoforms have been identified, with distinct α, β, and γ chain compositions. Rotary shadowing has revealed laminins 1–4,10 (Drosophila-like) and 11 to be approximately cross-shaped molecules, the three short arms of which are formed by the amino-terminal portions of the α, β, and γ chains. Laminins 5–9 appear as approximately Y-shaped molecules in rotary shadowing due to reduced N-termini in the laminin α3 and α4 chains. The laminin α4 chain is N-terminally truncated, lacking domains IV–VI, while the α3 chain exists as two splice variants, one of which lacks N-terminal portions of the chain (boxed region). No rotary shadowing information is available for laminin-12. In all laminin isoforms, α, β, and γ chains unite in a coiled-coil α-helix in the central long arm and are disulfide-linked at the center of the cross and at the end of the long arm. Globular domains of the short arms are separated by EGF-like repeats. The large α-chain-derived globular domain at the end of the long arm, characteristic of α chains, is composed of five subdomains called G-globules (G1–G5) that are stabilized by disulfide bridges.

amounts from neutral salt extracts of the Engelbreth-Holm-Swarm (EHS) murine basement membrane tumor [322]. Much laminin is bound into the EHS tumor basement membrane in a calcium-dependent manner and can be rapidly extracted with EDTA, in conjunction with a 150 kDa subsidiary protein, nidogen/entactin [323,324]. This method has also allowed the extraction of various native laminin isoforms from murine [324,325], human [320,326–328], bovine [325,329], and chick tissues [330,331], from several cell lines [315,318,332–335], and from *Drosophila melanogaster* [336,337] and sea urchin [338].

Rotary shadowing electron microscopy of isolated laminins has shown them to be either cruciform, with three short arms (36 nm), each terminated by two globular domains, and a long central arm (77 nm) terminated by a single large globular domain, or approximately Y-shaped and lacking the central short arm (Fig. 2c). A current model [132] suggests that the laminins are disulfide-linked heterotrimers. All three chains align in the long arm and separate at the center of the cross, each to form one short arm [24,25,312,313] (see Fig 2c). Between 10% and 15% (wt/wt) of laminin is carbohydrate, mainly in N-linked form. Some 15 complex processed forms have been found, some containing unusual oligomeric lactosamine extensions [339], the functional significance of which is unknown.

Most laminin α, β, and γ chains share homologous structures, which include globular domains (domains IV and VI), cysteine-rich rod-like domains containing EGF-like repeats known as laminin-like EGF repeats (domains III and V), and domains forming the α-helical coiled coil of the long arm of the molecule (domains I and II), which is also revealed by circular dichroism measurements [340]. In addition, all laminin α chains identified to date contain a large C-terminal globular domain with five internal repeat motifs (G domain) (Figs. 1D, 3). However, variations occur in the extent of expression of these domains in different laminin chains. For example, laminin γ2 lacks domain VI and portions of IIIb [341,342], whereas laminin β3 lacks terminal domain VI [343], resulting in truncated molecules. In the case of laminin α3, the gene is differentially spliced, resulting in either a full-length polypeptide, laminin α3B, or a polypeptide that lacks domains VI, V, and IVb, laminin α3A [344]. The laminin α4 chain lacks the N-terminal globular domains (domains IV and VI) and portions of the rod-like region (domains V and IIIb) [315]. Truncation may also come about through proteolytic processing occurring after secretion, as shown for the laminin α3 chain [345] and the α2, β1, and γ3 chains of laminin-12 [320]. In contrast, in laminin α5, domain V is exceptionally long [317]. In all laminin chains, both the NH-proximal region of domain II and the COOH-proximal region of domain I have interchain disulfide bridges necessary for holding the heterotrimer together.

Structure-Function Relationships in Laminin Isoforms

Laminins are very large molecules, the α chains ranging in molecular weight from 165 kDa for laminin α3A to approximately 400 kDa for laminin α1 and α5 chains, while β and γ laminin chains vary from 105 to 210 kDa. The heterotrimeric molecules, therefore, are in the order of 500–900 kDa. To define the function of particular structural domains in these large molecules, protein fragments prepared by limited proteolysis have been employed in *in vitro* assays [346–348]. However, dissection of the biological functions of the various laminin domains has been complicated by the interweaving of α, β, and γ chains. Many activities disappear when the chains are separated, and slight perturbation of the tertiary structure, such as by heating [154], results in irreversible loss of function. Nevertheless, biologically active proteolytic fragments can be prepared, in particular by elastase digestion [346,348,349] (Fig. 1D). Peptic (P) and chymotryptic (C) fragments closely corresponding to some elastase-derived fragments have also been investigated [350–352]. To date, only laminin-1 can be isolated in sufficient quantities to permit proteolytic fragmentation; hence, laminin-1 fragments are the best characterized and most widely employed in *in vitro* assays. However, because of conservation of domain structure in all laminin chains known to date, many of the structure-function relationships defined with laminin-1 fragments are thought to be conserved in other isoforms.

Elastase digestion of laminin-1 produces eight major fragments [350–353]. E8 represents the lower 35 nm of the long arm, comprising the C-terminal 226 residues of the β1 chain, the C-terminal 246 residues of the γ1 chain, and that portion of the α1-chain C-terminal to residue 1886, but lacking the C-terminal E3 fragment, of 55 kDa [132,353]. The fragment E1-4 (or E1-X) consists of the three short arms of laminin [132,353]. It is heterogeneous (hence 'X'), often lacking one or more arm-terminating globular domains. E1 is E1-4 but lacking all six short-arm globules. Fragments E4, E5, and E6 are globular preparations, possibly from the termini of the short arms. P1 is produced by peptic digestion of fragment E1-4 and lacks the terminal globular domains of the short arms (domains VI).

The most important functions of laminins in basement membranes involve their ability to self-assemble into a network structure and their ability to interconnect with other basement membrane components and cell-surface receptors. Studies involving laminin-1 fragments have identified the domain structures crucial for these different functions. Domains VI of the short arms of the molecule are necessary for self-assembly [354,355], and EGF-like repeats in domain III are crucial for laminin assembly into basement membranes via nidogen-1 binding, while the G domain (fragments E8 and E3) of the laminin α1 chain contains various cell-binding sites [356] as well as heparan sulfate proteoglycan-binding sites (fragment E3).

Laminin Self-Assembly and Incorporation into the Basement Membrane

Purified laminin-1 can polymerize in the presence of Ca^{2+} in a concentration- and temperature-dependent manner [354]. This self-assembly process requires the N-terminal globules of domains VI of the short arms of laminin-1 (Fig. 1D) because the inner cross-fragment, P1′, which lacks the α1, β1, and γ1 chain N-terminal globules, cannot bind to itself or to intact laminin-1 molecules, while both fragments E4 and E1′ can inhibit polymerization of laminin-1. Self-assembly of laminin-2 also requires the N-terminal globules of domains VI, as may be deduced from the dy^{2J}/dy^{2J} mouse, in which the laminin α2 chain lacks domains VI and assembly into the basement membrane is impaired [357], resulting in a mild muscular dystrophy. A similar condition has also been identified in humans [358]. In the case of laminin isoforms that have significantly truncated short arms, such as laminin-5, covalent linkage to laminins-6 and-7 allows cross-linking to the collagen type IV network and appropriate integration into the basement membrane [359].

The laminin network is thought to be interconnected to the collagen type IV lattice via nidogen-1, a sulfated glycoprotein of 150 kDa ubiquitously localized in basement membranes of embryonic and adult tissues. Rotary shadowing and electron microscopy (Fig. 2c) have revealed that nidogen-1 consists of three globular domains (G1–G3), with two of the globules (G2 and G3) connected by a rod-like segment containing four EGF-like domains and one thyroglobulin-like domain [360] (Fig. 3). The connection between G1 and G2 is both shorter and thinner (Fig. 1E) and presumably represents a flexible link which provides protease-sensitive sites that are readily cleaved by tissue proteinases such as thrombin, plasmin, leukocyte elastase, and matrix metalloproteinases [361]. This indicates a low conformational stability of the link and a prominent role in nidogen-1 catabolism, which may be of importance for basement membrane remodeling. Nidogen-1 binds with high affinity to the γ1 chain of laminin-1 (in a 1:1 molar ratio) via its C-terminal G3 domain [360,362], resulting in highly stable complexes. Although naturally occurring complexes of collagen type IV and nidogen-1 have not so far been identified, studies with recombinant nidogen-1 have identified possible interactions between the G2 domain of nidogen-1 and triple-helical binding sites in the C-terminal NC1 region of collagen type IV. However, other binding sites also appear to exist in nidogen-1 for collagen type IV, indicating a variability in this association.

A gene that codes for a second nidogen isoform, nidogen-2, has been cloned from a mouse osteoblast cell line [363]. Although deduction of the amino acid sequence of nidogen-2 revealed only 27.4% homology to nidogen-1, many structural similarities exist between the two proteins, and the domain structure of nidogen-1 is conserved. The distribution of nidogen-2 is also very similar to that of nidogen-1; however, its interaction with laminin-1 appears to be of lower affinity than that of nidogen-1 (R. Timpl, unpublished data). The data suggest that the functions of nidogen-1 and-2 are distinct, but this remains to be resolved.

Cellular Interactions

The first indication that laminins have a role in cell adhesion came in 1980 from work on the PAM212 mouse epithelial-derived cell line [364]. Since then, laminin-1 and its proteolytic fragments have been widely used in *in vitro* assays, and a wide range of cellular functions have been ascribed to laminin-1. It is important to keep in mind, however, that the *in vivo* distribution of laminin-1 is restricted (see Table 2), suggesting that many of the functions attributed to it are carried out by other isoforms *in vivo*. Despite some overall structural similarity between different laminin isoforms, their tissue-specific and developmentally regulated distribution patterns suggest that they have distinct functions. Apart from laminin-1, only laminins 2 [324], 5 [365] and 10/11 [335] can be purified, which has permitted their use in *in vitro* functional assays. However, difficulties in isolation procedures limit the number of experiments possible with these isoforms. Hence, the majority of the data discussed below are based on studies with laminin-1.

Laminin-1 supports the adhesion of many different types of cells; it also stimulates cell proliferation [155,366] and the migration of myoblasts and fibroblasts [155,348,367], is essential for neurite outgrowth [333] and epithelial cell development [368], can induce the differentiation of myoblasts to myotubes [369,370], and prevents apoptosis of cells [371]. Most of these functional activities of laminin-1 are located in the G-domain of the laminin α1 chain in fragments E8 or E3. Various cells have also been shown to attach to E1 and P1 in an RGDS-dependent manner [154,333,349,370,372–375] or to the E1-4 fragment in an RGDS-independent manner [154]. Which, if any, of these sites are used for cell attachment *in vivo* is as yet unknown, although it is believed from monoclonal antibody mapping studies that the orientation in native basement membranes is variable, with the E8 domain of laminin often being capable of pointing outward from the basement lamina toward the cells [376].

Growth and Differentiation

Laminin-1 has drastic effects on cell morphology, but it also affects cell growth. Myoblasts are spindle-shaped and polarized on laminins 1 and 2, while on fibronectin they are stellate [377]. Endothelial cells either on laminin-1 or within laminin-rich gels shut down division and form capillary-like structures; on other matrix molecules, such as fibronectin, they proliferate and flatten [378]. Laminin-1 stimulates an increase in cell numbers in a dose-dependent manner (compared to other matrix molecules) of fibroblasts [379], fibrosarcoma cells [380], Schwann cells [332,333,381–383], and epidermal cells [384–386] when offered as a substrate. Laminin-1 and its E1 fragment have been shown to move quiescent fibroblasts from the G_o phase into the cell cycle with kinetic and molar dose dependencies similar to those of EGF [366]. However, the EGF-like homologies in laminin-1 [362,387] evidently play no role in this process, and fibroblasts do not use E1 as an attachment substrate. Thus, the triggers for attachment and growth probably work through different receptors. The relationship between EGF-like cysteine-repeat motifs and growth promotion is not clear [377].

In accordance with its *in vivo* distribution, laminin-5 is more efficient than laminin-1 in promoting adhesion of primary keratinocytes and some other epithelial cells in *in vitro* assays [365]. Laminin-5 also has a different requirement for integrin receptor molecules (see below) as compared to laminin-1, suggesting distinct signaling pathways for these two laminin isoforms.

In vitro studies have identified both laminin-1 and laminin-2 as important factors in myogenesis, even though only laminin-2 is present in developing and mature muscle fiber basement membranes. Skeletal muscle precursor cells attach, proliferate, migrate, and differentiate to myotubes preferentially on these laminins as compared to collagen type I or fibronectin [155,348,370,377]. However, the addition of exogenous laminin-2, but not laminin-1, can restore the ability of laminin α2-deficient myoblasts to fuse into myotubes [388], and mouse myoblasts spread, differentiate, and form myotubes significantly faster on laminin-2 than on laminin-1 [370].

Preliminary data with mixtures of laminin-10/11 have shown that normal and malignant human epithelial cell lines [335,389] and a multipotent hematopoietic cell line (FDCP-mix cells) [390] bind preferentially to this isoform mix as compared to laminin-1. As laminins 10 and 1 are both expressed in epithelial cell basement membranes [318,391], the differential interaction of epithelial cells may reflect different intracellular signaling pathways for the two isoforms. However, this requires further investigation. The significance of multipotent hematopoietic FDCP-mix cell adhesion to laminin-10/11 remains to be established.

Locomotion

Laminin-1 stimulates the locomotion and associated rapid change in morphology of a number of cell types [348]. In particular, the E8 fragment of laminin-1 has been shown to be critical for the locomotion of primary skeletal muscle myoblasts, myoblast cell lines [367], and neural crest cells [392]. Similarly, laminin-2 has strong migration-promoting effects on myoblasts [393]. In marked contrast, fibronectin has no effect on myoblast locomotion but does promote myoblast adhesion [155,348,367,394], while the E1 fragment of laminin-1 promotes adhesion of neural crest cells but not their locomotion [392]. The E8 fragment does not stimulate locomotion when offered in suspension. Boyden chamber assays also show that laminin-1 [15], its E1 fragment, and the peptide YIGSR (Tyr–Ile–Gly–Ser–Arg) [395] will stimulate the movement of various transformed cell lines, compatible with laminin-1 exerting a haptotactic effect on the cells, as has been suggested for myoblasts [348].

Neurite Outgrowth

Sites in the E8 fragment of laminin-1 promote the outgrowth of neurites from peripheral ganglia [349,396]. Activity is detectable with coating in the 10 ng/mL range; cell attachment requires 10–50 times as much. Laminin-1 itself does not promote the adhesion of neurites, but the outgrowth is a laminin-1-stimulated motile phenomenon requiring a polyornithine-precoated adhesive substrate. Laminin-1, however, is not expressed by peripheral nerves and it has been shown that laminin-2 [370] has the same neurite-outgrowth-promoting activity as laminin-1 in *in vitro* assays [397]. Because laminin-2 is expressed by peripheral nerves, it is likely that *in vivo* the neurite outgrowth function is mediated by laminin-2. It has been shown that the laminin α4 chain is also strongly expressed by peripheral nerves [315,398]; however, no data are available yet on the neurite-outgrowth-promoting activity of isoforms containing this laminin chain because such isoforms have not been purified.

Epithelial Cell Polarization and Survival

Kidney epithelia polarize in coordination with the transient expression of laminin α1 chains within an α1/γ1 chain-rich and unpolarized background [368,399]. Inhibition of epithelial cell interactions with laminin-1, either using anti-laminin-1 antibodies or antibodies to known laminin-1 receptors (see "Matrix Receptors" below), in kidney and salivary gland organ cultures have identified two sites on the laminin-1 molecule that are important for early epithelial cell development [368,399–401]. A site within the E8 fragment of laminin-1 interacts with the α6β1 integrin, while the E3 fragment interacts with α-dystroglycan [402]. Further, the differentiation and survival of mammary epithelial cells grown on plastic or in collagen type I gels has been shown to require interactions with the E3 fragment of laminin-1, and inhibition of such interactions leads to apoptosis (reviewed in [371]).

Modern Methods for Identifying Laminin Functions

Data on inherited diseases involving laminin gene defects, and on laminin α2, β2, α5, and γ1 knockout mice, have provided some clues to the function of some of the laminin isoforms. In epidermolysis bullosa junctionalis, a blistering skin disease (see also Chapter 15, this volume), mutations in the human genes coding for laminin α3, γ2, and β3 chains, which comprise the laminin-5 isoform, have been described, demonstrating that this protein is essential for the attachment of keratinocytes to their basement membrane [403–408]. In the *dy/dy* mouse strain, a mutation has been described in the gene coding for laminin α2, resulting in significant reduction in expression of the protein and a marked muscular dystrophy [409,410]. When the laminin α2 chain gene is eliminated in mice, the muscular dystrophy is more severe and the animals die at 10 weeks of age [411]. In humans, various congenital muscular dystrophies have been shown to result from mutations in the laminin α2 chain gene similar to those identified in the mouse models. The data indicate that laminin α2 is necessary for the terminal differentiation and/or function of muscle fibers and also for the functioning of the peripheral nervous system. Eliminating the expression of the gene coding for laminin β2 has resulted in mice with impaired synapse formation and glomerular dysfunction [412], whereas laminin α5 [413] and γ1 [414] null mice die early in embryogenesis, suggesting that these chains are crucial for early embryogenesis. Due to the early manifestation of the phenotype of the γ1 and α5 null mice, it is not possible to identify sites of functional significance of laminins containing these chains at later stages of development or in the mature animal. Further experiments involving *in vitro* assays using the isolated laminin isoforms and *in vivo* elimination of laminin chains in particular tissues or at particular stages of development are therefore necessary to establish the functions of the different laminin isoforms.

COLLAGENS

With up to 25% (wt/wt) carbohydrate bound to their triple-helical structure, as in type VI collagen [415–417], and distinct cell-adhesive properties, collagens certainly represent the most abundant and ubiquitous class of adhesive glycoproteins in vertebrate tissues [5,7,418–420]. Although structural and pathological aspects of collagens are extensively reviewed in this volume, we feel that it is appropriate here to focus on their adhesive character and to compare structural and functional features of collagen-cell interactions with those of other adhesive glycoproteins.

Since it became clear from cell and organ culture studies using collagenous substrata that collagenous proteins serve not only as an inert structural support of connective tissue but also control multiple cellular parameters, such as adhesion, migration, cell shape, cytoskeletal architecture, and gene expression, efforts have been made in many laboratories to elucidate the biological significance and molecular basis of such interactions. The cell physiological activity of collagen was firmly established during the 1960s and 1970s; for example, fibroblasts were shown to exhibit a completely different, elongated morphology when cultured within hydrated collagen lattices as opposed to monolayer culture [421–423]. Not only is their morphology altered, but their metabolic activity in terms of collagen synthesis and matrix production is also reduced to physiological levels [424]. Denatured collagen (gelatin) has been shown to promote significantly the differentiation and fusion of skeletal myoblasts [425], to induce the differentiation of embryonic chick cornea epithelium [426], and to stimulate chondrogenic differentiation of somitic mesenchyme [427,428]. Three fundamentally different modes of collagen recognition are apparent: a conformation-independent way, with cells recognizing both triple-helical and denatured collagen, such as hepatocyte adhesion to collagen [429], a triple-helix-dependent mechanism, and a type of recognition requiring fibrillar structure, such as platelet aggregation [430]. Furthermore,

changes in cell shape, such as mesenchymal-epithelial transformations, capillary formation of endothelial cells [431], or the differentiation and polarization of mammary epithelial cells [432,433], require a three-dimensional lattice of collagen fibrils. Interestingly, such lattices are also able to induce the reverse phenomenon, namely the epithelial-mesenchymal transformation of lens epithelial cells associated with a fundamental change in gene expression from type IV to type I collagen synthesis [434].

The chain of molecular events involved in such complex phenomena, which begins with cell-collagen binding and transmembrane signaling and leads to changes in cytoskeletal arrangement and gene expression, remains to be elucidated. Progress in this direction has been achieved by dissecting complex organ culture systems into well-defined cell culture systems using purified collagens and by the identification of integrins and other collagen-binding cell-surface components. Yet collagens (particularly fibrillar collagens), as basic proteins, bind to numerous acidic glycoproteins and proteoglycans, raising questions about the physiological significance of *in vitro* interactions of cells or isolated cell-surface components with highly purified collagen molecules adsorbed to plastic dishes, as compared with interactions with native collagen fibers *in vivo*. Similarly, it will be difficult to establish finally whether, and which, cell-surface components binding to collagen *in vitro* are functionally involved in any of the cellular responses to extracellular collagen.

The analysis of the role of individual collagens has not kept pace with the identification of new collagen types, these having reached the respectable number of at least 20 genetically distinct proteins with triple-helical structures (for an overview, see Chapter 2, this volume, and [5,7,419,420]). Most significant information on the function of collagen has been obtained using collagen type I in the form of native or denatured molecules or as hydrated, three-dimensional gels consisting of native fibrils. More recently, cell interactions significantly different from those with type I collagen have been found with collagen types IV and VI [131,435,436]; for this reason, we will focus in the following on these three collagens.

Fibrillar Collagens

Collagen type I, as the most abundant collagen of the vertebrate organism, is not only the major component of connective tissue but, as a constituent of reticular fibers (together with collagen types III and V), pervades almost every tissue and thus is potentially in contact with most cell types [437]. Therefore, it is not unexpected that most cell types, including fibroblasts, skeletal and cardiac muscle cells [5], hepatocytes, osteosarcoma cells, and numerous other cell lines [2,3,8], should adhere to native, denatured, or fibrillar collagen *in vitro*. Because of its affinity for gelatin, fibronectin enhances cell adhesion to denatured collagen [44,438], but adhesion to native collagen can also occur in the absence of any serum adhesion factor [429,438,439].

It is now clear that not only do different cell types use different mechanisms for interaction with extracellular collagen, but also that the same cell may simultaneously use multiple mechanisms of collagen binding. For example, platelet aggregation and activation is stimulated only by native, fibrillar type I, II, or III collagens [430], whereas hepatocytes attach both to native collagen and to denatured collagen peptides [429,440].

Receptors for Fibrillar Collagens

The major collagen receptors are cell-surface heparan sulfate proteoglycans (syndecans and glypicans (for a review, see Chapter 4, this volume, and [122,441,442]) and integrins (see below). Syndecans and glypicans consist of an extracellular core protein domain carrying heparan sulfate side chains and a hydrophobic, transmembrane domain linked to the cytoskeleton [443]. At least four genetically distinct syndecans have been described; when incorporated into liposomes, these have been shown to bind specifically to fibronectin as well as to native collagen types I, III, and V (but not IV) [121] through their heparan sulfate side chains.

The major collagen-binding integrins are $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha10\beta1$ [18,444–447]. $\alpha2\beta1$, or VLA-2 (very late antigen 2), the first defined collagen-binding integrin, was initially identified as the platelet receptor (GP Ia-IIa) for collagen. It binds to collagen types I, II, III, and IV [444,448]. The interaction of $\alpha2\beta1$ with collagen types I–III is Mg^{2+}-[321,338] but not RGD-dependent [448]. Although RGD is a frequent sequence in collagens, most of these sites are cryptic and do not interact with integrins unless exposed by denaturing procedures. Arg, Gly, and Asp residues are, however, recognized by $\alpha1\beta1$ when located on three different α chains in type IV collagen in adjacent positions [435,449]. Similarly, conformation-dependent binding sites for $\alpha1\beta1$ integrin are also present in the CB3 peptide of $\alpha1$(I) chains [450].

Reports indicating that the binding site for $\alpha2\beta1$ integrin in collagen types I and II includes a KDGEA (Lys–Asp–Gly–Glu–Ala) motif located in CNBr peptide CB3 or KDGET (Lys–Asp–Gly–Glu–Thr), respectively, [451] could not be verified [452,453]. Studies by Knight et al. [453] provide strong evidence for an integrin-binding sequence GFQGER (Gly–Phe–Gln–Gly–Glu–Arg) located within CNBr peptide CB3 of the $\alpha1$(I) chain in the vicinity of the KDGEA (Lys–Asp–Gly–Glu–Ala) sequence.

In previous attempts to identify collagen receptors, several collagen-binding membrane or membrane-associated proteins have been isolated by affinity chromatography on collagen adsorbents, yet, for many of them, their physiological role as receptors is still unclear. From platelet membranes, an $\alpha1$(I) collagen receptor of 68 kDa has been isolated and characterized [454]. Annexin V (or anchorin CII) was originally isolated from chondrocyte membranes as a collagen-binding protein that binds to native collagen types II and X after incorporation into liposomes [455,456]. Antibodies have located the protein on the chondrocyte surface [457,458] and inhibit chondrocyte adhesion to type II collagen. Annexin V is a major calcium channel of matrix vesicles [459] and, like other annexins (calpactins, lipocortins), has a strong affinity for phospholipids and calcium [456]. It is located preferentially in calcifying regions of cartilage and in bone, suggesting a role in matrix vesicle-nucleated calcification. As in most connective tissue cells, however, the main collagen receptors of chondrocytes belong to the integrins. Chondrocytes express $\alpha1\beta1$ and $\alpha2\beta1$ integrins [452], and more recently a new type II collagen-binding integrin, $\alpha10\beta1$, has been found in chondrocytes [446].

Type IV Collagen

In contrast to the fibril-forming collagen types I, II, III, and V, type IV collagen molecules assemble into a large network, cross-linked at their N- and C-termini and between triple-helical domains [23,460] (Fig. 2e); (see also

Chapters 2 and 25, this volume). As a constituent exclusively of basement membranes, type IV collagen is likely to undergo specific interactions with basement-membrane-associated cells, such as epithelial and endothelial cells, myoblasts, and adipocytes. In fact, *in vitro* cell-attachment assays have demonstrated a specific adhesion and spreading on type IV collagen of keratinocytes [461], hepatocytes [440], endothelial cells [462], myoblasts [369,463], and a number of tumor cells [464]. A cell-binding site recognized by integrins α1β1 and α2β1 has been localized in the central triple-helical domain, within cyanogen bromide-derived peptide CB15 [435,447,449]. It involves Arg, Gly, and Asp residues, which, however, are not aligned in a continuous sequence but located on three different chains in adjacent positions.

Type VI Collagen

Type VI collagen was originally identified as a major, cysteine-rich glycoprotein constituent of microfibrils [416, 417]. The collagenous portion obtained after pepsin extraction represents only about one-third of the molecular mass (see also Chapter 2, this volume, and [465]). The molecule consists of three different chains: the α1(VI) (110 kDa), the α2(VI) (140 kDa), and the α3(VI) (240 kDa) (Fig. 3). Each chain ends with a large N-terminal (235 residues) and C-terminal (430 residues) globular domain connected by a triple-helical region of 335 amino acid residues.

The protein is rich in carbohydrates: 90% of the hydroxylysine residues are glycosylated; the globular domains contain several N-glycosylation sites. Interestingly, the globular domains contain three homologous repeats of 180 (chick) or 200 (human) residues each, which show similarity to the collagen-binding sequences of von Willebrand factor and cartilage matrix protein [466–468]. It is possible that these collagen-binding motifs are responsible for the particular type of assembly of type VI collagen monomers into tetrads (Fig. 2F) through their interaction with the triple-helical domains of neighboring molecules [469].

A strong cell-adhesive character of type VI collagen has been observed using the isolated subunits GP 140 [α2(VI)] and GP 250 [α3 (VI)] [131]. Close interaction between cells and type VI collagen has also been concluded from the electron-microscopical localization of type VI collagen in microfibrils adjacent to fibroblasts [470] or chondrocytes [471]. *In vitro* a large number of normal fibroblasts and tumor cells, including fibrosarcoma, rhabdomyosarcoma, and astrocytoma cells, have been shown to attach and spread on type VI collagen substrates [131]. The cells also attach and spread on the isolated α2(VI) and α3(VI) chains but not on the α1(VI) chain. Adhesion to denatured chains can be impaired by fairly low concentrations of RGDS (Arg–Gly–Asp–Ser) or RGDT (Arg–Gly–Asp–Thr) peptide. This is consistent with the existence of a total of 11 RGD sequences in all three α chains of type VI collagen [466], although probably not all RGD motifs are involved in cell binding. For example, the α2(VI) chain of chick type VI collagen contains seven RGD sequences, in contrast to the three RGD sequences in human α2(VI).

MATRIX RECEPTORS: INTEGRINS AND THE DYSTROPHIN-GLYCOPROTEIN COMPLEX

All of the matrix proteins we have described can bind to specific transmembrane receptors from a superfamily of cell-surface proteins termed "integrins" [10,472]. The discovery of the integrins represents a watershed in the field, drawing together the cell exterior and interior and integrating several widely divergent fields of research. Many reviews address these molecules in greater detail than our space permits [447,473–477], but our chapter would be incomplete without a brief introduction to them.

Integrins are αβ, heterodimeric glycoproteins, both chains of which are transmembrane polypeptides, with a single α-helical transmembrane domain. Some eight β and seventeen α chains are known to date (Table 3). Mixing particular α and β chains confers a certain degree of ligand specificity on the complex. For example, by combining the β1 chain with any of 11 α chains, cells can produce receptors specific for laminins, the fibronectin RGDS domain, collagens I and IV, or vitronectin (among other matrix molecules). Both α and β subunits appear to be involved in both divalent cation (especially Ca^{2+} and Mg^{2+})-dependent ligand recognition and interaction with the cytoskeleton [447]. In addition, there is evidence that both the specificity [107,447,478,479] and the affinity of ligand binding [480] of integrins can be

TABLE 3. Integrin α and β Subunit Combinations and Their Ligands

Subunits		Ligands
β1	α1	collagen, laminin-1 (E1-X)
	α2	collagen, laminin-1 (E1-X)
	α3	laminins 1,5, and 10/11, fibronectin
	α4	VCAM-1, fibronectin, (CSIII and HepII)
	α5	fibronectin (RGD), denatured collagen
	α6	laminin-1 (E8)
	α7	laminin-1 (E8), laminin-2
	α8	fibronectin (RGD), vitronectin, tenascin-C, osteopontin
	α9	collagen, laminin-1, tenascin-C, VCAM-1
	α10	collagen
	α11	collagen, unknown ligands
	αv	vitronectin, fibronectin, osteopontin
β2	αm	C3bi, fibrinogen, factor X, ICAM-1, ICAM-2
	αL	ICAM-1,2, and 3
	αD	C3bi, ICAM-1 and 2
β3	αIIβ	fibrinogen, fibronectin (RGD), vitronectin, von Willebrand factor, thrombospondin
	αv	vitronectin, fibronectin, von Willebrand factor, thrombospondin, fibulin, fibrillin, denatured collagen, PECAM-1
β4	α6	laminin-1 and laminin-5
β5	αv	vitronectin, osteopontin
β6	αv	fibronectin
β7	α4	MAdCAM-1, VCAM-1, fibronectin (CSIII)
	αE	E-cadherin
β8	αv	vitronectin

modulated from within the cell to suit local conditions and so change the way that cells respond to their surroundings. In general, the β1 and αv integrins are principally involved with cell-matrix interactions, whereas the β2 integrins, which are found only on leukocytes, mediate cell-cell interactions.

Fibronectin-Binding Integrins

One of the most studied adhesive receptors is the α5β1 integrin, which was successfully isolated and cloned as a fibronectin-specific receptor [481,482] following the identification of a major cell-binding site in fibronectin, in the RGDS sequence [50,483,484]. By passing detergent extracts of cells over a fibronectin affinity column and eluting with RGDS, M_r 140,000 membrane proteins were obtained that could be incorporated into liposomes and bound specifically and in an RGD- (but not RGE-[Arg–Gly–Glu]-) dependent manner to fibronectin-coated surfaces. Antibodies have localized α5β1 by immunofluorescence microscopy to focal adhesion plaques under cells in tissue culture and specifically block cell adhesion to fibronectin. Other integrins that bind to fibronectin belong either to the β1 (α3β1, α4β1, α8β1) or the αv (αIIbβ3, αvβ1, αvβ3, αvβ6) families [447]; however, unlike α5β1, these integrins also recognize other ligands (Table 3) [477,485,486]. Integrins α8β1, αvβ1, αvβ3, αvβ6, and αIIbβ3, which are structurally closely related to the α5β1 integrin, also use the RGD sequence in fibronectin for binding. Binding of α3β1 and α4β1 to fibronectin does not involve the RGD sequence. Various binding sites for the α4β1 integrin on the fibronectin molecule have been mapped within the IIICS region; two distinct sequences have been identified that mediate cell adhesion alone (REDV (Arg–Glu–Asp–Val) or RGDV (Arg–Gly–Asp–Val)) sequences in human and rat fibronectin, respectively) or adhesion and spreading (LDV [Leu–Asp–Val] sequence), while a third binding site (IDAPS [Ile–Asp–Ala–Pro–Ser]) is located in the second heparin-binding domain, termed Hep II [478].

Integrins Containing the αv Subunit and αIIbβ3

The αv subunit can associate with five different β subunits (Table 3) to form various receptors, all of which bind to their ligands in an RGD-dependent manner. Apart from αvβ6, which recognizes only fibronectin, all αv integrins recognize predominantly vitronectin. The αvβ5 and αvβ8 integrins selectively recognize vitronectin and not fibronectin, whereas αvβ1 recognizes both vitronectin and fibronectin, and both αvβ1 and αvβ5 also recognize osteopontin. Integrin αvβ3 has many different ligands, including vitronectin, fibronectin, fibrinogen, fibrin, von Willebrand factor, thrombospondin, tenascin-C, osteopontin, fibrillin, and denatured collagen. Furthermore, αvβ3 binds to the cellular receptor PECAM-1 (platelet endothelial cell-adhesion molecule-1) on endothelial cells, enabling αvβ3 to take part in cell-cell interactions between leukocytes and vascular endothelial cells [477]. The αv subunit has a widespread distribution *in vivo*, whereas its β subunit partners have spatially and temporally regulated expression patterns, conferring tissue specificity on the αv-series integrins.

The αIIbβ3 and αvβ3 integrins belong to a subset of integrins termed cytoadhesins. The αIIb subunit is restricted to megakaryocytes and platelets, in which it associates exclusively with the β3 subunit. On resting platelets, αIIbβ3 binds only fibrinogen, which triggers platelet activation and aggregation, finally resulting in clot formation. Activation of platelets by ligand binding or other stimuli also activates αIIbβ3, resulting in increased affinity for various RDG-containing ligands, including fibronectin, vitronectin, and von Willebrand factor (see Table 3).

Collagen-Binding Integrins

Integrins α1β1 and α2β1 have been shown to be the major receptors for native collagens and, depending on cell type, can also bind to laminins 1 and 2, but with low affinity (Table 3). More recently, α9β1 [194] and α10β1 have been reported as collagen receptors, while α11β1, which occurs principally on myogenic cells (D. Gullberg, personal communication), can also bind collagen among other yet to be defined ligands.

α1β1 binds collagen type IV with higher affinity than the fibrillar collagen types I, II, and III, whereas the opposite is true for α2β1 [449]. The two integrins recognize neighboring but distinct sites on collagen type IV [435]. Unfolding of the triple-helical collagen structure abolishes binding by α1β1 and α2β1 but exposes linear RGD sequences recognized by α5β1 and αvβ3. Integrin α2β1 is the major collagen receptor on platelets and, together with αIIbβ3, is a differentiation marker for platelets and plays an important role in platelet aggregation. α2β1 is further found on fibroblasts and various other cell lines and tissues. α1β1 is found on cells that are in close contact with the basement membrane, such as endothelial cells, smooth muscle cells, and hepatocytes, as well as astrocytes and neural crest cells and their derivatives.

Laminin-Binding Integrins

Several integrin receptors have been implicated in cell attachment to laminin isoforms, including α1β1, α2β1, α3β1, α6β1, α7β1, and α6β4 (Table 3). Some integrins, such as α6β1, α7β1, and α6β4, are specific for laminins, while the others behave as promiscuous receptors, binding other extracellular matrix components.

Integrins α6β1 and α7β1 appear to be the major receptors for the E8 fragment of laminin-1 on epithelial and myogenic cells, respectively [487–490]. In addition, α7β1 on myogenic cells binds to laminin-2, and elimination of this integrin in mice leads to a mild muscular dystrophy [491], suggesting interaction between α7β1 and laminin-2 *in vivo* [492,493]. More recently, α6β1 has also been shown to mediate the binding of normal and transformed epithelial cells [389] and multipotent hematopoietic cells [390] to laminin-10/11.

Integrin α6β4 is — besides α3β1 — the major laminin-5 binding integrin and occurs in association with hemidesmosomes in the skin and in other stratified epithelia. In epidermolysis bullosa junctionalis, a blistering skin disease (see Chapter 15, this volume), mutations in the human genes coding for laminin α3, γ2, and β3 chains, which comprise the laminin-5 isoform, have been described, demonstrating that this protein is essential for the attachment of keratinocytes to their basement membrane [403,408]. Mutations or deletion of the α6 or β4 subunits in mice result in a phenotype similar to that observed in epidermolysis bullosa junctionalis [476], suggesting that the endogenous ligand for α6β4 is indeed laminin-5.

Minor laminin receptors include α1β1, α2β1, and α3β1. The binding sites of α1β1 and α2β1 have been mapped to domain VI of the laminin α1 chain [494,495]. The α3β1 integrin was isolated by affinity chromatography using a human laminin preparation, which, at the time, was thought to be laminin-1 but is now known probably to have been laminin-10/11, as the ligand and Rugli rat cells as the integrin source. Further, α3β1 has been shown to interact with laminin-5 [365] and laminin-10 [335], and anti-α3β1 antibodies weakly affect cell attachment to laminin-1

fragments E8, E3, and P1 [479,496], suggesting that this receptor is promiscuous in its binding interactions.

Dystrophin-Glycoprotein Complex

α-Dystroglycan of the dystrophin glycoprotein complex, which was originally characterized in myogenic cells but is now known to be expressed on various cell types, is the major nonintegrin laminin-1 receptor that binds to the E3 fragment [497,498]. More recently, α-dystroglycan has also been shown to react with the equivalent of the E3 fragment (domains G4–G5) in the laminin α2 and α5 chains [498,499]. Apart from the laminins, α-dystroglycan interacts with perlecan, a basement membrane heparan sulfate proteoglycan [498], and with agrin at the neuromuscular junction [500]. The data suggest that this molecule plays a critical role in interconnecting the extracellular matrix to the cytoskeleton.

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Chapter 6

Keratins

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INTRODUCTION

Keratins are everywhere, from being the major components of household dust to common contaminants of laboratory protein analysis. Before being shed from the body surface, these proteins reach levels of 80% or more of the total cell protein in terminally differentiated keratinocytes of the skin. The skin is the largest organ of the human body, adapted to its barrier function with a "disposable" epidermal covering that naturally has a high turnover. Keratins are therefore likely to constitute a significant component in connective tissue sampling of the skin. The importance of keratins in skin derives from the physical pressures that keratinocyte cells of the epidermis are subjected to in normal everyday living. Keratins provide the reinforcement that keeps epithelial cells in the skin intact under pressure. A view is emerging that the family of proteins to which the keratins belong may have a broader function in stress resistance in many different tissues, and may be involved in the etiology of a number of disorders arising from cell fragility.

Keratins are the major filamentous proteins of epithelial cells, and occur at their greatest density and complexity in the keratinocytes of the epidermis. They make up a system of filaments that run through the cytoplasm of cells and connect at the cell periphery into cell-to-cell (desmosome) and cell-to-substrate (hemidesmosome) attachment plaques. Thus, the keratin-desmosome interaction creates a three-dimensional network through the epithelium, which must be of significant importance in the structural properties of the whole tissue. This chapter briefly outlines what is understood about the keratin intermediate filaments in relation to their tissue expression patterns, their structure and the consequences of faults in keratin proteins, and what can be deduced from all this about their function.

KERATINS AS INTERMEDIATE FILAMENTS

Keratins (often referred to as cytokeratins, and also sometimes as prekeratins or α-keratins) are members of the superfamily of intermediate filament proteins. These are cytoskeletal proteins that are expressed in one form or another in nearly every cell type of the vertebrate body. As a group of proteins they are widespread throughout the animal kingdom, ancient in evolutionary terms and substantially conserved in mammals. The whole human intermediate filament gene family is thought to consist of at least 50 members, expressed in different tissues (Fig. 1). All these proteins are intracellular; most are cytoplasmic like the keratins, and form long sinuous filaments of about 10 nm in diameter. Intermediate filaments fall into six classes (type I to type VI) on the basis of amino acid sequence data. Keratins make up the type I and type II groups which together account for about three-quarters of all the intermediate filaments known in human tissues.

Pairs of keratins are expressed together, a type I protein with a specific and (mostly) predictable type II partner, each pair being characteristic of a particular range of epithelial cell types. Intermediate filament protein types III–VI are found in non epithelial tissues. The type III filament proteins are expressed predominantly in mesenchymal cells, and include vimentin (present in many cell types including fibroblasts, endothelial and hemopoietic cells), desmin (in muscle cells), glial fibrillary acidic protein (or GFAP, in astroglia), and peripherin (in parts of the nervous system). Type IV intermediate filament proteins include the neurofilament proteins NF-L, NF-M, NF-H and α-internexin, expressed in neurons. The filament protein, nestin, is the largest intermediate filament protein known [1] and is also now grouped with the neurofilaments. The type V proteins are the intranuclear lamins, probably the oldest of the intermediate filament classes in evolutionary terms [2]. They consist of two groups, A- and B-type lamins, and have a broader range of tissue expression than any of the cytoplasmic intermediate filaments. Amongst the B-type lamins, lamins B_1 and B_2 are constitutive and B_3 lamin is found in germ cells. In contrast, the A-type lamins (lamins A and C, which are two alternatively spliced products of the lamin A gene) are absent from many undifferentiated or dedifferentiated cells [3,4]. Membership of the type VI group fluctuates from year to year as this category usually houses unusual or recently discovered intermediate filament proteins that do not easily fall into one of the other groups. Currently, this includes the lens intermediate filament proteins filensin and CP49 [5], and probably the large muscle intermediate filament proteins synemin and paranemin (See Fig. 1).

Keratins were initially identified by biochemical analysis using two-dimensional gel electrophoresis. They are classified individually by number after a system proposed by Moll et al. [6], which depends on their charge and molecular weight. Keratins 1–19 span the extremes (K20 is a later

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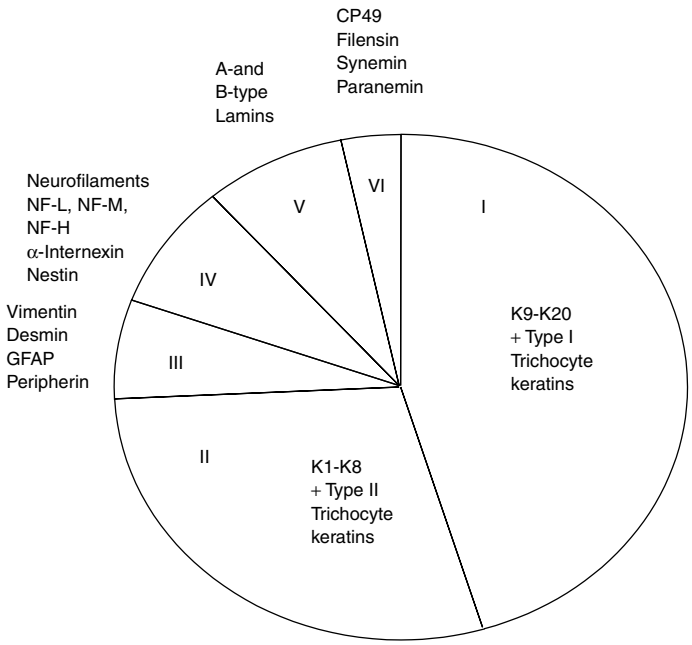

Figure 1. The six types of intermediate filament-like proteins identified in human tissues. This division is based on amino acid primary sequence data, but is supported by intron profiles observed in genomic DNA. Nearly three-quarters of these proteins are keratins, in the type I and type II classes. Types I and II are characteristic of epithelial cells, while type III are characteristic of various mesenchymal cells (vimentin occurs in many cell types, desmin in all muscle cells, GFAP [glial fibrillary acidic protein] in astroglial cells, and peripherin predominantly in peripheral neurons). Type IV filament proteins are all neuronal, type V (nuclear lamins) are in all tissues, and type VI currently includes two filament proteins identified in the eye lens, plus synemin and paranemin.

addition): K1 is the largest and most basic, and K19 the smallest and one of the more acidic. K9–K20 plus up to ten trichocyte keratins are in the type I group, and K1–K8 plus at least another ten trichocyte, or hair, keratins make up type II. The description of keratins by molecular weight is no longer used as a large number of related proteins coexist within a similar range of sizes and charges. The use of specific monoclonal antibodies has become an important way of distinguishing between different intermediate filament proteins and different keratins, both by immunoblotting and by *in situ* immunohistochemistry techniques. Today, nearly all of the human keratins have been sequenced at the DNA level, providing the ultimate identification. Among the latest members of this gene family to be characterized are the multiple genes encoding K6 proteins [7] and, most recently, several further genes for hair keratins [8]. The final count of intermediate filaments is probably still not complete.

KERATIN EXPRESSION IN SKIN

The major keratins show a characteristic tissue distribution (Fig. 2), even down to the resolution of subpopulations of cells within a tissue showing specific phenotypes. This must reflect physiological differences between these cell populations. Some keratin pairs, which can be described as *primary* or essential keratins, are constitutively expressed as the baseline keratin phenotype in appropriate tissues. These primary keratins are keratins 5 (K5) and 14 (K14) and keratins 8 (K8) and 18 (K18). The *secondary* keratins are differentiation-specific, and their expression can be modulated by a variety of external factors. Analysis of primary keratin distribution patterns confirms the existence of distinct categories of epithelial cells, *simple epithelial* cells, and *basal* or *stratifying epithelial* cells [9].

Simple epithelial cells. Simple epithelial cells retain contact with the basal lamina and also retain a free apical surface, and express K18 and K8, the primary simple epithelial keratins. In skin, such cell types are found in the secretory cells of the sweat gland. The other cell type found in skin which expresses these simple epithelial keratins is the Merkel cell, a type of solitary neuroendocrine cell found around the dermo-epidermal interface and thought to be involved in touch perception. K8 and K18 are the first keratins to be expressed in embryogenesis (Fig. 3) and persist as the only keratins in a small number of adult epithelia (e.g., hepatocytes). Occasionally, they have even been seen in some types of mesenchymal cells [10]. They appear to be the keratins expressed closest to the interface between epithelial and mesenchymal cells, and oscillations between expression of K8 and K18 and expression of vimentin are characteristic of epithelial-mesenchymal transitions in development and elsewhere. A switch to expression of simple epithelial keratins is commonly observed in metastatic non-melanoma skin cancers, although the significance of this is not yet clear. Keratins are very long-lived and stable proteins, and even these simple epithelial keratins, thought to be the most soluble and plastic of the keratins, have been calculated to have a half-life of around 100 h [13].

Basal epithelial cells. The basal cell type is seen in complex epithelial tissues from glandular epithelia to stratifying squamous epithelia. It is characterized by basal lamina contact in the absence of a free apical surface and is identified by a different keratin phenotype. Basal cells synthesize and

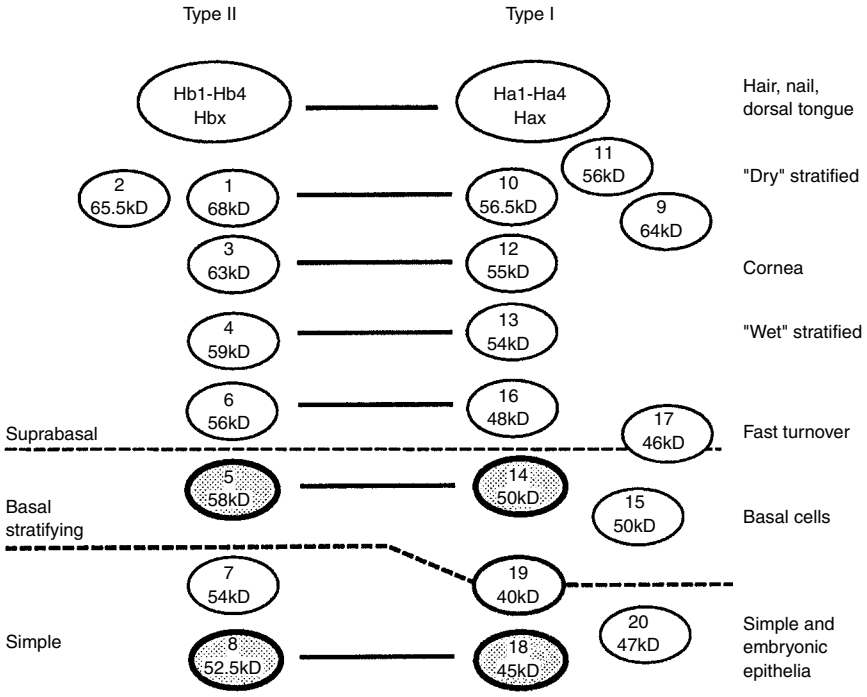

Figure 2. Summary of keratin classification, based on Moll et al. [6], giving the relative molecular weights observed on gel electrophoresis for each polypeptide. The major coexpressed keratin pairs are shown, based on Sun et al. [121], and their characteristic tissue distribution is indicated as deduced from many biochemical and immunohistochemical studies. Shaded keratins are the primary (essential) keratins for simple and stratified epithelia.

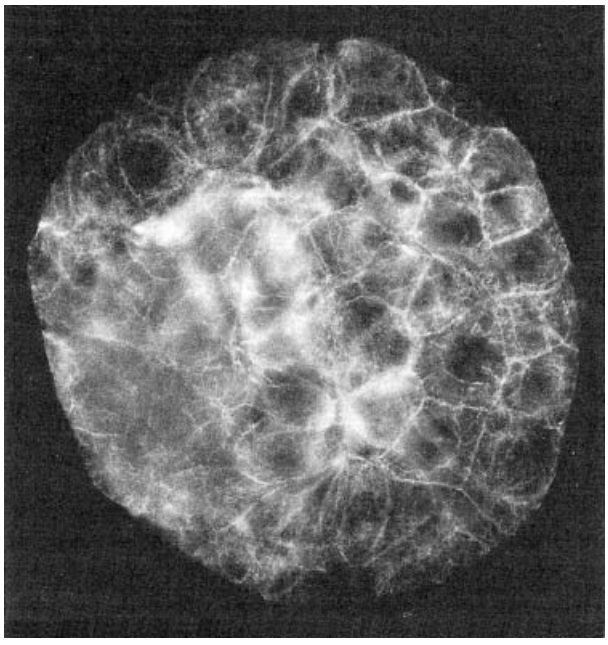

Figure 3. Simple epithelial or embryonic keratins K8 and K18 are the first to be expressed in the embryo. K8 and K18 persist into adult tissues in all simple epithelial cells, and even as the sole keratins in some tissues such as hepatocytes. They are shown here by immunofluorescence using an antibody to keratin 18 (LE65), in all cells of the preimplantation mouse blastocyst. (Unpublished material from collaborative experiments with Martin Johnson.)

express K14 and K5, the primary keratins of stratifying epithelia. Keratin filaments in this type of cell are shown in tissue culture in Figure 4. Basal cells appear to synthesize these keratins only while they are in contact with a basal lamina, although K5 and K14 persist in the tissue cells at biochemically detectable levels at least as far up as the stratum granulosum [9,12], corresponding to a transit time of 7–14 days.

Suprabasal cells. When basal cells begin to lose contact with the basal lamina and move upwards, as in the course of epidermal differentiation, a change occurs in keratin synthesis: production of keratins 5 and 14 slows or ceases [11] and secondary, or differentiation-specific, keratins are synthesized instead. Thus, a clear difference is usually seen in keratin expression between basal cells and the suprabasal cell layers. As a cell begins to leave the basal layer compartment there are several options for a particular cell type, depending on its body location and program of differentiation. Keratinocyte-like cells may begin to express keratins K1 and K10 in cornifying differentiation, as in skin and cornifying regions in the mouth, or K4 and K13 in non cornifying stratification such as occurs in mucosal epithelia of the eyes, ears, and nose, and in oral, urogenital and anal epithelia [14]. Corneal epithelial cells express K3 and K12 [15]. Areas of epithelium that are rapidly turning over express K6 and K16 (buccal epithelium, psoriatic lesions, cultured keratinocytes, hair follicles) [16], secondary keratins which can be simultaneously present with another keratin phenotype [17]. Trichocyte keratins are found in hair follicles and hair, nail bed and nail, Hassall's corpuscles in the thymus, and in filiform papillae on the dorsal surface of the tongue.

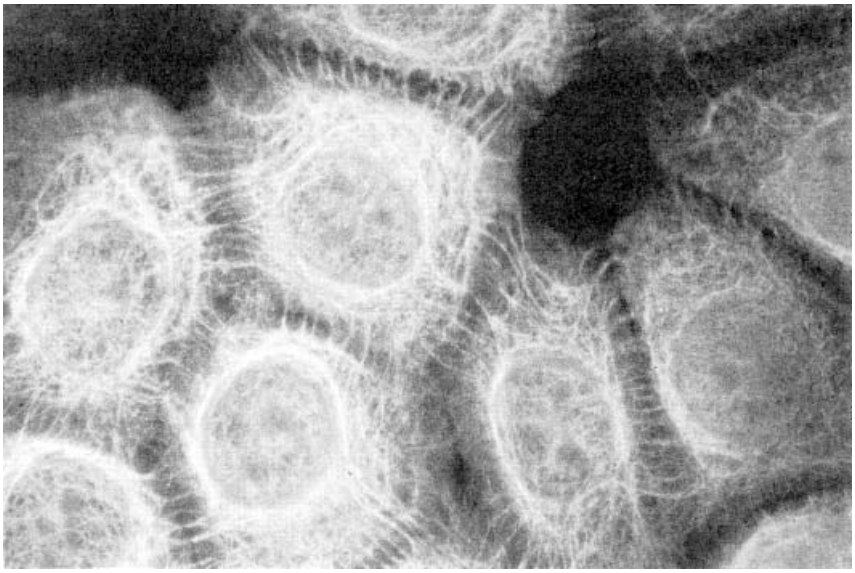

Figure 4. Keratinocytes express the greatest range of keratins at their highest organizational complexity. Keratin filaments are visualized here in tissue culture, by immunofluorescence of a squamous cell carcinoma-derived line, TR146 cells [122]. The position of the desmosomes is indicated, although they are unstained, by the symmetrical focussing of keratin filament bundles in neighbouring cells onto points around the cell periphery.

The precise localization of these keratins within tissues has come from the use of monospecific antibodies to individual keratins. As well as these major keratins, a number of other keratins are also expressed in these and other tissues: keratin 19 is present in a subset of cells in a wide range of simple and stratified epithelia [18], often at differentiation interfaces [19]. Keratin 15 is found in basal cells [20,21]. K17 is found in basal and suprabasal cells and is induced in response to stress, like K6/K16. There are now known to be two K6 genes, K6a and K6b, one of which appears to be strictly suprabasal while the other can be expressed in basal cells and may co-polymerize with K17 [22]. Keratin 20 is expressed by certain simple epithelia in the gastrointestinal tract [23] and in some neuroendocrine cells including Merkel cells in skin [24]. Keratins K2e (in epidermis) and K2p (in hard palate) occur in some cornifying epithelia [25]; keratin 7 occurs in a subset of glandular epithelia [26], and keratin 9 is expressed in regions of pressure or abrasion, such as palm and footsole skin [27,28].

Keratin expression in epithelia is therefore rather complex and the regulatory principles governing individual expression patterns are not yet fully worked out. It is possible that there are as many distinct keratin expression phenotypes among epithelial tissues as there are physically distinct epithelia and that these two parameters may be related.

HOW KERATIN FILAMENTS ASSEMBLE

When a type I protein and a type II protein are present within the same environment under physiological conditions, polymerization takes place very rapidly in the absence of any cofactors or polymerization factors. It has, therefore, been difficult to study the process of sequential assembly of intermediate filaments. However, intermediate filaments all share the same general pattern of molecular structure — a central domain, which is a long, mostly α-helical region, flanked by non-helical amino-terminal (head) and carboxyl-terminal (tail) domains. Thus, although their precise amino acid sequences vary considerably and their encoding DNA sequences vary even more, information acquired about the assembly of one intermediate filament protein is often applicable to another. Analysis of the protein secondary and tertiary structure of keratins, and models for how these proteins pack into filaments, have therefore evolved from a wide variety of studies in many laboratories.

The keratins appear to be more complicated structurally than other intermediate filament classes as they only polymerize into filaments if both type I and type II proteins are present (Fig. 5b). This obligatory heterotypic polymerization superficially contrasts with other intermediate filament protein types that all appear to have at least some capacity for homotypic polymerization. It has been shown in many laboratories that single vertebrate keratins of either type are unstable, and are rapidly degraded in the absence of a copolymerizing complementary keratin of the other type [29–31].

The evolution of heteropolymerization in keratins may be associated with greater physical resilience of the filament network so formed, or with a greater insensitivity of the system to potentially damaging sequence changes, in tissues where these filaments are particularly important. This could also be one mechanism used to balance the quantities of two coexpressed keratins *in vivo*.

The Rod Domain

The earliest structural analysis of intermediate filaments came from studies on wool keratins [32] and suggested that the main components in these structures were α-helical proteins. This was reinforced by secondary structure predictions from amino acid sequences as they became available [33], initially for type III proteins and shortly after for a type I keratin (K14). Although intermediate filaments are indeed predominantly α-helical, their dimeric molecular structure is quite different from that of the trimeric collagen family (see Chapter 2, this volume).

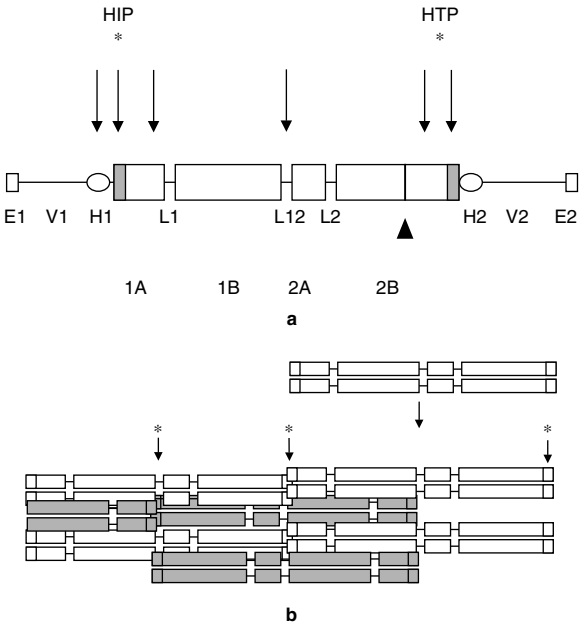

Figure 5. (**a**) Diagram of the basic structure of a keratin protein. In common with other types of intermediate filaments, keratin protein monomers consist of a central rod domain in four α-helical segments (1A, 1B, 2A, 2B) connected by nonhelical linker stretches (L1, L12, L2). The shaded triangle indicates the stutter, or helix polarity reversal feature, in helix 2B. The shaded blocks at either end of the rod domain are the conserved helix boundary peptides, the helix initiation peptide (HIP) and the helix termination peptide (HTP). This rod domain is flanked by nonhelical end domains, the amino-terminal head domain (E1, V1, H1) and the carboxyl-terminal tail domain (H2, V2, E2). Phosphorylation sites are predominantly in the H1 and H2 domains. See text for further description of the end domains. Arrows indicate the locations of mutation clusters seen in epidermolysis bullosa simplex (EBS) in K5 and K14; starred arrows (HIP, HTP) indicate the most frequent sites of mutations, especially in the HIP region, usually associated with severe Dowling-Meara EBS. The other four arrows are common mutation sites in the milder Weber-Cockayne EBS, especially the L12 site; (see also Chapter 15, Epidermolysis Bullosa, this volume). (**b**) Some features of current models of keratin assembly. The rod domain assembles in parallel in-register heterodimers (type I and type II) which then proceed by poorly-defined intermediate stages to a 10 nm thick intermediate filament consisting of about 32 polypeptide chains, or 16 dimeric strands. Whatever the order of assembly, the end result is that rod end overlaps occur within one strand and adjacent strands are anti-parallel, some with a half-length stagger alignment. This almost certainly brings 2–3 of the mutation cluster sites (starred arrows) into close proximity, identifying an important interaction within the polymer.

The basic structure of a keratin intermediate filament protein is shown in Figure 5a. A single polypeptide chain of an intermediate filament protein is unstable and once synthesized in the cell dimerizes rapidly to form a coiled-coil two-chain molecule. This will be a type I-type II heterodimer in the case of keratins [34,35]. This instability and dimerization is due to the presence of a curving strip of hydrophobic residues running down the length of the coiled α-helical cylinder. Formation of the coiled-coil sequesters the hydrophobic faces of two interacting polypeptides, with their hydrophobic side-chains interdigitating like the teeth of a zip, away from the aqueous environment of the cytoplasm. This tendency to form a coiled-coil is a characteristic that can be simply and confidently predicted from the amino acid sequence [36]. One can look for the presence of suitably hydrophobic residues (usually leucine, valine, isoleucine or methionine, or bulky residues such as phenylalanine or tryptophan: remembered in single letter code as "FAMILY VW") occupying the first and fourth positions of every seven residues over a stretch of sequence.

The α-helical conformation is the most common secondary structure seen in proteins [37], and the "leucine zipper" mode of dimerization is well known in many proteins. What makes the intermediate filament α-helical structure unusual is its great length. The rod domain extends for a fairly consistent minimum of 310 amino acids, starting and ending with two important conserved sequence motifs of about 15 amino acid residues, the helix boundary motifs, at either end of the rod. The rod domain also contains four interruptions of the helix. Three of these are unlikely to be α-helical in conformation and are referred to as linkers (L1, L12 and L2), dividing the rod domain into helical subdomains 1A, 1B, 2A and 2B (nomenclature of subdomains following Steinert [38]). The fourth interruption is also a highly conserved feature, or "stutter" (possibly a helix polarity reversal at which the handedness of the helical coil changes), in the middle of helix 2B. These features are all shown in Figure 5.

From a single polypeptide chain, a filament of around 8–10 nm in diameter is built up like rope by repeated coiled-coiling, forming dimers then tetramers, to protofilaments of 2–3 nm and then 4.5 nm, and then to the intermediate filament (around 10 nm in diameter). The final filament is composed of around 32 polypeptide chains assembled at any point as 16 dimeric strands forming coiled-coil protofilaments. Information on how the rod domains of these dimeric molecules pack to form a filament has come from a variety of sources. The smallest subunit of these proteins that can be identified is a tetramer formed of two antiparallel dimers, demonstrable by negative staining [39]. Herrmann et al. have shown that a "unit length" filament is formed as an early assembly stage by a variety of intermediate filament proteins, but that, if unchecked, keratin polymerization proceeds exceptionally rapidly from this stage to full-thickness long filaments [40]. Early binding studies [41] and transfection experiments [42,43] defined certain regions as essential for filament assembly, which substantially coincided with overlaps predicted from paracrystal analysis. Interactions between the rod domains are probably due primarily to numerous alternating charged patches on the surface of the helix [44], together with higher specificity "docking" sites at certain points. Sequence conservation in the helix boundary motifs, together with the dramatic effects on filament stability of deleting or mutating these motifs [45,46], highlights the importance of these rod ends. Analysis of near-neighbor fragments by sequencing dissociated peptides after cross-linking and partial proteolysis [47] suggests an overlap between the C-terminal end of one rod subunit and the N-terminal end of the next rod. These studies and earlier paracrystal analyses [48] suggest that the proteins assemble as alternate antiparallel strands, giving an overall apolar filament.

The nonhelical regions are also needed for correct filament assembly. It is not known why the diameter of intermediate filaments is so constant, but work from Aebi's laboratory [49] suggests that the end domains are involved in limiting the filament thickness, as removing them results in abnormally

thick structures. The linker regions that interrupt the α-helical rod domain (L1, L12, and L2) are highly conserved in their sizes and locations, and also show partial sequence conservation. Linkers may function as hinge regions to give the filament flexibility, since they are close to predicted boundaries or gaps between the staggered overlaps of the helical domains. A size restriction may be necessary to avoid steric hindrance of packing between the helical domains.

Head and Tail Domains

The nonhelical ends of the filament proteins can be divided into three subdomains (Fig. 5a). The extreme ends tend to be highly charged (the E1 and E2 domains on heads and tails respectively; [38]). Working inwards, there is then a section of high variability, V1 in the head and V2 in the tail. Secondary structure predictions here have suggested the presence of "omega loops" [50] or "glycine loops" [51] centered on the Phe- or Tyr- residues between runs of glycine-rich (in epidermal keratins) or serine-rich (in simple epithelial keratins) repeats. The abundance of glycines in the head and tail domains of epidermal keratins 1 and 10 argues for high flexibility in these domains. Immediately adjacent to the rod domains lie the H1 (head) and H2 (tail) domains, but these are not present in type I keratins. These shorter regions usually contain phosphorylation target sites within their sequences [52–54], suggesting that their net charge can be altered in response to signals from within the cell which could thus trigger cycles of polymerization/depolymerization and filament remodeling.

The end domains, at least the head domains, appear to play a significant part in regulating correct filament assembly [55,56]. Forced expression of keratins from which either head or tail domains had been deleted from one or both of the pair, suggested that tail domains were necessary for efficient filament network formation, and that at least one of the two interacting keratins needed both end domains intact in order to produce normal-looking filaments [31,57]. This suggests important functions for the head and tail domains within the filament itself, either as docking sites or spacers in setting up the initial polymer, or as regions that stabilize the forming filament by binding ahead or behind or sideways onto another strand or subunit. Sequence variation could result in different qualities of the final filament network, by altering for example the rigidity or flexibility of the filaments, or the ease with which the filaments could be dissociated or remodeled. For example, the large number of such repeat loops, as potential head-to-tail binding sites, on keratins 1 and 10 may result in a very strong bonding within the filaments, as opposed to the fewer repeats in simple epithelial keratins which are also easier to dissociate *in vitro*. The unusual presence of cysteine residues in the head and tail domains of trichocyte keratins [58,59] could allow for disulphide cross-linking to make a more rigid network in hair and nail cells.

It is very unclear where the head and tail domains lie in the polymerized filament. Several lines of evidence have suggested that the nonhelical domains of the head and tail may protrude outside the body of the polymerized filament. Light proteolysis of intact epidermal filaments was shown to yield fragments with the amino acid composition of terminal domains [60]. By immunoelectron microscopy the end domains can be seen, in neurofilaments at least, to protrude from the body of the filament: regular extensions at right angles to the filament are observed [61], and terminal-specific antibodies can be shown to bind to such extended protrusions [62]. Also, by immunohistochemistry of skin sections, K14 can be stained in the suprabasal layers of the epidermis when antibodies to the tip of the "tail" are used [9], but other sorts of K14 antibodies are often negative. It seems that parts of the molecule are no longer accessible outside the basal layer, possibly because of the laying down of secondary keratins over the top of them [63]. It seems possible, therefore, that the keratin end domains (and those of other intermediate filaments) could be in a good position to interact with other cellular components outside the filaments themselves, opening the way for a much wider range of potential functions.

Many intermediate filaments are known to be reversibly phosphorylated at sites within the head and tail domains. The H1 and H2 regions contain phosphorylation sites and regulation of filament assembly by modulation of H1 phosphorylation may be the reason that the head domains are so important. Hyperphosphorylation of the H1/H2 domains drives many intermediate filament proteins toward depolymerization [52–54], which may be necessary to allow cell separation during mitosis [64,65].

It appears, therefore, that the head and tail domains contain the key to the tissue specificity of intermediate filament expression. While the amino acid sequence of the α-helical rod domain is rather conserved, the head and tail domain sequences vary substantially, especially among the keratins. However, when terminal domains are compared between members of a coexpressed type I-type II keratin pair, a degree of relatedness in amino acid composition is seen. Thus, the end sequences are most closely related between members of a coexpressed pair, but the sequences in the rod domain are most similar between members of the same intermediate filament type class; it is in the end domains that we see the variability related to differentiation. The head and tail domains could primarily function to stabilize the filament itself or to modulate the physical qualities of the filament network, or to anchor the filament to other parts of the cytoskeleton. These domains could also function, alternatively or additionally, to detect and react to cell signals that require mobilization of the cytoskeleton. There is even evidence that selective keratin expression can influence the proliferative capacity of the keratinocyte [66–68]. As other possible binding sites are now emerging — such as the potential of a motif in the head domain of type II keratins to interact with desmoplakin [69] — the truth may be that they do all these things. As the analytical systems move from transfection of cultured cells into the complexity of transgenic mice, many of these ideas are being reinforced. A recent example is seen in a study in which the addition of the K14 tail made the behavior of an anomalous transgenic K16 substitute more like the K14 it was replacing [70].

HERITABLE DISORDERS OF KERATINS

The identification of a heritable disorder is usually very informative in understanding the true function of a protein and this has proven to be the case with keratins. The discovery of keratin mutations underlying a range of epidermal fragility disorders has made it very clear that a major function of these structural filament proteins is indeed to provide the tissue with the resilience it needs to withstand "wear and tear" in that particular body site. A number of fuller reviews of this field have been published recently [71–74] (see also Chapters 15 and 24, this volume).

Epidermolysis Bullosa Simplex

The inherited skin disorders in the epidermolysis bullosa group, in which skin blistering occurs upon minimal trauma, are divided clinically into three subgroups: junctional epidermolysis bullosa and dystrophic epidermolysis bullosa which arise from connective tissue abnormalities and result in sub epidermal blistering, and the epidermolysis bullosa simplex (EBS) forms which give rise to intra-epidermal tissue separation. Individuals with EBS have fragile skin that blisters on mild physical trauma, such as rubbing or scratching; the blistering is caused by breakdown of the basal cells of the epidermis leading to a fluid-filled blister under an intact blister roof of suprabasal cells. EBS of the Dowling-Meara form (EBS-DM, also known as EBS-herpetiformis; MIM 131760) is characterized at the electron microscopic level by the occurrence of cytoplasmic, non membrane-bound electron-dense bodies [75], composed of basal cell keratins K5 and K14 [76]. As these clumps of keratin protein are observed in both blistered skin [75,77] and non blistered skin of affected individuals [76], it was reasoned that they cannot be caused by the blistering but may predispose towards it. The keratin clumps are reminiscent of filament disruption induced in several other circumstances, such as the microinjection of antibodies [78], cold shock [79,80], and mitosis [64,81,82], or the transfection of cultured cells with aberrant keratins [42]. Tonofilament clumping was also seen following the introduction of a truncated keratin 14 gene into transgenic mice, in which skin loosening, reminiscent of human Dowling-Meara EBS, was produced [43]. When the molecular basis of EBS was first discovered, it was by three independent groups, producing convergent results from three different approaches. Epstein et al. identified a point mutation in helix 2 of keratin 14 (Leu384Pro) in one family [83], Coulombe et al. [84] identified two Dowling-Meara EBS patients with different point mutations in helix 1A (Arg125Cys in one, and Arg125His in the other), and our laboratory identified the mutation Glu475Gly in the helix termination peptide of keratin 5 [85]. These studies precipitated wide-scale investigations into other cases of EBS and other possible keratin disorders in which clinical phenotypes suggest epidermal fragility.

It is now clear that mutations are not randomly distributed along the protein but are clustered in particular regions. The rod ends are the most frequent locations of pathogenic mutations, particularly the helix initiation peptide of K14, and especially codon 125 encoding arginine (R125). Something like 70% of all mutations identified in cases of the severest form of EBS, the Dowling-Meara form, involve this codon. A closer look at the DNA sequence reveals a highly mutable CpG dinucleotide in this codon which would account for its high mutation rate, but this must also represent an extremely sensitive point in the protein structure at which there is little tolerance of variation. Such information is valuable in working out how subunits interact with one another during filament polymerization. The involvement of the rod ends in those mutations which most severely compromise the function of the keratin cytoskeleton fits very well with biochemical evidence for an overlap between the start of helix 1A and the end of helix 2B when protein subunits are packed together in a filament (see Fig. 5).

Nearly all of the mutations reported for Dowling-Meara EBS cases are dominant negative missense mutations. They can occur with equal effect in either K5 or K14, in either the helix initiation peptide or the helix termination peptide. What makes a difference to the clinical severity is when they are found outside the helix boundary motifs, in which case the phenotype is usually less severe and not usually diagnosed as the Dowling-Meara form. The severe Dowling-Meara form of EBS is only one clinical variant of the disorder. Weber-Cockayne EBS (EBS-WC; MIM 131800; also known as EBS localisata) is a milder form in which the skin fragility tends only to affect hands and feet. While Weber-Cockayne EBS is also caused by mutations in K5 and K14, and also is nearly always inherited as an autosomal dominant trait, the position of mutations within the protein sequence is more variable. Looking at data from many cases of Dowling-Meara and Weber-Cockayne EBS, it is apparent that the mutation clusters are non overlapping. While EBS-DM is associated with mutations in the two rod ends, the helix initiation motif and the helix termination motif, EBS-WC is linked to mutations in four regions elsewhere. These are (i) the H1 domain just before the helix initiation motif, (ii-iii) two regions in the rod domain which lie internal to the helix initiation motif and helix termination motif, and (iv) the non-helical linker domain, L12, that connects helix 1 to helix 2 [86]. All these mutation hotspots are shown in Figure 5.

Another variant of EBS is the Koebner form (MIM 131900; also known as EBS-generalisata), which is associated with generalized blistering all over the body. While the clinical presentation of Koebner EBS is the most variable and its diagnosis possibly the most subjective, some cases of Koebner EBS have turned out to be recessive forms in which K14 expression is lost altogether [87]. Recessive "knockout" forms of EBS seem to be quite variable in phenotype [87–90], and are the result of mutations causing premature termination of the mRNA, which usually leads to nonsense-mediated decay of the whole message and the lack of any detectable gene product. The survival of individuals completely lacking K14 has been a surprise, since this is one of the most widespread keratins, which occurs as a major protein in all basal cells, and in both stratified and complex epithelia. Interestingly, no K5 knockouts have been reported to date, suggesting that such a phenotype might be lethal.

There are other variants of EBS with different associated clinical hallmarks [91]. In a handful of EBS cases genes other than keratins have been implicated. The best understood of these is a rare disorder in which EBS is associated with muscular dystrophy (MIM 226670), which is caused by mutations in plectin [92-95], an intermediate filament-associated protein involved in hemidesmosome formation in the epidermis. In theory, any weak link in the structural chain of filament-cell junction could cause cell lysis resembling that in EBS. In our laboratory we have still been unable to identify keratin mutations in 20% of the apparently unequivocal EBS cases referred to us, so there probably are some other proteins involved in EBS-like skin disorders.

Bullous Congenital Ichthyosiform Erythroderma

Bullous congenital ichthyosiform erythroderma, or BCIE (MIM 113800; also sometimes loosely referred to as epidermolytic hyperkeratosis), is another skin fragility disorder caused by mutations in keratins, but in this case it is the suprabasal cells that are fragile. In sections of affected skin, the basal cell layer is intact while the suprabasal layers of cells are ruptured to varying degrees. The clinical picture, however, is very different from that of EBS: skin may blister in an infant, but in adults blistering is not often seen. Instead, the epidermis can become extremely thick and scaly, especially

in the flexure regions. This difference in phenotype is clearly due to the different cell population affected with fragility, and it is the striking thickening of the skin (hence the term *ichthyosiform*) arising from keratinocyte hyperproliferation that is the prevalent clinical feature, rather than the clusters of blisters seen when basal cells are very fragile, as in the severe forms of epidermolysis bullosa simplex. Prenatal diagnosis has been successfully carried out following the identification of mutations in K1 and K10 in this disorder [96,97].

The cause of the suprabasal cell fragility in BCIE is almost always a dominant negative mutation in either K1 or K10, i.e., the two major secondary keratins of cornifying stratified squamous epithelia [98–101]. Keratins 5 and 14 are never found to be affected — not surprisingly, since the basal cell layer is intact. The locations of the mutations within the protein structure are related to the mutation sites seen in cases of EBS, i.e., the helix boundary peptides, but it is interesting to note that mutations at the sites associated with milder forms of EBS are hardly ever seen in BCIE. The most likely explanation for this is the persistence of the K5/K14 filament substructure in the suprabasal cells of the epidermis, which may act as a template upon which the K1/K10 proteins polymerize, providing some reinforcement even to a filament weakened by a mutation in one of these secondary keratins. Thus, mutations at the "milder" sites would go undetected and only those mutations most severely disruptive to filament stability would have pathological consequences.

Thickening of the epidermis is a frequently observed phenomenon in many keratin disorders, but it is most usually seen on the palms and soles, which remains one of the unexplained features of these diseases. One possible mechanism may be that the breakdown of the keratinocytes may be chronically triggering a low-level wound response in the epidermis, since it is known that cytokines released by ruptured keratinocytes provide some of the signals that induce the epidermal response in wound healing.

Ichthyosis Bullosa of Siemens

Another genodermatosis now known to be caused by mutations in a keratin is ichthyosis bullosa of Siemens (MIM 146800). This is caused by mutations in keratin 2e [102–105], a late-expressed epidermal keratin [106] found in the upper layers of the epidermis. The histological phenotype of this disorder is cytolysis of the uppermost layers of the stratified epidermis only, which presents clinically as mild, very superficial blisters. The physical fragility of the cells fits exactly with the known expression pattern of the mutated keratin, but the clinical characteristics are sufficiently distinct to have given this form of keratin disorder a completely different classification.

All the above keratin disorders affecting skin keratinocytes dramatically demonstrate the critical structural role of keratins in reinforcing the epidermis, and adapting this epithelium to withstand one of the harshest environments to which cells are ever exposed.

Pachyonychia Congenita

The range of disorders associated with keratins is now very wide and not all of them strictly affect the epidermis, yet they all have their own story to tell and they all contribute a bit more to our understanding of how keratins work in cells. A rather bizarre group of genetic disorders associated with the epidermis is the spectrum of defects incorporating pachyonychia congenita and steatocystoma multiplex (MIM 18450).

As its name implies, pachyonychia congenita (PC) is characterized by abnormally thick nails on hands and feet. There are two clinical forms of PC: type 1, or Jadassohn-Lewandowsky type (MIM 167200), associated with thick nails, palmoplantar keratoderma and white plaques in the oral mucosa, and type 2, the Jackson-Lawler type (MIM 167210), with thick nails, pilosebaceous cysts, pili torti and, occasionally, natal teeth. The range of phenotypic characteristics was puzzling at first, but by extrapolating from the existing knowledge of keratin expression, the genes for the "stress" keratins K6, K16 and K17 were quickly identified as candidates that might be harboring detrimental mutations. The first mutations were found in keratin 16 for PC1 and keratin 17 for PC2, the expression range of these two genes fitting well with the places in which cellular weakening was indicated by the appendageal distortion [107]. Again, these mutations are dominant negative autosomal mutations, and are located in the helix boundary regions of the affected keratins.

These disorders are now shedding light on a number of other puzzles associated with keratins, one of which being the functional difference between the two (or more) K6 genes, K6a and K6b, and another being the identity of the polymerization partner of K17. K6a and K6b are indistinguishable at the protein level, but mutations found in K6a in PC1 [108,109] and K6b in a case of PC2 [22], led to the suggestion that K6b may be the partner for K17 [22] (and K6a possibly the partner of K16). The appearance of "K6" in basal cells (K6α in mouse [7]) would fit with the occasional appearance of K17 in basal cells also, while K16 is more cleanly restricted to the suprabasal layers.

Another contribution of these disorders may turn out to be in relation to the problem of phenotypic variation. Like the EBS phenotypes, the stress keratin mutation disorders also show a range of severity. However, unlike EBS, these variants appear not to be associated with the position of the mutation in the protein. Variation in severity is associated with quite different clinical markers. For example, in contrast to PC2, nail defects associated with K17 mutations in steatocystoma multiplex are minor [110,111], and hence the cysts influence the name of the disorder whereas they appear secondary to the nail defects in PC. In other cases the focal palmo-plantar keratoderma dominates. It seems likely that these disorders are part of the same spectrum and yet all the mutations have been found in the helix boundary motifs, not in the "milder" cluster regions associated in K5/K14 with EBS-WC. Maybe the lack of mutations in secondary sites is because, as with K1/K10, the cells are reinforced by an underlying framework of the primary keratins K5/K14, and so mild cluster sites do not result in any pathological abnormality. If this is the case, a mild phenotype must be due to the nature of a mutation rather than its position; i.e., substitutions that do not distort or loosen the tertiary structure of the protein, and therefore do not interfere too much with filament packing, produce a mild phenotype. The existence of multiple mutations at a single residue associated with phenotypes of quite widely differing severity [112] suggest that this is the only possible explanation.

Keratin Disorders in Tissues Other than Skin

As well as the disorders described above, there is a growing number of other disorders which have also been associated with mutations in keratins. Keratin disorders affecting stratified squamous epithelia but not manifested in skin include Meesmann corneal dystrophy (MIM 122100), in

which small blisters on the front of the cornea of the eye are associated with mutations in K3 and K12 [113], and white sponge nevus (MIM 193900), in which a type of benign oral leukoplakia is caused by mutations in keratins K4 and K13 [114,115].

All the extracutaneous keratin mutations identified to date have occurred in conserved regions of the α-helical domains, which would be predicted to be critical for filament structure. As with the first examples of pathological mutations in human keratin genes, the EBS disorders, the lack of an effective keratin cytoskeleton renders the epithelial cells susceptible to cytolysis on physical trauma; again, these are disorders of cell fragility.

The only significant group of keratins not yet associated with a type of genetic disorder is the group of simple epithelial keratins, K7, K8, K18 and K19. Since these are mostly expressed in internal tissues, it may be that we are not yet able to spot any such disorders before they escalate into clinical conditions with many other consequences and different overriding clinical characteristics.

Not surprisingly, disease-associated mutations are also now beginning to turn up in the non keratin intermediate filaments. These include desmin-derived myopathies in muscle tissues [116,117], lamin-associated disorders in Emery-Dreifuss autosomal dominant muscular dystrophy (MIM 181350) [118] and partial lipodystrophy (MIM 151660) [119], and possibly some neuropathies such as amyotrophic lateral sclerosis [120]. It is likely that several more such disorders are still waiting to be discovered.

CONCLUSIONS

All the observations described above indicate a fundamental structural role for the keratins, and one that is essential in tissue differentiation. The central question in intermediate filament biology is why is such heterogeneity necessary. Are we simply observing evolutionary redundancy?

Our inability to demonstrate, in a testable experimental context, a defined role for intermediate filaments in cells and tissues that requires such heterogeneity is probably because the essential function of intermediate filament proteins lies in the fully differentiated tissue, which we cannot adequately reproduce in a controlled tissue culture situation. From the structural integration of muscle cells to the maintenance of the attenuated axonal cytoplasm in a functional state in the neuron, a case for a supporting cytoskeleton can be seen, with distinctly different requirements in each tissue. The case is strongest of all for the keratins, since the structural role of epithelia as boundary tissues is unequivocal, and the association of keratin filaments with desmosomes gives a three-dimensional integrity across the whole tissue that helps it to resist deformation. Disorders are now being identified in which EBS-like phenotypes are associated with mutations not just in keratins but in keratin-associated proteins that link keratins to the cell-cell junctions [92,93].

This three-dimensional network also has the built-in potential to transduce mechanical signals over long distances. Across the wide variety of different situations in which an epithelial sheet has to perform a structural supporting role while contributing to optimal tissue shape compatible with function, the physical requirements of the component cells will vary substantially. Physical requirements such as plasticity or rigidity may or may not be accompanied by other functional demands, such as the production of specific secretory products as in glands. Biochemical properties of intermediate filament proteins observed *in vitro* are likely to reflect biological differences *in situ*. A more easily disruptable filament system (keratins 8 and 18) might be more appropriate in secretory cells, in which there is a lot of cytoplasmic vesicular traffic, and a less easily disruptable filament system (keratins 5 and 14) might be better fitted to the physically stressed epidermis [9].

There is no longer any doubt that different keratins make filaments with different physical properties. There is probably sufficient variability in the amino acid sequences of the head and tail domains to account for this variation. The coiled-coiling of the rod domains clearly forms the backbone of the keratin filament, although there are still many details to be worked out as to how this takes place. The tissue-specific modulation of the filament quality, and the remodeling of the filament network through the life of the cell, is probably effected by the head and tail domain sequences that are attached to and protrude from these long protein polymers. We hardly have any more information on the function of these end domains than we do on the rod domain, but at least we are learning where to look. The keratin filaments of the cytoskeleton must have tremendous strength and resilience, as they are the subcellular components that allow us, for example, to stand on the epithelium of our feet without bursting the keratinocytes. A closer study of these microscopical ropes and their role in cellular architecture and engineering might well have some valuable lessons for our larger scale human projects.

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Chapter 7

Extracellular Matrix Degradation

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INTRODUCTION

The degradation of connective tissue matrix is a normal event in the physiological remodeling associated with morphogenesis and growth, as well as in processes such as angiogenesis, cell migration, cervical softening, uterine involution, and wound healing. The resorption is brought about by both resident connective tissue and infiltrating cells and is tightly regulated by a complex interplay of cell–cell and cell–matrix interactions involving the production of enzymes, activators, inhibitors, and regulatory molecules such as growth factors. In recent years, the nature of these entities, as well as the modes of cellular degradation of matrix components, have become more clearly defined. It also seems that the accelerated breakdown of connective tissue occurring in pathological situations, such as the arthritides, periodontal diseases, and tumor invasion and metastasis, is mainly the result of a failure in the normal regulation of degradative processes. This chapter aims to present an overview of knowledge of the nature of the enzymes contributing to matrix turnover, their origin and role, and of the factors that control their production and function. The studies reviewed provide much of the foundations for future work that will be needed to define which disorders of the degradative processes are inheritable.

The endopeptidases (proteinases) are thought to be the key enzymes in degradative processes, because the protein components of most matrices are the predominant determinants of tissue structure and function: exopeptidases and glycosidases play a secondary role. In *in vitro* experiments matrix macromolecules can be degraded by endopeptidases from the four major classes (metallo, serine, cysteine, and aspartate active site residues; [1]; Tables 1 and 2). Their relative roles *in vivo* vary from situation to situation depending upon the tissue environment and the types of cells involved, particularly whether inflammatory cells are present. The current view is that the initial step in matrix degradation is an extracellular proteolytic process, which may range from fine modifications of glycoproteins associated with the cell to a clipping of the cross-linked insoluble collagen and elastin fibers that are the basis of the matrix (Fig. 1). This process may be supplemented by mechanical wear and tear, injury, and bacterial infection.

Of particular interest is the fact that connective tissue cells synthesize and secrete a group of MMPs, which can synergistically digest the major macromolecules of connective tissue matrices [2–5] (Table 2). Subsequent to such initial proteolytic attacks on the matrix, the fragments generated may be phagocytosed by local cells for intracellular degradation or degraded further extracellularly. Except in special circumstances, such as bone resorption or other situations where the cell is in close apposition to its matrix, extracellular degradation is thought to occur at neutral pH. Consequently, proteinases of the metallo- and serine families will be optimally functional and could be responsible for the initial phase of the degradative process. Their individual and synergistic actions on different types of matrix protein are discussed below. The cysteine proteinases, which are more active at acid pH values, are secreted in some situations and may act within local acidic environments extracellularly: their function, coupled with the other lysosomal hydrolases, appears to be confined to the subsequent degradative events within the phagosome into which they are incorporated with phagocytosed matrix fragments (Fig. 1). The extent of matrix turnover occurring extracellularly, relative to intracellularly, is extremely variable and often reflects differences in cell types present in the tissue. Interactions among cells, the effect of auto- and paracrine mediators, and the effect of the matrix on the production and activity of proteinases, are all discussed in detail in the sections below.

Abbreviations used: APMA, 4-aminophenylmercuric acetate; bFGF, basic fibroblast growth factor; cAMP, cyclic adenosine 3′,5′-monophosphate; EGF, epidermal growth factor; IGF, insulin-like growth factor; IL-1, interleukin 1; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin 10; IL-18, interleukin 18; α_2-M, α_2-macroglobulin; MMP, matrix metalloproteinase; MT MMP, membrane-type matrix metalloproteinase; PTH, parathyroid hormone; PA, plasminogen activator; PAI, plasminogen activator inhibitor; PDGF, platelet-derived growth factor; PMN, polymorphonuclear leukocyte; PGE_2, prostaglandin E_2; α_1-PI, α_1-proteinase inhibitor; M_r, relative molecular mass; SFD, Sorsby's fundus dystrophy; TIMP, tissue inhibitor of metalloproteinases; t-PA, tissue plasminogen activator; TGF-β, transforming growth factor-β; TNFα, tumor necrosis factor α; u-PA, urokinase-like plasminogen activator; uPAR, urokinase-like plasminogen activator receptor.

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TABLE 1. Proteolysis of the Extracellular Matrix

Proteinase Class	Matrix Substrates	pH Range
Serine:		
Plasmin	Fibrin, fibronectin, aggrecan, pro MMPs	Neutral
Tissue plasminogen activator	Plasminogen	"
Urokinase-type plasminogen activator	Plasminogen	"
Neutrophil elastase	Most ECM components, pro MMPs	"
Proteinase 3	Similar to elastase	"
Cathepsin G	Aggrecan, elastin, collagen II, pro MMPs	"
Plasma kallikrein	pro u-PA, pro MMP1, MMP3	"
Tissue kallikrein	pro MMP8	"
Tryptase	Collagen VI, fibronectin, pro MMP3	"
Chymase	Many ECM components, pro MMP1, MMP3	"
Granzymes	Aggrecan	"
Cysteine:		
Cathepsin B	Collagen telopeptides, collagen IX, XI, aggrecan	5.0–6.5
Cathepsin L	As cathepsin B, elastin	4.0–6.5
Cathepsin S	As cathepsin B, elastin	6.0–6.5
Cathepsin K	Collagen telopeptides, elastin	Neutral
Calpains	Proteoglycan, fibronectin, vitronectin	Neutral
Aspartate:		
Cathepsin D	Many phagocytosed ECM components	3.0–6.0

Abbreviations: ECM, extracellular matrix; pro MMPs, pro-matrix metalloproteinases.

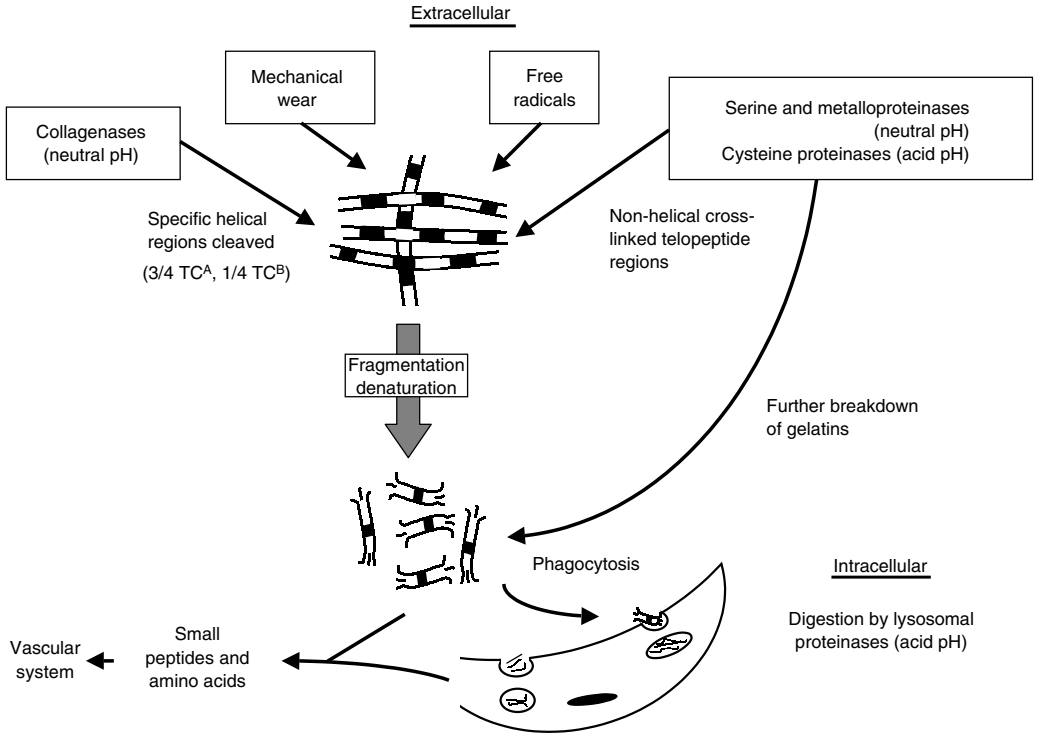

Figure 1. Pathways for the degradation of the collagenous components of connective tissues. Initial fragmentation of cross-linked insoluble collagen occurs extracellularly through proteinase cleavage, physical wear, and the action of free radicals in inflammatory situations. Telopeptidase activity, which weakens the cross-links, can result from the actions of metallo- or serine proteinases at neutral pH or cysteine proteinases at acid pH values. Cleavage within the fibrillar collagen triple helix has been attributed to specific collagenases only, resulting in TC^A and TC^B fragments (three-quarter- and one-quarter length fragments, respectively). In the case of fibrillar collagens, collagenase activity is more efficient against solubilized monomers generated by telopeptidase activity. The cleaved helix fragments (TC^A and TC^B) spontaneously denature at 37 °C and become susceptible to proteinases with gelatinolytic activity, including serine, metallo-, and cysteine proteinases. Collagen fragments, in some instances of very large size, are phagocytosed by cells and further processed by lysosomal enzymes, notably cysteine proteinases

TABLE 2. Proteolysis of the Extracellular Matrix

MMP No.	Enzyme Description	M_r Latent	M_r Active	Matrix Proteins Degraded
MMP1	Interstitial collagenase-1	55,000	45,000	Collagens I, II, III, VII, VIII, X; gelatin; aggrecan; versican; proteoglycan link protein; α_1-proteinase inhibitor; α_2-M; pregnancy zone protein; ovostatin; nidogen; myelin basic protein (MBP); pro TNF; L-selectin; MMP2; MMP9
MMP8	Neutrophil collagenase	75,000	58,000	Collagens I, II, III, V, VII, VIII, X; gelatin; aggrecan; α_1-proteinase inhibitor; α_2-antiplasmin; fibronectin
MMP13	Collagenase-3	60,000	48,000	Collagens I, II, III, IV; gelatin; plasminogen activator inhibitor 2; aggrecan; perlecan; tenascin
MMP3	Stromelysin-1	57,000	45,000	Collagens III, IV, IX, X; gelatin; aggrecan; versican; perlecan; proteoglycan link protein; nidogen; fibronectin; laminin; elastin; fibrinogen antithrombin-III; α_2-M; ovostatin; α_1-proteinase inhibitor; MBP; proTNF; MMP1; MMP7; MMP8; MMP9; MMP13
MMP7	Matrilysin	28,000	19,000	Collagens IV, X; gelatin; aggrecan; proteoglycan link protein; fibronectin; laminin; entactin; elastin; MBP; α_1-proteinase inhibitor; proTNF; MMP1; MMP2; MMP9
MMP10	Stromelysin-2	57,000	44,000	Collagens III, IV, V; gelatin; aggrecan; elastin; proteoglycan link protein; fibronectin; MMP1; MMP8
MMP11	Stromelysin-3	51,000	44,000	α_1-proteinase inhibitor
MMP12	Macrophage metalloelastase	54,000	45,000/ 22,000	Collagen IV; gelatin; elastin; α_1-proteinase inhibitor; fibronectin; vitronectin; laminin; proTNF; MBP
MMP19		56,000	48,000	Collagen IV; gelatin; aggrecan; laminin; fibronectin; COMP; tenascin
MMP20	Enamelysin			Amelogenin
MMP2	Gelatinase A	72,000	66,000	Collagens I, IV, V, VII, X, XI, XIV; gelatin; elastin; fibronectin; aggrecan; versican; proteoglycan link protein; MBP; proTNF; α_1-proteinase inhibitor; MMP9; MMP13
MMP9	Gelatinase B	92,000	86,000	Collagens IV, V, VII, X, XIV; gelatin; elastin; aggrecan; versican; proteoglycan link protein; fibronectin; nidogen; α_1-proteinase inhibitor; MBP; proTNF
MMP14	MT1 MMP	66,000	56,000	Collagens I, II, III; gelatin; elastin; fibronectin; laminin B chain; vitronectin; aggrecan; dermatan sulphate proteoglycan; fibrin(ogen); MMP2; MMP13; proTNF
MMP15	MT2 MMP	72,000	?	MMP2; gelatin; fibronectin; tenascin; nidogen; laminin
MMP16	MT3 MMP	64,000	52,000	MMP2
MMP17	MT4 MMP			proTNF; fibrin(ogen)
MMP24	MT5 MMP	63,000		MMP2
MMP23		44,000		Not known

Abbreviations: COMP, cartilage oligomeric matrix protein.

DEGRADATION OF EXTRACELLULAR MACROMOLECULES

Fibrillar Interstitial Collagens

The triple helical structure of the fibrillar collagens (e.g., types I, II, and III) is extremely resistant to the action of most proteinases and their durability is enhanced by being in tightly apposed fibrils, which can become heavily cross-linked in mature collagens. The presence of covalently bound carbohydrate and fibril interaction with glycoproteins of the surrounding matrix (ground substance) also make fibrillar collagens particularly difficult to degrade. The family of MMPs includes a number of enzymes that uniquely cleave the interstitial collagens at a single locus within the native helical structure at about three quarters of the distance from the N-terminal end (Fig. 1) [6]. These are the interstitial collagenases 1 and 3 (MMP1 and MMP13) and neutrophil collagenase 2 (MMP8). The membrane-associated metalloproteinase, MT1 MMP (MMP14), can also cleave collagen in this specific fashion. The collagenases show differing abilities to cleave the major forms of the fibrillar collagens, MMP1 favoring type III collagen, MMP8 type I, and MMP13 type II. Collagen I is slowly cleaved by gelatinase A (MMP2) and collagen III is susceptible to degradation by stromelysin 1 (MMP3) and gelatinase B (MMP9; Table 3). Cleaved portions of the helix quickly lose their triple helical

conformation and the denatured products are susceptible to the action of a number of less specific proteinases, such as the gelatinases. Gelatinase A is very widely distributed and is constitutively produced by many cell types in culture. Gelatinase B can also be produced by mesenchymal cells in culture after induction by cytokines and other agents and is a major product of monocytes and tumor cells: it is packaged in a granule fraction in PMNs. Recently, metal-dependent proteinases involved in the processing of the procollagen precursors of fibrillar collagens were fully identified [7]. These are members of families that are related to, but distinct from, the MMPs. The recessive disorder of animals and humans, dermatosparaxis, is due to a deficiency of the enzyme procollagen N-proteinase, resulting in the accumulation of collagen with N-propeptides in place and consequent skin fragility [8] (see also Chapter 9, this volume).

Serine proteinases such as elastase and cathepsin G from PMNs are very active against denatured collagens at neutral pH [9]. Lysosomal proteinases will degrade gelatin at acid pH [9] but there is no clear evidence for their generalized extracellular activity. The nonhelical telopeptide regions at the ends of the helical portions of interstitial collagens are important in both intra- and intermolecular cross-linking of the individual tropocollagen molecules, as well as of the aggregated fibrils. These nonhelical regions are susceptible to the action of a number of proteinases, particularly elastase and cathepsin G, as well as stromelysin 1 [10]. Similarly, at an appropriately acid pH, cathepsins B, L, K, and S may act against these regions [9,11–13].

Other Collagens

As described in Chapter 2, a plethora of collagens with specialized functions has now been characterized. The degradation of type IV collagen, which forms the basic network in basement membrane structures, is considered to be particularly significant, because this may be required for the intra- and extravasation of hemopoietic and tumor cells [14] and is central to capillary remodeling and growth during angiogenesis [15]. Type IV collagen consists of individual helical molecules with a large number of globular interruptions (Fig. 2) and is disulfide bonded into a network structure [16] (see also Chapter 2, this volume). It is quite susceptible to the action of a number of proteinases *in vitro*, including pepsin (Fig. 2) and plasmin [17]. The gelatinases A and B (MMP2 and MMP9, previously called type IV collagenases) derived from connective tissue cells and from PMNs and monocytes/macrophages have been shown to cleave isolated soluble type IV collagen within the helical portion of the molecule (Fig. 2), although there may be some doubt about the extent to which such type IV preparations are native and hence about the ability of these enzymes to cleave type IV collagen *in vivo* [18–20]. Collagen V is a component of hybrid fibrils in the extracellular matrix and is known to be cleaved by gelatinases A and B [21]. On the other hand, collagen VI is relatively resistant to MMP attack. Everts et al. [22] have shown that fibroblasts phagocytose type VI collagen prior to lysosomal degradation. Its extracellular degradation may be due to the action of collagenase 1, or of serine proteinases, as shown by Kielty et al. [23] for neutrophil elastase, cathepsin G, chymase, and tryptase.

Collagen VII, which forms anchoring fibrils in the skin, is susceptible to collagenase 1, gelatinase A, stromelysin 1, and gelatinase B [24]. Collagen IX, which aligns along the surface of collagen II fibrils, has a number of noncollagenous interruptions to the triple helix and is highly susceptible to proteolysis. The actions of collagenases 1 and 3 and stromelysin 1, against collagen IX have been documented [25,26]. Collagen X is cleaved by collagenases 1 and 3, stromelysin 1, and gelatinase A [26–28]. Collagen XI is cleaved by gelatinase A [29] and collagen XIV by gelatinase B [30]. It has recently been shown that type XVIII collagen can be proteolyzed at many sites adjacent to the NC1 domain but the enzymes involved were not identified. Such activity could generate the angiogenesis inhibitor, endostatin [31]. Other connective tissue cell MMPs, stromelysin 1 and

TABLE 3. Collagen Types and Proteinases that Degrade them at Physiological pH and Temperature

	Matrix Metalloproteinase Group				
Collagen Type	Collagenase MMP1,8,13	Stromelysin MMP3,7,10,12	Gelatinase MMP2,9	Membrane type (MT) MMP14,16	PMN Elastase
I	+	−	+ (2)	+	
II	+	−	+ (2)	+	
III	+	+ (3)	+		+
IV	−	+	+	−	+
V	−	−	+		
VI	+				
VII	+				
VIII	+				
IX	−	+	−		
X	+		+		+
XI	−		+		
XII					
XIV					
XVIII		+			
XX		+			

+ = cleaves; − = no cleavage; (2) = MMP2 only; (3) = MMP3 only.
This table is not comprehensive in that not all MMP-collagen combinations have yet been analyzed.

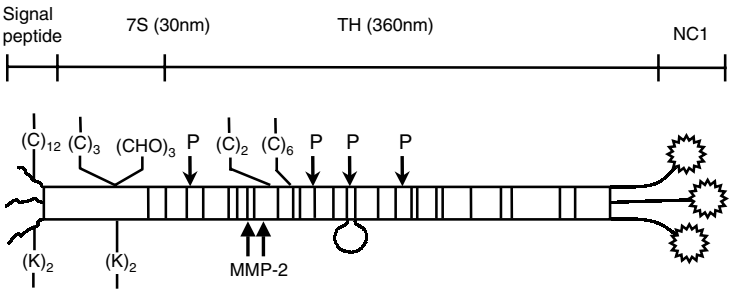

Figure 2. Type IV collagen structure and degradation. The type IV collagen molecule is most frequently composed of two α1(IV) and one α2(IV) chains that form a triple helix (TH) with an N-terminal domain that associates with other N-terminal domains to form the ''7S'' structure, and a C-terminal NC1 domain. The triple helix has many non-triple helical interruptions, indicated by black bars, which give the molecule great flexibility. However, there is substantial disulphide bonding with adjacent molecules that considerably modifies proteolytic susceptibility [20]. The major pepsin cleavage sites are indicated by arrows (P). The regions of gelatinase A cleavages within the molecule which are N-terminal to a disulfide-bonded region, are indicated by larger solid arrows (MMP-2). Type IV collagen is insoluble in basement membranes because of the formation of irregular polygonal networks of cross-linked supramolecular aggregates. Such structures are much more proteolytically resistant than soluble preparations. Cross-linking through cysteine is depicted by C and through lysine by K. CHO = carbohydrate binding site

matrilysin (MMP7), will efficiently degrade type IV collagen, apparently by attack at the nonhelical regions associated with cross-linking of the molecules [32,33], producing large but soluble fragments. The serine proteinases of PMNs, elastase and cathepsin G, can also degrade type IV collagen by attack on the nonhelical regions [34,35]. It is likely that the lysosomal cysteine proteinases act similarly at more acid pH values.

Proteoglycans

The protein cores of proteoglycans of differing types are generally susceptible to the action of many proteinases. The variation in cleavage patterns between types has not been systematically analyzed but most of the work carried out on chondroitin sulfate proteoglycan aggregates (e.g., cartilage aggrecan) seems likely to be generally applicable. Cleavages between the G1 and G2 globular domains, adjacent to the hyaluronate binding region are thought to be of the most significance, releasing a nonaggregating proteoglycan monomer, and various proteinases are now known to act in this region (Fig. 3). A predominant cleavage site is Glu373–Ala374, susceptible to the action of the metalloproteinase(s), ''aggrecanase'' [36], which is now thought to be a family of activities based on the soluble metalloproteinase disintegrins [37,38], termed ADAMTS [39], which contain thrombospondin 1 repeats (*a d*isintegrin *a*nd a *m*etalloproteinase: *t*hrombospondin). It should be noted that chondrocyte membrane aggrecanases have also been described [40]. Cleavage at Asn 341–Phe342, effected by many of the MMPs [41] and by cathepsin B [42] is also common. To compare the relative frequency of generation of these cleavages, antibodies to the neoepitopes have been used. Both types of digestion product could be isolated from osteoarthritic cartilage and were detectable by immunohistochemistry in rheumatoid, osteoarthritic, and normal cartilage. No specific distribution pattern could be defined and it was deduced that aggrecan turnover in all situations could be due to the classical MMPs or aggrecanase [43–45]. The topic is still under active investigation and it was recently shown that very early aggrecanase cleavages occur between G2 and G3 within the chondroitin sulfate-rich region (Fig. 3) [46]. The link protein that stabilizes the interaction of the aggrecan core protein with hyaluronan is also susceptible to proteolysis [47].

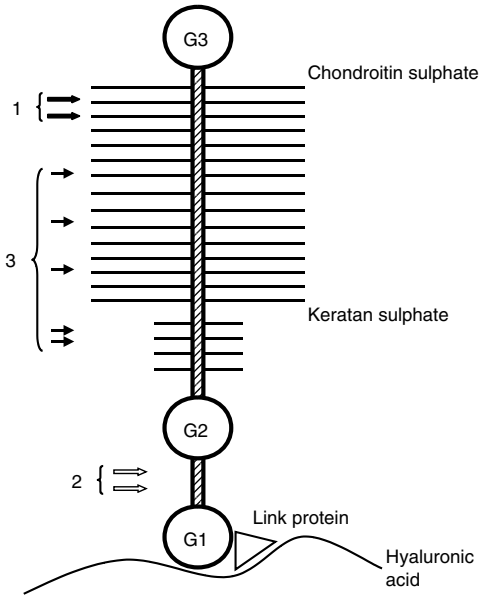

Figure 3. Proteinase degradation of proteoglycan core protein. The G3 domain is rapidly proteolyzed by an unknown mechanism, but early aggrecanase cleavage sites in the chondroitin sulphate-rich region have been identified (1). Major cleavage sites in the G1–G2 region (2) have been ascribed to either the aggrecanase or the MMP family, and generate large, nonhyaluronic acid-binding aggrecan monomers. Subsequent cleavages (3) can lead to further breakdown of both chondroitin sulfate and keratan sulfate-bearing regions of the core protein

Further cleavages between clusters of glycosaminoglycan side chains associated with extended regions of the core can occur, though these vary more widely. These cleavages generate small peptides with attached carbohydrate chains that are no longer trapped in the collagen network. In the case of aggregating cartilage proteoglycans, the fragment containing the hyaluronic acid binding region remains in the matrix, bound to hyaluronate. This may be due in part to protection of this region by link protein and hyaluronate [48].

The G3 domain appears to be very rapidly proteolyzed because as many as half of the aggrecan monomers in cartilage extracts have lost this domain. The lysosomal proteinases may be functional at pericellular sites, generating glycosaminoglycan peptides to be pinocytosed by the cell. It is possible that intact proteoglycan could be taken up by some connective tissue cells; some fibroblasts are able specifically to bind and internalize proteoglycans [49]. Dermatan sulfate proteoglycans appear to undergo pinocytosis more efficiently than heparan sulfate proteoglycans and via a different route. The internalized proteoglycan fragments are further degraded in the lysosomes, this involving the cathepsins, notably cathepsin D (reviewed by Heinegard and Paulsson [50]). There is sparse evidence for extracellular degradation of hyaluronic acid, although three hyaluronidase isozymes have been shown to be expressed by chondrocytes [51]. Pinocytosis through a specific receptor and intracellular degradation, and diffusion from the tissue and breakdown in the liver, have been described [52].

There is no evidence for cleavage of glycosaminoglycan side chains at extracellular sites, except in the case of heparan sulfate. Heparatinases and heparanases are known to be produced by platelets [53], activated T lymphocytes [54], neutrophils [55], and some tumor cells [56]. These enzymes are likely to be endoglucuronidases, similar to the well-studied platelet enzyme [53] and play an important role in the initiation of extracellular cleavage of this proteoglycan. Because heparan sulfate is involved in cell–cell and cell–matrix interactions, as well as binding to fibronectin, types I, III, and V collagens, and growth factors [57], its degradation may represent a significant part of the signaling mechanism of cells.

The intracellular digestion of glycosaminoglycan chains occurs through the initial action of lysosomal endoglycosidases, with further degradation by exoglycosidases and sulfatases that work from the nonreducing terminus (reviewed by Poole [58]). Our understanding of the degradation of glycosaminoglycans has been derived largely from studies of patients with genetic abnormalities, the mucopolysaccharidoses, resulting in either a deficiency or an absence of a specific enzyme in these pathways (reviewed by Neufeld and Muenzer [59] and in Chapter 22, this volume). Small leucine-rich proteoglycans, which interact with the surface of collagen fibrils, appear to be much more resistant to proteolytic degradation; it has been proposed that they help to stabilize the collagen network [60].

Elastin

As discussed in detail in Chapter 3, elastic fibers are comprised of two morphologically and chemically distinct components, predominantly an amorphous material (90%), which is the elastin, and fibrillin-containing microfibrils. This latter component is readily degraded by a variety of proteinases [61,62] but the amorphous elastin is susceptible to few enzymes. The serine proteinase elastase secreted by PMNs is a powerful general proteinase and degrades elastin moderately well by nonspecific electrostatic binding and attack of alanine rich regions [63]. This enzyme functions optimally at neutral to alkaline pH.

Metallo-elastase activities have been described in association with macrophages and some connective tissue cells [64–67]. It has also been shown that stromelysin 1 and gelatinases A and B produced by most connective tissue cells can degrade elastin, albeit slowly [66,68]. Whether their activity would be of significance *in vivo* is still difficult to judge, but a prominent role for MMPs in the degradation of elastin by human macrophages in the lung has been proposed [69]. The cysteine proteinase cathepsin L has been shown to have potent elastinolytic activity at pH 5.5, whereas cathepsin B has weak activity at this pH value [70,71]. Chapman and colleagues [65,72] have used a model system of macrophages growing on elastin substrates to demonstrate a major role for acid cysteine proteinases in elastin degradation. Their results also indicate that the action of the cysteine proteinases is dependent upon the action of neutral proteinases, notably plasmin, if the elastin substrate is associated with other glycoprotein matrix components. Their data suggest that matrix elastolysis mediated by cysteine proteinases occurs extracellularly and by analogy with bone resorption [73] they propose that acidification occurs at the cell-substratum interface.

In summary, the normal turnover of elastin in tissues is very slow and may be mediated by the MMPs produced by local cells. In situations of more rapid breakdown, notably pathological conditions such as emphysema, more potent proteinases may be involved, including cathepsin L from macrophages and leukocyte elastase from PMNs.

Fibronectin, Laminins and Other Extracellular Glycoproteins

Connective tissue cells produce many adhesive glycoproteins, some of which are specific to cell types while others, such as fibronectin, laminins, and vitronectin, have a widespread distribution (see Chapter 5, this volume). Characterization of these proteins and the definition of their roles have developed rapidly in recent years and detailed analyses of specific degradation patterns have been carried out. The majority of these glycoproteins are proteinase sensitive; they are susceptible to the action of many serine proteinases, such as PA [74,75] and PMN elastase and cathepsin G [76], MMPs including stromelysin [32,33], and the MT MMPs. A number of these molecules mediate cell–cell and cell–matrix interactions, and small changes in their levels, which could be effected by changes in their rates of degradation, may represent important signaling mechanisms *in vivo* [77]. Plasmin is thought to play a significant role in the removal of the glycoprotein components of the matrix. *In vitro* studies, including trophoblast and macrophage cultures on defined extracellular matrices, have shown that removal of glycoproteins by plasmin can be rate-limiting for the subsequent degradation of the collagen and elastin components [65,78,79].

PROTEINASE BIOCHEMISTRY

As outlined in the Introduction, members of the four major classes of proteinase are active in the degradation of connective tissue matrix macromolecules. This section briefly summarizes their biochemical properties in relation to their known roles in matrix turnover.

Matrix Metalloproteinases

Zinc dependent endopeptidases of the MMP (matrixin) family may be derived from many different cell types. At least 22 different human homologues have been identified and many are now well characterized, particularly in terms of their ability to degrade extracellular matrix proteins (reviewed in Nagase and Woessner [80]). The

MMPs have been classified into subgroups based on their structural similarity and substrate specificity. All the MMPs, apart from matrilysin (MMP7), have three common domains: the N-terminal propeptide, catalytic, and C-terminal domains [81]. The propeptide has the conserved sequence PRC V/N PD in which the cysteine residue plays a critical role in liganding to the active site zinc as part of the latency conferred by propeptide interactions with the catalytic domain [3]. The catalytic domain has the zinc binding motif HEXGHXXGXXH, in which the three histidine residues ligate the catalytic zinc. The C-terminal domain has a structure related to that of the hemopexin family and confers varying properties on different MMPs, including substrate, matrix and inhibitor binding. The two gelatinases (A, MMP2 and B, MMP9) have a fourth domain consisting of three fibronectin-like type II repeats with gelatin and collagen binding properties. The MT MMPs have a small C-terminal transmembrane domain and cytoplasmic domain such that the "traditional" MMP structure forms the ectodomain. Most recently, MMP23 was identified with cysteine-rich, proline-rich, and interleukin/receptor-like regions, which replace the hemopexin domain [80]. MMPs all require Ca^{2+} for stability and exhibit a preferred cleavage specificity for the N-terminal side of hydrophobic residues [82].

Collagenases and Gelatinases

MMPs with the ability to cleave fibrillar triple helical collagen [83,84] include collagenases 1 and 3 (MMP1 and MMP13), collagenase 2 (MMP8), and MT1-MMP (MMP14). Collagenase 2 is largely confined to neutrophils, although reports have been made of its expression in cartilage. The other collagenases are very widely distributed in most mammals; a definitive identification of MMP1 has yet to be made in rodent tissues, in which MMP13 is usually predominant. The collagenases have varying abilities to degrade other matrix components, but collagenase 3 seems to be particularly versatile [26]. Gelatinases A and B degrade denatured collagens or gelatins; they have been proposed to be of importance in the degradation of basement membrane type IV collagen, although this activity is confined to solubilized material [20]. Collagen V is also degraded [21] by gelatinases. They have activity on other matrix components including elastin [66,68], aggrecan [41], and cartilage link protein [47]. One of the most interesting recent observations is the cleavage of laminin 5 by gelatinase A, hence promoting epithelial cell migration [85]. Gelatinase A is very widely expressed, notably by mesenchymal cells in culture, and gelatinase B is found in neutrophils and macrophages. Mesenchymal cells may be stimulated to produce gelatinase B, which is constitutively produced by a number of tumor cell types.

Stromelysins, Matrilysin, Metalloelastase, and Membrane-Type Metalloproteinases

The stromelysins make up a subgroup of the MMP family, the archetypal members being stromelysin 1 and 2 (MMP3 and MMP10). They are biochemically similar with a broad ability to cleave matrix components including aggrecan, fibronectin, nidogen, laminin, and collagen IV [10,68]. Stromelysin 1 has also been shown to degrade collagens III, IX, and X as well as the telopeptides of collagens I, II, and XI. Stromelysin 3 (MMP11) is structurally much less related to stromelysins 1 and 2 but the mouse enzyme displays a very weak proteolytic capacity against similar substrates [86]. The human enzyme is only known to cleave α1-proteinase inhibitor [87] and specific synthetic peptides [88]; hence it is clear that its natural substrate(s) has not yet been identified. Stromelysin 3 is widely expressed in association with tissue repair [89] and tumor–fibroblast interactions. It is also expressed in atherosclerotic lesions [90] and co-localized with CD40 and it is suggested that MMP11 targets serpins that regulate cellular functions in atherosclerosis. Matrilysin (MMP7) has potent proteolytic activity, digesting a similarly wide range of matrix proteins as the stromelysins [68,91]. This is the only known MMP lacking the C-terminal domain, and therefore shows only weak interaction with TIMPs. Matrilysin is associated with ductal epithelia and may be secreted luminally by polarized cells [92].

Macrophage metalloelastase (MMP12) has been identified as an important MMP for the degradation of elastin [66] and has been implicated in destructive diseases such as abdominal aortic aneurysms [93]. However, it also has a broad matrix substrate specificity and is important in macrophage migration through tissues [94–97]. MMP12 has also been found in hepatocellular carcinoma [98], where it has been invoked as the enzyme responsible for the generation of angiostatin; lack of MMP12 and angiostatin correlated with poorer patient survival. The MT MMPs also exhibit a broad ability to cleave matrix proteins when studied in a soluble form. It is conceivable that their activity will be more limited at the cell surface. The catalytic domains of MT MMPs 1, 2, 3, and 5 are similar in substrate specificity, cleaving fibrin, fibronectin, tenascin, nidogen, aggrecan, and perlecan [84,99]. They all have the significant ability to initiate propeptide processing and activation of progelatinase A. MT1 MMP can be prepared with the C-terminal domain intact; in its soluble form it is able to cleave fibrillar collagen [84,99]. MT4 MMP appears to differ somewhat from the other MT MMPs and is not well characterized. MT MMPs 1-4 are widely expressed as evidenced by the detection of their corresponding mRNAs in many tissues, although MT5 MMP seems to be largely confined to the brain [100]. Correlation of MT1 MMP mRNA expression with that of gelatinase A and/or TIMP2 has been observed in some tissues [101,102]. Other MMPs, including MMP19 and 23, have been described, though they are not yet well characterized.

Activation of Latent Metalloproteinases

An important regulatory feature of the MMPs is that they are generally secreted in latent proforms and require activation. The activation of these precursors may be regarded as a critical step of matrix degradation as is subsequent inactivation by the action of endogenous inhibitors (see below). The other factor thought to modulate extracellular MMP activity is the ability to bind to specific extracellular matrix components, as discussed above, which increases the local concentration of the enzyme.

In vitro, the MMPs can be activated chemically by agents that modify cysteine residues, such as the organomercurial APMA. This acts to disrupt the cysteine-active site zinc interaction between the propeptide and the catalytic domain [21,103]. Disruption of cysteine-zinc binding can also occur when the conformation of the proenzyme is perturbed (e.g., the SDS activation of progelatinase A and progelatinase B during zymography). Most mechanisms involve a step-wise proteolytic processing of the propeptide [21]. These cleavages may be totally autolytic but often involve a second proteolytic activity that initiates the processing by cleavage of the propeptide in the N-terminal region, destabilizing the cysteine-zinc interaction.

Many of the MMPs share common activators that include other MMPs, leading to the concept of activation cascades (Fig. 4) [104]. The generation of plasmin by the action of PAs on plasminogen seems to be of particular importance for the activation of procollagenases 1 and 2, and prostromelysins 1 and 2. The u-PA is especially significant because it is functional in association with a specific cell surface receptor where activity can be focused (Fig. 4). The MT MMPs are also cell associated and appear to act as another focus for MMP activation cascades. MT MMPs 1–3 and 5 can all initiate the activation of progelatinase A and hence other pro MMPs (Fig. 4) [26,100,105].

Further regulation of the MMPs occurs by the action of the specific inhibitors, the TIMPs, which are described in more detail below. The tight regulation and relatively low activity of the MMPs may be a reflection of their physiological role in tissue turnover and remodeling. In disease states in which the control mechanisms become uncoordinated, the ubiquitous action and nature of MMP activities may be one of the factors leading to inexorable destruction of the extracellular matrix.

Mammalian Reprolysins and Astacins

Both of these groups of metalloproteinases fall within the metzincin superfamily with the MMPs. The mammalian reprolysins (or ADAMs) are proteins related to the snake venoms and contain *a d*isintegrin and *a m*etalloproteinase-like domain. They may be divided into two groups, membrane integrated, and soluble but cell-associated. The membrane-bound ADAMs frequently have fully fledged proteinase domains and are clearly implicated in the turnover of other cell surface proteins [106]. Some studies have suggested roles in the turnover of extracellular matrix but such a role is likely to be confined to cell bound molecules. A recent study suggests roles as fusion proteins for ADAMs 12 and 9 (meltrin-α and -β) in the formation of osteoclasts and multinucleated giant cells [107]. The major soluble ADAMs are the ADAMTS family that have C-terminal thrombospondin repeats, which may be involved in cell binding [39]. Two of these, ADAMTS-4 and ADAMTS-5, have been identified as aggrecanase and termed ADMP-1 and ADMP-2 respectively [37,38]. ADAMTS-3 is the procollagen N-proteinase involved in the processing of newly synthesized tropocollagen into fibrils (see Chapter 2, this volume).

The astacins are also multidomain proteinases and are still relatively understudied. The astacins discovered so far include procollagen C-proteinase (bone morphogenetic protein-1), which cleaves the carboxyl terminal propeptides of the fibrillar collagens during assembly [108] and others involved in cell differentiation and pattern formation during development. Meprin is an astacin expressed by intestinal epithelial cells and carcinomas and may have a role in the breakdown of basal lamina proteins [109,110]. It is a heterodimeric protein with α and β subunits and may be membrane anchored via the α subunit. Differential intracellular processing generates a soluble form that may be released into the lumen of the colon.

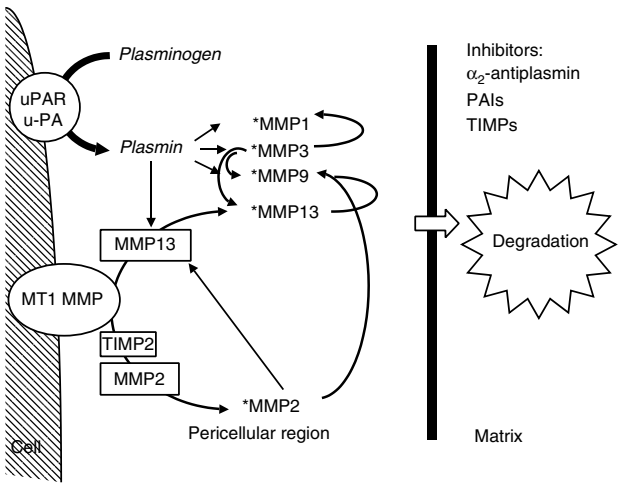

Figure 4. Cell surface-associated activation cascade for matrix metalloproteinases. The essential feature of this cascade is the production of proteinases and inhibitors by cells activated by cytokines, growth factors, and hormones. Whether or not matrix degradation occurs depends finally on the relative amounts of the metalloproteinases and inhibitors and also on the complex interplay with serum inhibitors. In many cases of tissue destruction, it is clear that plasminogen is essential for the cascade (see text): it is likely that u-PA plays a key role in the production of plasmin from plasminogen, which in turn is involved in the activation of collagenases and stromelysins. *indicates activated MMP. Solid lines denote activatory pathways

Serine Proteinases

Plasminogen Activators and Plasmin

The PAs specifically generate plasmin from its plasma zymogen plasminogen and are thought to be key regulators of connective tissue turnover [75]. Plasminogen is present in high concentrations in plasma [111] and binds to cells and components of the extracellular matrix [112]: thus its activation by PAs could be rate-limiting. Plasmin has activity against a number of connective tissue macromolecules including fibronectin, proteoglycan core proteins, other glycoproteins, and type IV collagen, as well as its major substrate, fibrin. Additionally, plasmin has been shown to initiate the autoactivation of many MMPs by a cleavage in the propeptide [21]. Both plasminogen and plasmin bind to extracellular matrix proteins that protect them from inactivation [113,114].

Two major PAs have been described, a t-PA and a u-PA. The single chain form of t-PA is an M_r 70,000 enzyme primarily associated with endothelial cells in tissues but variably expressed by other cell types, including fibroblasts, chondrocytes, and tumor cells. It binds specifically to fibrin and is thought to be the major generator of plasmin for fibrinolytic processes. u-PA is an M_r 55,000 enzyme, consisting of two disulfide-linked subunits, M_rs 30,000 and 24,000, and can be expressed by most cell types. It binds to a specific plasma membrane receptor on fibroblasts and some macrophage-like cells and may be more involved in generating plasmin for extracellular matrix remodeling processes [71,75,115]. Plasminogen (M_r 90,000) is cleaved by both PAs at an Arg–Val bond near the carboxyl terminus, resulting in an autocatalytic cleavage near the amino terminus of the enzyme. The PAs are chymotrypsin-like enzymes and are also initially secreted as proenzymes. Their activities are closely regulated by specific inhibitors, discussed below.

Elastase and Cathepsin G

The serine proteinases neutrophil elastase, proteinase 3, and cathepsin G are located in the azurophil granules of mature circulating PMNs. They are synthesized by premyelocytes during development in the bone marrow and packaged in the granules for future function in mature cells. These enzymes are potent nonspecific proteinases and

their roles are thought to be as bactericidal agents and in the degradation of immune complexes by intraphagosomal processes [116]. They may also be important in promoting inflammation, because the granule contents are frequently secreted into the extracellular environment. Elastase and cathepsin G can together degrade all the components of the tissue matrix. Both enzymes, however, are highly basic and bind tightly to the cell surface and the matrix, properties that may be important in determining the extent of their activity. Elastase, an M_r 29,000 glycoprotein, exists transiently in a proform and is then rapidly activated to the mature form [117]. It functions at neutral to alkaline pH, efficiently degrading not only elastin but also the nonhelical cross-linked regions of all collagens, including type IV [34,35]. Most glycoproteins of connective tissues are highly susceptible to elastase degradation. Proteinase 3 (myeloblastin) has a similar substrate specificity to elastase, degrading elastin, fibronectin, vitronectin, and type IV collagen [118]. Cathepsin G, M_r 22,000, is also active against collagens, solubilizing both insoluble types I and II, as well as degrading proteoglycan core protein and other matrix glycoproteins [119]. The activity of both enzymes is tightly regulated by a number of inhibitors, as discussed below.

Mast Cell Proteinases

Chymases are mast cell secretory proteinases with chymotrypsin-like specificity, and may have a role in matrix catabolism in some situations [120]. Chymase has a broad spectrum of activities, including activation of the proenzymes procollagenase 1, prostromelysin 1 [121], and progelatinase B [122], which can be synthesized by mast cells [123]. This group of proteinases varies to a large extent in its catalytic and physicochemical properties according to origin. Tryptase is a trypsin-like serine proteinase that is resistant to inactivation by circulating inhibitors and has been shown to be an activator of procollagenase 1 and prostromelysin 1 [21]. It has limited proteolytic activity toward type VI collagen, fibronectin, and fibrinogen [124]. Functional differences between tryptases from different tissues have been reported and the genetic heterogeneity of human tryptases documented [125]. Both chymase and tryptase as well as cathepsin G are stored in granules within the mast cell as inactive precursors and activation appears to occur during the secretory process.

Kallikreins

Plasma kallikrein, which consists of two disulfide-linked chains of M_r 36,000 and 52,000, acts on kininogens to generate bradykinin. It activates procollagenases 1 and 2 and prostromelysin 1 [21]. Tissue kallikrein (M_r 46,000) generates lys-bradykinin from kininogens [126] but is also an activator of procollagenase 2 [127] and progelatinase B [128].

Cysteine Proteinases

The cysteine proteinases that have been shown to degrade connective tissue proteins *in vitro* include cathepsins B, L, K, and S and the calpains. Although these enzymes function optimally at acid pH and have a major role in intracellular (lysosomal) digestion of phagocytosed material as well as in specific processing events, they may have extracellular activity in situations where the pH is acidic [71]. Both cathepsins B and L are secreted in significant amounts by stimulated connective tissue cells and macrophages, frequently in a proform that is stable at higher pH values [42,129,130]. With some cell types these enzymes have been shown to be associated with the outer surface of the plasma membrane, but it is not clear whether they are associated with specific receptors [131].

Cathepsins B and L have M_rs of about 28,000 and 25,000, respectively, in their active forms. They are related to the plant proteinase papain, all possessing an active site cysteine as well as an essential histidine residue, and cleave internal bonds in proteins and peptides. Cathepsin L is the most potent of the lysosomal endopeptidases, although the recently studied cathepsin S has a similar spectrum of activity and is more active at neutral pH values [132]. Cathepsin B is less active as an endopeptidase and can function as a peptidyl dipeptidase. It is likely that the normal physiological functions of cathepsins B and L are intracellular, with a major role in the lysosomal degradation of proteins in cells [133]. Although cathepsins B and L are generally stored in lysosomal granules, they can sometimes be found extracellularly and often in a latent form requiring activation. Both the active and latent extracellular forms of cathepsin B have higher M_rs than the lysosomal enzyme and are thought to be precursor forms that can be activated by proteolysis. They are stable at alkaline pH and some forms may be poorly inhibited by endogenous inhibitors such as the cystatins and α_2-M. Procathepsin L has been isolated from transformed mouse cells and activated human and mouse macrophages in culture, but is not active unless exposed to low pH. Both cathepsins B and L degrade cartilage proteoglycan and collagen [11]. Type I collagen is cleaved in the N-terminal telopeptide region causing depolymerization of the covalently cross-linked mature fibrils, thereby generating monomers [9]. Cartilage collagens, types II, IX, and XI, are also cleaved within the nonhelical domains. Cathepsin L is a particularly active matrix-degrading proteinase: it can degrade elastin and may have a role in elastin degradation in the lung, where macrophages are abundant [134]. Cathepsin S, M_r 24,000, has a similar substrate specificity to cathepsins B and L [132] but is more stable at pH values close to neutral. It has collagenolytic and elastolytic properties at neutral pH. It is less abundant than cathepsins B and L although it is still an important component of the lysosomal degradation mechanism. Cathepsin S maps to chromosome 1q21 and has been implicated in Alzheimer disease and Down syndrome [135].

Cathepsin K (previously O) has been isolated from cDNA libraries from osteoclasts and spleen [136] and has M_rs in the mature form of 43,000 and 29,000. It plays a key role in osteoclast-mediated bone resorption, efficiently degrading type I collagen at pH values of 4.5–6.0 as well as gelatin, elastin, and osteonectin. Strong evidence for the function of cathepsin K has been derived from the study of patients with the recessive autosomal disease pycnodysostosis (see Chapter 19, this volume). The disease is linked to chromosome 1q21 [137] and characterized by dwarfism, and severe bone abnormalities such as osteopetrosis. Collagen fibrils have been found to accumulate in cytoplasmic vacuoles in the osteoclasts of such patients [138]. Cathepsin K has subsequently been mapped to the pycnodysostosis location on chromosome 1q21 and has been shown to contain additional and altered stop codons in patients with the disease [139]. It was also shown that cathepsin K is localized at the ruffled border of osteoclasts and the collagen in resorption lacunae implying in part an extracellular localization and function [140]. Studies ablating the gene for cathepsin K in mice have resulted in a confirmatory osteopetrotic phenotype [141]. Osteoclast cathepsin K is

up-regulated by all-*trans* retinoic acid, a potent stimulator of osteoclastic activity [142] and may account for matrix degradation in other retinoid sensitive tissues. Specific extracellular inhibitors of the cysteine proteinases would further regulate their activity, as discussed below. To date, the only other cells reported to synthesize cathepsin K are bronchial and alveolar epithelial cells [143]; because cathepsin K is highly collagenolytic and elastinolytic, it is possible that this proteinase may be important in remodelling processes in the lung.

Calpains are cytosolic, Ca^{++}-dependent cysteine proteinases with neutral pH optima. μ-Calpain and m-calpain require micromolar and millimolar Ca^{++}, respectively, for activation [144]. These enzymes can degrade proteoglycan, fibronectin, and vitronectin [145]. Calpains have been shown to be raised in synovial fluids and tissues of patients with rheumatoid and osteoarthritis and in some animal models of arthritis. Calpastatin, a specific cellular inhibitor of calpains, has been identified as an auto antigen in some rheumatoid patients [146].

Aspartic Proteinases

Lysosomal cathepsin D is the major aspartic proteinase involved in matrix degradation. It is now accepted that its role is confined to the breakdown of phagocytosed material. Procathepsin D is sorted to lysosomes via the mannose-6-phosphate receptor and is stored in its M_r 34,000 mature form. Its optimal proteolytic activity against most substrates is at pH values as low as 3.5 and it is, therefore, most suited to a function within phagosomes [147].

PROTEINASE INHIBITORS

The abundance and diversity of proteinase inhibitors that function in connective tissue metabolism indicate the importance of efficient regulatory mechanisms for degradative events. The *in vivo* levels of proteinase inhibitors generally exceed the levels of their target proteinases. With the exception of α_2-M, the naturally occurring inhibitors act specifically against the individual classes of proteinases and are considered according to this classification.

α_2-Macroglobulin

This plasma inhibitor is unique in that it inactivates proteinases from all the major mechanistic classes [148]. α_2-M (M_r 780,000) consists of four identical polypeptide chains, which exist as two dimers covalently linked by S–S bonds, which can noncovalently associate to form a tetramer. Inhibition is effected by a "trap" mechanism in which a "bait" region of the molecule, containing preferred cleavage sites for all classes of endopeptidases, is cleaved. Upon cleavage of the bait region the conformation of the α_2-M tetramer is modified, trapping the enzyme within the molecule. The interaction of α_2-M with MMPs has been studied in detail and shows that the bond cleavage in the bait region is variable: collagenase preferentially cleaves in this region at the amino-terminal side of large hydrophobic residues [149]. Each subunit of α_2-M contains an intrachain β-cysteinyl-γ-glutamyl thioester which becomes reactive on proteolysis of the "bait" region and can form a covalent link with the proteinase. This is not essential for the inhibitory mechanism, however. α_2-M is thought to be largely confined to the blood supply since its large M_r usually precludes penetration within the tissue matrix. Consequently, it serves an important role as the humoral regulator of proteinase activity. The liver is the major site of synthesis, but there are reports of α_2-M synthesis by lung fibroblasts in culture [150] and by tissue. There is tissue penetration where capillaries are leaky, as in inflammatory situations, but the role of α_2-M, if any, within matrices is still not clear [148].

Inhibitors of Matrix Metalloproteinases

One major group of inhibitors of the connective tissue MMPs has been described in detail to date, the TIMPs. Four TIMPs have been described; TIMP1 is an M_r 28,000 glycosylated protein [151], TIMP2, an M_r 22,000 protein [152], TIMP3, an M_r 21,000–27,000 protein that binds to extracellular matrix components [153], and TIMP4, an M_r 22,000 protein that is related to TIMP2 [154]. The TIMPs have a similar basic structure of six disulfide-bonded loops that can be divided into two subdomains. All the TIMPs can inhibit all the MMPs, with a few rare exceptions, forming 1:1 complexes [155–157]. In addition to their ability to bind to active MMPs, complexes between specific TIMPs and pro MMPs have been identified, notably between TIMP2 and progelatinase A and TIMP1 and progelatinase B. These complexes are formed through interactions between the COOH subdomain of the TIMP and the C-terminal domain of the enzyme. The complex between TIMP2 and progelatinase A has been implicated in the cellular activation mechanism for progelatinase A, involving MT MMPs [104].

TIMP1 is produced by many cell types and can also be up-regulated by factors such as inflammatory cytokines, growth factors, retinoids, and progesterone. TIMP2 is constitutively produced by many cells in culture and little is known of its regulation. TIMP3 is induced by a number of growth factors and dexamethasone; its production appears to be cell cycle regulated [158]. Interestingly, point mutations have been identified in the TIMP3 gene that are associated with the hereditary macular degenerative disease, Sorsby's fundus dystrophy (see below). TIMP4 may have an important role in the proteolytic balance of the vasculature [159]; it is increased after vascular injury in rats.

Inhibitors of Serine Proteinases

Human plasma contains a superfamily of serine proteinase inhibitors, the serpins, which are synthesized in the liver [160]. They are generally glycoproteins (M_rs 40,000–60,000) with a single polypeptide chain, which act by presentation of a potential cleavage site for the target enzyme. These include α1-PI (previously called α1-antitrypsin), which is an important regulator of neutrophil elastase activity and may also act against plasmin. Indeed, α1-PI is the major serum inhibitor of PMN elastase and its deficiency is often associated with the early development of panacinar emphysema (see also Chapter 17, this volume). In emphysema, elastase-α1 proteinase inhibitor complexes can be detected in tissue fluids and the concept of protease:antiprotease imbalance has been invoked. Interestingly, mouse macrophage metallo-elastase (MMP12) can degrade native α1-PI at a single methionine residue [161] but does not degrade the oxidized form. This region of α1-PI has more recently been found to be sensitive to cleavage by many MMPs and other proteinases. Thus, it is possible that MMPs could disrupt the balance between serine proteinases and their inhibitors *in vivo*.

The major physiological plasmin inhibitor in plasma is α2-antiplasmin, an M_r 67,000 member of the serpin family that binds to both plasminogen and plasmin at lysine binding sites. α2-Antiplasmin subsequently complexes with the active site of plasmin at a second reactive site (reviewed in Lijnen and Collen [162]). The concentrations of such proteinase

inhibitors within connective tissue spaces are likely to be similar to those in plasma under normal conditions and they are thought to be effective in regulating plasmin activity. However, in inflammatory situations and other pathological processes, the free plasma inhibitor level may fall.

The PAIs are potentially some of the most important regulators of connective tissue degradation because of their control of the rate of plasmin generation in local extracellular environments. Although PAs do complex with other types of serine proteinase inhibitor, many may not be effective *in vivo*. Four specific PAIs have been described, all members of the serpin family (reviewed in Kruithof [163]; Andreasen et al., [164]). The endothelial-type, PAI1, originally isolated from cultured endothelial cells [165], is an M_r 54,000 glycoprotein produced by a number of cell types in culture; it inhibits both u-PA and t-PA but forms a more stable complex with the latter. It is present in platelets and is the primary inhibitor of PAs in plasma [166]. PAI2 is also present in plasma and has an M_r of 47,000 in the nonglycosylated form; an M_r 67,000 glycosylated form was originally described from placenta but is expressed by a number of cell types including macrophages [167]. The third inhibitor, PAI3, is identical to the inhibitor of activated protein C and is less well understood [163]. The fourth inhibitor, known as protease nexin I, was originally found in fibroblasts [168] but is produced by other cultured cells such as endothelial cells. Secreted PAI1 is initially deposited pericellularly and can be recovered from extracts of extracellular matrix [169]. This association maintains inhibitor stability, whereas the free form becomes rapidly inactive, suggesting that its major function is to regulate pericellular proteolysis. The precise function of PAI2 is not clear, although it may have a role in inflammatory reactions. It is more stable than PAI1, is more effective against u-PA than t-PA and is a plasmin inhibitor [163]. Reaction with PA leads to inhibitor inactivation with the loss of an M_r 15,000 peptide. Protease nexin I is a less specific inhibitor of both u-PA and t-PA; it has a broad spectrum of action and is more effective as a thrombin inhibitor. However, it has been shown to inhibit u-PA-mediated matrix degradation [170].

Acid-stable serine proteinase inhibitors have been isolated from mucus secretions, seminal plasma, and salivary glands and show a degree of immunological cross-reactivity. The inhibitor from human parotid secretions, mucus proteinase inhibitor or secretory leukocyte protease inhibitor, SLPI, has been isolated, characterized, and sequenced [171]. SLPI has an M_r of about 12,000 and is a potent inhibitor of the neutrophil proteinases, elastase, and cathepsin G. It is a two domain protein with the C-domain conferring proteinase inhibitory properties. SLPI is thought to play an important role in the defence of different tissues against these enzymes and it has been shown to inhibit proteolysis at the neutrophil-substrate interface [172]. An M_r 15,000 form of SLPI has been found in cartilage [173]. An inhibitor termed elafin has been isolated from human skin and is comprised of 57 residues with 38% similarity to the inhibitory domain of SLPI. It inhibits neutrophil elastase and proteinase 3, but not cathepsin G. Aprotinin, which is present in mast cells and cartilage is a kunitz-type pancreatic trypsin inhibitor that inhibits plasmin and kallikreins [174].

Inhibitors of Cysteine Proteinases

A super-family of cysteine proteinase inhibitors, the cystatins, is largely responsible for the extracellular regulation of the activity of these enzymes [175]. The members of the super-family have been placed into three groups. One comprises the type 1 cystatins (stefins), of low M_r (11,000) and containing no disulfide bonds or carbohydrate groups. They are synthesized without a signal sequence and are confined to the cell. Two species are known; type A, found in high concentrations in various epithelial cells and neutrophils, and type B, which is more widely distributed. Both type 1 cystatins have been found in extracellular fluids [176]. Type 2 cystatins are more complex, with two disulfide loops and an M_r of 13,000. Human cystatins C and S have been described [175]. Cystatin C has been found in cells [177] and is a major secretory product of human alveolar macrophages *in vitro* [178]. Type 3 cystatins (kininogens) are the most complex, containing three type 2-like domains. They are major plasma proteins functioning not only as cysteine proteinase inhibitors but also in vasodilation and coagulation events. Both types 2 and 3 cystatins are synthesized with signal peptides and are secreted products.

Barrett [175] has assessed and discussed the relative physiological roles of the cystatins. Cathepsins H and L are tightly bound by these inhibitors and their intra- or extracellular activities are likely to be adequately controlled. Cathepsin B, which is present in greater amounts, is more weakly inhibited, but cystatins C and S would probably be effective extracellularly.

PROTEOLYTIC MATRIX RESORPTION IN PHYSIOLOGICAL, PATHOLOGICAL AND EXPERIMENTAL SITUATIONS

Proteinase Cascade Model of Tissue Breakdown

It is clear that no one proteinase can be considered of overriding importance in matrix turnover. A complex cascade of proteolytic events best explains how matrix destruction takes place generally (Fig. 4); in each specific situation an interplay of proteinases will be seen. Overlapping specificities are clearly demonstrated by research showing that the phenotypes of MMP-mutant mice are generally mild with respect to development and physiology, but that the deletion of specific, abnormally expressed MMPs can alter disease onset [67,95]. It is also evident that proteolysis is usually localized and that extracellular matrix degradation takes place at or near the cell surface [4,77], often where activation of MMPs occurs. We envisage that in situations without invading cells, resorption is often initiated by specific attack on matrix macromolecules by activated MMPs synthesized by stimulated tissue cells. This initial attack on the integrity of extracellular macromolecules facilitates further extracellular degradation and also adds to any intracellular degradation by phagocytosis. These processes may be augmented by the infiltration of inflammatory cells, mechanical damage, free radicals, and bacterial infection.

In most situations there is a very substantial overlap between the various proteinases, in that one specific enzyme is not absolutely essential. With some cell types, such as human monocyte-derived macrophages, both MMPs and cysteine proteinases contribute to resorption [179], as they do in bone. However, there are instances where only one proteinase may be involved in specific roles, such as the central role of enamelysin (MMP20) in the process of tooth enamel formation [180] and the high elastase and general proteolytic activity of MMP12, which is produced essentially only by macrophages [94] and seems to be regulated differently than other MMPs [181]. Also, gelatinase B (MMP9) is found to be strongly expressed in invading trophoblasts, with no

expression of gelatinase A (MMP2). Many aspects of the individual actions of MMPs have been reviewed recently (see Parks and Mecham [182]). With regard to inhibitors, it seems that TIMP3 has a major role in the control of extracellular proteolysis associated with implantation in the early embryo, in which TIMPs 1 and 2 are not expressed [183], and in fetal development [184]. A protective effect against breakdown is suggested by the observation of extensive colocalization of TIMP2 with type IV and fibrillar collagen in the lung [185].

A second general theme is that an imbalance of proteinases over inhibitors seems to occur in rapid tissue degradation. Early evidence that tissue destruction in disease processes might result from an imbalance of MMPs over TIMPs came from a study showing a reduction in TIMP synthesis compared with that of MMPs in synovial explants from a rabbit model arthritis (reviewed in Murphy and Reynolds [186]). Studies with human joint tissues from normal individuals and patients with arthritis indicated close similarities with the rabbit model and supported the idea that resorption is closely linked to insufficient TIMPs to counteract increases in MMP production. Extracts from osteoarthritic cartilage have considerably elevated levels of MMPs and only moderately increased levels of TIMP activity, leading to an imbalance [187]. A detailed study of osteoarthritis and rheumatoid arthritis confirmed that an excess of MMPs over TIMP activity in the synovium and cartilage may make a significant contribution to cartilage loss [188]. Because active enzymes were found, it is clear that TIMP levels must be insufficient to counteract the enzymic activities. High levels of TIMP-MMP complexes have been found in the synovial fluid aspirated from patients with septic arthritis [189], with active MMPs present but no free proteinase inhibitory activity. It was suggested that MMPs are released from PMNs and complex all the available TIMP activity, thus leading to an imbalance with consequent rapid cartilage destruction.

Stromelysin 1 (MMP3) is very prominent in synovial biopsies from patients with rheumatoid arthritis and osteoarthritis [190] and, even if it is not the major enzyme concerned in cartilage destruction, it may be overpowering any TIMP activity. Such an imbalance is also likely to be true for periodontitis-related conditions. Pourtaghi et al. [191] showed that antimicrobial therapy raised TIMP levels and lowered MMP3 levels in gingival crevicular fluid. In other situations, members of the other classes of proteinases, such as cathepsin B, can contribute to TIMP destruction [192].

The balance between matrix deposition and turnover is fundamental in wound healing. Bullen et al. [193] found that TIMP1 levels were lower in fluids from chronic than from healing wounds, and MMP9 is probably involved in the early stages [193–196], although Moses et al. [197] suggested that MMP2 is the more important gelatinase. Berend et al. [198] found that a high ratio of MMP1 to TIMP1 in human chondrosarcoma may be indicative of a more invasive and aggressive tumor: the ratio could therefore be useful in diagnosis and therapy, and this area is explored in more detail below. MMP13 expression is also found in chondrosarcomas [199] and was detected along with bFGF in all specimens examined. Other pathological conditions, such as the matrix breakdown surrounding loose artificial hip joints [200,201] and the formation and stability of atherosclerotic plaque [202], may also be related to an imbalance of MMPs over TIMPs.

Although molecular and biochemical studies and *in vitro* studies have made substantial contributions to the understanding of matrix breakdown, our knowledge of the specific roles of proteinases and inhibitors *in vivo* is less extensive. A major problem is that there are few techniques available that can detect the often very low levels of these entities at the sites of resorption. Biochemical assays are too insensitive and *in situ* hybridization often lacks resolution. One approach has been to prepare specific antisera for use in immunolocalization studies on cells and tissues *ex vivo*, particularly using model systems of rapid matrix destruction. Even with specific antisera and an indirect second step for amplification it has often proved difficult to localize MMPs and TIMPs, and many early immunofluorescence localization studies on tissues showed low or no staining. Consequently, a method was developed for the accumulation of antigens by using short-term culturing of cells and tissues and inhibiting cellular secretion of the antigen with the ionophore monensin (e.g., the localization of TIMP1; [203]). Such treatment results in the intracellular accumulation of antigens in the Golgi apparatus and in secretory vesicles. With this technique, unique patterns of synthesis have been observed in many situations, consistent with specific roles for the individual MMPs and other data. For example, the results of immunolocalization studies on the rabbit growth plate [204] demonstrate a unique pattern in each developmental area, both spatially and temporally, of synthesis for each MMP and TIMP1. Most of the published work has concentrated on localization at the light microscope level but MMP1 has been immunolocalized to collagen fibrils by electron microscopy [205], and MMP3 to rheumatoid synovioblasts [206]; the fixation methods needed often cause problems with loss of antigenicity for MMPs and TIMPs [207].

Another interesting point is that in many instances of rapid resorption, active MMPs can be immunolocalized bound to extracellular components in tissue *ex vivo* [208–210]. A specific antibody to mammalian MMP1 was used to immunolocalize collagenase in a porcine synovial model system [209]. When synovial preparations were cultured with colchicine for 2 days, latent MMP1 could be detected within the cells and fluorescence was associated with the extracellular fibrils. It was concluded that this was active collagenase because latent collagenase does not bind to collagen. The addition of cortisol, an anti-inflammatory steroid, to cultures so treated, resulted in the abolition of the diffuse fluorescence of the active collagenase bound to collagen and a large reduction in the number of cells containing latent collagenase. MMP1 has also been immunolocalized on collagen fibrils in frozen sections of VX2 tumor taken *ex vivo* [210]. Such tumor cells, when transplanted into nude mice, were shown to contain MMP1, thereby demonstrating that the VX2 tumor cells synthesize it *in vivo*. Only recently have monoclonal antisera been prepared that will detect active MMPs and TIMPs specifically [211] and can be used in immunochemical studies [212]. Activated MMP9 was detected by immunohistochemistry in fixed, wax-embedded sections from a series of esophageal cancer cases known to contain the enzyme in tumors themselves [211].

In much recent work, *in situ* hybridization and *in situ* zymography [213] have become the norm for demonstrating expression, especially when no antisera are available. However, it must be pointed out that mRNA levels do not necessarily equate with protein expression. A study showing the expression of MT1 MMP in mouse tissue during embryogenesis, using *in situ* hybridization and immunolocalization

in combination with gelatin zymography, well illustrates the power of the present techniques [214]. Not only was the presence of MT1 MMP shown, but also its localization with respect to MMP2 and TIMP2. MMP7 was shown to be expressed by epithelial cells in a tissue-restricted pattern in the mouse very clearly by *in situ* hybridization using a riboprobe [215].

Modulators of the Synthesis of Matrix Metalloproteinases and Their Inhibitors

One of the striking features of the family of MMPs is that they are "inducible," i.e., their synthesis and secretion seem to be controlled by factors that alter gene expression. The expression of MMPs and TIMPs by connective tissue cells is regulated by complex interactions between a number of cytokines, growth factors and hormones [216], some of which are specific to cell type and others more ubiquitous. Many factors are the products of monocytes/macrophages and their production in inflammatory situations is a key step in initiating MMP synthesis and tissue degradation [217]. IL-1 [218,219] seems to be particularly important in the up-regulation of MMPs, and roles for TNF [220–222], growth factors such as bFGF [223–225] and PDGF [226,227], and prostaglandins as stimulators have been documented.

MMP2 production by human fibroblasts was found to be stimulated by TGF-β [228], whereas MMP1 synthesis was decreased by this factor [223,228]: TGF-β also increases TIMP1 production [223,228]. It is likely that MMP2 is controlled differently from the other MMPs, which may be related to the fact that it has a different chromosomal localization [229] to that of MMP1 and MMP3 [230] and is activated differently. In murine macrophages, it has been found that MMP2 and MMP9 are differentially regulated [231]. Different cell types show differential signalling pathways in response to TGF-β [232] and that there are cell-type activities of AP-1 components. TGF-β may be especially important because it can decrease the cytokine-induced synthesis of MMPs and increase that of TIMP1, and is probably physiologically important since many connective tissue cells make this factor and extracellular matrices can be large stores of it [233]. IL-10 can result in a decrease in MMPs and an increase in TIMPs [234], which may be mediated by the inhibition by IL-10 of prostaglandin production [235]. Interaction between multiple factors is important when considering cells in tissues *in vivo* rather than in artificial culture models. Inhibitory effects of IGF-I, IL-4, [236] and IL-13 [237] on MMP expression have also been found.

Studies with human gingival fibroblasts suggest that IL-1 may act to stimulate MMP1 by first stimulating nuclear factor (NF) κB proteins; these then interact with sites on the collagenase gene promoter. This pathway of activation may play a pivotal role in the pathogenesis of periodontitis [238] and IL-1α may be more important than IL-1β[239]. The MT MMPs can also be up-regulated by several growth factors [240]. IL-1α seems to be the mediator of other inducers of collagenase, such as phorbol 12-myristate 13-acetate [241].

MMPs are often synthesized coordinately in simple experimental situations but their expression in tissues *in vivo* is far more complex. From studies of the distribution of MMPs and TIMP1 in the development of the rabbit growth plate [204] and mandibular condyle [242] it is clear that each MMP has its own pattern of expression. Recent work suggests that MMP9 is particularly important in growth plate vascularization and ossification [243]. Not only are there unique patterns of MMP expression in normal situations but also, for example, in pathological tissues, such as rheumatoid synovium and tumors. These results are consistent with the idea of specific roles for the individual MMPs, and the role of TIMPs may be to tightly control MMP activity at both the level of activation and subsequent substrate degradation. As with other proteinase-inhibitor systems (notably the plasminogen activators) MMPs may be active only at specific locations where inhibitor concentrations are reduced compared with enzymes.

Corticosteroids are potent inhibitors of MMP synthesis [244–246] and many other agents have been tested for possible therapeutic use (in addition to TGF-β) to ascertain if they might either block MMP synthesis or increase TIMP activity. Retinoids can decrease MMP production and increase TIMP [244,246–248] and MMP synthesis can be down-regulated by interferon-γ [249] in fibroblasts, although opposite effects are found with cultured keratinocytes [250]. Interferon-γ and -β, but not -α, inhibit invasiveness and MMP2 production of renal carcinoma cells [251]; surprisingly the same workers found no effects on fibroblasts. Interferons α and γ up-regulated MMPs 2 and 9 in human melanoma cells [252] but only if treatment time was short.

Most cells, especially connective tissue cells and endothelial cells, synthesize TIMPs constitutively. Although activity is elevated by treating cells *in vitro* with phorbol esters [253], corticosteroids under some conditions, TGF-β bFGF [223], EGF, IL-6 family members and retinoids [246], less is known about the control of the basal levels of TIMPs *in vivo*. Many fibroblasts stimulated with IL-1 synthesize increased amounts of TIMP1 in parallel with MMPs, but with other cells, such as rabbit uterine cervical fibroblasts [254], IL-1 decreases TIMP synthesis. It has been reported that IL-6 can increase the synthesis of TIMP1 and can act synergistically with IL-1 [255], although Silacci et al. [256] found that IL-6 and its soluble receptor blocked IL-1-induced collagenolytic activity in parallel with the induction of TIMP1 in chondrocytes and synoviocytes; TIMP2 and TIMP3 levels were not affected. MMP9 seems to be involved in the HIV-induced invasiveness of monocytes and it was suggested that this enzyme has an important role in HIV pathogenesis [257]. IL-8 may play an atherogenic role by inhibiting local TIMP1 expression [202].

In murine embryonic development, TIMP1 is expressed at sites of osteogenesis in the limbs, ribs, digits, skull and vertebrae [258]. TIMP2 is often constitutively produced by cells and does not generally show a response to cytokines. However, where expression is regulated, it is often in the opposite direction to TIMPs 1 and 3; e.g., the expression of TIMP2 is repressed by TGF-β in various cell types. TIMP3 has been shown to be induced by factors such as EGF, PDGF, TGF-β, phorbol ester, and dexamethasone [258], and it has been shown that it is up-regulated in the G_1 phase of the cell cycle [259]. TIMP4 [154,260], the human form of which was cloned from a cDNA library and expressed as a 22-kDa recombinant protein, is more closely related to TIMPs 2 and 3 than to TIMP1.

TIMPs have growth-promoting activities [2,261] for many cells. Precisely how growth promotion is or is not related to MMP inhibitory activity is not known. The growth stimulatory activity of TIMP2 was shown to be cell type-specific and to require the presence of a cofactor, insulin [262]; tyrosine phosphorylation is crucial for growth signalling by TIMPs 1 and 2, and it has been suggested that MAP kinases are involved [263]. Also, an involvement of

a cAMP-dependent mechanism has been proposed [264], probably involving a cAMP-dependent protein kinase. Guedez et al. [265] have also shown that TIMP1, but not TIMP2 or a synthetic inhibitor of MMPs, suppresses apoptosis in B cells, suggesting a role in tissue homeostasis. A TIMP1 transgene rescued mammary epithelial cells from apoptosis when they were expressing an auto-activating MMP3, an effect which may be related to the cleavage of basement membrane entactin [266]. TIMP3 overexpression, however, can uniquely promote apoptosis in many cancer cell types [267]. Clark et al. [268] described yet another puzzle over the activities of TIMPs: they showed that TIMP1 applied to human fibroblasts could stimulate collagenase secretion but were unable to define any receptor-mediated event.

It has been reported that the expression of proteinases and proteinase inhibitors in human periodontal ligament cells can be modulated by fibronectin and fibronectin fragments, molecules found in the extracellular matrix. Kapila et al. [269] found an up-regulation of MMPs 1 and 3, and u-PA in these cells *in vitro*, and suggested that regions of the fibronectin molecule may modulate many functions of ligament cells and contribute to periodontal disease. The binding of fibroblasts to fibronectin involves integrins on the cell surface [270], and their precise role in signaling changes in MMP expression is still being defined [270–272]. α1 and α2 integrins mediate the invasive activity of mouse mammary carcinoma cells [273], principally through regulation of MMP3, whereas α6 integrins regulate cell motility. Integrins are important in signaling [273], and there is evidence that signal transduction through chondrocyte integrin receptors up-regulates MMP expression and that this may be mediated through induction of IL-1 [274]. Thus, binding of adhesion molecules may play a role in escalating the effects of IL-1 induction of MMPs. Tremble et al. [275] have found that one of the pathways that transduces signals activated by the $\alpha_5\beta_1$ fibronectin receptor acts through promoters that contain AP-1- and PEA3-responsive DNA sequences (see also below). Fibronectin and integrins induce a variety of downstream effects, including the altered expression of many proteins, and the signalling mechanisms involving kinases have been reviewed [276]. The role of the extracellular matrix in signaling and orchestrating cell behavior, especially in cancer, has also recently been reviewed [277].

The extracellular regulation of MMP activity is a key feature of their function, and TIMPs are not the only factors involved. The regulation of zymogen activation is a critical level of control of enzyme activity. The MMPs have latency conferred on them by their propeptide domains, which effectively block the active sites in their catalytic domains. *In vitro*, the MMPs can be activated by chemical agents that modify cysteine residues, such as the organo mercurial APMA. Activation may also occur when the conformation of the proenzyme is perturbed, as is evident from the SDS-activation of proMMP2 and proMMP9 during zymography. *In vivo*, activation probably takes place upon the enzymic cleavage of the propeptide. This cleavage may be an autolytic step, as occurs with proMMP2 at high concentrations *in vitro* [278], but more usually requires a second proteolytic activity. Many of the MMPs share common activators and many are activated by other MMPs. This has given rise to the concept that the MMPs are involved in many activation cascades [279]. An important cascade is initiated by the action of PAs on plasminogen, which results in active plasmin. This proteinase can activate several MMPs, although MMP2 is not involved in the plasmin cascade and is regulated differently to the plasmin-activated MMPs. Pro-MMP2 is not cleaved by trypsin, chymotrypsin, plasmin, thrombin, elastase, cathepsin G, plasma kallikrein, or MMP3 [280,281]. The mechanism involving MT MMPs (see above) appears to be the most likely pathway for MMP2 activation and can lead to the activation of MMP13 and MMP9 (see Fig. 4) [105]. MT1 MMP can itself be activated by the u-PA pathway extracellularly [282,283] and be found in a soluble form. However, MT1 MMP also seems to act as a pericellular fibrinolysin, even in PA- or plasminogen-deficient mice. Thus, in neovascularization there are multiple complex proteinase cascades in matrix resorption [284]. In inflammatory conditions it has been shown that a variety of bacterial proteinases can activate MMPs and thus increase tissue destruction [285].

Modulators of the Synthesis of Plasminogen Activators and Their Inhibitors

Those factors considered above for MMPs and TIMPs often have similar effects on the control of PAs and inhibitors, but there are some differences. Most published data indicate PA production is increased by IL-1, an effect that may be mediated, at least in part, by PGE_2 and cAMP [286]. The precise changes in t-PA and u-PA synthesis are different for different cell types and cytokine combinations (see, e.g., [287,288]). u-PA activity is suppressed by anti-inflammatory glucocorticoids [289] in several cell types, whereas PAI1 is increased substantially [290], as is also found with IL-6 [291]. PAI1 and PAI2 are members of the large superfamily of proteinase inhibitors named serpins; these serine proteinase inhibitors have roles far beyond the scope of this review [160]. It must be pointed out, however, that PAI2 is not required for either normal development or survival; mice that are deficient in PAI1 and PAI2 do not express any overlap of function between the two [292].

TGF-β increases PAI1 [228,293,294], and with this and a number of other agents there is often an inverse relationship between PAs and PAIs, as is often the case with MMPs and TIMPs. For example, PAIs 1 and 2 are TNF inducible [294a]: inhibitor levels go up as t-PA activity goes down in human fibrosarcoma cells. IL-1 can induce PA inhibitory activity by cultured endothelial cells [295].

Of considerable interest have been many studies on the regulation of plasminogen activation, especially the cloning of the cell receptor for u-PA and the work establishing that activation of plasminogen takes place via cell-associated u-PA [115,296]. Bound u-PA is less susceptible to inhibition. These studies have parallels with the activation of gelatinases and clearly proteolysis near the cell-surface is an important area of study. It is of interest that the binding of PAI1 to extracellular matrix has been demonstrated to be mediated by vitronectin [297].

Plasminogen added to chondrocyte cultures causes an increase in IL-1-induced matrix degradation, implicating PA in this process; indeed, IL-1 induces u-PA and PAI1 in these cells. Similarly, plasminogen added to IL-1-stimulated cartilage explants potentiates increased collagen and proteoglycan loss, and this can be blocked with PA inhibitors, or MMP inhibitors. Hence, there is a cascade of enzyme activation with PA activating plasminogen, and plasmin then activating MMPs [298,299]. Furthermore, intra-articular injection of protease nexin-1 blocks IL-1 or FGF-induced proteoglycan loss from rabbit knee, and tranexamic acid (TEA), another antiplasmin agent, used orally, has the same effect [300]. However, it is of interest

that loss of plasminogen gene function (t-PA and u-PA) in mice does not result in a lethal phenotype [301], although there is impaired wound healing [302], emphasizing that in the proteolytic cascades there must be redundancy of a high order.

In synovial fluid, u-PA, PAI1, and uPAR are all raised in rheumatoid arthritis (RA) compared with osteoarthritis (OA), and in OA compared with normal, but there is no direct association with clinical parameters [303]. There is also evidence for local production of u-PA and PAI1, with synovial fluid levels raised compared to plasma. At the invasive pannus front in RA synovium, u-PA, uPAR, PAI1, and PAI2 are all induced compared with normal tissue [304,305]. The *in vivo* role of the u-PA/plasmin system in the activation of MMPs during aneurysm formation has been well documented [306], along with the ancillary action of MT1 MMP in the activation of MMP2 and MMP13 [307].

Mechanisms of Induction of MMPs and TIMPs

The genes encoding MMPs 1,3,7,9, and 10 have a TATA box and a phorbol ester-responsive element (TRE), a sequence that binds to Fos and Jun (AP-1) and that is thought to mediate the induction of these MMPs by inflammatory cytokines such as IL-1 and TNF-α [308]. MMP2 seems to be constitutively produced by cells in culture and is not generally significantly modulated by cytokines. The 5′-flanking region has no TATA box or identifiable TRE sequence, but does contain a p53-sensitive sequence and an adenovirus ElA-responsive element resembling the AP-2 binding site and two "silencer" regions [309]. Glucocorticoids, retinoids, and TGF-β repress the expression of at least MMP1 and MMP3 in connective tissue fibroblasts [310,311]. Agents may induce the expression of one MMP, while repressing expression of another. Furthermore, the effect of any one agent may differ with cell type; this makes it difficult to predict the effects of blocking cytokine/growth factor action. In the case of gelatinase B (MMP9), a dual regulation of IL-1-mediated expression has been found in mesangial cells [312]. These workers demonstrated that both tyrosine kinase-mediated NFκB stimulation and c-Jun/AP-1 activation are essential for MMP9 induction; inhibition of either pathway blocks the cellular response. Stimulation of MMP9 promoter activity by *ras* oncogene requires multiple transcription factors including AP-1 sequences and closely spaced PEA3/ets sequences [313]. An src-related tyrosine kinase has been shown to be involved in transcription factor activation of the MMP1 gene by IL-1 [314]

The MMP3 gene has an AP-1 binding site, but Quinones et al. [315] found that other regions were involved. Recently Borghaei et al. [316] have attempted to identify the transcription factors and cis elements that might be involved in the IL-1 induction of MMP3. They found an upstream complex binding region that they termed the stromelysin IL-1 responsive element site, binding to which site is also induced by TNF. At least two DNA-binding proteins are involved; transfection experiments demonstrated that proteins binding to this site act as repressors of IL-1-induced expression of stromelysin. The suppression of SL expression by IFN-γ in human fibroblasts is mediated through the AP-1 element [317]. Recent work shows that both AP-1 and a cis-acting Cbfa1-like factor are required for the induction of MMP13 in mouse bone by parathyroid hormone (PTH) [318]; noteworthy is the fact that Cbfa1 is also a regulator of bone development [319]. TGF-β up-regulates MMP13 expression in fibroblasts in contrast to the down-regulation of MMP1 [320]. The signaling pathway involves protein kinase C and tyrosine kinase activities, and an AP-1 site. Protein kinase C is also involved in the regulation by vitamin D_3 of MMP3 expression by chondrocytes [321].

The promoter regions of TIMPs 1, 2, and 3 have potential Sp1-binding sites and share other features that are suggestive of housekeeping genes; however, TIMPs 1 and 3 are known to be highly stimulus-responsive [157]. The TIMP1 promoter has an AP-1 and a PEA3 motif in close proximity, reminiscent of the inducible MMP genes described above; this gene appears to have important regulatory sequences downstream of the transcription start sites. The murine TIMP3 gene has six upstream AP-1 sites that may be involved in basal expression of the gene. The data from the murine and human genes are in conflict here; the murine gene also has a putative p53 binding site that appears to be nonfunctional. The TIMP2 gene has a promoter proximal AP-1 motif [322], but this appears not to have a major role in basal expression of the gene [258]. Mertens et al. [323] have shown that gelatinase A expression at high levels is mediated by a unique interaction of two developmentally regulated transcription factors, AP-2 and YB-1, within a discrete 40-base pair enhancer element (RE-1) located in the 5′-flanking region of the gene. There is evidence for unique cell type-specific expression of MMP9; Fini et al. [324] found that although rabbit fibroblasts have AP-1-like elements, corneal epithelial cells also have the capacity for activation by the transcription factor AP-2.

Rat TIMP1 is expressed in rat hepatocytes and is up-regulated by IL-6. An IL-6/oncostatin M response element has been characterized [325] that has two functional binding sites for AP-1 and STAT3; binding to both sites is needed for full responsiveness. TIMP1 production is also stimulated by oncostatin M and other members of the IL-6 family in human articular cartilage [326]. Oncostatin M up-regulates TIMP3 gene expression in articular chondrocytes [327], and the involvement of tyrosine kinases and mitogen-activated kinase cascades are implicated in the signaling mechanism [328]. However, Gatsios et al. [329] found that oncostatin M preferentially up-regulates TIMP1 rather than TIMP3 in human synovial lining cells. Specific methylation events contribute to the transcriptional repression of the mouse TIMP3 gene in neoplastic cells [330].

SPECIFIC EXAMPLES OF PHYSIOLOGICAL MATRIX BREAKDOWN

Degradation of Bone Matrix

MMPs have been implicated in bone turnover, either via removal of osteoid by osteoblast-derived enzymes to allow osteoclastic bone resorption to take place, or more directly as osteoclast synthetic products. They have been found at sites of both endochondral and membranous bone formation [331], and appear obligatory for the migration of preosteoclasts to the sites of resorption [332]. Interstitial collagenase (MMP1) can be produced by both human osteoblasts and osteoclasts [333], although others have found that osteoclasts are not a source of high levels of it compared with osteoblasts and stromal cells [334]. In fetal rat calvarial osteoblasts, a variety of growth factors have been shown to alter expression of interstitial collagenase (MMP13 in rodents), and also to modulate bone resorption in calvarial explant assays; EGF and IL-1α have been shown as important up-regulators of rabbit MMPs, notably MMP1 [335]. Other inducing agents include PDGF [336], PTH, bFGF [337], and IL-6 [338]; suppressing factors include IGF-I and -II [339], TGF-β, and bone morphogenetic protein BMP-2. Some of these factors

(bFGF, TGF-β and BMP-2) also induce expression of TIMPs 1 and 3 [340]. Human osteoblasts have been reported to express at least MMPs 1,2, 3,9, 10,13, and 14 [341]; MMP14 has been identified in rabbit osteoclasts [342]. Synthetic MMP inhibitors suppress bone resorption *in vitro* [343] and TIMPs 1 and 2 can also do this [344].

In human development, MMP13 is expressed in mineralizing skeletal tissue, hypertrophic chondrocytes [345] and osteoblasts involved in ossification, rather than MMP1 [346]. In osteoblasts, MMPs 2 and 14 are colocalized with MMP13. In postnatal tissues, MMP13 is expressed at sites of remodeling such as bone cysts and ectopic bone and cartilage formation, while in rheumatoid arthritis patients strong expression is seen in cartilage. MMP13 has, therefore, been proposed to function in the degradation of type II collagen in primary ossification, skeletal remodeling, and joint disease [347]. Similar patterns of expression are seen in developing rat and mouse bone [348]. Rat osteoblasts possess a scavenger receptor that removes MMP13 from the extracellular space [340] and is a further control mechanism. The abundance and functioning of this receptor in rat bone and intracellular degradation are controlled by PTH [349]. In endochondral bone formation, it has been proposed that the complete degradation of type X collagen requires both the action of MMP13 from the chondrocyte and that of cathepsin B from the osteoclast [28].

Everts et al. [350] studied in detail the degradation of collagen in the bone-resorbing compartment underlying the osteoclast. This degradation clearly involves both MMPs and certain cysteine-proteinases and the work extended earlier studies showing that both classes of proteinase are important. Indeed, evidence continues to build that there are extracellular and intracellular pathways for collagen degradation, and bone resorption well illustrates the great complexity of degradative processes in specific tissues [351]. Specific inhibitors of a cysteine proteinase (cathepsin B) and metalloproteinases (collagenase and gelatinases A and B) can block bone resorption *in vitro* [352,353]. In bone resorption by osteoclasts in a murine calvarial assay, studies with specific inhibitors suggested that cathepsins L and S might act both intracellularly and extracellularly, but that cathepsin B only acts in the intracellular compartment, possibly via activation of other proteinases [352]. Gelatinase B has been implicated along with collagenase [351] as of importance in osteoclastic bone resorption [343,354]. Expression of MMPs has been noted in osteoclasts, particularly MMP9 [355,356], and also that of MMPs 1,2,3,13, and 14 [354]; indeed, some inhibition of bone resorption by MMP inhibitors has been shown even on osteoid-free bone [343]. Interestingly, stromelysin 2 (MMP10) has been found strongly associated with human osteoclasts in contrast to MMP3 [357]. In rat osteoclasts, cathepsin B and L immunostained along lacunae, with strong extracellular staining of both on collagen fibrils and the bone matrix under ruffled borders, and with a stronger signal for cathepsin L than cathepsin B [358]. A further ultrastructural study using specific inhibitors also suggested that cathepsin L plays a key role in bone resorption by cultured osteoclasts [359].

Recently a new cysteine proteinase, cathepsin K, has been discovered in osteoclasts. Cathepsin K has 56% sequence homology with cathepsin S; it has a pH optimum of 6–6.5 similar to cathepsin S, but has activity over a broader range, remaining largely active at neutral pH. It degrades elastin, collagen telopeptides, and gelatin better than the other cysteine proteinase cathepsins [13,360]. Uniquely, cathepsin K can act as a collagenase by attacking type I collagen in the helical region, reminiscent of the action of bacterial collagenase [361]. Inhibitors of cathepsin K have been shown to reduce bone resorption *in vivo* and *in vitro* [362], and an antisense approach blocked osteoclastic bone resorption *in vitro* [363]. It has been reported that only cathepsin K, and not cathepsins B, L, or S, is abundantly expressed in human osteoclasts, and that this enzyme is responsible for osteoclastic bone resorption [364]. Interestingly, an osteochondrodysplasia, pycnodysostosis (see Chapter 19, this volume), is caused by mutation in the cathepsin K gene [139]. Families with pycnodysostosis demonstrate a molecular heterogeneity of mutations, some of which seem to involve the active site binding to type I collagen [365]. Retinoic acid stimulates the expression of cathepsin K along with increased resorption when tested on rabbit bone cells from long bones, including osteoclasts [142]. Targeted disruption of the gene for this proteinase in mice leads to severe osteopetrosis [141].

Many of the seemingly disparate data on MMPs and cysteine proteinases have been resolved by the studies of Everts et al. [366]. These workers have documented site-specific differences in osteoclasts in calvaria (membranous bone) and long bones (endochondral bone). A major finding is that osteoclasts in long bones depend on cysteine proteinases (higher expression of cathepsins B and K) for resorption, whereas the activity of calvarial osteoclasts depends on MMPs and cysteine proteinases. These important findings may have wider implications since the site-specificity of cells has not often been considered.

Osteoblasts also produce u-PA, t-PA, PAI1, and uPAR, the synthesis of which is regulated by osteotropic hormones and PGE_2 [367,368]. In general, u-PA expression parallels that of MMPs. It has been proposed that PAs are involved in bone matrix degradation directly [369]; perhaps they could function as activators of proMMPs or latent growth factors in bone remodeling, or u-PA may be acting as a mitogen via its growth factor domain. The direct resorptive activity of osteoclasts does not require t-PA or u-PA [370], but the latter may be involved in migration of the preosteoclasts to the mineral surface [371]. In osteosarcoma cells there seems to be a u-PA/MMP cascade that leads to bone turnover [372], which is strongly stimulated by IL-1α.

The family of cysteine proteinase inhibitors, the cystatins, has been described in detail [175]. Their role in controlling the turnover of cartilage and bone is largely unknown, although cystatin C is reported to inhibit bone resorption in different *in vitro* systems through the inhibition of osteoclastic proteolytic enzymes and to be produced by bone cells [373].

Suture and Soft Tissue Remodeling

Orthodontic treatment is based upon the ability of fibrous joints and bone to undergo remodeling in response to mechanical forces. An organ culture system in which fibrous joints from the rabbit cranium can be deformed mechanically under controlled conditions showed that tension not only stimulates the synthesis of structural macromolecules but also MMPs [374]. By use of immunocytochemistry, localized areas of remodeling involving MMP1 and TIMP1 are seen to occur in response to mechanical force [375,376]. Sutural fibroblasts under tension synthesize significant quantities of type III collagen, as well as type I, suggesting an important role for the biomechanical environment in the expression of collagen phenotype.

Everts et al. [377] have highlighted the importance in many tissues of an intracellular route for fibrillar collagen digestion. Their research suggests that in situations where there is a high turnover of matrix (such as inflammation), the MMPs are especially important in a mainly extracellular pathway of degradation. Where turnover is low (normal remodeling), an intracellular pathway for resorption, particularly for collagens, is of major importance. In this case, they propose that membrane-bound gelatinases may be involved in partially digesting collagen fibrils prior to uptake into the lysosomal system and digestion by intracellular cysteine proteinases [378]. Evidence from experimental studies from these and other workers suggests that the normal turnover of collagen in the periodontal ligament may take place largely by this intracellular pathway (for review see Everts et al. [377]) and that cathepsin B, particularly, plays an important role [379]. Type VI collagen is present in most soft connective tissues, where it plays a role in the organization of the collagen meshwork. Everts et al. [22] have shown that type VI collagen degradation may be by way of the phagocytic route, with collagenase, serine proteinases, and lysosomal enzymes all involved.

Van der Zee et al. [380] have demonstrated that soft connective tissue can be a store of procollagenase, which can be activated under certain conditions. Thus, a collagenase-induced breakdown of collagen could take place without there necessarily being any *de novo* synthesis of this MMP. If these findings in an experimental system [380] were true for chronic inflammatory diseases in general, then an explanation would be available for the cyclic breakdown of tissues in active arthritis and periodontitis.

SPECIFIC EXAMPLES OF PATHOLOGICAL MATRIX BREAKDOWN

Periodontal Tissue Destruction

Many older studies focused on the action of proteinases released by bacteria, PMNs, and macrophages rather than tissue-derived proteinases. Evidence now suggests that invasion of the gingival tissues by intact bacteria occurs in more severe and advanced forms of periodontal disease, but many observations support the view that bacterial invasion is not a conspicuous feature of periodontitis. Periodontal pathogens could mediate connective tissue degradation in periodontal disease by the ability of antigens from their cell walls to stimulate cytokine production by circulating mononuclear phagocytes [381]. These cytokines and prostaglandins could in turn induce MMP synthesis by resident cells, thereby initiating degradation [382–384] of ligament attachment and an increase in bone resorption. Each of the major cell types of periodontal tissue is capable of expressing a unique complement of MMPs when stimulated [385]. Bacterial antigens also induce many arachidonic acid products that also can be involved in inducing proteolytic destruction of gingival tissues. Clearly the continued presence of bacteria is essential for the maintenance of inflammation and altering host defence.

Heath et al. [382] found that antigens from the walls of bacteria commonly present in dental plaque (both Gram negative and Gram positive) can induce matrix breakdown by a mechanism that involves a primary interaction with circulating mononuclear cells. Although human gingival fibroblasts *in vitro* responded directly to lipopolysaccharide (LPS) preparations in terms of PGE_2 production, they were only induced to synthesize collagenase in response to supernatants from mononuclear cells stimulated with either LPS or lipoteichoic acid. It was concluded that if a cytokine-mediated mechanism does play an important role in connective tissue destruction during periodontitis, then the disease can probably be triggered by a large variety of bacteria or by combinations of different bacteria. The particular cytokines responsible remain incompletely characterized but much circumstantial evidence and some direct evidence suggests that IL-1 is important (see below); cytokines, in turn, can stimulate PGE_2 production, although not all of the actions of the interleukins are mediated via prostaglandins. Indeed, there are many cytokine loops including one in which PGE_2 can stimulate osteoclast formation via endogenous IL-1β expressed through protein kinase A [386]. There is evidence from work with gingival fibroblasts that CD40 ligation can restrain up-regulated MMP production, and may be an important mechanism in restraining tissue damage [387]. An additional pathway for increased destruction is suggested by the observations of DeCarlo et al. [388] that quiescent mucosal epithelial cells and fibroblasts can be stimulated to degrade type I collagen by a thiol-proteinase released by *Porphyromonas gingivalis*.

A relationship has been established between latent and active forms of collagenase extracted from gingival tissues and the degree of inflammation [389]. Collagenase activity has been identified not only in gingival explant culture supernatants but also in gingival crevicular fluids at levels that correlate with disease activity. Collagenase activity can be found in the crevicular fluid of patients with periodontitis in much larger amounts than in control subjects [390,391]. This collagenase seems to be only partly tissue derived and mostly from PMNs [391] in the inflammatory infiltrate. More recent work has shown that although MMP8 from PMNs is the main collagenase in adult periodontitis, MMP1 is more prominent in localized juvenile periodontitis [392]. MMP9 from neutrophils is prominent in crevicular fluid and gingival tissue specimens from patients with periodontitis [393]. TIMP1 levels can be measured only in healthy individuals or in clinically healthy sites [390]. In clinical studies, Lee et al. [394] showed a good correlation between active MMP8 and disease activity, whereas Hayakawa et al. [395] found significantly lower TIMP1 levels in whole saliva in diseased subjects compared with healthy normals. Thus, it seems that periodontal disease activity does indicate an imbalance of proteinases over inhibitors. Hayakawa et al. [395] showed that reciprocal changes in TIMP1 and collagenase levels were observed after therapy. It has been suggested that a relative imbalance of MMP3 over TIMPs may be a significant marker of periodontitis-affected tissues [396], as it often is in arthritis [190], and an increased level of MMP3 does correlate moderately well with clinical indices [397]. MMPs from PMNs may also increase matrix destruction by inactivating α_1-proteinase inhibitor [398], which would normally control serine proteinase activities such as PMN elastase.

The first report of collagenase immunolocalization in human gingival biopsy specimens from patients with periodontitis showed that the enzyme was frequently associated with inflammatory cells, but its precise cellular origin was uncertain [399]. These studies were extended by identifying cells producing MMPs and TIMP1 in gingival biopsy specimens from patients with periodontitis [400]. MMPs 1,2, and 3 and TIMP1 were all immunolocalized in gingival tissues, both from patients with periodontitis and from patients undergoing crown-lengthening procedures. On histological and morphological grounds it was deduced

that the MMPs may be synthesized by both fibroblastic cells and macrophages. Cells secreting MMPs and TIMP1 were often observed at sites that histologically showed signs of connective tissue remodeling. A study using *in situ* hybridization [401] demonstrated that TIMP1 was broadly expressed in gingival tissue in periodontitis but that TIMP2 was predominantly expressed in tissue adjacent to the pocket epithelium. Pinchback et al. [402] found that much staining of proteolytic enzymes, particularly collagenases and inhibitors in advanced periodontitis is associated with vascular structures. Their results on biopsies from patients at a late stage of the disease suggested that an up-regulation related to the vasculature is an integral component of destructive periodontitis with chronic inflammation. An increase in mRNA for MMP1 was found in gingival biopsies from patients with inflamed gingiva, whereas only very low levels of message for MMP8 were measured [403].

Many data demonstrate the crucial importance of the inflammatory mediators IL-1 and TNF in connective tissue resorption. Thus, attention has particularly been given to these cytokines in periodontal diseases. IL-1 activity was early identified in gingival crevicular fluid samples from clinically inflamed sites in human subjects [404] and in peripheral blood monocyte culture supernatants from periodontitis patients [383]. It was found that there was an increased release of IL-1β and TNF-α by cultured peripheral blood mononuclear cells from patients with chronic periodontal disease, compared with controls [405]. These and many other studies amply demonstrate that in periodontal disease there is a stimulated release of cytokines, especially by circulating mononuclear cells, and that these modify the host response. This area of research has been well reviewed [406,407]. The cytokine-inducing components of periodontopathogenic bacteria are probably much wider than has hitherto been thought [408]. This area of research could give important clues to the most important agents for cytokine induction so that specific blocking agents could be aimed locally to block pro-inflammatory cytokine expression in the periodontium.

Already there is a considerable literature on the use of tetracycline analogues, such as doxycycline, in the treatment of periodontal disease and other inflammatory conditions [409,410]. Low doses prevented collagen breakdown and alveolar bone loss in animal studies and gave a reduction in pocket depth and attachment loss in human clinical trials [410,411]. Originally, it was thought that tetracycline antibiotics might primarily act as inhibitors of MMP8, but recent work suggests that doxycycline may act generally on latent MMPs by binding enzyme-associated Ca^{++}, rendering them more susceptible to proteolysis and loss of enzymic activity [412]. Also, it seems that MMP13 is much the most sensitive collagenase to inhibition by tetracyclines [413]. Another explanation for the mechanism of action of tetracyclines is that they might interfere with the cytokine induction of MMPs. Amin et al. [414] showed that they can inhibit the expression of inducible nitric oxide synthase; the overproduction of nitric oxide has been implicated in several inflammatory diseases, in which cytokines are in turn induced by this molecule. All the foregoing explanations support a mechanism of action at the post-translational level, but Jonat et al. [415] found a dose-dependent transcriptional inhibition by tetracycline on MMP3 expression by fibroblasts. The antimicrobial activity of tetracyclines is not needed for the efficacy of this group of compounds [410], but a major advantage is that tetracyclines have been used safely for many years. Other work indicates that gelatinases, especially MMP2, are important in tissue destruction in periodontitis [416]. Thus, the potential of specific MMP inhibitors that will become available could be investigated as therapeutic agents.

Synovial Tissue Breakdown, Joint Destruction, and Arthritis Models

As discussed above, there is now much evidence that cytokines, particularly IL-1 and TNF, are major stimuli for the production of MMPs in inflammatory conditions. Ghivizzani et al. [417] have shown that intra-articular expression of human IL-1β following gene transfer to rabbit synovium produces all the major pathological alterations associated with rheumatoid arthritis. Recently, it has also been shown that IL-18 (IFN-γ-inducing factor), which can be produced by human articular chondrocytes and has structural homology with IL-1, may also contribute to cartilage degradation, but it is less potent than IL-1 [418]. In cartilage breakdown, proteoglycan loss is rapid and reversible but, in contrast, collagen loss is essentially irreversible. Most *in vitro* studies have tended to correlate loss of proteoglycan with cytokine-induced cartilage destruction and fewer studies have been made on the extent of cartilage destruction as assessed by other parameters. Henderson and Pettipher [419] injected IL-1 and TNF into rabbit knees; in the case of IL-1 they found glycosaminoglycan release, but there was no such effect with TNF. The loss of glycosaminoglycans from cartilage *in vitro*, most often used as an assay of cartilage resorption, may be partly dependent on enzymes [420] but not entirely dependent on MMPs: it is not clear whether such experiments have physiological relevance. It is interesting, however, to note that the IL-1 stimulated loss of proteoglycans *in vitro* can be partially inhibited by TGF-β [421,422].

The enzyme responsible for proteoglycan release from cartilage was once thought to be MMP3, partly because of its abundance [190], but analysis of proteoglycan fragments released from resorbing cartilage implicates another enzyme, "aggrecanase," the identity of which, until recently, has been uncertain. A good correlation between inhibition of proteoglycan loss and inhibition of MMP3 has been reported, although high concentrations of MMP3 inhibitors were needed (approximately 1000 times greater concentration required to inhibit proteoglycan loss than inhibition of MMP3). A strong correlation was found between inhibition of collagenase activity and inhibition of collagen release from cartilage explants; no correlation was observed with inhibition of MMP3 or gelatinase activity [423]. Proteolysis of the aggrecan core protein occurs within the interglobular domain; amino-terminal sequencing of cleavage products has identified two main sites of proteolysis [36,424]. Cleavage at the Asn 341–Phe 342 bond has been attributed to the action of the MMPs [425]: cleavage at the Glu 373–Ala 374 bond appears to be predominant in rheumatoid arthritis and also in stimulated cartilage explants *in vitro*. MMP8 [425] and MMP13 [44] cleave aggrecan, and although MT1 MMP cleaves aggrecan it does not produce the fragments typical of arthritis [426]. Using a model system of cartilage degradation, Kozaci et al. [427] concluded that MMPs 3,8, and 13 are unlikely to contribute to proteoglycan degradation, but that collagenases and gelatinases have major roles in type II collagen breakdown. MT1 MMP is expressed in human articular cartilage; the level of its mRNA is not modulated by IL-1 [428], suggesting that regulation of MT1 MMP in the arthritides is not a significant factor. The

likely role of aggrecanase has already been discussed above. However, MT1 MMP expression by rheumatoid synovial fibroblasts is increased by TNF-α, resulting in secretion of active MMP2 [429]. Because MT1 MMP exhibits broad-spectrum proteolytic activity, it may itself be involved in matrix degradation as well as having a role as an MMP2 activator [430].

The identity of the collagenase responsible for cartilage collagen loss is currently not entirely clear. MMP1 and MMP13 can be made by human chondrocytes, with studies showing that MMP8 may also be expressed by these cells and be up-regulated by IL-1 [431]. There is some evidence that specific MMP13 inhibitors can block IL-1-induced collagen loss and an enhanced cleavage of type II collagen in osteoarthritic cartilage seems to be correlated with MMP13 activity [432]. Human chondrocytes synthesize MMP13, and this is strongly up-regulated by IL-1β and TNF-α [433,434]; its induction requires c-Fos, which binds to the AP-1 site. MMP3 can cleave collagen types II, IX, X, and XI of cartilage [25], and collagen type XI is also gelatinase susceptible [435]. MMP2, but not MMP9, can degrade human collagen type II almost as effectively as MMP8 [436], and MMP2 could be important in degradation at the pannus-hard tissue junction in rheumatoid arthritis.

Immunohistochemical studies of connective tissues have shown that gelatinase is frequently expressed in both normal and pathological situations and it has been demonstrated in stromal cells of the hyperproliferative synovium in an antigen-induced rabbit model arthritis [437]. MMP1 mRNA has been localized in synovium from patients with rheumatoid arthritis by use of *in situ* hybridization [438] and supporting evidence given that the collagenase was associated with the type A macrophage-like synovial lining cell. MMP3 has also been shown to be secreted by invasive synoviocytes in rheumatoid synovium [439] and is a marker of invasive arthritis in a rat model [440].

Using a collagen-induced arthritis model, Carmichael et al. [441] have shown that systemic administration of recombinant TIMP1 can suppress the pathology. These studies again suggest MMP inhibitors may be lacking in some diseases in which tissue breakdown is a feature and also that they may be potential therapeutic agents. TIMPs 1 and 2 have been shown to prevent collagen release in an *ex vivo* cartilage resorption model, suggesting the involvement of collagenase MMPs in this event; the same inhibitors were unable to prevent proteoglycan loss, again indicative of the nonMMP nature of aggrecanase activity. Synthetic MMP inhibitors can prevent both proteoglycan and collagen loss, but achieve the former only at high concentrations at which their specificity may be doubtful [442].

Considerable interest has been generated by the use of cysteine proteinase inhibitors in biological systems. Inhibition of IL-1-stimulated aggrecan degradation was found with a lipophilic inactivator of cysteine endopeptidases [443]. Inhibitors of cathepsins B and L were found to decrease the severity of joint destruction in a rat model of arthritis [444].

Tumor Invasion and Metastasis

The progression of tumors is driven by the expression of oncogenes and the loss of suppressor genes, leading to malignant traits such as the ability to invade into and grow in ectopic tissue environments. Although serine proteinases and MMPs have long been associated with tumor invasion and metastasis, much of the earlier work was centered on the role of proteinases in the destruction of extracellular matrices so that invasion could proceed [445]. There is now much evidence for a wider role of MMPs in tumor development, metastasis, and neovascularization [446]; they are crucial for creating and maintaining an environment that supports the initiation and growth of primary and metastatic tumors [447,448] and are involved in interactions with stromal cells. There are strong parallels between cancer invasion and tissue remodeling [449]. Many of the MMP activities expressed by tumor cells are similar, if not identical, to those produced by normal cells. Proteinases and their inhibitors can influence the bioavailability of growth factors and consequently modulate tumor growth, angiogenesis [450], which is a key step in the growth of large tumors, and apoptosis; they are involved in cell migration and the shedding of cell surface proteins. Also, there are many interactions between the proteinase classes and there are often cascades of proteolytic activities. Studies with proteinase-deficient mice do not define a phenotype, and support the idea that there are very considerable overlaps of proteinase activities. Thus, different tumors express different patterns of enzyme expression and complex cascades are a common theme.

Basement membranes constitute a reservoir of latent growth factors, such as bFGF, that can be released upon proteolysis of perlecan in the basement membrane [451], and could promote tumorigenicity [452]. MMPs and serine proteinases could be involved in their destruction and so influence their bioavailability in both positive and negative ways. Another illustration of the cooperation between MMPs and serine proteinases is the study of Montgomery et al. [453] showing that the degradation of matrix by melanoma cells is essentially a two-step process. The first stage involves the u-PA/plasmin system in the degradation of glycoproteins that cover collagen fibers; then, activated MMPs are involved in the destruction of the collagen network. There is also evidence that the u-PA system modulates invasive capacity and proliferation in prostatic tumor cells [454], with MMP activation being found. MT1 MMP correlates well with MMP2 activation potential after epithelial to mesenchymal transition in human breast carcinoma cells [455]. Other workers propose that MT1-MMP arises from stromal cells and that activation might be a perifibroblastic event [456]. It was postulated that MT1 MMP on the surface may mediate a paracrine loop for the utilization of stromally produced MMP2. Also, cell surface-associated MMP2 is more effective than soluble activated enzyme in remodeling of the matrix by tumor cells [457]. Invasion of interstitial matrix barriers by carcinoma cells seems more dependent on MMPs than the u-PA/plasmin system [458], in as much as MMP inhibitors are much more effective at reducing invasion than inhibitors directed against the plasminogen/plasmin axis.

The requirement for tumor cells to degrade basement membranes led to the characterization of MMP2 (originally named type IV collagenase) [459] and became the focus of many studies attempting to correlate proteinases with the various stages of invasion. The main component of basement membranes is type IV collagen, with laminin, entactin, and perlecan, and so proteinases that degrade these molecules have received most attention. With a number of tumors there is a moderate correlation between the expression of MMP2 and metastasis [460]. In breast cancer, MMP expression represents a tumor-induced host response [461],

with unique patterns of expression in different cell types. MT1 MMP is expressed in many tumor cells *in vitro*, but *in vivo* it is mainly localized in stromal cells [462]. It is therefore tempting to associate this proteinase with MMP2 activation and matrix breakdown as invasion proceeds. High levels of MT1 MMP expression were found to be associated with the degree of invasiveness of cervical cancer cells [463]. Polette et al. [462] observed that MT1 MMP was expressed in fibroblasts in focal areas in preinvasive lesions, and others have documented the proteolysis of matrix proteins being associated with plasma membrane protrusions, termed invadopodia [464]. Proteolysis of matrix by invadopodia facilitates breast cancer cell invasion by MMPs [465]. Nakahara et al. [466] showed that MT1 MMP is required for cell invasion and localizes to invadopodia in human melanoma cells overexpressing it. MMP13, along with MT1 MMP and MMP2, is associated with advanced local invasion in human squamous cell carcinomas of the larynx [467]. The other gelatinase, MMP9, is also often correlated with invasion (see also below) and is critical for breaching the vascular wall, a rate-limiting step for intravasation and consequently metastasis [468]. Cells with low surface u-PA are incapable of intravasation, even if MMP9 is abundant; thus intravasation needs the cooperation of u-PA/uPAR and MMP9 [468]. Bound u-PA is needed for the induction of brain tumor cell migration [469], independent of u-PA-mediated proteolysis. u-PA is expressed in stromal cells, in contrast to its receptor, which is found in cancer cells at invasive foci in human colon adenocarcinomas [470]. The mRNA for the inhibitor PAI1 is located in endothelial cells in the tumor stroma [471]. Such studies demonstrate the complex interaction between cell types as well as proteinase types. In some instances, it has been shown that there is a correlation between the metastatic potential of tumor cells and their secretion of MMP activities degrading type IV collagen [459].

There is evidence that MMPs have a role in the early stages of tumor progression. Stromelysin 3 (MMP11) expression promotes tumor "take" in nude mice, seemingly by favoring cancer cell survival in a tissue environment initially not permissive for tumor growth [472]. There is also evidence that it is involved in tumor implantation [473], seemingly by way of extracellular matrix-associated growth factors. Matrilysin (MMP7) production is increased in the early stages of colorectal tumorigenesis [474], which seems to be correlated with increased *in vivo* tumorigenicity. MMP7 expression in the mammary epithelium may contribute to early-stage mammary tumorigenesis [475]. Interestingly, synthetic inhibitors of MMPs cause a delay in human breast cancer regrowth, as well as reducing metastasis, in a nude mouse xenograft model [476]. In a model system it was shown that the expression of the *ras* oncogene up-regulated MMP3 and increased invasiveness and the degradation of collagen synthesized by vascular endothelial cells [477]. In other model systems, oncogenes (*fos*, *jun*, and *ets*) were shown to control the transcriptional regulation of MMP1 as well as MMP3 [478], and an Ets-related protein (E1A-F) was found to up-regulate MMPs [479].

It has now become clear that increased production of proteinases associated with malignant carcinomas often comes from the host stromal cells, induced by the tumor cells. Data from a study of invasive and noninvasive human meningiomas [480] first suggested this, and similar data were obtained with mouse fibrosarcoma cells [481] and with the Lewis lung tumor. All these data suggesting that tumor cells stimulate stromal cells to synthesize MMPs and aid cancer dissemination led Biswas and colleagues to characterize a factor that, in culture, stimulates fibroblasts to produce high levels of collagenase [482]. This factor was originally called TCSF (tumor cell-derived collagenase stimulatory factor) but is now named EMMPRIN (extracellular matrix metalloproteinase inducer). It is a glycoprotein and a member of the immunoglobulin superfamily that is located on the outer surface of many human tumor cells, but which is absent from most normal cells except keratinocytes [483]. Fibroblast MMPs 1,2, and 3 are all stimulated by it and it seems likely that it is acting via cell–cell interactions. EMMPRIN and MMP9 have been found to be strongly expressed in neoplastic melanocytes [484] and MMP9 may be involved in early invasion. Recent work demonstrates that EMMPRIN stimulates collagenase transcription specifically through MAPK p38 [485]

Other systems show that direct contact of tumor cells with stromal cells may lead to proteinase expression [486]. These workers found that even methanol-fixed metastatic rat embryo fibroblasts could induce MMP9 in normal cells. Co-culture of human breast adenocarcinoma MCF-7 cells with fibroblasts enhanced the production of MMPs, and soluble and membrane-bound factors were implicated [487]. Takahashi et al. [488] have characterized a gene *RECK*, which encodes a membrane-anchored glycoprotein with EGF-like repeats and serine proteinase inhibitor-like domains. *RECK* mRNA is expressed in human tissues but not in tumor-derived cell lines and transformed cells. Remarkably, restoring the expression of *RECK* in malignant cells suppresses invasive activity and decreases MMP9 secretion. This work suggests yet another pathway by which oncogenes act. Precisely how the suppression works is currently being investigated. Finally, in this context, it has been shown that MMP9 can bind to the cell surface hyaluronan receptor CD44 [489], thus providing a mechanism to explain how CD44 promotes tumor growth and metastasis by localizing proteolytic activity. CD44 cleavage seems to be mediated by MT MMPs [490], and thus is involved in a complex interaction between tumor cell and matrix that controls migration. CD44 is involved in the up-regulation of MMP2 in human melanoma cells [491], because binding of a monoclonal antibody uniquely increased expression of MMP2 and invasion in *in vitro* test systems.

Cathepsin B can facilitate tumor progression directly through degradation of components of the basement membrane and extracellular matrix [492]. Overexpression of cathepsin B mRNA, increased cathepsin B staining and elevated cathepsin B activity were found in different human cancers. These occurred especially at the invasive edges of cancers. Cathepsin B can also facilitate tumor progression indirectly through activation of other latent proteases and/or degradation of protein inhibitors of other proteases. Thus, cathepsin B may be an integral component of the proteolytic cascade linked to malignant progression of human tumors.

TIMPs may play an important role in the control of tumor MMP activity, since it has been shown with some experimental tumors that there is a marked decrease in TIMP1 production by malignant as compared to benign tumors. Khokha [493] showed that by transfecting B16-F10 melanoma cells to overexpress TIMP1 their tumorigenic and metastatic abilities were suppressed. This work built on earlier studies showing that anti-sense RNA to TIMP1 induced a reduction in murine TIMP1 levels and conferred

oncogenicity on Swiss 3T3 cells [494], and that TIMP1 inhibited B16-F10 cells from invading [495]. Overexpression of TIMP2 can also prevent local invasion and inhibit tumor growth [496]. TIMPs 1 and 2 can be up-regulated by activating cyclic adenylase activity with 8-bromo-cAMP [497] and this treatment suppresses the invasive phenotype of HT1080 cells. Recent work [498] suggests that with some tumors loss of TIMP3 is related to the acquisition of tumorigenesis and may be related to aberrant promoter-region methylation; the most frequent TIMP3-related methylation was found in renal cancers, which originate in the tissue that normally expresses the highest TIMP3 levels. However, there is some contrary evidence for the role of TIMPs since it has been observed that TIMP1 levels are higher in breast carcinomas than in fibroadenomas [499], and high levels of TIMP1 indicate a poor clinical outcome. High levels of TIMP2 expression in the tumor stroma are associated with a poor outcome in invasive bladder cancer [500]. In these situations, could it be that the TIMPs are acting as growth promoters? In a model system in which collagenase and gelatinase have been shown to be important, TIMP1 prevented the invasion of bovine capillary endothelial cells (stimulated with bFGF) into human amnion basement membrane [501]. TIMP1 has also been shown to inhibit the metastatic potential of a highly metastatic rat embryo cell line, transfected by c-Ha-ras1 in order to induce secretion of gelatinases [502]: high doses of TIMP1 injected intraperitoneally into nude mice prevented the metastasis to the lung of cells injected intravenously into the animals.

It has also been shown that collagen degradation and invasion by human melanoma cells can be reduced by transfection with an antisense RNA for MMP1 [503]. Durko et al. [503] found that, in addition to preventing degradation of type I collagen, there was inhibition of invasion through type IV collagen despite the presence of functional MMP2. Suppression of MMP7 inhibits colon cancer invasion *in vitro* [504]. Intestinal tumorigenesis is suppressed in mice lacking MMP7 [505], which is highly expressed in early-stage human colorectal tumors: it was postulated that MMP7 may function in a capacity independent of matrix degradation.

Fibrosis

The theme of matrix destruction based on an imbalance of proteinases over inhibitors has a mirror-image in the consideration that an excess of inhibitory activity could lead to accumulation of matrix. Evidence has been produced that cells from patients with scleroderma simultaneously produce less MMP3 but more TIMP1 than do normal fibroblasts [506] and a potential role for PN1 (protease nexin 1) overexpression in the pathogenesis of scleroderma has been suggested [507]. These abnormalities could play an important part in the molecular pathology of the disease, which is characterized by excessive matrix in skin and internal organs. It seems likely that TGF-β might be a major mediator. An excess of TIMP1 may be involved in the pathogenesis of liver fibrosis [508] and chronic asthma [509].

Interestingly, synthetic metalloproteinase inhibitors can result in the condition of "frozen" shoulder in patients treated with them [510]. There are many resemblances in this condition to Dupuytren's contracture, which is characterized by abnormal proliferation of fibrous tissue within the palmar fascia of the hand [511]. The localized nature of Dupuytren's contracture and the likely involvement of cytokines such as TGF, suggest that proteinases/inhibitors may be abnormal in this condition.

INHERITED DISORDERS LINKED TO MATRIX PROTEOLYSIS

Sorsby's Fundus Dystrophy

Sorsby's fundus dystrophy (SFD) (MIM 136900) is a rare autosomal dominantly inherited macular disorder. It is characterized by loss of central vision owing to subretinal neovascularization and degeneration; there is also a thickening of Bruch's membrane. The disease locus was mapped to chromosome 22q13-qter, the same region as the gene encoding TIMP3 [153,512]. Mutations were found to affect the C-terminal region (exon 5) of the mature TIMP3 protein [512–514]. All mutations result in an additional cysteine residue, suggesting strongly that mutations in *TIMP3* are causally responsible for the SFD phenotype and that there is a critical role for a free thiol group. The mutations in different families seem to have arisen independently [514], but all could lead to an altered regulation of proteolytic activity. TIMP3 is an inhibitor of angiogenesis [515] and mutations could disrupt the balance in the tissue. There are now five known different disease-associated mutations in the TIMP3 gene involving missense mutations and a splice site mutation with different clinical features [516].

Recent work [517] has shown that a mutant TIMP3 (Ser181Cys) [512,518], found in SFD, retained its ability to localize to the extracellular matrix and inhibit MMPs. It has been hypothesized that TIMP3 mutations contribute to disease progression by accumulation of mutant protein rather than by loss of functionality. This interpretation would fit with the late onset of symptoms, the absence of pathological features in other tissues with high TIMP3 expression, and immunolocalization studies showing that TIMP3 is a component of the increased extracellular matrix in Bruch's membrane in SFD [519,520]. It should be noted, however, that up-regulation of TIMP3 occurs in other degenerative diseases without mutations [521].

Promoter Polymorphisms

MMP3 gene expression was found in atherosclerotic plaques by *in situ* hybridization [522] and the involvement of MMPs in cardiovascular disease has been reviewed [523]. A common naturally occurring variant in the promoter of the *MMP3* gene has been identified [524], with one allele having a run of five adenosines (5A) and the other having six adenosines (6A), the latter having lower promoter activity. The 6A allele is associated with the progression of atherosclerosis and it was suggested that lower MMP3 activity might favor matrix deposition. In a clinical therapeutic study, it was confirmed that the 5A allele was associated with a beneficial effect on disease progression [525]; this raises the possibility that patients with coronary artery disease who are homozygous for the 6A allele (25–30% of the population) may be at particular risk of rapid disease progression. The same polymorphism has been associated with acute myocardial infarction [526].

A highly polymorphic marker has been found in the *CLG4B* gene [527], which encodes MMP9. This gene had been proposed as a candidate gene for abdominal aortic aneurysms, but, based on the reported results, this does not seem likely, even though macrophage-derived MMP9 levels are elevated in diseased aortic tissues [528]. Functional polymorphism in the regulatory region of MMP9 has recently been characterized in detail [529], but further work is still needed before it is clear that genetic variation does have a

profound influence on MMP9 expression and the severity of coronary atherosclerosis.

Of interest is a recent paper by Rutter et al. [530] showing that a single nucleotide polymorphism in the *MMP1* promoter creates an Ets binding site and augments transcription. Members of the Ets family of transcription factors are present within *MMP* promoters and are potent positive regulators. These workers [530] found that the polymorphism is not a mutation and that its frequency is increased in tumor lines. The data point to a mechanism for more aggressive matrix degradation and could have wide applicability.

Dawson et al. [531] found a common polymorphism in the promoter of *PAI1* and that the site might be of functional importance in regulating the expression of this inhibitor. There is also evidence that genetic polymorphism of cathepsin D is strongly associated with the risk of developing sporadic Alzheimer disease [532]; cathepsin D might be involved in the intracellular cleavage of amyloid protein and polymorphism could give rise to altered enzyme function.

CONCLUSIONS AND PROSPECTS FOR RESEARCH AND THERAPY

Clearly an exciting area for research is to use the information available to design therapeutic advances based on proteinase inhibition at multiple levels [533]. Proteinase inhibitors are now very attractive for a wide range of degradative diseases [534] and their possible use in cancer therapy has been reviewed by DeClerck and Imren [535]. The low-molecular-weight MMP inhibitors in clinical trials are mainly hydroxamic acid derivatives that do not fully mimic TIMP actions, and much more specific inhibitors are now being designed. Also, it is possible to think beyond MMP inhibitors working on active proteinases, to consider blocking agents that either prevent induction of MMPs and PAs or prevent the translation of messages or inhibit MMP activation. Also, we can consider the up-regulation of natural inhibitors such as TIMPs, and many combinations of inhibitors could also be useful. For example, although it has been shown that inhibitors of prostaglandins can reduce inflammation, no effort has yet been made to test therapy based on inhibitors of phospholipase, which controls a rate-limiting precursor, arachidonic acid. Combination therapies of nonsteroidal anti-inflammatory drugs with proteinase inhibitors might be effective, as has been proposed in periodontal disease [410]. Chemically modified tetracyclines (see also above) also show great promise in animal models of bone and gingival destruction [536].

Arner et al. [537] showed in a model system that a series of isothiazolones could inhibit cartilage proteoglycan degradation without decreasing synthesis: they concluded from their studies that the activation step for MMPs was the likely target of these drugs, without their having any activity against active enzymes. Thus, interfering with the activation of MMPs may offer a control point in many diseases involving the family of MMPs. Perhaps another good area for exploration would be to try to up-regulate TIMP production and restore a better balance in the tissue. A possible way that this might be done is suggested by the work of Fang et al. [538], who found that they could stimulate new bone formation *in vivo* by the technique of direct transfer of osteogenic plasmid genes. There seems every possibility that a similar approach could be used for agents that would up-regulate TIMPs. Another route is suggested by Cheng et al. [539], who used adenovirus-mediated gene transfer of TIMP2 to smooth muscle cells. Such cells expressed inhibitor and had reduced invasiveness *in vitro*, and the transfection delayed neointimal development *in vivo* after carotid injury. Fernandez et al. [540] have shown an inhibition of endothelial cell migration and invasion of extracellular matrix by gene transfer of TIMP1 using replication-deficient recombinant adenovirus technology. Also using a retroviral vector, Pelletier et al. [541] showed that a local increase in IL-1 receptor antagonist could reduce the progression of experimentally induced lesions in dogs.

Another recent advance should complement protease inhibitors. Human heparanase has been cloned by two groups [542,543]: the mRNA and the protein were shown to be preferentially expressed in metastatic cell lines and some human tumors. Transfection of low metastatic murine cells with heparanase cDNA gave rise to a highly metastatic phenotype *in vivo* [543], and mRNA for heparanase in rat tumor cells correlated well with metastatic potential [542]. Interestingly, both groups found that there was only one heparanase sequence, and so this enzyme must be the dominant endoglucuronidase in mammalian tissues. Specific inhibitors should soon be available that might suppress angiogenesis as well as halting the ability of metastatic cells to reach target organs.

RECENT DEVELOPMENTS

The field of proteases has developed apace and a number of new 'players' in the extracellular matrix degradation arena have been identified. These are comprehensively documented in the MEROPS database: *http//www.merops.co.uk*

In relation to extracellular matrix turnover, much new work has been achieved on the matrix metalloproteinases. Genetic manipulation of mice has yielded far more specific information about the significance of these enzymes [544].

There has been increased interest in the role of MMPs in tumor invasion and metastasis, especially whether inhibitors could be a powerful new therapy. It is now clear that membrane MMPs are involved in tumor cell adhesion and locomotion [545,546] and metastasis [547], as well as in normal cell migration [548] and angiogenesis [549]. Among the other families of MMPs it is clear that stromelysin is a powerful mammary tumor promoter [550,551].

The possibility of overexpressing TIMPs has been discussed in detail [552], and in an experimental nude mouse model, the intramuscular delivery of TIMP-4 DNA suppressed the growth of Wilms' tumor cells [553]. Tumor invasiveness can also be inhibited by the group of chemically modified tetracyclines [554]. New probes for imaging MMP activities in intact tumors have been developed, together with novel near-infrared fluorescence imaging technology [555].

The realization that peritumoral fibroblasts synthesize most of the MMPs in human tumors, rather than the cancer cells themselves, has increased interest in the role of extracellular MMP inducer (EMMPRIN) in tumor dissemination. Recent work on this glycoprotein, which is present on cancer cell plasma membranes, has demonstrated that it can enhance fibroblast synthesis of MMPs [556]. The authors concerned propose that EMMPRIN plays an important role in cancer progression by this pathway of increasing synthesis of MMPs.

Sorsby fundus dystrophy has continued to intrigue. Although a common molecular phenotype has been described for the TIMP-3 mutation [557], this condition has been found without any mutation in TIMP-3 [558]. It still seems

likely that Sorsby fundus dystrophy is connected to an overproduction of TIMP-3 and it will need further research to rule out whether the patients reported by Assink et al. [558] represent a related disease.

Of great interest has been the finding that the mutation of the *MMP2* gene in humans causes a multicentric osteolysis and arthritis syndrome [559]. The precise details of how MMP2 is involved remain unclear, but the results do demonstrate how disruption of matrix turnover can have dramatic effects on connective tissues. The inherited osteolyses, or "vanishing bone" syndromes, are a group of rare disorders (MIM 166300, 259600, 259610, 277950) of unknown etiology characterized by destruction and resorption of affected bones: clearly new insights will be forthcoming. It seems likely that gelatinase A (MMP2) is a key proteinase for controlling collagen turnover in many tissues [560].

It has also been suggested that a genetic variation of cathepsin D is a major risk factor for Alzheimer disease [561], backing up earlier work by the same authors.

Cathepsin K has been an intensively researched area, recently, since it is clear that this proteinase, produced by osteoclasts, is the dominant cysteine proteinase in bone. It has been clearly linked to osteopetrosis and pycnodysostosis (see Chapter 19, this volume) [562].

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Chapter 8

Osteogenesis Imperfecta

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SUMMARY

- **Osteogenesis imperfecta (OI) is a clinically and genetically heterogeneous disorder characterized by osseous fragility and fractures, accompanied by other connective tissue alterations, and thought to affect about 1 per 10,000 individuals in all its forms.**
- **The vast majority of affected individuals have mutations in one of the two genes, *COL1A1* and *COL1A2*, that encode the two chains of type I collagen, the major protein of bone. On clinical, genetic, and radiological grounds, now modified by biochemical and molecular genetic studies, four major types of OI have been delineated.**
- **OI type I, characterized by bone fragility, near-normal stature and blue sclerae, usually results from mutations in the *COL1A1* gene that lead to low or absent levels of the products of one allele, generally the result of premature termination codons.**
- **OI type II is lethal in the perinatal period and, like the other forms of OI, usually results from heterozygosity for mutations in either of the two type I collagen genes that perturb the normal sequence of the triple helix, particularly by mutation of single glycine codons or by mutations that lead to exon skipping.**
- **OI type III, the progressive deforming type, is characterized by severe growth retardation and bone deformity, accompanied by marked limitation of mobility, and life expectancy may be diminished.**
- **OI type IV is less severe, with adult stature ranging from about 4 feet (122 cm) to normal, and ambulation is generally independent although sometimes assisted.**
- **Although inheritance is generally autosomal dominant, recurrence in sibships with unaffected parents is seen with several types of OI and usually reflects parental mosaicism for the mutation.**
- **Confirmation of the diagnosis by analysis of collagens produced by cultured dermal fibroblasts or by direct gene analysis is readily available.**
- **Prenatal diagnosis is available for most forms of OI using a variety of strategies.**
- **Although treatment has been largely mechanical through assisted ambulation and internal fixation of bones, more recent studies suggest that additional medical strategies may hold promise.**

INTRODUCTION

The major clinical feature that defines osteogenesis imperfecta (OI) is bone fragility. Fractures may be rare or frequent (more than 200 prior to puberty), and bone fragility may be accompanied by reduced life span, short stature, bone deformities, dentinogenesis imperfecta, hearing loss, altered scleral hue, and other evidence of connective tissue abnormalities [1,2] (Table 1). In almost all individuals, OI results from mutations in one of the two genes (*COL1A1* and *COL1A2)* that encode the chains of type I collagen (Fig. 1, Tables 2 and 3).

History, Prevalence, and Classification

OI in all its forms probably affects more than 1 per 10,000 individuals, although precise accounting is difficult because of unfamiliarity with the disease among health care personnel, poor reporting of some forms, and, in some centers, evaluation of incidence at birth, which often misses the more mildly affected individuals. The condition is seen in all ethnic and racial groups, probably with equal frequency. There may be some forms of OI that are more common in isolated regions, presumably because of the accumulation of a rare recessive allele [3–5]. OI has been in existence for more than two millennia, and at least one skeleton from among those preserved in Egypt has the morphology compatible with a deforming variety of the disease [6]. A partial skeleton from the 7th century in Britain also has similar features [7]. Seedorf [8] cites the 11th century example of Ivar Benlos (Ivar the Boneless), the son of King Regnar Lodbrog, who was carried about on a shield and had little skeleton, as an example of OI in Denmark.

Lethal forms of osteogenesis imperfecta were recognized during the late 18th century and early 19th century by Lobstein [9] and by Vrolik (see discussion by Seedorf [8]), who lent their names to the syndrome [2]. By the middle of

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TABLE 1. Classification of Types of Osteogenesis Imperfecta

Type	Clinical Manifestations	Inheritance	Molecular Defects
I (Mild)	Blue sclerae, bone fragility, normal or near-normal stature, no deformity, hearing loss in adolescence to adult years	AD	*COL1A1* Null
II (Perinatal lethal)	Short, deformed extremities, soft thoracic cage and skull with severe bone fragility, absent calvarial mineralization, bony compression, platyspondyly, and beaded ribs. Dark sclerae. Death in the perinatal period.	AD (New mutations) AR (Exceedingly rare)	*COL1A1* and *COL1A2* Substitutions for glycine Exon skipping Partial gene deletions C-terminal propeptide mutations that interfere with chain association
III (Deforming)	Bone fragility and progressive deformity, marked short stature, light sclerae, dentinogenesis imperfecta, hearing loss in early adult life and shortened survival, on average.	AD AR (Rare)	*COL1A1* and *COL1A2* Substitutions for glycine Exon skipping *COL1A2* mutations that prohibit chain association Homozygous by descent for point mutations in the triple helix
IV (Mild deforming)	Bone fragility, mild to moderate short stature, mild bone deformity, dentinogenesis imperfecta, light colored sclerae, hearing loss in early adulthood.	AD	*COL1A1* and *COL1A2* Substitutions for glycine Exon skipping Partial gene deletions
V*	Bone fragility, mild to moderate short stature, no dentinogenesis imperfecta, radio-ulnar synostosis. Hyperplastic callus formation	Probably autosomal dominant	Not known

*Details of recent studies that distinguish OI type IV and OI type V are discussed in Recent Developments.

the 18th century, the familial nature of OI was recognized and one family with autosomal dominant inheritance had been described by Ekman [10] (cited by Seedorf [8]). The association of OI with blue sclerae [11] and with hearing loss [12,13] was identified in the early part of the 20th century. The postulate that severe (congenita) forms differed from the milder varieties (tarda) based on the time at which fractures first occurred was proposed by Looser in 1906 [14]. Although often helpful in predicting long-term morbidity, this division frequently provided a false sense of security because it could be difficult, several years later, to distinguish some individuals initially classified in the tarda group from those in the congenita group. By the mid 1920s, Bell [15] was able to assemble a large number of families in Britain with OI and recognized that there was additional heterogeneity. Seedorf [8] realized that there were at least two milder phenotypes, one in which there was little deformity, generally normal height, and blue sclerae, and a second in which deformity was the rule. Ibsen [16], who recognized the heterogeneity described by Seedorf, was able to compile a substantial number of additional families.

Beginning in the late 1970s, Sillence and his colleagues [1,17], using radiographic, genetic, and clinical criteria, developed the classification currently in use. Although this division of OI into four major types (I–IV) was rapidly adopted by geneticists, it has not achieved similar popularity with orthopedic surgeons because of the difficulty in classifying many affected individuals. The Sillence modification of the Danish classification [8] has been used to categorize most of the patients who have been investigated at the biochemical and genetic levels. The biochemical studies themselves and recent linkage studies support the broad validity of the classification but confirm that it is incomplete. Although biochemical and genetic studies will provide the basis of the most rational classification, even such a detailed biochemical classification probably will never predict correctly the evolution of OI in every affected individual because of the still unexplained variability of expression seen in many families.

The value of a classification scheme and the criteria used to develop it depend on the use to which it is to be put. From a clinical point of view, classification is needed to provide information about natural history and recurrence risks and to determine whether therapies will change natural history. If these requirements can be met by clinical data alone, then no additional elements are necessary. At the present time, the clinical data alone do not provide some important information. For example, it is not possible to distinguish on clinical evaluation alone the apparently rare autosomal

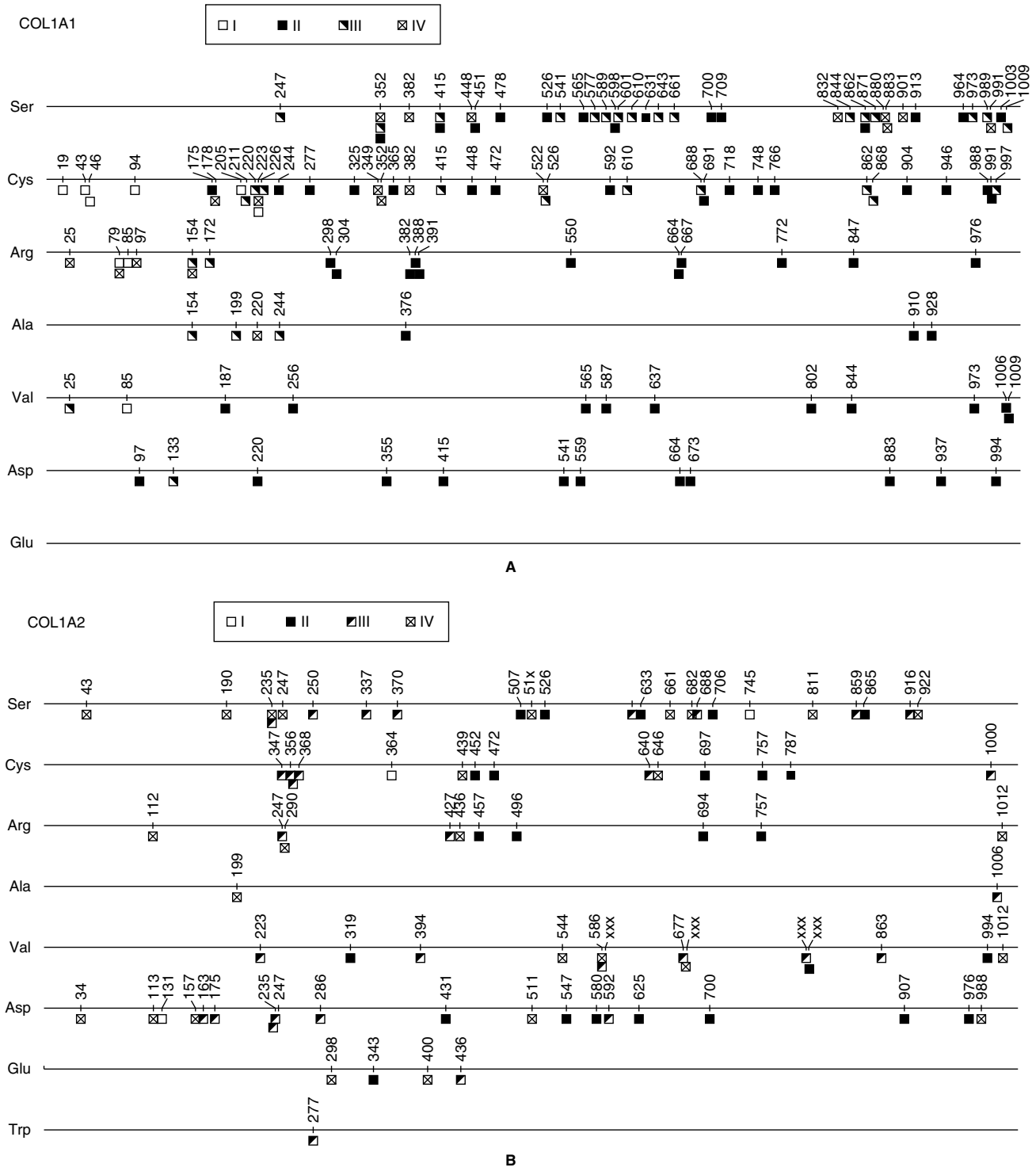


Figure 1. Point mutations in the triple-helical domains of the *COL1A1* (**A**) and *COL1A2* (**B**) genes that result in substitutions of glycine residues by other amino acids. The numbered residues are within the triple-helical domain of each chain; the first glycine of the triple helix is designated as residue number 1. There are 1,014 residues in the triple-helical domain of each chain. The substituting residue is listed at the left end of each line, the substituted glycine and its position are indicated by number, and the phenotype is indicated below the line. The source references for each published mutation can be found in the Database of Human Type I and Type III Collagen Mutations (*http://www.le.ac.uk/genetics/collagen/index.html*). All other mutations are from our own unpublished studies.

TABLE 2. Splice Site Mutations in the *COL1A1* Gene and Their Outcomes*

Phenotype	Mutation	Effect	Reference
OI type III	IVS8 + 1G → C IVS8 + 5G → A	Redefinition of exon 7 with skip of exon 8, skip of exon 8, retention of IVS 8; retention of IVS8 and IVS 7; use of cryptic acceptor in exon 8	[57] [59]
OI type IV	IVS9 − 1G → T	Skip exon 10	Unpublished
OI type I	IVS12 + 1G → A	Null allele, mechanism unknown	[19]
OI type I	IVS13 − 9G → A	Creates a new acceptor site, which is preferentially used, adds 7 nt to the mRNA and a frame shift in exon 14	Unpublished
OI type II	IVS14 + 5G → A	Exon skip in about half the products of the mutant allele	[133]
OI type II	IVS14 + 2T → A	Skip exon 14	Unpublished
OI type I	IVS16 − 2A → G	Low-abundance exon 17 skip; most of the products are rapidly degraded	Unpublished
OI type I	IVS17 + 1G → C	Rare skip of exon 17; most products are functionally null	[96]
OI type I	IVS18 + 1G → A	Use of cryptic donor site in exon 18 at G − 8 leads to frame shift and unstable mRNA	[56]
OI type I	IVS18 − 1G → A	Null allele, mechanism unknown	[19]
OI type II	IVS20 + 5G → C	Exon 20 skip	Unpublished
OI type I	IVS20 − 2delAG and del first 5 nt of exon 21	Use of downstream acceptor site in exon 21 results in frame shift	Unpublished
OI type II	IVS21 − 3C → N	Exon 22 skip	Unpublished
OI type I	IVS22 + 3A → G	Null allele; mechanism unknown	[19]
OI type I	IVS23 + 1G → C	Inclusion of 44 bp of intron 23 Inclusion of entire intron 23 Both contain stop codons	Unpublished
OI type I	IVS25 − 1G → A	Null allete, mechanism unknown	[19]
OI type I	IVS26 + 1G → A	Inclusion of intron 26	[322] [323]
OI type II	IVS26 − 2A → C	Exon 27 skip	[96]
OI type II	IVS29 − 2A → C	Exon 30 skip	Unpublished
OI type I	IVS36 + 1G → A	Use of cryptic donor site 44 bp upstream in exon 36; frame shift in exon 37	Unpublished
OI type II	IVS43 − 2A → G	Skip exon 44	[324]
OI type I	IVS44T + 2 → C	Use of alternative donor site in exon 44 8 nt upstream from usual site	Unpublished
OI type II	IVS47G + 1 → A	Exon 47 skip	Unpublished
OI type III	IVS47C − 3 → G	Low-abundance exon 48 skip; unclear whether some is spliced normally or is lost	Unpublished
OI type I	IVS48G + 1 → A	Unstable mRNA; mechanisms not identified. May use G − 17 in exon 48, G + 4 in intron 48, or include 91nt intron	[56]
OI type I	IVS49T + 2 → C	High-abundance mRNA that uses a nontraditional donor site GAG/GGC within the upstream exon; mRNA is abundant and stable, but frame shift occurs in product	Unpublished

*All unpublished mutations described in this table come from the laboratory of PH Byers.

recessive forms of OI that give rise to progressive bone deformity from those that result from dominant mutations. Furthermore, the hypothesis [1,18] that the severe lethal forms of OI were due to autosomal recessive mutations could not explain the unexpectedly low rate of recurrence of the condition among siblings, just as new dominant mutations could not explain what appeared to be an unexpectedly high recurrence rate.

From the point of view of molecular genetic studies, it should be possible to distinguish mutations in the *COL1A1* gene from those in the *COL1A2* gene, to distinguish among various types of mutations, and to distinguish among mutations in different domains of the two genes. Although hypothesized [5], no mutations that cause OI have been identified in genes other than those of type I collagen. It has become practical, although still expensive, to identify

TABLE 3. Splice Site Mutations in the *COL1A2* Gene and Their Outcomes*

Phenotype	Mutation	Effect	Reference
OI type IV	IVS9 + 1G → T	Skip exon 9	Unpublished
OI type IV	IVS9 + 5G → A	Skip exon 9 (may be more complex)	Unpublished
OI type IV	IVS9 del + 4–7	Skip exon 9	Unpublished
OI type I/IV	IVS9 del + 3–13	Skip exon 9	[236]
OI type I/EDS	IVS9 + 5G → A	Skip exon 9	[325]
OI type IV	IVS12 + 2T → G	Skip exon 12	[237]
OI type IV	IVS12(+1) – (+19)del	Skip exon 12	Unpublished
OI type IV	IVS14 + 2T → C	Skip exon 14	Unpublished
OI type IV	IVS14 + 2T → A	Skip exon 14	Unpublished
OI type IV	IVS15 – 2A → G	Skip exon 16	Unpublished
OI type IV	IVS16 + 1G → A	Skip exon 16	[238]
OI type IV	IVS16 + 2T → C	Skip exon 16	[239]
OI type I	IVS19 – 1G → C	Skip exon 20	[240]
OI type IV	IVS21 + 5G → C	Skip exon 21 Normal splice of mutant allele	Unpublished
OI type IV	IVS21 + 5G → A	Skip exon 21 (only)	Unpublished
OI type I/DI	IVS21 + 5G → A	Skip exon 21	[241]
OI type IV	IVS26 + 3A → G	Skip exon 26	[242]
OI type II	IVS27 – 2A → G (Other allele is null)	Skip exon 27	[97]
OI type II	IVS30 + 1G → A	Skip exon 30	Unpublished
OI type II	IVS32 + 1G → A	Skip exon 32	Unpublished
OI type IV	IVS33 + 4G → A	Skip exon 33 Use of cryptic donor site in intron 33 that results in insertion of 18 nt and 6 amino acid residues	[243]
OI type II	IVS33 + 5G → A	Skip exon 33	[135]
OI type II	IVS37 + G → T	Skip exon 37	Unpublished
OI type IV	IVS37 – 2A → G	Skip exon 38 Probably minor outcome; major use of cryptic site and frame shift	Unpublished
OI type II	IVS41 – 3T → G	Skip exon 42	Unpublished
OI type IV	IVS43 + G → A	Skip exon 43, include intron 43	Unpublished

*All unpublished mutations described in this table come from the laboratory of PH Byers.
EDS, Ehlers Danlos Syndrome; DI, Dentinogenesis Imperfecta.

mutations in the *COL1A1* and *COL1A2* genes with sufficient speed to make them clinically useful [19]. This strategy can be particularly useful for prenatal diagnosis early in pregnancies at risk and to identify individuals who are mosaic for mutations and thus at risk of having infants with much more severe phenotypes than those with which they themselves are affected. From a clinical point of view, it is often not significant whether the mutation occurs in *COL1A1* or *COL1A2*; it is the mode of inheritance and the ability to determine whether the next child is affected that are important.

Most geneticists continue to use the Sillence classification as it has been modified by the use of molecular genetic and biochemical studies during the last two decades. The Sillence classification is used here, while trying to point out both its limitations and its advantages. Thus, as now construed, osteogenesis imperfecta can be divided into four major groups — types I, II, III, and IV — that differ in clinical presentation, radiographic picture, mode of inheritance, and, for the most part, biochemical basis of the connective tissue disorder (see Tables 1–3 and Figs. 1 and 2). It is likely that biochemical, morphologic, and molecular genetic studies will add additional types of OI in future years [20], but there are not yet sufficient published criteria to warrant their addition in this chapter.

The greatest limitation of the Sillence classification, even as modified from the original here (and of any other classification, for that matter), is that it attempts to convert what is essentially a continual variable of clinical phenotypes into discrete variants. In this situation, a "stereotyped" or idealized prototype is taken as the model. As will be clear, the linear nature of the type I collagen molecule and the possibility of literally thousands of discrete causative mutations in each of two genes (*COL1A1* and *COL1A2*) are more compatible with a continuously variable range of phenotypes than with discrete entities. Nonetheless, to the extent that phenotypes vary around a few discrete nodes, classification in this manner can be useful for the clinician and for the family.

From the perspective of a classification of OI based on gene of origin and nature of the mutation, several different elements emerge. There are four general classes of mutation: (1) those that alter the amount of type I procollagen produced; (2) those that alter the structure of the triple-helical domain of type I procollagen by virtue of substitutions of the canonical third position glycine residue, small deletions or duplications, or exon skipping; (3) those that alter residues in the carboxyl-terminal propeptide and influence chain association; and (4) those that alter the chain composition of

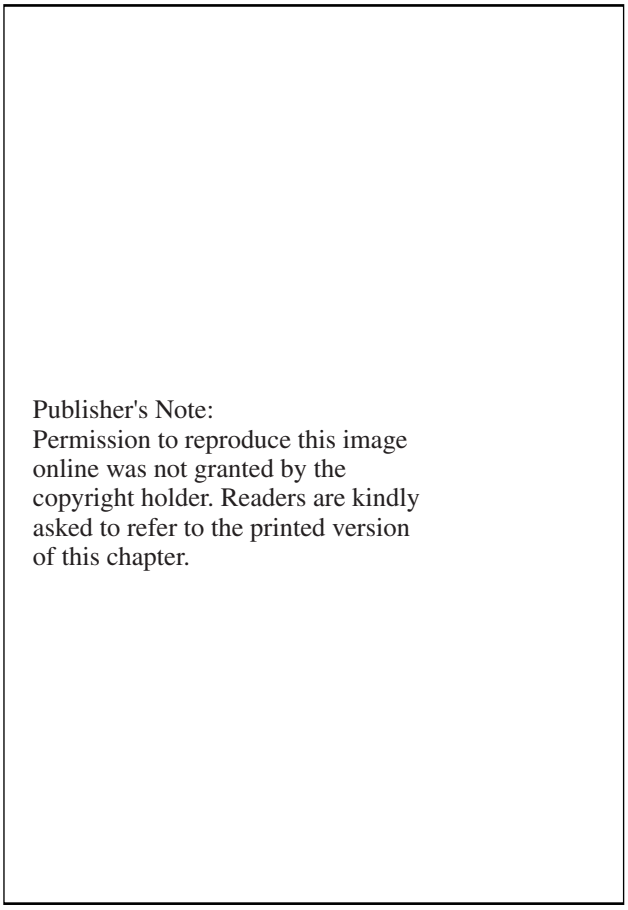


Figure 2. Molecular mechanisms of osteogenesis imperfecta. Virtually all individuals with OI have mutations that affect either the amount of type I procollagen synthesized (Group A) or the structure of the chains of type I procollagen (Group B). In the first group (**A**), ''excluded'' mutations, the decreased synthesis of type I procollagen usually results from a decrease in the amount of steady-state mRNA for the proα1(I) chain. Because type I procollagen molecules must contain at least two proα1(I) chains, that chain becomes the limiting molecule in procollagen production. Almost all the mutations that result in decreased *COL1A1* mRNA are those that lead to premature termination codons in the mRNA, although a mutation in a *COL1A2* allele that resulted in the destruction of all procollagen molecules into which the abnormal chain was incorporated could produce the same end result. In the second group (**B**), ''included'' mutations, mutations may occur in either the *COL1A1* or the *COL1A2* gene to alter the structure of proα chains. The gene in which the mutation occurs affects the proportion of abnormal molecules and is probably reflected in the phenotype. (Reprinted from [71].

type I procollagen by diminishing the production of proα2(I) chains. The clearest distinction in phenotype is between those mutations that decrease the production of type I procollagen and those that produce abnormal molecules. The former result in the mild OI type I phenotype, whereas the latter produce the more severe deforming and lethal varieties. For mutations within the triple-helical domain, the nature of the substituting amino acid, the chain in which it occurs, and the position in the chain are all factors that contribute to the severity of the phenotype (see extended discussions below). It is clear, however, that not all the determinants of the phenotype are known. The lack of complete understanding of the variables in this pathway may be best understood if it is remembered that OI represents the physiological response of an organism to mutations in a major structural element of body form, type I collagen. Such a concept emphasizes the role of evolution in defining the complexity of interactions of these molecules and the role that variations in other molecules play in determining body form.

OSTEOGENESIS IMPERFECTA TYPE I

Clinical Features of Osteogenesis Imperfecta Type I

OI type I (MIM 166200) is inherited in an autosomal dominant fashion and, typically, affected individuals have blue sclerae, normal teeth, normal or near-normal stature, and may experience a few or more than fifty fractures (usually of long bones) before puberty [1,21].

Individuals with OI type I rarely have fractures *in utero* or in the perinatal period, although femoral bowing can lead to early prenatal diagnosis. Occasionally, fractures occur during diaper changing, but their usual onset is when children begin to stand and walk. Fractures may affect any bone, but it is common to see involvement of long bones, the ribs, and the small bones of the hands and feet. Avulsion fractures of the olecranon and transverse fractures of the patella, which are seen in people with OI type I, result from sudden contractions of the triceps and quadriceps, respectively [22]. Fracture frequency remains roughly constant through childhood and then decreases dramatically after puberty. Although many hormonal changes occur in the peripubertal period and no doubt contribute to the change in fracture rate, animal models suggest that bone thickness increases to compensate for fragility [23]. Fractures heal normally and without deformity, given good orthopedic care.

The incidence of OI type I has been estimated at between 1 in 15,000 and 1 in 20,000 but, because of the relatively mild presentation, the incidence may be underestimated. There appears to be no decrease in fertility and little, if any, change in the longevity of affected individuals (that is, genetic fitness approaches 1). At birth, blue sclerae are readily apparent and may be darkly colored, lightening gradually to the blue-gray present in affected adults. Radiographic bone morphology is generally normal at birth, although mild osteopenia or femoral bowing may occur in some newborns. Affected individuals are usually within the normal range for height although often shorter than their unaffected family members. Vertebral body morphology in the adult is normal initially but often develops the classic ''cod-fish'' appearance, which is accompanied by loss of height in later decades (Fig. 3). Fracture frequency may increase following the menopause in women and in the sixth to eighth decades for men. In about half the families with OI type I, affected individuals have early-onset hearing loss, beginning in the late teens and leading gradually to profound loss by the end of the fourth to fifth decades [24–29]. Early hearing loss is generally conductive and may be readily treated by surgical techniques. Later, a sensorineural component appears to emerge [28]. Despite advances in prosthesis design and in the technique of replacement of the fractured and fused bones of the middle ear, which provide significant restoration of hearing for many affected individuals [27,30–35], hearing loss may continue following a period of improvement after surgery. Early hearing loss is typically high-frequency in type, and tympanometry identifies a characteristic bifid compliance curve [24].

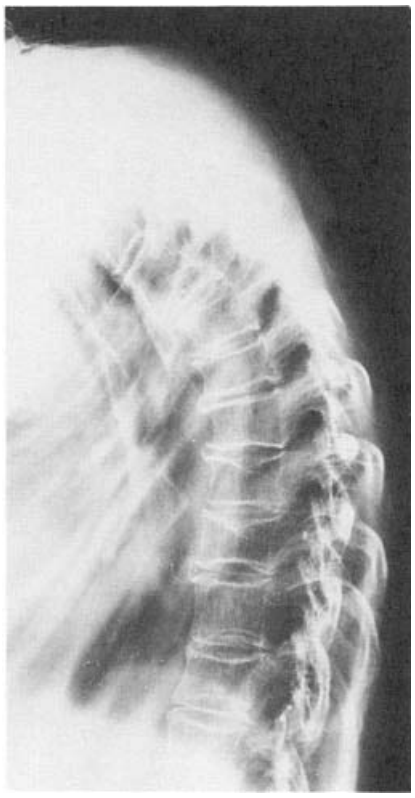


Figure 3. Vertebral body deformity (''cod-fish'' vertebrae) in a 48-year-old woman with OI type I. The lateral spine X-ray demonstrates marked osteopenia, kyphosis, and deformation of the vertebral bodies by the intervertebral discs. The radiograph is courtesy of Drs. David Rowe and Jay R. Shapiro.

Additional clinical findings in people with OI type I may include mild joint hypermobility and increased bruising. Mitral valve prolapse and aortic valvular insufficiency have been identified in some individuals with OI type I, but it is not clear that these disorders are significantly more frequent than in the general population [36]. A small group of people who have OI type I have been found to have slightly larger than normal aortic root diameters, apparently without significant risk of progression or dissection [36,37]. Rare individuals with OI type I may have vascular dissection [38]. Although type I collagen is a major structural element of arterial walls, the effect of diminished amounts of it is not a universal predisposition to aneurysm formation or dissection, both of which appear to be uncommon.

In some classifications, OI type I is subdivided on the basis of the absence (IA) or presence (IB) of dentinogenesis imperfecta (DI) [39]. Even mild dentinogenesis imperfecta is very uncommon when OI type I is defined by the biochemical finding of diminished type I collagen production and no evidence of abnormal molecules [40]. Most individuals with DI make abnormal type I procollagen molecules, and some with relatively mild forms of OI may also have blue or blue-gray sclerae [40]. Blue sclerae are not the sole clinical criterion for OI type I, and the inclusion of a form of OI type I with DI may reflect an undue reliance on scleral hue as a distinguishing feature of OI types.

Biochemical and Molecular Bases of OI Type I

Null Alleles (Silent Alleles or Mutations Leading to Excluded Proteins) are Most Common

Several forms of OI were among the earliest of the inherited disorders of collagen biosynthesis and structure to be studied using cultured dermal fibroblasts from affected individuals [41,42] (for detailed reviews of collagen protein and gene structure, see Chapter 2, this volume). Cells cultured from patients who, in retrospect, would be considered to have OI type I synthesized less type I procollagen than did those from controls, but the mechanism by which production was decreased was not determined. Concordant with these studies, skin from many individuals with OI, including those with mild, dominantly inherited OI and blue sclerae, had a low ratio of type I to type III collagen [43]. It soon became clear that the synthesis of type I procollagen by cells cultured from individuals with OI type I was about half the normal level, while that of type III procollagen was normal [44] (see Fig. 4 as an example). The structure of the secreted type I procollagen was normal, and the decrease in type I procollagen production resulted from the synthesis of only half the usual amount of the proα1(I) chains of type I procollagen (see Fig. 2).

The mechanism by which the synthesis of proα1(I) chains is decreased has come under increasing scrutiny. In the vast majority of instances, the mechanism reflects about half normal steady-state levels of *COL1A1* mRNA in cells. A variety of mutations that result in decreased synthesis of proα1(I) chains — deletion of an allele, promoter and enhancer mutations, splicing mutations that lead to frame shifts and premature termination codons, as well as point mutations that change codons to termination codons, all have the potential to reduce amounts of steady-state *COL1A1* mRNA, although deletion of the entire allele and promoter/enhancer mutations have yet to be described [45]. Mutations that prohibit proα1(I) chain assembly into molecules also result in the same biochemical picture and similar clinical phenotypes.

Examination of genomic DNA from fifteen individuals with OI type I demonstrated no evidence of change in copy number of the *COL1A1* gene in individuals [46]. Furthermore, linkage studies in 38 additional families have

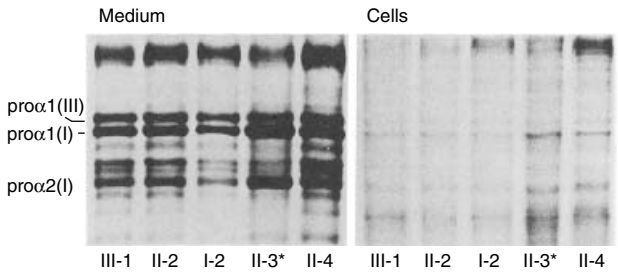


Figure 4. Decreased synthesis of type I procollagen by dermal fibroblasts from individuals with OI type I. Cells were incubated with [^{3}H]proline overnight in the presence of ascorbic acid. The medium and cell-layer proteins were harvested separately and examined under reducing conditions by SDS-polyacrylamide gel electrophoresis. Individual II-3 is not affected; all the others have OI type I. The pedigree is presented in Fig. 5A. In the cells from affected individuals, a mutation in one allele of the *COL1A1* gene prevents the protein product of the mutant allele from associating with other molecules (see Fig. 5). (Reproduced from Willing et al. [60].)

TABLE 4. Mutations in the *COL1A1* gene that result in OI type I*

Exon	Mutation	Effect	Reference
9	777C → T	Arg42Ter	[19]
17	1200C → T	Arg183Ter	[19]
19	1362C → T	Arg237Ter	[50,53]
31	2208C → T	Arg519Ter	[50,19]
48	3540C → T	Arg963Ter	[56,322]
	IVS12 + 1G → A	Null allele	[19]
	IVS16 − 2A → G	Low level skip of exon 17 Mostly null	[49]
	IVS17 + 1G → C	Low level skip of exon 17 Mostly null	[96]
	IVS18 + 1G → A	Use of cryptic G at −8 in exon 18 leads to premature termination codon in exon 19 and null	[56]
	IVS18 − 1G → A	Null	[19]
	IVS19 − 12G → A	Presumed null; may create a new acceptor site that would add 10 nt to the mRNA and frame shift	[19]
	IVS22 + 3A → G	Presumed null	[19]
	IVS25 − 1G → A	Presumed null; may use a cryptic acceptor site in exon 25	[19]
	IVS26 + 1G → A	Inclusion of intron 26 that contains a premature termination codon	[19,321]
	IVS26 + 1G → T	Presumed intron 26 inclusion	[19]
	IVS48 + 1G → A	Null, mechanism unknown	[56]
11	775delG ACA<u>G</u>CT	Frame shift with termination 6 codons downstream	[50]
12	808delT GGT<u>T</u>TC	Frame shift with termination codon in exon 26	[19]
17	1127delC GG<u>C</u>CCCCCT	Frame shift with termination codon in exon 24	[50]
17	1127insC GGC***C***CCCCCT	Frame shift with termination codon in exon 18	[50]
17	1128delT GGCCCCCC<u>T</u>	Frame shift with termination codon in exon 24	[50]
22	1486–1487delCC TT<u>CC</u>CT	Frame shift with termination codon in exon 23	[56]
24	1666insC GGCCCCCCC***C***T	Frame shift with termination codon in exon 28	[19]
25	1719–1720insAC GCC***AC***CTG	Frame shift with termination codon in exon 25	[19]
27	1866delT CCCCC<u>T</u>GGC	Frame shift with termination codon in exon 46	[53]
31	2073delT GGTCCCCCC<u>T</u>	Frame shift with termination codon in exon 46	[19]
36	2451–2452insT GGCCCCCCT***T***	Frame shift with termination codon in exon 36	[50]
37	2510-2525del	Frame shift with termination codon in exon 46	[50]
37	2517insC GGT***C***CCCCCT	Frameshift with termination codon in exon 38	[50]
38	2613delT GGTCCCCC<u>T</u>	Frame shift with termination codon in exon 46	[19]

TABLE 4. (*Continued*)

Exon	Mutation	Effect	Reference
43	3079delG CGAGAC	Frame shift with termination codon in exon 46	[19]
47	3400insC GAGA***C***CCT	Frame shift with termination codon in exon 47	[56]
47	3401delA GAGACCT	Frame shift with termination codon in exon 47	[19]
47	3402delC GAGCCT	Frame shift with termination codon in exon 49	[56]
49	3589–3590delGA TTCGAC	Frame shift with termination codon in exon 49	[56]
49	3782–3794del	Frame shift with termination codon in exon 50	[50]

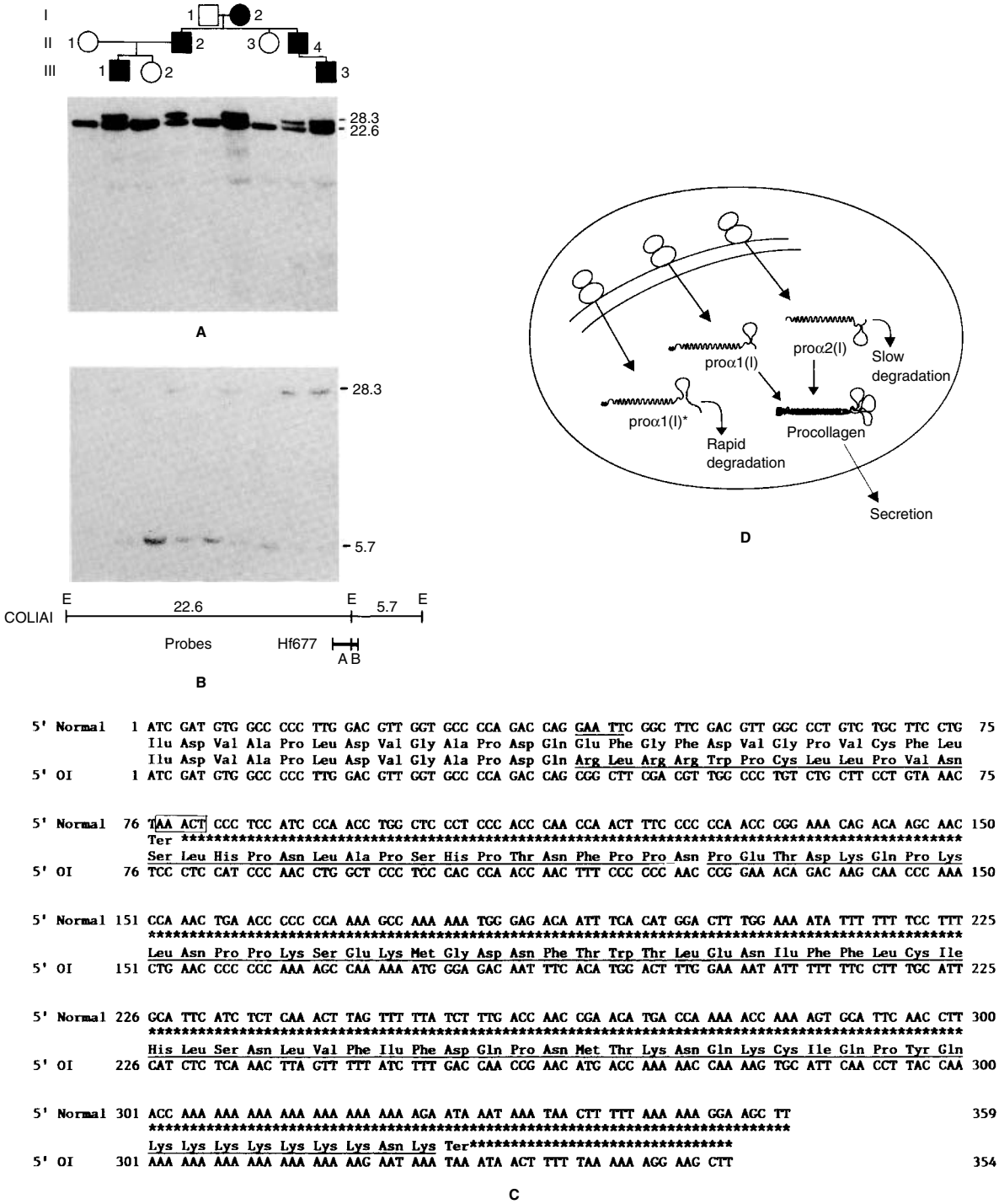
*Underlined nucleotides (e.g., CC) are deleted from the genomic sequence; italicized and bolded nucleotides (e.g., ***AC***) are inserted into the genomic sequence. Ter, termination or stop codon.

demonstrated no evidence of deletion of those regions of the *COL1A1* gene used for linkage analysis [47] and confirmed that most individuals with the OI type I phenotype have mutations linked to the *COL1A1* gene. There are three common types of mutation that result in a reduced level of cytoplasmic *COL1A1* mRNA. These are single base substitutions that create a premature termination codon, insertion or deletion of nucleotides in numbers not whole integer multiples of three so that frame shifts and downstream stop codons are produced, and splice site mutations that result in insertions or deletions that produce frame shifts or intron inclusions with internal stop codons [19,48–52] (Table 4). It appears, on the basis of quite limited data, that single codon mutations to nonsense codons, small insertion/deletions (perhaps at "hot spots" characterized by runs of single nucleotides [19]), and splice site mutations are probably equally represented as the causative mutations. Although such mutations are often referred to as "truncation" mutations, with the expectation that they encode shortened proteins, the major impact is instead very marked reduction in stability of the mRNA due to a process known as "nonsense-mediated decay." Although a small amount of this mRNA can often be amplified from cells [53], more detailed studies demonstrate that the majority of it is retained within the nucleus, where translation does not occur. If the new stop codon is separated from the normal termination codon by one or more introns, the mRNA is usually very unstable, although the precise mechanism of decay is unclear [54,55].

Splice site mutations constitute a special category because similar mutations at other intron/exon boundaries can result in exon skipping and very different phenotypes. For example, the same mutation in adjacent introns 47 and 48 (IVS + 1G → A) results in OI type I (intron 48) [56] or lethal OI type II (intron 47) (PH Byers, unpublished). The intron 48 mutation results in the use of alternative donor sites that produce frame shifts, premature termination codons, and unstable mRNA. In contrast, the intron 47 mutation results in exon skipping and deletion of an in-frame cassette (see below for discussion of disease mechanism). Although this pattern of mutation outcome has been seen in many genes, the explanation has been elusive. It now appears that the most likely reason for differences in outcome of these mutations is related to the order in which the intron is normally removed with respect to the surrounding introns [57,58]. It appears now that mutations in rapidly removed introns generally result in skipping of the adjacent exon, whereas mutations in slowly removed introns result in the choice of alternative acceptor or donor sites, or intron inclusion, most of which lead to frame shifts and mRNA instability [57,58]. In some instances, an intron can be removed in a different order in different splicing pathways. When this occurs, multiple outcomes may appear and change the expected phenotype [57,59].

Mutations that Abolish Proα1(I) Chain Association can Produce OI Type I

The most completely characterized mutation in the *COL1A1* gene that results in the defective production of type I procollagen as a result of a defect in chain association is a 5 bp deletion near the 3′ end of one *COL1A1* allele [60]. The mutation results in a reading frame shift 12 amino acid residues from the normal terminus of the chain and predicts an extension of 84 amino acids beyond the normal termination site (Fig. 5). Although the abnormal mRNA can be translated *in vitro*, it is very unstable in the intact cell. A similar mutation in the murine *Col1A1* gene also results in the synthesis of a very unstable chain that can be stabilized by the addition of proteosome inhibitors, suggesting that these chains are synthesized and rapidly degraded in the proteosome, a cytoplasmic entity responsible for turnover of many proteins [61]. Because the proα chains are normally synthesized within the rough endoplasmic reticulum, proteosomal degradation would require retrograde transport, something seen with several other defective proteins. The possibility that a protease residing in the endoplasmic reticulum, rather than the 26S cytoplasmic proteosome, is responsible for degradation has not been entirely excluded because the inhibitors of proteosomal degradation are not entirely specific. Although the end effect of this mutation, a lack of adequate proα1(I) chains, is the same as if the mRNA were unstable, it makes clear that many different mutations in the *COL1A1* gene could produce the OI type I phenotype by resulting in the

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5' Normal    1 ATC GAT GTG GCC CCC TTG GAC GTT GGT GCC CCA GAC CAG GAA TTC GGC TTC GAC GTT GGC CCT GTC TGC TTC CTG  75
               Ilu Asp Val Ala Pro Leu Asp Val Gly Ala Pro Asp Gln Glu Phe Gly Phe Asp Val Gly Pro Val Cys Phe Leu
               Ilu Asp Val Ala Pro Leu Asp Val Gly Ala Pro Asp Gln Arg Leu Arg Arg Trp Pro Cys Leu Leu Pro Val Asn
5' OI        1 ATC GAT GTG GCC CCC TTG GAC GTT GGT GCC CCA GAC CAG CGG CTT CGA CGT TGG CCC TGT CTG CTT CCT GTA AAC  75

5' Normal   76 TAA ACT CCC TCC ATC CCA ACC TGG CTC CCT CCC ACC CAA CCA ACT TTC CCC CCA ACC CGG AAA CAG ACA AGC AAC 150
               Ter **********************************************************************************************
               Ser Leu His Pro Asn Leu Ala Pro Ser His Pro Thr Asn Phe Pro Pro Asn Pro Glu Thr Asp Lys Gln Pro Lys
5' OI       76 TCC CTC CAT CCC AAC CTG GCT CCC TCC CAC CCA ACC AAC TTT CCC CCC AAC CCG GAA ACA GAC AAG CAA CCC AAA 150

5' Normal  151 CCA AAC TGA ACC CCC CCA AAA GCC AAA AAA TGG GAG ACA ATT TCA CAT GGA CTT TGG AAA ATA TTT TTT TCC TTT 225
               **********************************************************************************************
               Leu Asn Pro Pro Lys Ser Glu Lys Met Gly Asp Asn Phe Thr Trp Thr Leu Glu Asn Ilu Phe Phe Leu Cys Ile
5' OI      151 CTG AAC CCC CCC AAA AGC CAA AAA ATG GGA GAC AAT TTC ACA TGG ACT TTG GAA AAT ATT TTT TTC CTT TGC ATT 225

5' Normal  226 GCA TTC ATC TCT CAA ACT TAG TTT TTA TCT TTG ACC AAC CGA ACA TGA CCA AAA ACC AAA AGT GCA TTC AAC CTT 300
               **********************************************************************************************
               His Leu Ser Asn Leu Val Phe Ilu Phe Asp Gln Pro Asn Met Thr Lys Asn Gln Lys Cys Ile Gln Pro Tyr Gln
5' OI      226 CAT CTC TCA AAC TTA GTT TTT ATC TTT GAC CAA CCG AAC ATG ACC AAA AAC CAA AAG TGC ATT CAA CCT TAC CAA 300

5' Normal  301 ACC AAA AAA AAA AAA AAA AAA AAA AGA ATA AAT AAA TAA CTT TTT AAA AAA GGA AGC TT                    359
               ************************************************************************
               Lys Lys Lys Lys Lys Lys Lys Asn Lys Ter**********************************
5' OI      301 AAA AAA AAA AAA AAA AAA AAG AAT AAA TAA ATA ACT TTT TAA AAA AGG AAG CTT                         354
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C

Figure 5. Molecular basis of OI type I in one family (pedigree at top). **A**, **B**: Southern blots of genomic DNA from family members. DNA was digested with *Eco*R1 and separated by electrophoresis in agarose before transfer to nitrocellulose. The filter was hybridized with a partial cDNA from the 3′ end of the *COL1A1* gene (A in map below) (**A**) or with a short fragment of the cDNA (B in map below) from the 3′-untranslated region (**B**). There are two *Eco*R1 sites 22.6 kb apart that encompass virtually the entire coding region of the gene (see map below **B**). The 3′ site is located approximately 30 bp from the 3′ end of the coding sequence (**B**), and a third *Eco*R1 site is located 5.7 kb further 3′ in the DNA. In this family, affected members have two distinct alleles: one is normal, and the other is about 6 kb larger than normal (**A**). The mutation in this family is the deletion of 5 bp within the 3′ intragenic *Eco*R1 site that fuses the 22.6 kb and 5.7 kb fragments (**B**). This mutation results in a frame shift and the synthesis of a chain predicted to be 84 amino acids longer than normal because of the carboxyl-terminal extension (**C**). The abnormal chains appear to be highly unstable and rapidly degraded within the cell (**D**). Figures **A**–**C** reproduced from Willing et al. [60].

synthesis of half the normal amount of a functional proα1(I) chain. In each instance, the synthesis of proα2(I) chains would be expected to be normal, but about half of them would not be incorporated into intact molecules (because proα2(I) chains alone cannot associate into trimers) and thus would be degraded.

Structural Mutations in COL1A1 and COL1A2 can also Produce OI Type I

In some families, a phenotype similar to OI type I is thought to result from mutations in the *COL1A2* gene, but the clinical criteria by which the diagnosis of OI type I is made are not always clear. Although less common than "null" allele mutations, there are several examples in which the synthesis of abnormal type I procollagen molecules can produce the OI type I phenotype (see Fig. 1 and 2). In one family, cells cultured from the affected mother and son, but not the normal daughter, synthesized α1(I) chains bearing a cysteine residue within the protease-resistant domain of the collagen molecule, a region from which that residue is normally absent [62]. Although it was initially thought that the substitution could be for a nonglycine residue in the triple helix, peptide sequence analysis and the sequence of the cDNA demonstrated that the mutation resulted in the substitution of a glycine by cysteine three amino acid residues carboxyl terminal to the end of the triple helix [63,64]. This mutation did not lead to overmodification of the triple helix, in contrast to mutations that produced substitutions for glycine within the triple-helical domain. The mechanism by which bone fragility was produced is not clear but could reflect the presence of a free sulfhydryl group in about half the type I collagen molecules (those that contain only a single abnormal chain). Other substitutions of cysteine for glycine within the triple-helical domain of the α1(I) chain at residues 94 [65], 46 [66], 43 [67], and 19 [40] also produce very mild forms of OI, perhaps compatible with OI type I.

One patient, with blue sclerae, a height of 4′ 10″ (147 cm), deformity as a result of a poor orthopedic result, and hearing loss, was the only affected member of her family. Her cultured dermal fibroblasts synthesized a proα2(I) chain in which 18 amino acid residues were deleted from the triple-helical domain [68]. Subsequent studies indicated that a point mutation resulted in the excision of the sequence of exon 12 (amino acids 91–108) from about half the *COL1A2* transcripts as a result of a point mutation at the consensus splice donor site. These findings suggest that other point mutations in the *COL1A1* gene, and perhaps in the *COL1A2* gene (as suggested also by linkage studies [47,69,70]), could produce similar phenotypes (see Fig. 1 and 2 and Tables 2 and 3).

Diagnosis, Management, and Treatment of OI Type I

OI type I is first suspected because of a family history or the finding of blue sclerae in the patient with fractures but normal body habitus. In the absence of a family history, the diagnosis is generally confirmed by studies of collagen synthesis by dermal fibroblasts [71] or by direct analysis of the type I collagen genes [19]. It is important to establish, or confirm, the diagnosis in infants for whom there is no family history because the biochemical or molecular genetic studies provide reassurance as to the relatively mild natural history, provide a way to determine the recurrence risk (small), and, in large part, eliminate concern that fractures arise from abuse [66] (and see below). A letter to the family and another to the referring physician are often vital forms of protection against harassment of the family. When first seen, each affected individual should have formal hearing evaluation, which should be repeated at approximately three-year intervals after adolescence. There are probably not increased risks of other disorders, and routine health care and preventive medicine protocols should be followed.

Currently, no treatment is known for OI type I that decreases the frequency of bone fracture or increases bone density [72,73]. An effective agent could increase the production of type I procollagen if the mutation that produced the phenotype resulted in diminished synthesis and secretion. Although a number of agents, such as ascorbate, can increase the production of collagens by cells in culture, none has been tested clinically. Alternatively, an agent that decreased bone turnover could be effective. Bisphosphonates, which alter bone resorption and alter the rate of fracture in osteoporosis, are potentially effective agents that need to be studied in a controlled format. Preliminary data presented at the Sixth International Symposium on Osteogenesis Imperfecta (Montreal, September 1999) by Sillence and others suggest that as children with OI type I go through puberty, usually they do not have the increase in bone mass and bone density that occurs in unaffected children. Use of intravenous pamidronate may increase bone density in this period. Only a small group of people have been studied in this context, and it cannot be emphasized too strongly that carefully controlled studies are needed to assess the efficacy of such agents. Further use of the drug in other forms of OI is discussed below.

The orthopedic management of individuals with OI type I is generally straightforward [74]. Families are instructed in the emergency care of fractures. The methods of fracture treatment are the same as in normal children. Immobilization is kept to a minimum, and lightweight casts are used routinely. Avulsion fractures of the olecranon and patella are fixed with biodegradable devices, thereby avoiding further surgery for removal of metallic implants [75]. Progressive scoliosis is treated by spinal fusion with instrumentation using methods similar to those used in treating idiopathic adolescent scoliosis [76,77]. Brace treatment is less effective than in children without OI because of the osteopenia of the rib cage. However, the prevalence of scoliosis and various aspects of its management will need to be re-evaluated once the outcomes of bisphosphonate treatment are established. Delivery of an affected infant by Cesarean section does not appear to diminish fracture rate and thus such intervention should be reserved for the usual obstetric reasons.

OSTEOGENESIS IMPERFECTA TYPE II (PERINATAL LETHAL FORM)

Genetics, Natural History, and Management

OI type II (MIM 166210), the perinatally lethal form of OI, affects between 1 in 20,000 and 1 in 60,000 newborn infants [1,78,79]. Prematurity and low birth weight are common. Affected infants have a characteristic facial appearance with dark sclerae, a beaked nose, and an extremely soft calvarium. The extremities are short, the legs are bowed, and the hips are usually flexed and abducted (frog-leg position); the thoracic cavity is generally very small. The radiological picture is heterogeneous [18,80], but all infants have markedly telescoped femurs, bowed tibias, and a virtual absence of calvarial mineralization. The ribs are generally beaded, although they may be broad throughout,

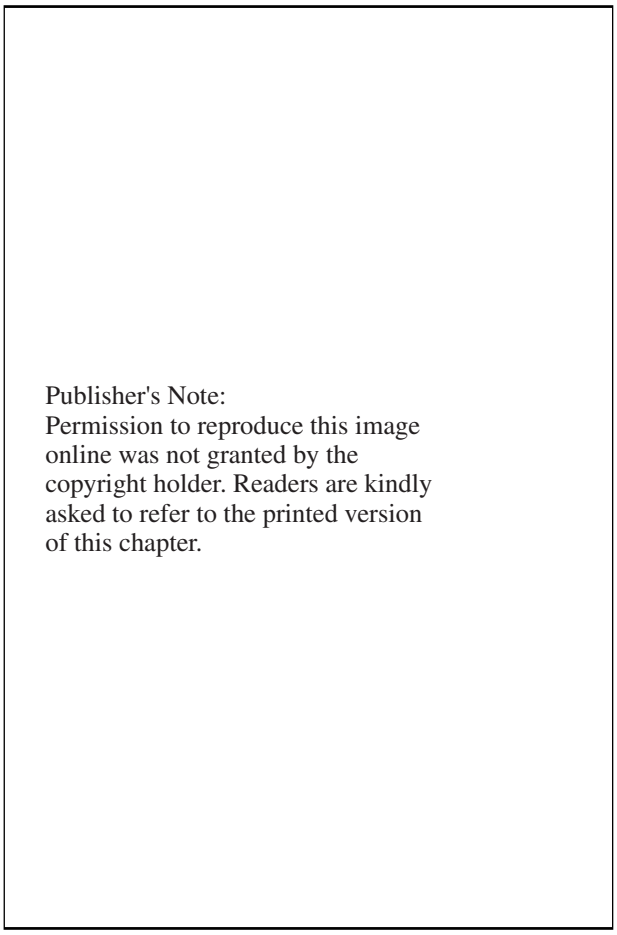


Figure 6. Radiographs of infants with OI type II. There is moderate variation in the radiographic features of infants with perinatal lethal OI. All have shortened femurs, ''beaded'' ribs, and decreased calvarial mineralization. Many also have platyspondyly. The identification of five ''groups'' by Byers et al. [80] and three types of OI type II by Sillence et al. [18] reflects the variation in radiographic presentation. There is little evidence that there are different genetic mechanisms. Only the extent of rib involvement appears to hint at prognosis, with survival longer in the presence of a larger rib cage and fewer fractures. (Figure reproduced from Byers et al. [80]).

and the vertebral bodies may be flattened (Fig. 6). Obvious fractures of the long bones with displacement are rare in the newborn period (probably because the bone is soft and compressed). More than 60% of infants with OI type II die during the first day, 80% die within the first month, and survival beyond a year must be extremely rare (Fig. 7) [80]. The only role for Cesarean section in the delivery of infants with OI type II is if any of the usual obstetric complications intervene.

Survival for infants with OI type II ranges from minutes to months [80]. Death usually results from pulmonary insufficiency, congestive heart failure, or pneumonia. The most difficult management decisions occur at the birth of an affected infant if the diagnosis is not clear. If the diagnosis is known, infants are offered supportive care and parental bonding is encouraged. Some affected infants can leave the hospital, and supportive care in the home is essential.

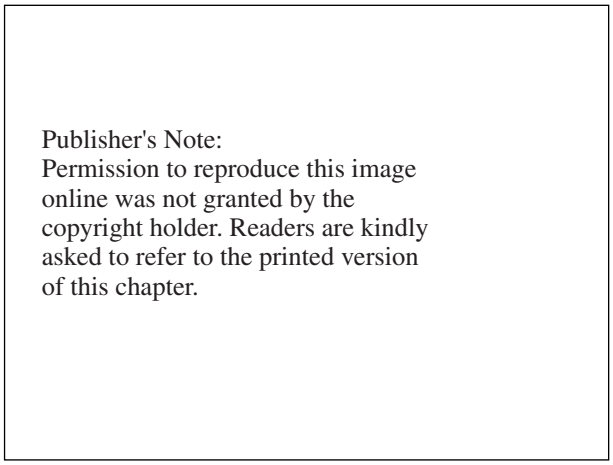


Figure 7. Survival curve for 45 infants with OI type II. More than 50% of infants with OI type II die within 24 hours of delivery. Very few survive beyond 2 months. Routine obstetric ultrasound examination leads to early second-trimester identification of affected fetuses in many instances. (Figure reproduced from Byers et al. [80].)

Bedding that contains soft foam can decrease the fracture frequency. Respiratory insufficiency may limit the ability of many affected infants to maintain adequate caloric intake.

With the increasing use of routine early second trimester ultrasound to assess fetal development, affected infants are being detected early in pregnancy, often leading to its termination [79,81–91]. If, however, no early-pregnancy studies have been done and the diagnosis is suspected only late in pregnancy, there has been some controversy about the mode of delivery. Most recently, it appears that an informal consensus is building against the use of Cesarean delivery. Such a move is supported by data indicating that Cesarean delivery does not prolong survival or change the number of fractures in infants thought to have OI type II.

There has been some uncertainty about the mode of inheritance of OI type II. Seedorf [8] identified some instances of sibling recurrence of OI type II in which parents were not related. Sillence et al. [1,18] identified one family with multiple affected children born to normal but consanguineous parents. Using the formula of multiple, independent ascertainment, they calculated a segregation ratio of close to 0.25, consistent with autosomal recessive inheritance. Furthermore, they cited several instances of sibling recurrence in consanguineous families to support the hypothesis of autosomal recessive inheritance of OI type II.

It now appears that the initial assessment of the mode of inheritance of OI type II was either incorrect or incomplete. Analysis of more than 200 families into which a proband with OI type II was born [80,91–96] has indicated that new dominant mutation is the usual explanation for the phenotype. Although there were no instances in most families, the small rate of recurrence was most consistent with parental mosaicism (either isolated gonadal mosaicism or combined somatic and gonadal mosaicism) (see below). Autosomal recessive inheritance could not be excluded as an explanation for recurrence in one family [80], and biochemical and molecular genetic evidence is consistent with that conclusion in at least one other family [97] and

also consistent with compound heterozygosity for mutations in type I collagen genes.

The largest study of OI type II found a recurrence risk of 6–7% among siblings [80]. A more recent study [96], in which recurrence following the birth of a first or second affected infant was analyzed, found a 2% recurrence risk after the first and a 22% recurrence risk after the second affected infant. The recurrence results from parental mosaicism for the mutation that is lethal in the infant who is heterozygous for the same mutation. We are aware of more than 20 families in which one parent is mosaic for a causative mutation, and several more have been detailed in the literature [80,98–107]. In almost all the families in which the mutation was identified, one parent was mosaic in both germline and somatic cells, indicating that the mutation occurred early in embryogenesis, before the allocation of cells to different lineages. On the basis of results to date, it appears that mutations occur equally in male and female embryos. In some instances, the extent of mosaicism is sufficient to result in mild features of OI (often compatible with OI type IV, see below) in the mosaic parent [103,104,106,107].

The hypothesis that OI type II was inherited in an autosomal recessive fashion reflected the attempt to explain recurrence of severe OI phenotypes among siblings and the presence of consanguinity in some of the families. It is worth noting that the subdivision of OI type II into A, B, and C [18] and the identification of four groups with OI type II [80] demand too much of the supporting data. In short, virtually all instances of OI type II represent the results of dominant mutations in the genes of type I collagen, and recurrence of the phenotype among siblings is accounted for by parental mosaicism for the mutation. Several options for prenatal diagnosis in subsequent pregnancies can now be offered to all families who have had a child with OI type II (see below).

OI type II needs to be differentiated from other lethal skeletal dysplasias — especially thanatophoric dysplasia (MIM 187600) and achondrogenesis (MIM 200600) — and the autosomal recessive form of hypophosphatasia (MIM 241500). Although the radiographic picture of OI type II is characteristic, the ultrasound features early in pregnancy may be difficult to distinguish from other lethal forms of skeletal dysplasia. The characteristic rib abnormalities and markedly decreased calvarial mineralization are the most helpful features. Experienced pediatric radiologists generally have little trouble with the diagnosis in newborns, although evaluation of X-rays of early fetuses may present problems. Nonetheless, many referral centers can provide diagnostic assistance. If there is any doubt, the diagnosis of OI type II can be confirmed by examination of the collagens synthesized by cultured fibroblastic cells from any of several tissues or by direct analysis of type I collagen genes. Alternatively, if no fibroblasts are available to study, pathological examination with the finding of undermineralized bone, osteoblastic cells with dilated rough endoplasmic reticulum, and increased osteoid can help to confirm the diagnosis [108].

Biochemical and Molecular Bases of OI Type II

OI type II is the most extensively studied form of OI and the best characterized at both the biochemical and molecular genetic levels. Cells from several hundred affected infants have been studied in culture (see below), and the molecular basis of OI type II has now been established in more than 100 infants (Fig. 1). A surprisingly wide array of mutations in type I collagen genes produce the OI type II phenotype. These include point mutations in both genes in the domains that encode the triple-helical regions and result in substitutions for glycine, multiexon rearrangements, small deletions or duplications, and single-exon skips in the triple-helical-encoding domains. Mutations in the *COL1A1* gene in the domain that encodes the carboxyl-terminal propeptide, which produce sequence changes that interfere with molecular assembly, can also produce the phenotype [109,110]. In almost all instances, the affected individual is heterozygous for the lethal mutations.

Rearrangements

All multiexon rearrangements within the *COL1A1* gene and most in the *COL1A2* gene have proved to be lethal. Among the cells initially studied by Pentinnen and his colleagues [41] was one from an infant that had the lethal form of OI [111]. Although it was recognized that the cells did not produce type I procollagen efficiently, the nature of the defect was unclear. Studies by others of the same cell strain led to the recognition that decreased production was a consequence of inefficient secretion of molecules that contained one or more proα1(I) chains from which the 84 amino acids from 327 to 411 of the triple-helical domain (encoded by exons 23–25) had been deleted [112–114]. Because the deletion endpoints were within introns, the resulting proα1(I) chain was predicted to have an intact Gly–X–Y triplet structure, 84 residues shorter than the product of the normal allele. Cells from this infant synthesized three populations of type I procollagen molecules: normal molecules and those that contained either one or two of the short chains. Molecules that contained the shortened chain had a decreased thermal stability (about 32 °C compared to the normal of 42 °C) and virtually none of these molecules was secreted [112,115].

Identification of a multiexon deletion among the first few cell strains from infants with OI type II to be studied created the expectation that such mutations would be encountered frequently. However, only two additional published examples of multiexon rearrangements within the type I collagen genes that cause OI type II have emerged. In the first, a 600 bp duplication within one *COL1A1* allele [113,114] that resulted from a recombinational event involving exons 17 and 14 produced proα1(I) chains in which 60 amino acids were duplicated [116]. The abnormal molecules synthesized by these cells were secreted only slightly less efficiently than the normal molecules, so that the lethal effect was probably the consequence of the presence of molecules in the matrix that could not copolymerize with the normal molecules to form fibrils. The other rearrangement identified was a 4.5 kb, seven-exon deletion from one *COL1A2* allele. About half the proα2(I) chains synthesized lacked 180 amino acids of the triple helix, residues 586–765 [117]. Molecules that contained the shortened chain were not secreted but remained in the lumen of the rough endoplasmic reticulum (Fig. 8), where they remained bound to prolyl hydroxylase for an extended period [118]. This cell strain provided a surprising insight into the manner in which the behavior of molecules synthesized by cells from individuals with OI might be altered. Although the deletion resulted in maintenance of the triplet motif of the triple helix (Gly–X–Y), molecules that contained the abnormal chain had undergone a marked increase in post-translational modification but only amino-terminal to the deletion junction. Thus, although the triple-helical structure was maintained, the stability of the triple helix amino-terminal to the junction was compromised. This demonstrated clearly

that even minor shifts in triple helix registration among chains are destabilizing and indicated that interactions in addition to hydrogen bonding involving hydroxyproline residues must contribute to helix stabilization (Fig. 9). It was further surprising that the secretion of, at best, very small amounts of the abnormal molecule was sufficient to result in the OI type II phenotype. If the block to secretion were complete, the phenotype would be expected to be OI type I (see above), suggesting that other factors may play a role in the generation of phenotype or that very small amounts of abnormal molecules in the matrix may be sufficient.

Point Mutations

The majority of cells from infants with OI type II are heterozygous for point mutations that result in the substitution of single glycine residues within the triple-helical domain of either the proα1(I) or proα2(I) chains (see Fig. 1). These mutations lead to increased post-translational modification (Fig. 10) as a result of decreased triple helix stability (Fig. 11), interfere with secretion (like those with more complex mutations illustrated in Fig. 8), and affect the formation of fibrils in the extracellular matrix.

The first demonstration that point mutations in the triple helix of the proα1(I) chain might be significant came with the recognition that cells from an infant with OI type II contained a cysteine residue (normally excluded) within the triple-helical domain of the α1(I) chain [119]. In this cell strain, one allele contained a single nucleotide substitution that changed the glycine codon at position 988 in the triple helix to that for cysteine [120]. All molecules that contained either one or two copies of the abnormal chain were less efficiently secreted than normal molecules, had undergone increased post-translational modification (lysyl hydroxylation and hydroxylysyl glycosylation) along the entire length of the molecule, and were less stable to thermal denaturation (they melted at 38 °C instead of the normal 42 °C). A similar pattern of behavior of abnormal molecules has now been noted in cell strains from many other infants with OI type II.

Because of the importance of glycine in every third position for the propagation and stability of the triple helix (see also Chapter 2, part I, this volume), it was postulated (as a result of these studies) that substitutions of most glycyl residues in the triple-helical domain of the α1(I) chain would most likely result in the same phenotype, provided they interfered sufficiently with stability. It is now clear that most substitutions for glycine within the triple-helical domain of either proα1(I) or proα2(I) chains result in the clinical picture of osteogenesis imperfecta. However, the severity of the phenotype is a reflection of the chain in which the mutation appears, the location of the mutation in that chain, and the nature of the substituting residue (see Fig. 1).

Almost 100 point mutations have now been characterized from infants with the lethal OI phenotype (Fig. 1). Single nucleotide substitutions in the glycine codon (GGN) give

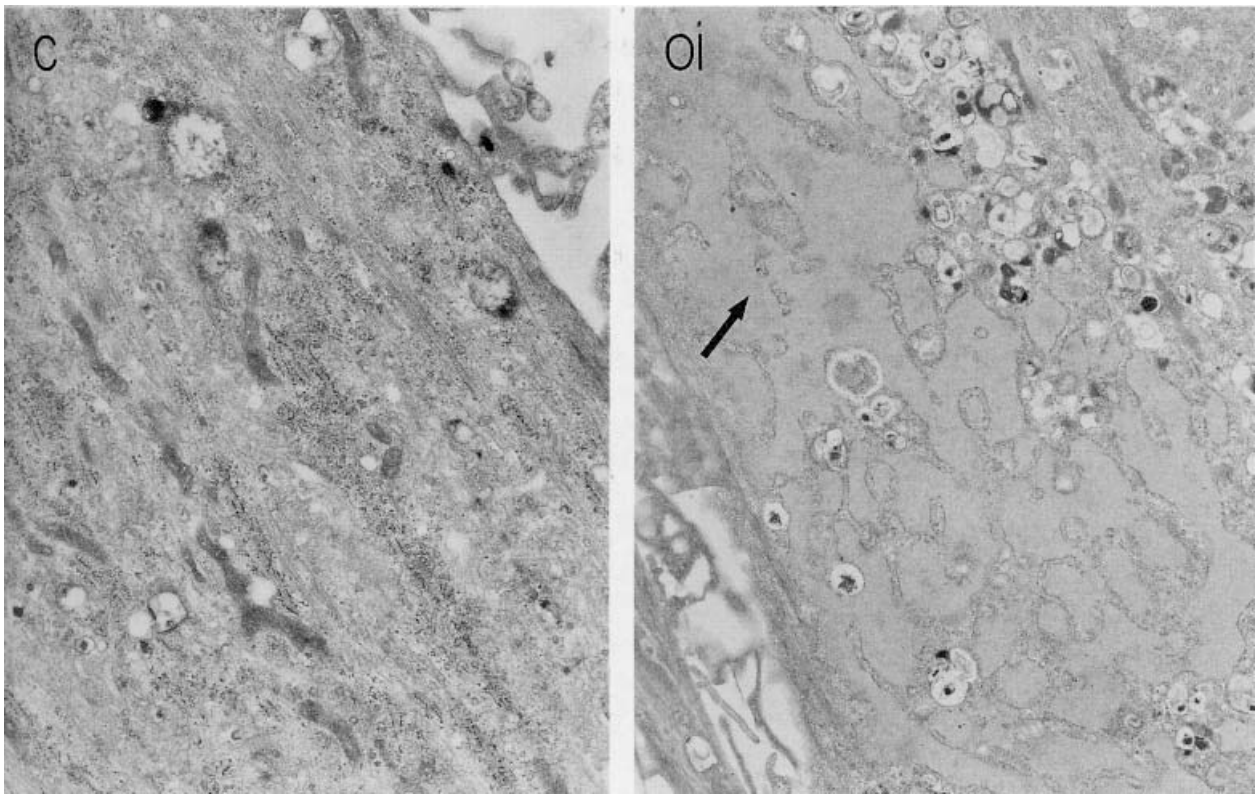


Figure 8. Electron micrograph of rough endoplasmic reticulum (RER) in cultured fibroblasts from an infant with OI type II. The RER (arrow) is engorged with abnormal type I procollagen. This infant had a 4.5 kb deletion from one *COL1A2* allele (see Fig. 9), and all type I procollagen molecules that contained the abnormal chain were not transported beyond the RER. Although this degree of RER engorgement is greater than that seen in cells from most infants with OI type II, cells from virtually all affected infants have defects in the secretion of type I procollagen. C, control, OI, osteogenesis imperfecta. (Figure reprinted from Willing et al. [117].)

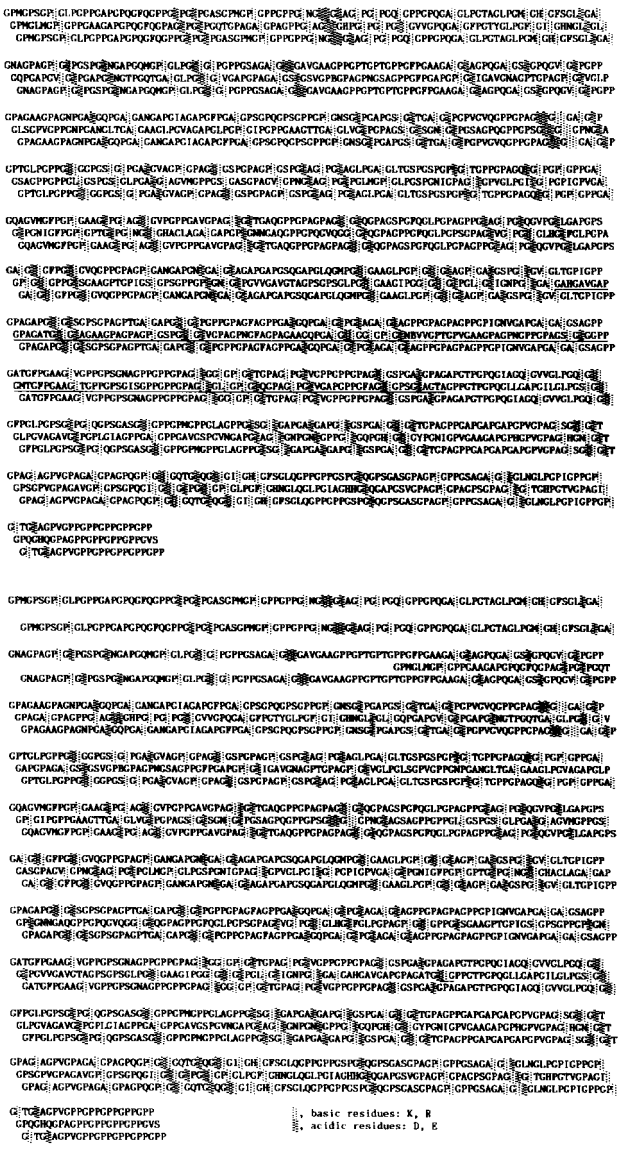


Figure 9. Alignment of the three chains and charge distribution in the triple helix of normal type I collagen (**top**) and when a shortened proα2(I) chain is included (**bottom**). The sequence of the α2(I) chain is the middle line in each row, and that of α1(I) is on the top and bottom lines. The amino acid residues in both chains are represented by the single-letter code. Every Y-position (Gly–X–Y) proline is ordinarily hydroxylated to hydroxyproline, and several Y-position lysyl residues are usually hydroxylated. All are shown as unmodified residues. The chains are staggered by one position, with α1(I) occupying the first and third positions and α2(I) the second (this conforms to most models of the triple helix). The portion of the α2(I) chain that is deleted from the bottom figure is underlined in the top (residues 587–765); the two residues that mark the deletion junction are underlined in the bottom figure. The first glycine of the triple helix is designated as position 1 in this figure, although both chains have additional residues in their precursor forms. The charged residues have been replaced by symbols, as indicated in the key. The alteration in relative position of charged residues in the abnormal molecule, as well as alterations in the relative content of tightly wound and loosely wound helix, probably accounts for the striking alterations in molecular behavior. Similar alterations with small deletions or insertions or exon skips would be expected. (Figure reproduced from Willing et al. [117].)

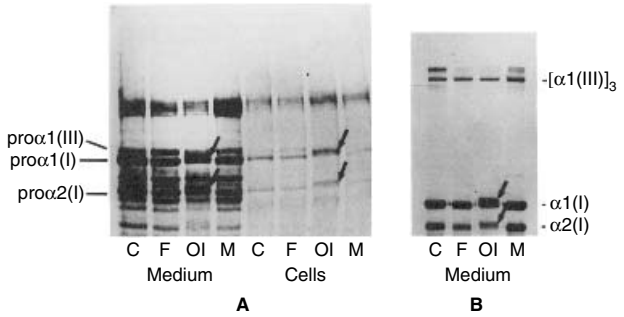


Figure 10. Collagens synthesized by dermal fibroblasts from an infant with OI type II (OI), the unaffected father (F) and mother (M), and a control (C). Cells were incubated with [^{3}H]proline overnight in the presence of ascorbic acid. The medium and cell-layer proteins were harvested and separated under reducing conditions on SDS-polyacrylamide gels (**A**). Cells from the affected infant synthesized some normal type I procollagen molecules and a second species that is inefficiently secreted and accumulates in the cells (arrow). The slower mobilities of the chains from the abnormal molecules (arrows) are best seen following digestion of the procollagens with pepsin (which removes the amino- and carboxyl-terminal propeptides) (**B**). The altered mobilities are the consequence of post-translational hydroxylation of additional lysyl residues within the triple helix and of subsequent glycosylation of the hydroxylysyl residues. The mutation in this cell strain is substitution of glycine at position 580 of the triple-helical domain of the α2(I) chain by aspartic acid as a result of heterozygosity for a point mutation [319]. (Figure reproduced from Bonadio et al. [321].)

rise to codons for serine, arginine, cysteine, tryptophan, and a stop codon (first-position substitutions), or aspartic acid, glutamic acid, valine, and alanine (second-position substitutions). If substitution were a random event, then the proportions of the substitutions should reflect glycine codon usage. In both genes, triple helix glycine codons occur as GGT, GGC, GGA, and GGG (in order of frequency from most to least common). Of possible substitutions, tryptophan (GGG→TGG) and glutamic acid (GGA→GAA or GGG→GAG) would be expected infrequently, and a stop codon would be expected to produce a "null" allele effect and thus an OI type I phenotype. To date, substitution of glycine in the α1(I) chain by glutamic acid has not been observed in OI type II, and only a single instance of glutamic acid for glycine has been seen in the α2(I) chain (Gly343Glu) [121]. It is not clear whether substitutions for glycine residues within the triple-helical domains of the two chains occur with the frequency expected on the basis of codon frequency, but it is clear that there has been some ascertainment bias. For example, the abundance of cysteine mutations in the α1(I) chain represents an ascertainment bias as a result of screening for identifiable protein abnormalities. About twice as many mutations have been identified in the *COL1A1* gene as in the *COL1A2* gene. The reason for this discrepancy is not clear but may reflect a different role for the α2(I) chain than the α1(I) in the triple helix.

Substitutions of glycine by residues with bulky side chains along the carboxyl terminal three-quarters of the length of the triple-helical domain of the α1(I) chain tend to have very severe or lethal consequences, but less predictably so in the α2(I) chain. Thus, in the α1(I) chain it is the rare substitution of glycine by arginine, alanine, aspartic acid, glutamic acid, valine, or tryptophan carboxyl terminal to residue 250 of

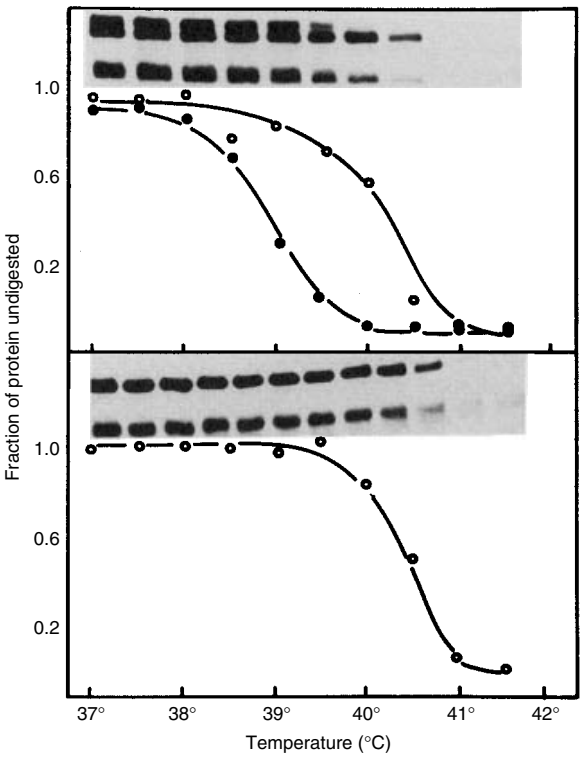


Figure 11. Thermal stability of collagens synthesized by dermal fibroblasts from an infant with OI type II. The infant is the same as that described in Fig. 10. Cells were incubated with [^{3}H]proline for 16 hours and the collagens secreted into the medium were harvested and treated with pepsin. Following dialysis and transfer to a neutral pH buffer, the sample was warmed at a rate of 5 °C/hour. At 0.5 °C increments, aliquots were removed and digested with trypsin. The proteins were separated by SDS-polyacrylamide gel electrophoresis, and the radioactivity in the bands was quantified by scanning densitometry. These data were used to generate the melting temperature curves in the top and bottom panels. The top panel shows results for the normal collagens (open circles) and the abnormal collagens (closed circles) made by the OI type II cells; the bottom panel shows results obtained with control cell collagens. The temperature at which half the collagen was digested (Tm) was 40.5 °C for the control collagen and the normal collagen produced by the OI type II cells but was 39.5 °C for the abnormal collagen molecules. (Reprinted from Bonadio et al. [321], with permission of the publisher.)

the triple-helical domain that would be expected to be nonlethal. If such mutations are found in nonlethal forms of OI, the possibility of mosaicism for the mutation needs to be considered.

One of the most striking features of point-mutation distribution is the nonlinear effect of substitutions of glycine by serine and cysteine in both the α1(I) and α2(I) chains. These substitutions result in alternating lethal and nonlethal zones or domains [122] along the chains from the carboxyl-terminal ends of the triple-helical domains up to residues 200 of the same domains. The explanation for these findings has not been readily forthcoming but probably reflects regional sequence variations in the chains. It has been postulated for more than 20 years that there are domains that can be recognized by their effects on folding and melting [123]. Bächinger and colleagues [124] examined sequence variation along the length of the triple helix of type I collagen and tried to correlate the position of mutations and their relation to regions of peak stability but were unable to create a coherent model. In the α1(I) chain, substitutions of cysteine for glycine can result in significant conformational changes that are seen as "kinks" in the molecules when they are examined by electron microscopy after rotary shadowing or when models are constructed [125–128]. Other substitutions appear not to create such structural changes when they occur in the α1(I) chain [128] but may do so if they affect the α2(I) chain [129], although too few examples have been examined to be sure. Modeling these substitutions with space-filling tools suggests that one form the molecule could take to create the bend is a loop-out of a single tripeptide [127]. Crystal structure of a model peptide in which alanine is substituted for glycine in the center of a repeating Gly–Pro–Hyp polypeptide suggests that with other residues a phase shift in the molecule can occur and be propagated toward the amino-terminal end of the molecule [130]. Examination of the structure of different α1(I) trimers that contain substitutions of glycine by serine that correspond to lethal or nonlethal positions suggests that the intrinsic pitch of the triple helix is an important factor in determining the clinical outcome of the mutation [131,132]. Although such studies are instructive, the use of single peptides limits their relevance to the molecular pathogenesis of OI because most type I collagen molecules are heterotrimers. The importance of these models for disorders in which the abnormal molecules form homotrimers is clear, but for abnormalities of type I collagen they need to be refined.

Exon-Skipping Mutations and Small Deletions

Heterozygosity for exon-skipping defects within the triple-helical domains of both type I collagen genes comprises the second-largest group of mutations that produce the OI type II phenotype (see Tables 2 and 3). Within the regions of both *COL1A1* and *COL1A2* that encode triple-helical domains, the exons are cassettes that begin with a glycine codon and end with a Y-position codon. Thus, mutations that lead to skipping of an entire exon result in the deletion of an integral number of Gly–X–Y triplets from the products of one allele. In general, they occur as a result of point mutations in the splice donor or splice acceptor sequences. In the *COL1A1* gene, mutations that result in skipping exons 14 [133], 20, 27, 30, 44, and 47 produce the OI type II phenotype (Table 2). In the *COL1A2* gene, there are no lethal exon-skipping mutations 5′ of exon 27 (Table 3). Skipping of exon 28 in *COL1A2* as a result of an acceptor site mutation resulted in the OI type II phenotype but may have been accompanied by a *COL1A2* null in the other allele [97,134]. Skipping of exons 30, 32, 33 [135], 37, and 42 in the *COL1A2* gene have all proved to be lethal (Table 3). One of the infants whose cells skip exon 14 of the *COL1A1* mRNA is homozygous for the IVS14 + 5G → A mutation for which its father was mosaic [133]. These data suggest that the infant had uniparental isodisomy for all or part of chromosome 17 and further, because some normal molecules were also made, that the mutation produced a leaky effect on splicing. Heterozygosity for a mutation at the canonical splice donor site in *COL1A1* intron 14 (IVS14 + 2T → A) produced the lethal OI phenotype, confirming the effect of skipping exon 14 in half the mRNA species (see Table 2).

Deletions of single Gly–X–Y triplets in the proα1(I) chain, residues 732–734 (unpublished results), 868–870 [136], or 874–876 [137], have also been identified in infants with the perinatally lethal form of OI. Similar mutations that result

in the deletion of triplets in the proα2(I) chain can have a similar effect, as can duplications of triplets or sextets within the proα1(I) chain [96,138]. Like the other mutations, they alter the extent of post-translational modification of the triple helix amino-terminal to the site of the mutation and thermal stability, and they interfere with secretion.

Mutations Outside the Triple-Helical Domain

Although less common than the other mutations, alterations in the sequence of the carboxyl-terminal propeptide of the proα1(I) chain can cause OI type II. Most such mutations interfere with the ability of proα1(I) chains to associate correctly with other proα1(I) chains or proα2(I) chains. Several single amino acid substitutions in the carboxyl-terminal propeptide have been identified [109,110] as well as a two amino acid deletion [109]. In addition, frame shifts that alter the sequence within the large terminal disulfide-bonded loop of the propeptide and alter the ability of the propeptide to fold normally are also associated with the OI type II phenotype [139]. These differ from premature termination codons that appear earlier or frame shift mutations that predict the loss of the entire carboxyl-terminal propeptide. This latter group may result in very unstable mRNA species (most likely) or, in some instances, result in chains that are unable to associate at all with their partners and usually result in the OI type I phenotype (see above). The mutations in the C-propeptide domain in the proα1(I) chain that result in the lethal OI phenotype are often associated with the induction of BiP, a chaperone resident in endoplasmic reticulum that recognizes unfolded proteins. In addition, such proteins are often very unstable and may be degraded by the cytoplasmic 26S proteosome [61]. In some cells, the secretion of normal type I procollagen is reduced to about 25% of the normal amount, which is apparently insufficient to sustain normal bone formation [139].

Homozygosity or Compound Heterozygosity is Rare

The vast majority of infants with OI type II appear to be heterozygous for mutations in one of the genes that encode the chains of type I procollagen, a concept that is reinforced by the finding of parental mosaicism in a small proportion of them. There are, nonetheless, a small number of infants in whom two mutant alleles appear necessary to produce the OI type II phenotype. In one, a *COL1A2* intron 27 splice acceptor mutation (IVS27 − 2A →G) resulted in skipping of exon 28 and deletion of the amino acids encoded by exon 28 [97]. In addition, the infant apparently received a *COL1A2* allele from one parent that had reduced mRNA synthesis capacity so that less than normal amounts were made [97]. As a result, all the proα2(I) chains synthesized (half the normal amount) were abnormal and the cells secreted a trimer of proα1(I) chains [134]. The second mutation has not yet been identified and parental studies are incomplete, so that a final evaluation of the need for two mutations remains to be confirmed.

A further example of homozygosity for a mutation provides additional insight into the mechanisms that may produce OI type II [133]. The infant described bore two copies of an allele in which there was a *COL1A1* donor site mutation in intron 14 (IVS14 + 5G → A). In cultured dermal fibroblasts, there appears to be alternative splicing in both alleles. As a consequence, normal mRNA and mRNA in which exon 14 had been skipped were present in equal abundance. Although homozygous for the mutant allele, this mutation mimics the effect of an intron 14 splice donor mutation (IVS14 + 2T → A) in which all the products of the allele result in exon skipping. Because the father of the infant with the leaky splice donor mutation (IVS14 + 5G → A) was mosaic for the mutation, it is not clear what the phenotype of the heterozygote would be, although it is likely that it would be mildly to moderately severe.

The clinical phenotype of OI type II produced by exon-skipping mutations or small genomic deletions is indistinguishable from that resulting from multiexon deletions, substitutions for single glycine residues in the triple-helical domain of either chain, or carboxyl-terminal propeptide mutations.

OSTEOGENESIS IMPERFECTA TYPE III

Natural History

The progressive deforming variety of osteogenesis imperfecta, OI type III (MIM 259420—note that although the MIM identification number would indicate that inheritance is recessive, the data suggest heterogeneity), is probably genetically heterogeneous (see below), although the majority of cases appear to result from dominant mutations in either of the two type I collagen genes. The phenotypes are usually recognized at birth or prenatally because of short stature and deformities resulting from *in utero* fractures. Radiographs at birth generally demonstrate an undermineralized calvarium with a large anterior fontanelle and Wormian bones; thin gracile ribs, which may have fractures, thin, gracile long bones, which frequently show evidence of intrauterine fracture with healing and of bowing, especially of the tibias; short and deformed (but generally not crumpled, "accordion") femurs; and generalized osteopenia (Fig. 12). If no fractures are present at birth, they usually appear during the first year of life, frequently with minimal trauma. If not evident at birth, long bone deformity generally becomes apparent during the first two years of life (Fig. 13). Between two and five years of age, "cystic" structures form in the distal metaphyses of many of the long bones ("popcorn epiphyses") (Fig. 14) but especially in the distal femurs [140]. These are areas in which the growth plate is disrupted, probably by recurrent microfractures, and herald the loss of long bone growth. The thin cortex of the long bones predisposes to angulation deformities and, as a result of the deformities, to fracture. Individuals with OI type III have the highest fracture frequency of all those with OI, and 200 fractures during a lifetime is not uncommon.

In a very small proportion of children with progressive deforming varieties of OI, exuberant, hyperplastic callus formation may be observed at the site of fractures, with gradual resolution over time [141–143]. It has been proposed that the presence of hyperplastic callus defines a different type of OI, OI type V [20], although the criteria for this diagnosis are not clear. These regions of hyperplastic callus raise concern about the presence of osteosarcoma [144,145] and, rarely, osteosarcoma may develop in individuals with OI [146–149].

Bone deformation at the base of the skull in individuals with OI type III is of concern because it leads to basilar impression or progressive incursion of the spinal column into the cranium with compression of the brain stem. Involvement of the cranial base is not limited to individuals with OI type III, and some with OI type IV may also be at risk [150,151]. Because of its higher incidence in this group, more aggressive screening may be needed to identify affected individuals. The diagnosis of basilar invagination is best made by MRI or computerized axial tomography because lateral films of the skull may not be informative in the early stages [152]. These

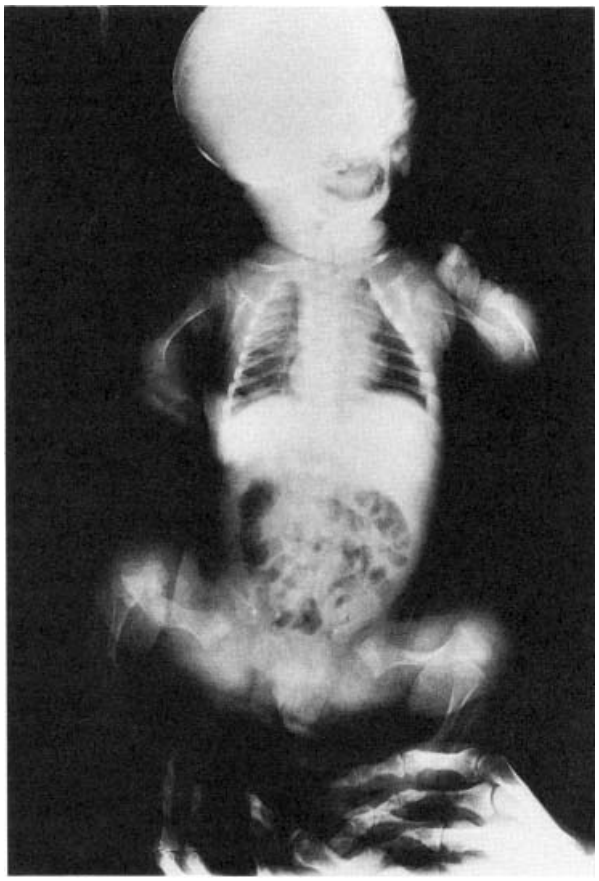


Figure 12. Radiograph taken shortly after birth of an infant with OI type III. Calvarial mineralization is decreased, the ribs have a near-normal size and configuration, and there is marked deformity of the long bones of the lower limbs. This infant is heterozygous for a mutation that results in the substitution of the glycine residue at position 526 of the α1(I) chain by cysteine. (Reprinted from Starman et al. [65] by copyright permission of the American Society for Clinical Investigation.)

findings may be evident in childhood and are occasionally associated with symptoms that can include headache, cranial nerve dysfunction [153], long tract signs, and respiratory depression with sleep apnea [154–157]. Death from respiratory compromise or brain stem compression may be one of the consequences [158,159]. Treatment for basilar invagination and the associated neurological impairment is currently surgical, and it is often suggested that both decompression of neuronal components and stabilization of the spatial relationship between the spine and the lower cranium are needed to prevent progression [152,153,157,160–163]. After surgery, prognosis remains uncertain, and further follow-up of those who have had surgery is needed. If medical therapy with bisphosphonates were successful in changing the mineral content of bone and protecting against deformation of the lower skull, it could be a valuable therapy, even if the effect on long bone morphology and fracture were more limited.

Another consequence of loss of bone rigidity is that some children and adults with OI type III are at risk of significant bowel obstruction as a result of protrusio acetabuli [164].

Children with OI type III, and some other forms of OI, are frequently described by their parents as having increased sweating, heat intolerance, and a preference for cooler environments. The metabolic basis of these observations is unclear, although increased bone turnover may be one factor as bisphosphonate treatment may reduce sweating in some children. As a result, however, concern has been raised about hyperpyrexia during surgery, and at least some instances of such a complication have been observed [165–167]. It is unclear, however, whether hyperpyrexia during surgery is a reflection of OI itself or of the concomitant presence of one or more predisposing mutations for malignant hyperthermia that is independent of OI. In general, this seems to be a rare complication of anesthesia in children or adults with OI, although the use of nonprovocative anesthetic agents may be the reason it is seen so infrequently.

Although head size may be large, with resultant ventricular enlargement, there does not seem to be a significant risk of loss of intellectual function. Instead, the large head size appears to reflect the lack of containment by the soft calvarium and may be present in 15–20% of children with OI type III [155,168]. Over time, a characteristic "triangular" facies develops, with a broad and bossed forehead and small chin.

Dentinogenesis imperfecta is common and involves both the primary and secondary teeth, although the earliest erupted teeth are often the most affected. Sclerae are often pale blue at birth and generally become of normal hue by puberty.

Short stature is the rule in OI type III and is one of the diagnostic features. An adult height between 90 cm and 110 cm is characteristic. Many children with OI type III develop significant kyphoscoliosis, which may progress to cause cardiopulmonary insufficiency, probably the major cause of early death in these individuals [159].

Life expectancy is not well-documented in OI type III but, to the extent that it is, premature death is the rule rather than the exception. There appear to be two major peaks of mortality, one in late childhood or adolescence, in which acute respiratory compromise is the cause of death, and a second in the third or fourth decade, in which cardiac decompensation is the rule [159]. On the other hand, long-term survival is also known.

Genetics of OI Type III

The mode of inheritance of the progressively deforming varieties of OI has been uncertain and, as a result, genetic counselling in families with affected individuals has been confusing. Initially described as a usually autosomal recessive condition [1,169], it is now clear that the major reason for the confusion is that the OI type III phenotype is genetically heterogeneous. Furthermore, parental mosaicism leading to recurrence of the phenotype among their children has added to the uncertainty about inheritance [170]. There are now well-documented instances of autosomal recessive inheritance [171–176], of parental mosaicism leading to sibling recurrence [170], and of autosomal dominant forms of OI type III [170,177].

Although autosomal recessive inheritance of the progressive deforming phenotype is probably rare in most populations [169], it may be the most common form of OI in the South African Black population [3]. Among the South African families, there is evidence of consanguinity and a relatively high frequency of affected siblings. Furthermore, biochemical studies have failed to demonstrate evidence of abnormalities in the structure or synthesis of collagen [5]. In this population, linkage studies using *COL1A1* and *COL1A2*

polymorphic markers have excluded these as candidate genes for mutations that produce the phenotype [5]. In most families in other populations, only a single child is affected with OI type III, compatible with the phenotype usually resulting from a new dominant mutation. This hypothesis has been amply confirmed by the birth of affected children to affected individuals in some families, and by biochemical findings in the majority of affected individuals [71].

Management and Treatment of OI Type III

Following the birth of a baby with OI type III, families need support to gain confidence in caring for their child [178]. Pain care is essential. Parents need advice concerning the emergency treatment of fractures. Lightweight, flexible splints are provided for the families to support new fractures.

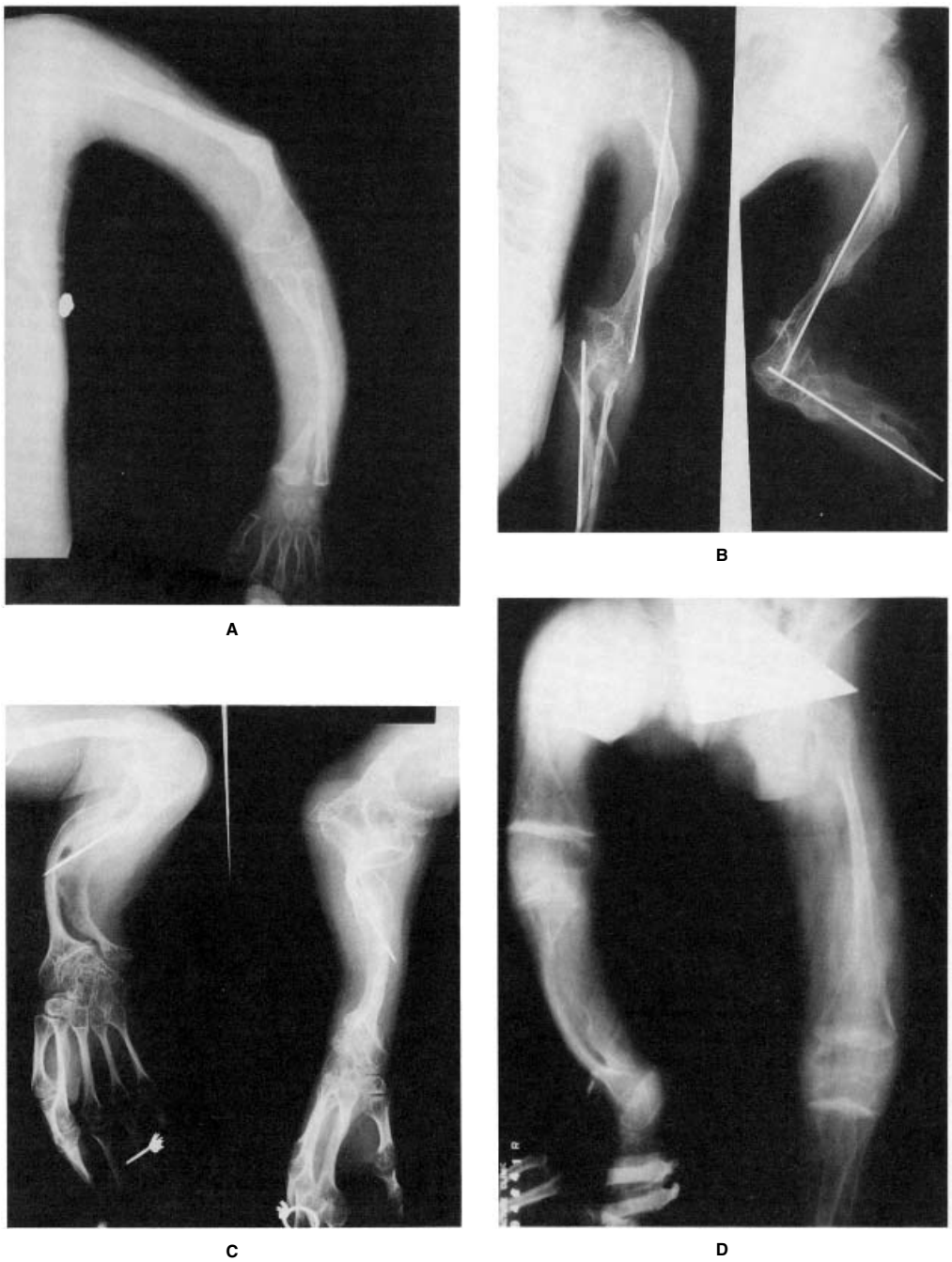
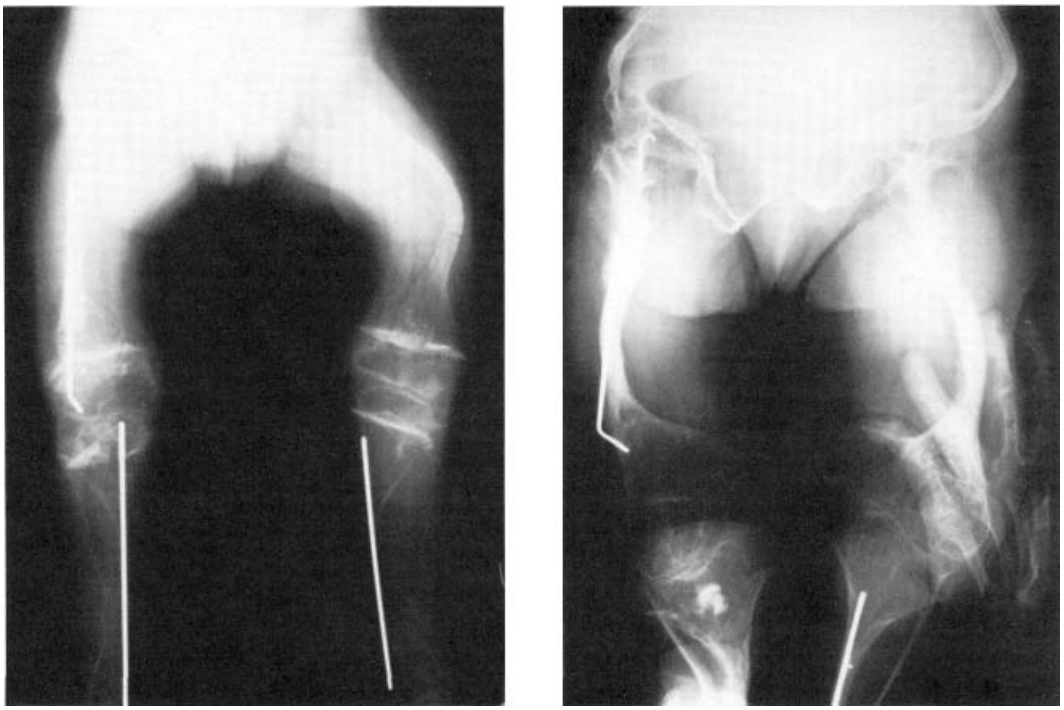


Figure 13. Progressively deforming nature of OI type III. The radiographs of the arms were taken at ages 2 years (**A**), 9 years (**B**), and 22 years (**C**), and those of the legs were taken at 3 years (**D**), 5 years (**E**), and 28 years (**F**). **A** and **B** are of the left arm, and **C** is of the right arm. The magnification of each radiograph is the same, indicating the paucity of growth of the long bones over many years.



E F

Figure 13. (*Continued*)

The prognosis for infants with OI type III, in the absence of treatment to improve bone mass, is poor because of severe bone fragility with progressive deformities of the long bones and spine, muscle weakness, and joint contractures [179–182]. The progressive deformities result from malunion of fractures, deformation of bones, and abnormal function of the growth plates. Progressive scoliosis is usual and may result in even more pronounced short stature and life-threatening cardiopulmonary decompensation [76,156,183]. Many, if not most, children do not walk because of the severity of their bone fragility, anatomic deformities, and muscle weakness. Consequently, most of these children use mobility devices such as wheelchairs. Increasing musculoskeletal and respiratory symptoms occur in adulthood along with the complications of basilar invagination (see above).

Until recently, any enthusiasm for medical therapy for children with OI type III was limited because of the absence of convincing demonstration of efficacy. Agents studied included anabolic steroids, sodium fluoride, magnesium oxide, and calcitonin, all of which failed to provide evidence of therapeutic value [184]. Growth hormone therapy over an extended period has enjoyed its enthusiasts, but prolonged increase in growth, increased bone mineralization, and decreased fractures have not been easy to detect in longitudinal studies in a relatively small population [185,186].

There has been an upswing of interest in the use of bisphosphonates, agents that interfere with bone resorption and decrease bone loss in postmenopausal women with osteoporosis and children with osteoporosis of unknown origin [187]. Beginning in the early 1990s, single patients with severe OI [188,189] or small groups of children [190–193] have been treated with these drugs. As with many small, uncontrolled trials, the enthusiasm for these drugs has been high. The small observational studies are of limited value, however, because, although they report apparent improvement in bone mass, they are compromised by the absence of controls, uncertainty about the endpoints, the use of internal longitudinal controls with, in some instances, very high starting values for fracture rates, and uncertainty about appropriate measures for success. In the largest and most comprehensive of these studies [73], 30 children aged between 3 and 16 years were studied for up to 5 years. They received intravenous pamidronate at intervals from 4 to 6 months, or, in some instances, more frequently. Bone mineral density, vertebral area, metacarpal cortical width, height, several biochemical measures of bone synthesis and turnover, and aspects of mobility and general well-being were examined. In this study, there was an increase in the bone mineral density of the lumbar spine in all groups, although absolute changes were difficult to assess because they were reported as Z scores and measured against pretreatment values. Vertebral area also increased. Fracture rates appeared to decrease slightly, but neither they nor growth rates appeared to change significantly. The authors reported quite remarkable changes in the sense of well-being among the children with decreased bone pain and an increased willingness to walk. Anecdotal descriptions of changes in the character of bone in some children being operated on suggest that alterations in bone composition could be beneficial. Like all the other studies reported, this one is observational, although it uses well-defined measures of change in bone and behavior. Currently, there is a large multicenter controlled trial in progress using the same type of protocol at the Shriners Hospitals in the United States,

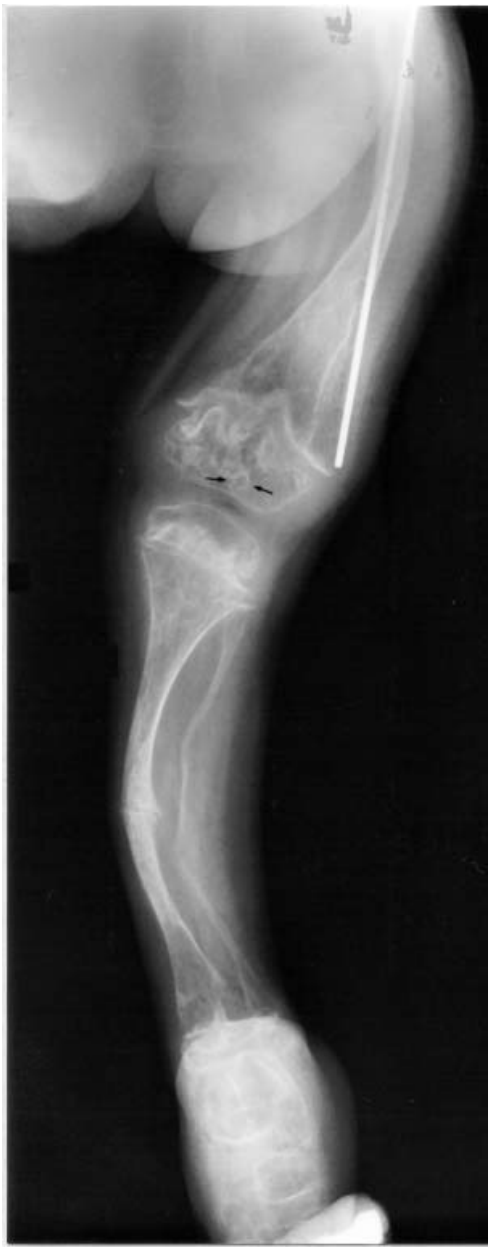


Figure 14. ''Popcorn'' epiphyses at the ends of long bones in a child with OI type III (Courtesy Dr.B. Steinmann).

with hopes of completion by the beginning of 2002. The outcome of these studies will be particularly important in determining the extent to which bisphosphonates can be valuable adjuncts to sustained physical therapy, traditional surgical intervention, and other supportive measures.

Physical therapy and the use of internal rodding or external bracing will continue to be mainstays of therapy to improve strength and joint mobility and to create confidence in standing, walking, and other physical activities [73,180]. The studies on bisphosphonates have generated considerable enthusiasm for their use, even in the absence of controlled studies. It is important that well-designed studies be done in the near future to determine whether many of the early problems with recurrent fractures and pain can be alleviated by the early administration of bisphosphonates, whether continuous or intermittent oral dosing can be used, and whether outcome is improved with treatment within the first few months of life. If the apparent improvement can be further documented in controlled studies, and studies of bone morphology and strength provide supportive data, then the roles of lower-limb bracing and intramedullary rodding of long bones of the upper and lower limbs will need to be re-evaluated. For example, could bracing or surgery be avoided in many children with OI type III, or could external bracing substitute for rodding, particularly in young children? If intramedullary rods are used, should they be nonextending or extending? [74,194–197] Should the rods be inserted before the commencement of bisphosphonate treatment or after treatment has improved the bone quality to a particular level? The prevalence of scoliosis will also need to be determined, and the brace and surgical management will also need to be re-evaluated. The influence of treatment with bisphosphonates on the incidence and progression of basilar invagination of the skull will need to be assessed. Further, the effect on hearing loss should be determined. Finally, although the action of the current forms of bisphosphonates appears to be limited to bone because of tissue-specific distribution of the drug and cell-specific limitations in uptake, some of these drugs appear to be potent inhibitors of protein prenylation [198,199] (see also Chapter 1, Part III, this volume) and thus their side effects need to be monitored closely.

Bone marrow transplantation, with the objective of transferring marrow stromal cells, has been proposed as a therapeutic strategy in children with severe forms of OI [138]. Initial studies to test the feasibility of whole bone marrow transplantation [138] have been met with both high enthusiasm [200] and considerable concern [201,202]. In the initial studies reported [138], three children with severe OI, each with a different *de novo* mutation in a type I collagen gene, had partial marrow ablation using cytotoxic drugs and then had a bone marrow transplantation from a compatible unaffected sibling donor. Two infants had relatively uncomplicated courses, whereas the third had significant complications, possibly related to the procedure. The authors reported some improvement in height/length and bone density, but the basis of these differences has been called into question [202]. At this point, although some cells may be directed to bone, the extent of engraftment using whole marrow seems far too small to support significant bone changes. The prudent course at this point seems to be to provide far better animal studies as a prelude to further studies in humans. At least one person with a form of OI has survived bone marrow transplantation for hematopoietic malignancy, indicating that OI itself is not a contra-indication to the procedure; however, no details of subsequent changes in bone are included in that report [203].

The concept behind the use of stromal cells is that they appear to contain multipotent stem cells that have the capacity to produce bone cells [204,205]. These osteoblastic precursor cells, the idea continues, could repopulate the marrow and may have a replicative advantage over the endogenous cells. The first human studies, done to test feasibility rather than efficacy [138], were carried out after very preliminary experiments in mice. In those studies in mice, biologically marked marrow cells were shown to be present in many tissues after intravenous administration of cultured adherent cells from marrow [206], but no attempt was made to determine whether bone could be repopulated

to a sufficient extent to change its character. In subsequent studies, mice that constitutively carried multiple copies of a shortened human *COL1A1* transgene were injected with stromal cells from normal mice, and bone and other tissues were assayed for the mouse *Col1a1* and the human *COL1A1* genes. These assays were difficult and nonlinear so that precise measurement of the effect was uncertain. The amounts of collagen and mineral in bone were measured, and the treated mice appeared to have slightly greater amounts of each [207]. The intrinsic variation within the target strain makes these results very difficult to interpret, however [208–210]. It is clear that cells derived from marrow that have the capacity to make bone can home to bone, but the efficiency of uptake, the extent to which they can be used to make new bone, and the age at which they show the greatest efficacy all need to be determined.

Biochemical and Molecular Genetic Defects in OI Type III

The issues of the mode of inheritance, the molecular basis of OI type III, and the number of genes in which mutations could produce OI type III remain incompletely resolved. It is clear that many individuals with OI type III have dominantly inherited disorders that result from mutations in the genes of type I collagen (see Fig. 1) [71,99,170,177,211–218]. In many instances, cultured cells appear to synthesize only normal type I procollagen molecules, although in some cases this reflects the position of the substitution in the chains of type I collagen (Fig. 15). Nonetheless, although the effects of mutations in type I collagen genes may be difficult to detect by the protein screening techniques normally used, the primary mutations may occur in genes other than those that encode the chains of type I procollagen. For example, the studies in Southern Africa (cited above [4,5]) could be consistent with a localized noncollagen mutation that results in OI type III. The intermediate form of hypophosphatasia, particularly with marked elevations of alkaline phosphatase in plasma, mimics some of the features of OI type III.

Autosomal Recessive OI Type III

The molecular basis of recessively inherited OI type III due to a mutation in a type I collagen gene has been determined in two families. In the first, the proband was born to phenotypically normal, first-cousin parents and was recognized to have OI at birth. He had bone fragility, short stature, decreased calvarial mineralization, and moderate bone deformity, which increased during childhood [171,172]. Type I collagen isolated from the proband's skin contained only α1(I) chains. Dermal fibroblasts cultured from the child secreted type I procollagen molecules that contained only proα1(I) chains because proα2(I) chains synthesized by the cells were not incorporated into procollagen molecules [173,174]. Both *COL1A2* alleles contained the same 4 bp deletion near the end of the exon that encodes the carboxyl-terminal end of the proα2(I) chain that changed the sequence of the final 33 residues of the chain [175]. The frame shift deleted the cysteine residue in carboxyl-terminal propeptide position 245 that normally bonds with a cysteine residue at position 80 of the propeptide to stabilize its structure. Other studies support the concept that alterations in this domain and, more specifically, loss of the cysteine residue near the end of the chain, alter the ability of such abnormal proα2(I) chains to be incorporated into type I procollagen molecules [219,220]. In the absence

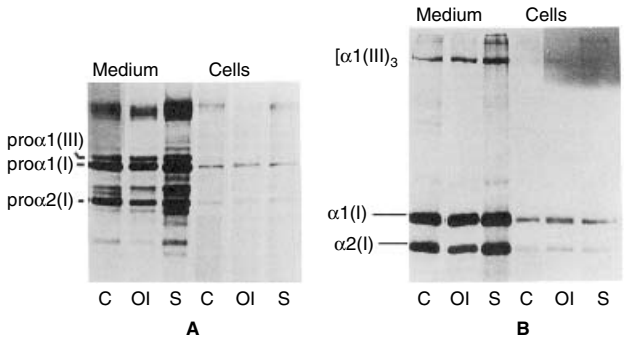


Figure 15. Collagens synthesized by cells from an individual with OI type III due to a point mutation that results in the substitution of the glycine residue at position 154 in the α1(I) chain by arginine. Cells were incubated with [^{3}H]proline overnight in the presence of ascorbic acid, and the medium and cell-layer proteins were harvested separately and analyzed by electrophoresis on SDS-polyacrylamide gels under reducing conditions (**A**) or under nonreducing conditions following partial proteolysis with pepsin (**B**). The proα chains of type I and type III procollagens have essentially normal electrophoretic mobilities (**A**). When the procollagens are treated with pepsin and the resulting α chains are analyzed, a subtle difference in the intensity of bands in the cell layer becomes apparent, compatible with the accumulation of a small amount of type I procollagen within the cells (**B**). These findings indicate that not all mutations in the genes that encode the chains of type I procollagen are readily identified at the protein level. C, control cells; OI, cells from the proband with osteogenesis imperfecta; S, cells from an unaffected sibling. (Reprinted from Pruchno et al. [245], with permission of the publisher.)

of normal proα2(I) chains, proα1(I) homotrimers formed. The chains in these molecules were overmodified along the length of the triple helix (although its thermal stability was normal [221]), and the molecules were secreted more slowly than the normal type I procollagen. About two-thirds of the normal amount of type I procollagen would be expected to be synthesized and secreted (the stability of the molecules being normal). It is not clear whether the relatively severe phenotype resulted from the presence in the matrix of only homotrimeric molecules that were overmodified or from the absence of the α2(I) chain. This mutation demonstrated, to the surprise of many, that the absence of α2(I) chains in type I collagen can be tolerated, albeit with consequences. Interestingly, an Ehlers-Danlos phenotype also results from homozygosity (or compound heterozygosity) for mutations in the *COL1A2* gene that lead to little or no mRNA from those alleles [222,223] (see also Chapter 9, this volume). The reason for the different phenotypes is yet to be resolved but could be a consequence of the synthesis of some abnormal proα2(I) chains in the OI instance.

In a second consanguineous family, two siblings with OI type III are homozygous by descent for a *COL1A2* mutation that results in substitution of the glycine residue at position 751 of the triple helix by serine (Gly751Ser). Heterozygosity for the same mutation results in mild features of OI in the parents, who have mild short stature, a triangular face, mild bowing of the lower legs, and generalized osteopenia with mild platyspondyly of the lower spine.

No noncollagen genes that could result in the OI type III phenotype, if mutated, have been identified.

OSTEOGENESIS IMPERFECTA TYPE IV

Natural History and Genetics

OI type IV (MIM 166220) is a dominantly inherited disorder characterized by normal or greyish sclerae, mild to moderate deformity, and stature that is generally short for the family and often below the 5th percentile for age [1,224]. Dentinogenesis imperfecta is common. Estimates of the prevalence of hearing loss among individuals with OI type IV are scanty. Analysis of dominant pedigrees identified through linkage studies suggests that hearing loss is a more constant feature of families in which linkage to polymorphic markers at the *COL1A1* locus is demonstrated, most of whom are likely to have OI type I as a result of *COL1A1* null alleles [47,50,56].

The majority of infants with OI type IV can be identified in the perinatal period. The presenting features at birth can include fractures that occurred either *in utero*, during labor and delivery, or in the newborn period. Occasionally, these fractures go unrecognized because of adequate healing and a failure to take X-rays. Lack of early recognition of these fractures puts these children and their families in jeopardy of accusation of child abuse when the "first" fracture occurs and the previous, now healed, fracture is detected by routine skeletal survey. Thus, early ascertainment of affected infants is important and can protect some families from suspicion of having abused their child. Not all infants with OI type IV have fractures or deformity at birth, and some may have only mild femoral bowing.

Although birth length is frequently normal, by two years of age height is generally below the 25th percentile and frequently below the 5th percentile, and growth follows the lower percentile tracks thereafter. It can be particularly distressing for families to see their children drop from the 50th percentile to the 5th or below before resuming a growth velocity appropriate for that size. Concerns about other causes of growth retardation are common at that time, for both family and physician, but in these families OI is generally the sole cause.

The fracture rate in individuals with OI type IV may be quite variable. Fractures during the first few months following birth are common and then frequently cease until the child begins to walk. Significant bowing of the femurs, which may have been present in the newborn, may gradually diminish as the child begins to bear weight, and the deformity present in the lower legs may also lessen. Fracture frequency may increase with ambulation and then, as in the other forms of OI, decrease following the onset of puberty. Fractures rarely cease altogether during adult life but are generally uncommon from 20 to 40 years of age, only to increase in the older age group, especially among postmenopausal women. In some individuals, the phenotype may be very mild, with minimal effect on stature, dentin formation, and hearing, such that the onset of fractures in the near postmenopausal period raises the issue of osteoporosis. Family studies in this group of women may be particularly useful.

Scoliosis occurs in perhaps as many as one-third of individuals with OI type IV and ranges from mild to severe [76,156,183,225,226]. As in OI type III, kyphoscoliosis may compromise cardiopulmonary function if severe. In individuals with OI type IV who have scoliosis, both bracing and placement of rods to prevent progression may be valuable in preventing later cardiovascular disease. Because the bones of the ribs are better able to resist deformation from external compression, and because the quality of the ligaments and bones in the spine appears better than in OI type III, the prognosis following bracing or surgery appears to be significantly better.

Although thought to be rare when initially segregated from other forms of OI by Sillence et al. [1], OI type IV is probably one of the more common varieties of OI. The family described by Ekman in 1788 [10] (cited by Seedorf [8]) probably had OI type IV. Many of the families described by Seedorf [8] with dominantly inherited OI characterized by mild deformity and short stature also fit into this group. A significant number of the individuals with dominantly inherited OI described by Bauze and colleagues [42] also had this form of OI.

Considerable confusion concerning the classification of individuals with this form of OI has periodically created havoc with the clinical literature and with biochemical studies. At the time Sillence and colleagues extended the OI classification, they limited OI type IV to those with white sclerae [1]. Subsequently, those with blue sclerae and almost any degree of deformity were often considered to have OI type I, no matter how illogical such a classification might have seemed on the surface. As more extensive biochemical and clinical studies have been completed, it has become clear that the criteria for inclusion within OI type IV should be extended so that scleral hue (probably one of the least helpful clinical signs for classification) does not assume such a large role [71].

In the absence of severe cardiopulmonary complications, the life span of individuals with OI type IV is probably near normal [159]. As a result, both sporadically affected individuals and large families with affected members having OI type IV are seen, raising, for some, the concern that there are both dominantly inherited and autosomal recessive forms of OI type IV. As pointed out by Sykes et al. [47], there is little evidence that there are any families with mutations in the genes of type I collagen in which OI type IV is inherited in other than an autosomal dominant fashion. Detailed biochemical and molecular genetic studies also support this hypothesis, and there is now evidence that the occasional event of sibship recurrence of OI type IV with normal parents reflects parental mosaicism for the mutation [227] (PH Byers et al. unpublished results).

The OI type IV phenotype covers a wide range, from the OI type III picture on the one hand to that of OI type I on the other. Intrafamilial variability can be dramatic [228,229]. There are at least two explanations for intrafamilial variation in expression: the genetic background on which the primary mutation is expressed (the other genes that account for variability have yet to be identified) and mosaicism. When mosaicism for a mutation that results in a severe or lethal phenotype in the heterozygote occurs in the proband with OI type IV, counselling will differ from circumstances in which the condition is inherited in an autosomal dominant fashion. The frequency of mosaicism is uncertain but needs to be considered in all instances in which the proband with a nonlethal form of OI is the first affected individual in the family [96,230].

Management of Individuals with OI Type IV

The objectives of treatment and management of individuals with OI type IV are to minimize the long-term complications of kyphoscoliosis and to assure independence and mobility [180,231,232]. As indicated above, the scoliosis that is often associated with OI type IV is generally

amenable to bracing, and progression can often be minimized. In the event that scoliosis progresses beyond 50–60°, surgical intervention is often a valuable adjunct to therapy.

In general, fractures are treated in the usual orthopedic manner. It is important to minimize the duration of immobility, to start physical therapy immediately upon removal of casts, to use lightweight casts whenever feasible, and to maintain as aggressive a program of physical activity as possible. On occasion, either recurrent fracture in the same location or angulation deformity of a long bone will necessitate surgical repair and stabilization, usually by placement of intramedullary rods. Fixation with screws and plates appears to offer few advantages and several disadvantages when compared with rod placement.

There is consensus that aggressive physical therapy directed at muscle strengthening and a program of early and assisted ambulation will provide the greatest likelihood of independent ambulation for individuals with OI type IV. The program is similar to that used for children with OI type III and relies on a regimen of physical therapy, graded activity, and external support for limbs at risk of fracture. The most common braces use a lightweight plastic "clam-shell" anterior and posterior support with hinged hip, knee, and ankle joints.

Dentinogenesis imperfecta often leads to fracture and excessive wear of fragile teeth. Advances in the design and application of plastic polymers to coat and shape teeth have provided some measure of therapy for individuals with dentinogenesis imperfecta. The early application of bonding agents appears to slow the rate of fracture of teeth and to impede the loss of tooth structure for children and probably offers some of the same advantages for secondary teeth.

Hearing loss is a feature of OI type IV, but profound loss of function in childhood is rare and should prompt the search for other causes. Children should be tested at least once prior to puberty, and adults warrant formal testing about every three to five years unless there is a clinical reason to suspect that hearing loss is progressing at a more rapid rate. As indicated above (see OI type III), the early phases of hearing loss can be managed with amplification devices, but the more profound loss encountered in later life usually requires surgical intervention to repair stapes fixation, the most common cause of deafness.

The role of bisphosphonates in the management of children with OI type IV is still undecided (see detailed discussion above) and awaits the outcome of controlled trials currently under way.

Biochemical and Molecular Genetic Bases of OI Type IV

Individuals with OI type IV were included in the studies of collagen content of skin [42,43] and collagen synthesis by cells from individuals with OI [41]. These studies suggested that there were alterations in the amount of type I collagen synthesized by cells or present in tissues. However, it was linkage analysis, using polymorphic markers within the *COL1A2* gene, that demonstrated definitively that the phenotype could result from mutations in or near the *COL1A2* locus [233]. Linkage heterogeneity was subsequently recognized among families with dominantly inherited forms of OI [47,69,70,234]. In these families, OI type IV was most often linked to polymorphic markers at the *COL1A2* locus, but in some families linkage to *COL1A1* was identified. The clinical criteria by which many of these families were classified were often not clearly defined. As a result, minor features such as scleral hue, rather than major features of bone deformity and stature, may have been used, with the resultant classification of families with OI type IV into the OI type I subset. The significant point from these studies is that mutations in either *COL1A1* or *COL1A2* can lead to the OI type IV phenotype.

As is true in other forms of OI, point mutations that result in substitutions for glycine residues in the triple-helical domains of the proα1(I) and proα2(I) chains, exon-skipping mutations, and a variety of other mutations that disrupt the normal sequence of the proα chains can all result in the OI type IV phenotype (Fig. 1 and Tables 2 and 3).

Point Mutations in Type I Collagen Genes

As discussed above with reference to substitutions of glycine residues that result in the OI type II and type III phenotypes, mutations that give rise to the OI type IV phenotype are scattered along the chains if they produce substitutions of glycine by serine or cysteine, but in the proα1(I) chain other substitutions are clustered close to

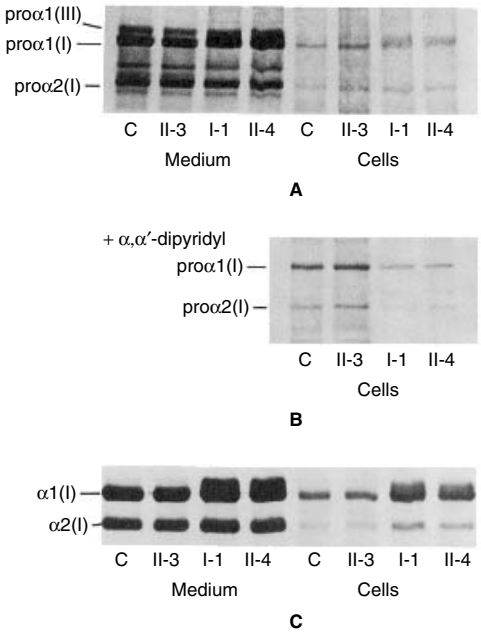


Figure 16. Collagenous proteins synthesized by dermal fibroblasts from individuals with OI type IV due to the substitution by arginine of glycine at position 1012 in the α2(I) chain. Cells were labeled with [^{3}H]proline for 16 hours in the presence of ascorbic acid. Medium and cell-layer proteins were harvested separately and analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions (**A**) or were digested with pepsin and the proteinase-resistant chains of the collagen triple helices examined under nonreducing conditions (**C**). Additional cells were incubated with [^{3}H]proline in the presence of α,α′-dipyridyl, which inhibits post-translational modification of procollagen molecules (**B**). In the presence of the inhibitor, the reversion to normal electrophoretic migration indicates that the altered mobilities seen in **A** and **C** do not reflect the insertion of peptidyl sequence. Cells from affected individuals I-1 and II-4 (parent and child) synthesize some normal molecules and some molecules that contain chains with slower than normal electrophoretic mobilities. The secretion of the abnormal molecules is delayed, as evidenced by the accumulation of molecules in the cell-layer compartments. (Reprinted from Wenstrup et al. [235], with permission of the publishers.)

the amino terminus (Fig. 1). The variability in the proα2(I) chain is a little greater, and one of the first point mutations identified in the *COL1A2* gene to produce OI type IV was shown to result in substitution of the glycine residue at position 1012 by arginine (Gly1012Arg) [235] (Figure 16).

Exon-Skipping Mutations that Result in OI Type IV

In the *COL1A1* gene, only the skipping of exon 10 has been shown to produce the OI type IV phenotype (Table 2). In the *COL1A2* gene, skipping of exons 9 [236] (PH Byers et al. unpublished results), 12 [237], 16 [238,239], 20 [240], 21 [241], and 26 [242] all result in the OI type IV phenotype (Table 3). This spread of mutations more 3′ in the gene and carboxyl terminal in the protein suggests that the type I procollagen molecule is more tolerant of changes in the proα2(I) chain than in the proα1(I) chain. Most of these exon-skipping events are the consequence of mutations in the canonical splice junction sequences. We (C Kuslich and PH Byers, unpublished results) have also observed splice site mutations that result, in part, in exon-skipping in more 3′ positions in the gene, but often the major product is unstable and only a small proportion of the transcripts contain the exon-skipped product. In these situations, the clinical phenotype reflects a complex mix of factors.

Other Mutations that Produce the OI Type IV Phenotype

As is true of the OI type II phenotype, it is likely that as the number of mutations characterized increases, variations on the more common themes will be identified. For example, an intron 33 point mutation that results in the use of a cryptic donor site 18 nucleotides into the intron results in the insertion of six additional amino acids in the triple helix, between residues 585 and 586 of the α2(I) chain, and produces the OI type IV phenotype [233,243]. In a second family, deletion of 9 bp removes a Gly–Pro–Hyp triplet at residues 1009–1011 of the α2(I) chain and produces a mild phenotype (GA Wallis, PH Byers, unpublished results).

MECHANISMS OF MUTATION IN COLLAGEN GENES THAT RESULT IN OSTEOGENESIS IMPERFECTA

For several reasons, collagen genes are good reporters of mutations. First, there is a high density of invariant and required glycine residues in the triple-helical domain (one-third of all amino acids). Substitution of either of the first two nucleotides of the glycine codon (GGN) changes the encoded amino acid to one that has a side chain that does not fit in the central core of the triple helix. Thus, the alteration of any of 22% (2 out of 9) of the nucleotides encoding the triple-helical domain will probably give rise to a phenotypic change in the heterozygote. Second, the large exon number, and sensitivity to exon loss regardless of position in the protein, provides more than 200 additional mutation-sensitive sites in each gene. Third, the need to maintain structure in the globular carboxyl-terminal domain to allow for interactions that generate a triple-chain molecule provides an unknown number of additional targets. Finally, because type I collagen forms fibrils from identical subunits, the presence of any sequence alteration in the externally facing residues of the triple-helical domain could interfere with the production of a normal fibrillar structure. In the 18 kb *COL1A1* gene, this means that mutation at 8–10% of the nucleotides has the potential to produce an OI phenotype. The *COL1A2* gene, which is about twice that size, would have correspondingly fewer reporter nucleotides.

Most characterized mutations that produce recognizable forms of OI are single nucleotide substitutions that alter a glycine codon. Some of the cataloged mutations have occurred independently in unrelated individuals. Some of the recurrent mutations appeared at CpG dinucleotides in the *COL1A1* gene, such as Gly79Arg [53,244] Gly154Arg [245,246], Gly352Ser [122,211,247], Gly415Ser [105,211], Gly589Ser [212,248,249], Gly688Ser [138,250], Gly862Ser [170,215,248,251], and Gly1003Ser [245]. In the *COL1A2* gene, recurrent mutations at CpG sites have been identified at Gly238Ser [40,96,217,252], Gly247Cys [250], Gly502Ser [253], and Gly859Ser [216,252]. Substitutions of alanine for glycine are markedly underrepresented in both genes (Fig. 1). Only a single substitution by tryptophan has been identified in either gene (Gly227Trp in *COL1A2*) (Fig. 1), which is probably consistent with glycine codon usage. With the exception of mutations that give rise to substitutions of glycine by cysteine in the proα1(I) chain, there is a paucity of mutations in the domains of both chains amino-terminal to residue 200 of the triple helix. This either represents different phenotypes that result from these mutations or, more likely, a lesser effect on protein electrophoretic mobility, which is used to screen cells prior to mutation analysis. With directed DNA analysis in the absence of protein studies, these gaps may be filled.

Translation of Mutation to Phenotype

The most complex task in the study of OI is to understand the relationship between the genotype (the specific mutation in a single host) and phenotype in a single person. The difficulty of this task is best recognized when we remember that the OI phenotype is the physiologic response to a mutation in a single collagen allele (most of the time). This response is mounted on the background of the entire genetic background, which is the product of millions of years of evolution in the context of normal collagen alleles. Thus, it should not be surprising that the molecular correlates of clinical severity may not emerge solely from identification of the nature and site of individual mutations.

At the molecular level, OI falls into two groups that have fundamentally different mechanisms of action — the production of structurally normal but diminished amounts of type I procollagen in one, and the production of abnormal molecules in the other. At the clinical level, this distinction marks the divide between OI type I and other types of OI. Yet, even among those with OI type I, there can be considerable differences in the clinical picture. OI type I almost always results from mutations in the *COL1A1* gene that lead to premature termination codons within the mRNA from one allele. There are several mutational events that can give rise to these premature termination codons. They include single nucleotide substitutions within codons, the insertion or deletion of nucleotides in amounts not divisible by 3 in coding sequences, and the creation of frame shifts as a consequence of splice site mutations. The several mechanisms by which these alleles are produced may explain some of the clinical variability between families and, in some instances, even among members of the same family. The stability of the mRNA produced by these different mechanisms has not been extensively explored, and it is possible that some protein product could be produced that does not become incorporated into molecules but could interfere with fibrillogenesis. With splice site mutations, it is not uncommon for several mRNA species to be produced. In some instances, these low-level transcripts may be stable, have intact association

domains, and, say, skip an exon. Although these products may be present at only the 2–5% level, they may be sufficient to modify the expected phenotype of the *COL1A1* null.

Although the expression of the two type I collagen genes is coordinated, mutations in the *COL1A1* gene that lead to low levels of mRNA do not appear to up-regulate the other allele, down-regulate *COL1A2* expression, or alter the expression of many other genes expressed in bone and other connective tissues. The effects of these mutations should be felt directly in the extracellular matrix, where about half the normal amount of normal type I procollagen molecules is secreted into an environment in which all other interacting molecules, including proteoglycans and other collagens, are made in their usual amounts. The use of emerging "chip" technology to identify genes, the expression of which is altered in cells from individuals with different forms of OI, may provide clues to the mechanisms of complex tissue alterations. Fibrillogenesis is a complex process [254,255] (see also Chapter 2, Part I, this volume) that is strongly influenced by multiple components in the matrix. In addition, mineralization is expected to occur on a highly organized substrate that must be altered when the amounts of the different components vary. The effects on tissue strength of decreased production of type I procollagen are not well-understood. Bonadio et al. [23] demonstrated that in the OI type I mouse model there is a marked decrease in bone strength, compatible with a tissue that has decreased amounts of type I collagen. It is not clear, however, that a decreased amount of collagen is the only effect. The striking morphology of collagen fibrils in the skin of individuals with OI type I, similar to that seen in skin from people with Ehlers-Danlos syndrome type I [256], suggests that altered ratios of the major components of the matrix may contribute to abnormal tensile strength. Thus, even the simplest of mutations is likely to have complex effects on the extracellular matrix, forcing us to recognize the interrelationships of the numerous macromolecules in the tissue. Although the pathway from mutations that result in decreased amounts of type I procollagen production to the end product of brittle but normally formed bones is not yet completed, the major factors have probably been identified and warrant further study.

The phenotypic effects of mutations that result in the generation of abnormal type I procollagen molecules are more deleterious than those of "null" mutations. There is, however, an enormous range in the clinical presentation of these mutations, which appears to reflect the gene (chain) in which the mutation occurs, the nature of the mutation, the location of the abnormal sequence in the protein, and the effects of the mutation on the behavior of the chain and the mature molecule into which it is incorporated.

In contrast to the gradient of effect of large residue substitutions, substitutions of glycine by serine and cysteine, residues with smaller, hydrophilic side chains, have a nonlinear effect on phenotype. These two substitutions have different effects within the molecule. There are no cysteine residues within the triple-helical domain of either proα1(I) or proα2(I) chains. Substitution of cysteine for glycine within proα1(I) chains leads to interchain, intramolecular disulfide bonds when two abnormal chains are in the same molecule. The disulfide bond can introduce a "kink" in the triple helix of some molecules and may partially stabilize an otherwise unstable molecule and provide a new site of nucleation for triple helix formation. In contrast, serine appears to disturb triple helix shape, the rate at which helix forms, or both, without introducing a stable shape change. Within both proα1(I) and proα2(I) chains, both substitutions result in alternating regions of lethal and nonlethal phenotypes. The density of mutations is not yet sufficient to determine whether two types of substitution in the same region have the same phenotypic effect because there are few examples of different substitutions at the same residue within the triple helix.

Substitutions for glycine residues alter the triple-helical structure [257] and delay triple helix formation. In molecules where the progress of helix formation can be monitored, triple helix appears to propagate normally from the carboxyl-terminal end to the site of the mutation, where it either ceases or slows at 37 °C [258]. The effect on helical structure has been suggested by both modeling studies and the analysis of peptides with substitutions [127,130,257]. In both situations, there is a change in the pitch of the helix in the region of the substitution that propagates an altered relationship among the chains to their amino-terminal ends.

Both the delay in helix formation [258] and the change in relationship among residues in the three chains help to explain why overmodification of lysyl residues occurs largely amino-terminal to the site of mutations, although there is evidence of a minor extent of retrograde destabilization. In the normal molecule, post-translational modification continues until the triple helix is formed and stable. In the presence of mutations in the triple helix, the disruption of the helical motif does not allow the chains to come into proximity. A new site of nucleation must be found amino-terminal to the disruption and, while this occurs, post-translational modification continues. The association of molecules that contain abnormal chains with the modifying enzymes is one factor that accounts for the delay in secretion.

The key to the transport of type I procollagen molecules from the endoplasmic reticulum is folded status [259]. The effects of mutations vary from the complete inhibition of transport to a virtually undetectable delay in secretion. Substitutions for glycine, and other mutations that alter helix integrity, are most likely to result in intracellular retention if they occur near the carboxyl-terminal end of the triple helix. Furthermore, the more bulky residues, such as valine, aspartic acid, glutamic acid, and arginine, generally have a greater effect. Substitutions of glycine by serine often result in little impairment of secretion despite their relatively carboxyl-terminal locations and extensive overmodification [260]. The reasons for the disparities in effect are not clear. Possibly, the larger residues exert a greater effect on folding and impair chain alignment more than the smaller ones, as might be expected. Alternatively, there may be a greater effect on changes in helical pitch with larger residues. The abnormal molecules are retained by association with modifying enzymes in the rough endoplasmic reticulum, prolyl and lysyl hydroxylases and protein disulfide isomerase being the best candidates [118,261].

Mutations that result in exon skipping, or the insertion or deletion of small multiples of triplets, also delay secretion, largely as a result of the retention of procollagen molecules in the rough endoplasmic reticulum. This has always been a finding that is difficult to explain because triplet integrity is maintained throughout the majority of the molecule. More recent studies suggest that the pitch of the helix differs along the extent of the molecule [257]. The logical interpretation of these data suggests that there is too little flexibility in these different helical motifs to allow propagation to occur efficiently. Thus, regardless of the size of any discontinuity (single triplet, single exon, or multiple exon), the failure

to propagate helix reflects variations in pitch. This appears to provide a unifying model for these different extents of deletion or insertion.

Studies of cells that synthesize abnormal collagens, and of bone from affected individuals, suggest that some abnormal molecules are incorporated into the matrix, although not into the most highly cross-linked, stable portion [262]. An *in vitro* study of purified molecules demonstrates that the inclusion of even a small proportion of molecules that harbor abnormal chains readily leads to the failure of fibril formation or the formation of abnormal fibrils [263]. These two different studies have not been readily reconciled because there are abundant fibrils formed in both tissues and cultured cells, even in the presence of known mutations. Thus, the precise manner in which these mutations produce a phenotype remains to be examined.

PRENATAL DIAGNOSIS OF OSTEOGENESIS IMPERFECTA

Several approaches to prenatal identification of fetuses affected with OI are available. These include mutation detection in fetal tissue (in some instances, regardless of whether previous affected individuals in the family have been studied) [88,96], analysis of linked *COL1A1* or *COL1A2* markers in at-risk families [96,264,265], analysis of proteins synthesized by chorionic villi [107], and analysis of proteins synthesized by cells grown from chorionic villi [96]. Ultrasonography or other radiographic approaches can provide specific diagnoses as well [84,86,87,91,111,266–268] and, because they are more general, could identify other abnormal conditions. The technique used for prenatal diagnosis depends on the information available in the family, the type of OI for which the pregnancy is at risk, and the gestational age by which the diagnosis must be established (Fig. 17).

Linkage analysis in appropriate families provides diagnosis at 11–12 weeks gestation if DNA is prepared from chorionic villus samples, or 16–17 weeks if amniocytes are used. Linkage studies, using linked markers in *COL1A1* and *COL1A2*, require that the family be sufficiently large to exclude one locus and that the couple for whom the studies are proposed be informative. With an increasing number of polymorphisms available at both loci, the proportion of families that is informative is high. In some instances, it may be sufficient to exclude one locus because the data now indicate that there is very little likelihood of genes other than *COL1A1* and *COL1A2* harboring mutations that produce the dominant OI phenotypes.

An alternative to linkage studies in small families in which it has not been possible to exclude a locus is analysis of the collagens synthesized by cells cultured from chorionic villus samples taken at 10 weeks gestation. Sufficient cells are available within 10 days after the biopsy in most instances, and the biochemical studies can be completed within an additional 10 days [96]. Successful use of such studies requires that an affected individual in the family has been studied previously and that any alteration in electrophoretic mobility of the abnormal collagens can be identified in the molecules synthesized by chorionic villus cells. Some families with a previously affected child with OI type III, or a parent with OI type III, representing a significant group of families by which prenatal diagnosis might be requested, may not be amenable to prenatal diagnosis because no abnormality can be identified. Further, there is significant variation in the relative amounts of type I and III procollagens synthesized by different chorionic villus cell strains, making the diagnosis of OI type I difficult unless other measures to determine that one *COL1A1* allele is transcribed at low levels can be used [96]. Amniotic fluid cells do not synthesize regular type I procollagen and so cannot be used for prenatal diagnosis by analysis of proteins.

Transabdominal ultrasound examination can identify fetuses with OI type II by 15–17 weeks gestation [266,269–271] and most fetuses with OI type III by 20 weeks gestation [91,94,272]. By 14–16 weeks, fetuses with OI type II have short femurs, a small thoracic cage with evidence of rib irregularity, and diminished calvarial mineralization. Increasingly, with the use of routine ultrasound screening of pregnancies, fetuses with OI type II are recognized prior to 20 weeks gestation. The findings in OI type III are limited to deformity and shortening of the long bones and decreased mineralization but do not become definitive until about 20 weeks gestation. In the absence of fractures or deformities, neither OI type IV nor OI type I can be identified prenatally by ultrasound examination.

DIFFERENTIAL DIAGNOSIS OF OTHER CAUSES OF OSTEOPENIA AND FRACTURE

The diagnosis of OI must be considered whenever osteopenia or pathological fracture is encountered clinically. Although osteopenia is commonly associated with bone

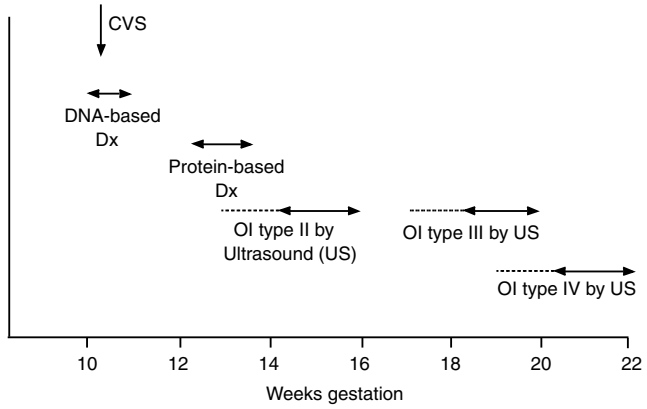


Figure 17. Options for prenatal diagnosis of different forms of OI and the gestational age at which they can be accomplished. CVS, chorionic villus sampling; Dx, diagnosis; US, ultrasound. (Reprinted from Pepin et al. [96], with permission of the publisher.)

fragility, not all bone fragility is characterized by osteopenia as various hyperostotic syndromes are also characterized by bone fragility.

The differential diagnosis to be considered depends, to some extent, on the age at which diagnosis is pursued. Early in gestation, for abnormal bone length identified at the time of routine ultrasound examination (e.g., 16–18 weeks), it would include the major forms of dwarfing: OI type II, OI type III in some instances, autosomal recessive hypophosphatasia (MIM 241500), achondrogenesis (MIM 200600,200610,200700), thanatophoric dysplasia (MIM 187600), and some forms of other lethal dwarfing conditions. By the time of the late mid-trimester and early third trimester, additional conditions, including OI type IV, occasionally OI type I, campomelic dysplasia (MIM 211970), and achondroplasia (MIM100800) become concerns because of limb-length abnormalities and deformity. At the time of birth, the radiographic picture of OI type II usually does not create difficulty, although on rare occasions the nature of the bone abnormality may not be so easily distinguished from hypophosphatasia. The complete absence of mineralization of some vertebral bodies in hypophosphatasia is a helpful clue. At birth, the milder forms of OI may be confused with campomelic dysplasia (in part because some disorders called campomelic dysplasia are, in fact, forms of OI), and the presence of bowing of the long bones should alert the physician to the possibility of OI. Fractures in the newborn period and during childhood often raise the question of nonaccidental injury (see below). At slightly older ages, fractures may raise the question of osteopetrosis (MIM 166600,259700), pycnodysostosis (MIM 265800), and other disorders of increased bone density. During childhood and adolescence, osteopenia may raise the question of juvenile osteoporosis (MIM 259750), which is characterized by decreased bone density, especially of the vertebral bodies, perhaps as a consequence of decreased collagen production. In adult life, familial varieties of "osteoporosis" may represent mild forms of OI.

The extensive range of connective tissue involvement that accompanies bone fragility in some individuals with OI sometimes leads to confusion about the diagnosis. For example, in one family, affected individuals presented with clinical findings that represented a combination of features of OI and the Ehlers-Danlos syndrome (see above, with discussion of OI type IV due to skipping of exon 11). The demonstration of the deletion of amino acid residues encoded by exon 11 from about half the proα2(I) chains synthesized, as a result of a 19 bp deletion that removed a splice junction, appears to account for some of the phenotypic findings. The deletion removes one of the lysyl residues involved in intermolecular cross-link formation, affects the structure of the amino-terminal end of the triple helix, where interaction with matrix mineralizing elements may occur, and changes the organization of the amino-terminal propeptidase cleavage site. Thus, a small deletion may alter many functional domains of the type I procollagen molecule with reflection in a complex phenotype (see Chapter 9, this volume).

Osteoporosis-pseudoglioma (MIM 259770) [273–275] (see also Chapter 26, Part V, this volume) and a separate syndrome of osteopenia, fractures, blue sclerae, and craniosynostosis (MIM 112240) [276] share some features of OI, but no molecular basis of either disorder has been established and no evidence of abnormal collagen synthesis or structure has been identified in either. Osteoporosis-pseudoglioma, a rare recessive disorder, has been localized to 11q12–13 [277], a region that has been extensively mapped [278], so the gene should be identifiable in the near future. Studies of collagens synthesized by cells grown from individuals with other connective tissue disorders in which bone fragility occurs, geroderma osteodysplastica (MIM 231070) and Hajdu-Cheney syndrome (MIM 102500), and in the syndrome of blue sclerotics with fragile corneas, have been normal (PH Byers, unpublished results, and B Steinmann, unpublished results).

Bruck syndrome (MIM 259450) (see also Chapter 26, Part IV, this volume), a rare disorder characterized by bone fragility and joint contractures [279–281], has many characteristics of a mild form of osteogenesis imperfecta. The results of analyses of bone-derived cross-linked collagen peptides in bone and urine are consistent with the absence of a lysyl hydroxylase specific for the lysyl residue in the N-terminal telopeptide of type I collagen. This disorder has been mapped to 17p12 [282], but none of the known lysyl hydroxylases map to that domain.

A form of neonatal hyperparathyroidism that results from heterozygosity for mutations in the calcium-sensing receptor gene has some features of OI [283], as does the syndrome of the calvarial "doughnut lesions" (MIM 126550) [284].

DENTINOGENESIS IMPERFECTA

Dentinogenesis imperfecta (DI) is a form of hereditary dentin abnormality [285,286] and may accompany most forms of osteogenesis imperfecta (DI type I), although it is uncommon in OI type I. In addition, isolated DI may be inherited in an autosomal dominant manner as a distinct entity (DI type II). The clinical manifestations of DI type I include discoloration of the teeth with a characteristic brown or blue-grey color. The primary teeth often appear opalescent when first emerging. Radiographically, a variety of findings characterize DI, including, most commonly, narrow pulp chambers and thin enamel with constricted necks of the teeth (for a review, see [285]).

DI type II (MIM 125490) is a dominantly inherited form of dentinogenesis imperfecta, genetically distinct from that which accompanies OI. Linkage studies have confirmed that the mutant gene is in close proximity to the *Gc* locus on the long arm of chromosome 4 [287] within a 2cM region at 4q21 [288], a region devoid of fibrillar collagen genes. Several genes that map to this locale have been excluded as harboring mutations, including dentin phosphoprotein [289] and osteopontin [290]. Teeth from individuals with isolated DI have been shown to lack phosphophoryn [291,292], a glycoprotein that appears to play a critical role in mineralization of the dentin matrix. However, no genetic studies have either excluded it as a candidate or confirmed mutations in the structure of the gene.

Treatment of the effects of DI type I is largely symptomatic, although there is increasing support for the early application of surface polymer coats to affected teeth to preserve surface as long as possible. For teeth that are rapidly ground to gum level, dental implants could be considered if the mandible and maxilla have sufficient bone matrix to provide adequate support.

OSTEOGENESIS IMPERFECTA AND ABUSE

Since the early 1980s, there has been increasing concern about nonaccidental injury to infants and children and awareness that such injury may lead to death. Physicians and social service agencies have been galvanized in efforts to minimize the possibility of injury to children and to intervene quickly in instances where it seems likely. In

the same period, there has been concern that the number of children with OI who have been removed from their homes because of mistaken accusations of parental abuse has increased. These concerns, in the absence of stringent data, represent a dilemma for the physician, the family, the social agencies, and those who offer diagnostic testing on a service basis. Paterson and McAllion [293] surveyed more than 800 individuals with OI in Britain and found that diagnosis had been reached only with some difficulty in about 15% of them and that, of these, in 15 families there had been accusations of abuse with the institution of formal proceedings. No similar data are available in the United States. In the majority of the 15 cases in Britain that advanced to the stage of hearings, the diagnosis of OI was clearly overlooked, but in a few, even physicians familiar with OI might have had difficulty because of normal teeth, normal bone densitometry, and normal sclerae. Paterson and colleagues [294] considered this group of children and proposed that they have an entity that they termed "temporary brittle bone disease." These are children in whom abuse cannot be directly demonstrated but who have a pattern of fractures that is similar to that seen in abused children. They do not have biochemical findings consistent with OI but may have other characteristics, such as prematurity, that may predispose to fractures. Miller and Hangartner [295] examined 33 infants whom they thought fit into this group because they had multiple fractures, parental denial of abuse, no bruising, and no further fractures following evaluation. They thought that prematurity, decreased fetal movement, and uterine constraint were correlated with this phenotype. In addition, they used radiographic absorptiometry or computed tomography to evaluate bone density in a subset and found it to be diminished, although relatively few controls were studied and the values were compared with adult controls. The concept as a whole has few supporters, and most investigators who are concerned about abuse find no evidence for the existence of such a distinct entity [296,297]. For the concept of transient brittle bone disease to gain credibility, positive diagnostic criteria will have to be developed.

There is continuing controversy over the best way to identify the child with OI in these circumstances. On the one hand, some students of the area [293,298] indicate that virtually any type of fracture can be seen in OI, whereas on the other most investigators assert that certain fractures, particularly metaphyseal chip fractures and "bucket-handle" fractures, are typical and virtually diagnostic of abuse [296,297,299]. It seems likely that fractures of these types are seen far more commonly following abuse but may not be absent from children with OI. In some situations, biochemical studies have been helpful in trying to determine whether a child has OI [300]. However, because cells from only about 90% of individuals with a clinical diagnosis of nonlethal forms of OI have abnormalities in the amount or structure of type I collagen synthesized, such studies may not offer definitive proof of the presence or absence of OI. Perhaps, as studies evolve to the evaluation of gene structure in addition to protein abnormalities, more certainty will accrue. In the interim, it appears that if questions arise concerning the etiology of fractures, the infant or child should be evaluated by geneticists for the possibility of OI or other disorders that can simulate abuse (such as bleeding diatheses and some other forms of connective tissue disorder, e.g., Ehlers-Danlos syndrome) [66] (see also Chapter 9, this volume). If there is any question about the diagnosis of OI, then biochemical or molecular genetic studies should be undertaken with the full realization that not all affected individuals will be detected. Ultimately, the differentiation of abuse from OI and other disorders will rest on clinical grounds, with the results of a variety of laboratory studies playing an adjunct role. It is also conceivable that children with OI can be abused.

ANIMAL MODELS OF OSTEOGENESIS IMPERFECTA

Animal models of human genetic disorders can be valuable tools in the identification of effective therapies. An ideal model of OI would have the same type of mechanics for walking and the same types of weight distribution as in humans, but no such animals are available. The only currently available models are in cattle and mice, the latter comprising both naturally occurring recessive models and created dominant models. It is only with models that mimic human defects that therapies can be evaluated prior to their institution in people. Unfortunately, such models have been difficult to create and of little appeal to most investigators. The need for stable models with reproducible phenotypes as a consequence of engineered mutations cannot be emphasized too strongly.

In isolated herds of cattle, one in Denmark [301] and one in Australia [302], calves with a severe form of OI (similar to OI type III in humans) have appeared. In the Australian herd, almost half the calves born as a result of insemination of cows with sperm from a single bull had OI, although the bull was phenotypically normal. Outbreeding suggested that the most likely explanation for the high rate of affected calves was that each bull was mosaic in the germ line (at least) for the mutation. In one of the calves from the Australian herd, there was heterogeneity of mobility of the type I collagen molecules isolated from bone [303]. This finding is most compatible with heterozygosity for a mutation in a collagen gene. No molecular genetic studies have been published.

A mouse with a recessively inherited phenotype of fragile bones (the fragilitis ossium mouse [*fro/fro*]) has been described [304]. The phenotype is similar to the OI type III picture, but the molecular basis of the disorder is unknown. Another mouse strain with bone fragility (*oim/oim*), demonstrating recessive inheritance, is homozygous for a single base-pair deletion near the 3′ end of the *Col1a2* gene that encodes the mouse proα2(I) chain [305]. The mutation changes the sequence of the final 48 amino acids of the chain such that the domains of the carboxyl-terminal propeptide involved in chain folding are defective. Although the chains are synthesized, they cannot be incorporated into heterotrimers and appear to be very unstable. The phenotype is similar to the recessively inherited form of OI in humans characterized by defective proα2(I) chain association (see above). Although these mice provide a model of a rare form of human OI, they are being used in studies of bone marrow transplantation [205] and as a model with which to test potentially therapeutic drugs [306,307].

A strain of mice in which a retrovirus inserted into the first intron of one *Col1a1* allele created a model of OI type I [308]. Mice heterozygous for the insertion event, known as the Mov13 mutation, have early-onset hearing loss, bones that are more fragile than those of controls [23], and skin that contains about half the normal amount of type I collagen. Embryos homozygous for the insertion die at about 12 days gestation from rupture of the heart and arterial vessels [309]. Tissues in these embryos contain no

type I collagen, and death appears to result from mechanical failure of the organs. Teeth, in contrast, synthesize type I procollagen in a normal fashion, indicating that the promoter used in dentin differs from that in bone and other tissues [310,311]. Transfection of the normal *Col1a1* gene into cells grown from embryos homozygous for the Mov13 mutation rescues proα2(I) chains that are otherwise rapidly degraded [312]. Transfection into Mov13 cells of a human *COL1A1* cDNA, which contained a point mutation that resulted in substitution of a single glycine, resulted in the rescue of proα2(I) chains, but all the type I procollagen molecules synthesized were overmodified [312]. Introduction of the same mutant collagen gene into normal fertilized mouse eggs resulted in a lethal phenotype characterized by bone deformity similar to that seen in the OI type II phenotype [313]. This experiment is consistent with the findings in human infants with OI type II that a point mutation in one type I collagen allele is sufficient to produce the lethal phenotype.

Several transgenic mouse strains have been developed from founders in which a human *COL1A1* "minigene," lacking the coding sequences of 41 central exons, was inserted [314]. Multiple chimeric founders had no bone abnormalities but gave rise to offspring with different phenotypic effects, ranging from neonatal lethal to mild-moderate involvement, that may have reflected the load of the transgene [315]. In additional experiments, levels of the transgene mRNA did not appear to reflect the phenotypic severity in inbred lines derived from the same founder [315]. Bone fragility in these animals seemed increased early in life, appeared to normalize in adult life, and then to increase again as the animals aged [316]. Mice from these strains have been used as test animals to determine whether whole marrow transplantation, or the use of marrow stromal cells, could ameliorate the phenotype, even though the variability among animals in the same strain [207] could complicate interpretation of the results. Such studies serve to underscore the need for an animal model with a single genetic alteration that reflects the human mutations.

In an attempt to create such a model, Forlino et al. [317] used a *Cre/lox* strategy to incorporate a specific mutation (Gly349Cys, known to produce an OI type III/IV phenotype in man [318]) into one mouse *Col1a1* allele. The specific mutation was created in a cassette that also contained a *Neo* gene in the opposite orientation flanked by LoxP sites in an adjacent intron. This strategy was used to try to create two types of mutation—a "null" in which the *Neo* cassette was retained to interfere with expression of the allele, and an allele in which the cassette had been removed after animals carrying the recombinant allele had been mated with others that expressed the *Cre* recombinase. Although cassette removal did not work quite as planned, a strain of mouse developed in which bony abnormalities similar to those seen in people with OI type III/IV were seen. Additional study will determine the extent to which these animals can be used to test a variety of intervention strategies that could be useful in people with OI. Perhaps a more standard approach to the creation of animals with directed mutations created by homologous recombination could give a variety of phenotypes without the intervening step.

CONCLUDING REMARKS

The clinical heterogeneity in OI is striking among individuals from separate families and within some families. The variation between individuals from different families is explained, in large part, by the diversity of mutations in the two genes that encode the chains of type I procollagen. The variation within families remains to be explained. The phenotypic effects of mutations reflect the genes in which the mutations occur, the nature of the mutations, their locations within the genes, and, for point mutations, the location of the substitution and the nature of the substituting residue. Mutations that affect the amounts of the mRNAs of the type I collagen genes are milder in their phenotypic effects than those that affect the structure of the protein. Rearrangements (multiexon insertions and deletions), exon deletions, smaller deletions, and point mutations all have a similar effect on the assembly of type I procollagen molecules and thus may have similar phenotypic effects. Both the genetic background on which a mutation occurs and the extent of mosaicism (in mosaic individuals) are important in determining the phenotypic outcome. Because of the large number of potential mutation sites, it is not surprising that there is a very wide, almost continuous, phenotypic spectrum. Indeed, it is somewhat surprising, given the nature of the mutations, that there are discrete phenotypes that are almost instantly recognizable. Although a great deal is known about the mutations, the manner in which they are translated into phenotype remains to be fully appreciated. As more mutations are characterized, the nature of the relationship to phenotype and the role of other genes will become clearer and other elements that modulate phenotype can be explored. Effective medical treatments may be emerging that will alter the course of OI in both severely and mildly affected individuals. These therapies have the potential to change the character of bone, alter surgical management, and, potentially, to minimize some of the most severe complications. With the creation of new animal models, the chance to test a variety of therapies will emerge and remove some of the uncertainties prior to testing in humans.

RECENT DEVELOPMENTS

Expansion of the OI Phenotype and Nosology

OI Type V

Very soon after this chapter was completed, a fuller clinical, radiological and histological description of a subgroup of individuals with a clinical picture like that of OI type IV, but subject to hypertrophic callus formation after fractures, was published, which the authors proposed be known as OI type V [327] (see Table 1). They identified seven patients of their own and recognized additional individuals described in previous reports [328–330]. One of the major clinical features that distinguished this group from those with OI type IV was limitation of pronation and supination of one or both forearms as a consequence of calcification of the interosseous membrane. Neither blue sclerae nor dentinogenesis imperfecta were part of the phenotype. On average, this group was shorter than the mean for unaffected individuals, although five of the seven were within the normal range. Four of the seven had developed hyperplastic callus in one or more sites by the age of two, and all had radiographically abnormal vertebral bodies. In three of the families there were other affected members, consistent with autosomal dominant inheritance. The natural history of this entity, however, is not yet well studied, as most of the affected individuals identified have been children. At least one adult used a wheelchair for mobility but the reason was not clear.

Biochemical analysis of collagens synthesized by cultured fibroblasts from affected individuals displayed no alterations

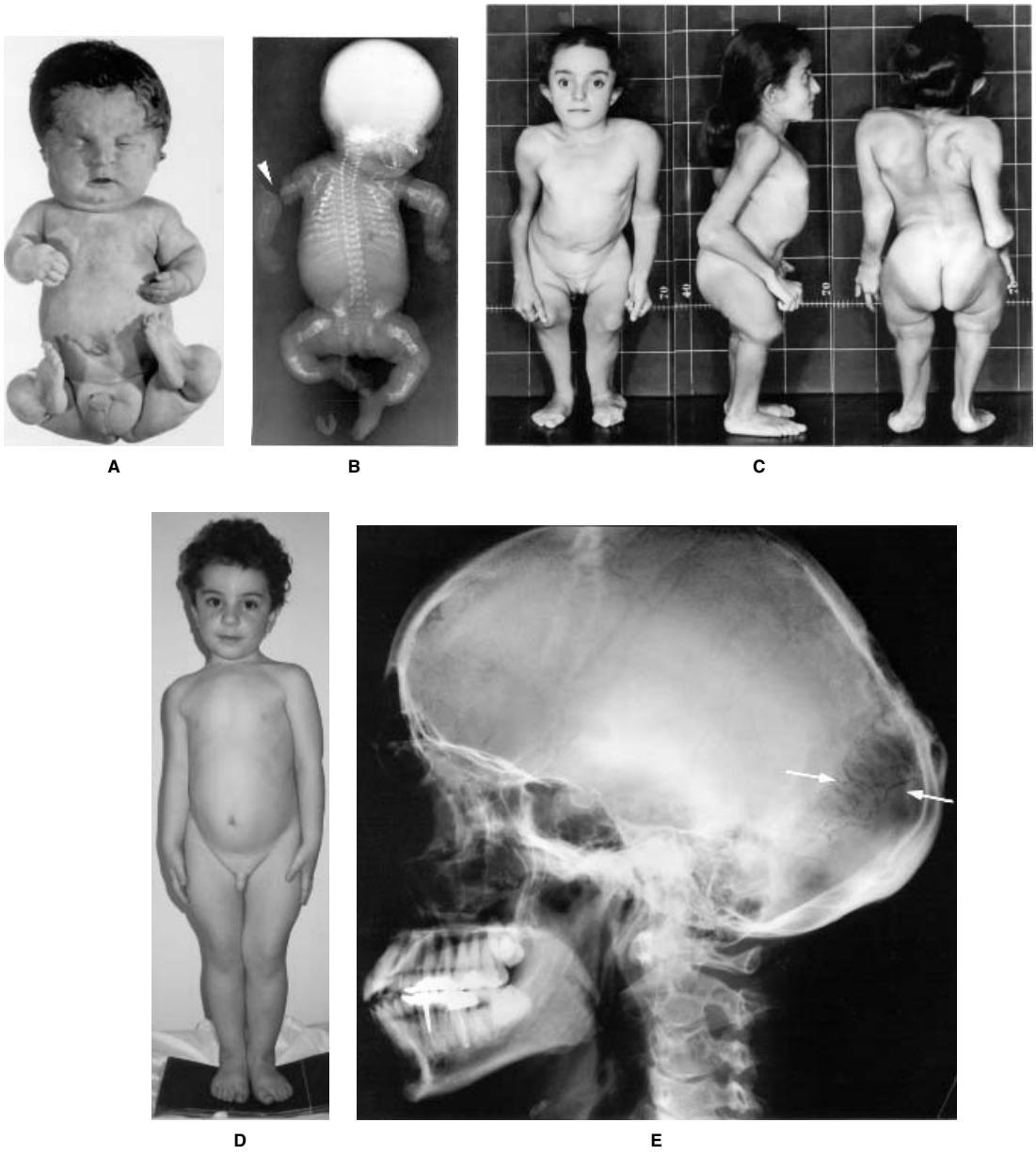
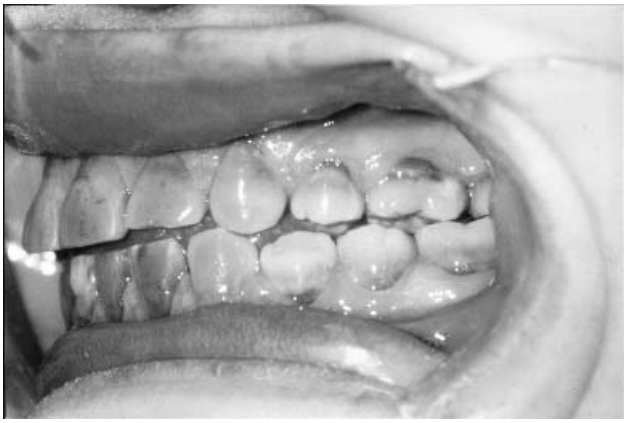
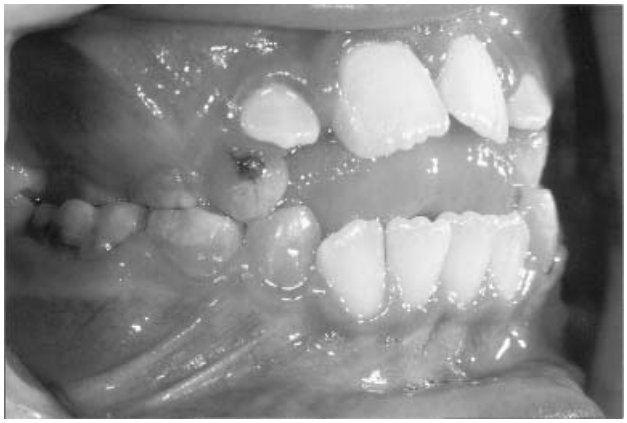


Figure 18. Clinical, radiological, and social aspects of osteogenesis imperfecta. **A**. Neonate (F.B., d.o.b. 20 Apr 1985) with OI type II, born at term with short stature (~36 cm), short and bowed extremities, a low birth weight (2070 g), a large (32 cm) and soft head resembling a balloon filled with water, and inguinal herniae; he died after 5 hours from respiratory failure. His condition was due to a 9 base pair deletion in *COL1A1* [136]. **B**. Radiological findings in a boy (A.K., d.o.b. 5 Jun 1979) with OI type II delivered by Cesarean section in the 32nd week of gestation because of premature rupture of the membranes and breech presentation. Note the right arm, which was torn off during this procedure (arrow), severe osteoporosis, deficient calcification of the skull, and innumerable fractures of the shortened and broad long bones and ribs. His condition was due to a point mutation in *COL1A1* resulting in a Gly988Cys substitution in one of the α1(I) chains [119,120]. **C**. A 14-year-old girl (D.L., d.o.b. 14 Jul 1959) (OI type III) with severe deformities and a height of only 87 cm and weight of 15.5 kg, not yet wheelchair-bound. **D**. A 4.5-year-old boy (OI type IV) with moderate short stature, varus deformity of the femora, and dentinogenesis imperfecta (''bad teeth''), so far without fractures. **E**. Lateral radiograph of the skull of a 48-year-old man (F.L., d.o.b. 10 Jan 1950) (OI type I) shows an ''overhanging occiput,'' Wormian bones along the lambda sutures (zone between arrows) (as in his affected monozygotic twin brother and his child), and basilar impression. **F**. Joint laxity in a 40-year-old woman with OI type IV. **G**. Dentinogenesis imperfecta in a 22-year-old woman (R.B., d.o.b. 17 Feb 1959) with OI type IV. Note the blue-grayish discoloration of all permanent teeth, as also seen in her affected sister; her father became edentulous at around 40 years of age. **H**. Dentinogenesis imperfecta in a 7-year-old boy (T.H., d.o.b. 5 Aug 1981) with OI type III to IV. Note the translucent, opalescent primary teeth and the white, normal-looking permanent teeth, which, radiologically and histologically, were not normal, as was also the case in his affected sister (A.H., d.o.b. 5 Aug 1979) [285,326]. The condition was due to a heterozygous point mutation in *COL1A2* resulting in a Gly688Ser substitution [230]. **I**. Stapes removed from a patient with OI (right) and a control (left). Note the broken plate of the stapes and its thin crura, which explains the conductive hearing impairment. **J**. Carole Piguet (d.o.b. 11 Mar 1962), with severe OI type III, is a well-known Swiss movie star and as helpless physically as the two physicians are professionally.

F



G



H

I

J

Figure 18. (*Continued*)

in either the amount or structure of the type I procollagen molecules produced. Screening of the *COL1A1* and *COL1A2* genes by CSGE identified no sequence abnormalities compatible with an OI phenotype [327], suggesting that the causative mutations were in other genes. However, no causative gene has yet been identified.

Natural History

Hearing loss is common in OI, but there has been some controversy about the age of onset of significant impairment and the ages at which affected individuals should be assessed. In a recent study of 45 children among 201 individuals in

Finland with OI, hearing loss was found to be rare [331]. The significant hearing loss noted in two children was thought to be the result of factors other than OI. Nonetheless, because intact hearing is vital for adequate school performance, formal assessments should be made prior to school and at any time school performance drops.

Treatment

The search for effective treatment of OI, with the aim of decreasing the likelihood of fractures, enhancing growth, and diminishing associated pain and diminished mobility, continues. The major threads of investigation include the use of bisphosphonates at ever younger ages, the assessment of bone marrow transplantation as a source of osteoblastic stem cells, and the use of intramedullary rods to increase bone strength and resistance to fracture and deformity.

Bone Marrow Transplantation

The clinical responses to bone marrow transplantation in children with severe osteogenesis imperfecta have recently been reassessed [332]. Five children have now received marrow transplants from related donors, of whom the follow-up study includes data on three. One was not in the original group, while two from the original group were removed from the study because osteoblast engraftment could not be demonstrated. All the children had clinical features of OI type III. The three children with low levels of donor osteoblast engraftment had a median 7.5 cm increase in body length six months after transplantation, compared with 1.25 cm for age-matched control patients. However, because crown-heel measurements were used for length, any change in limb deformity could have contributed to apparent growth. The children also gained 21–65 g total bone mineral content by three months after transplantation. With extended follow-up, the patients' growth rates either slowed or reached a plateau, although the bone mineral content continued to rise. Further studies are needed to determine the role of allogeneic bone marrow transplantation in severe forms of osteogenesis imperfecta, and to understand if a very small population of cells can contribute to the claimed growth.

Bisphosphonate Treatment

Although the results of a controlled study of bisphosphonate infusion with contemporary controls have not yet been reported, a second observational study in very small children has been published [333]. These children ranged from just over 2 months to about 20 months of age at the time of study and were followed for a year. A historical control group with a similar clinical range was also assessed. In both the treated group and the controls, height appeared to increase to an equal extent. Absolute vertebral area increased in the treated group, but diminished in the untreated group. At the same time, the Z score values for bone mineral density followed the same pattern. Fracture frequency was more variable in the treated group, but it is not clear if the criteria for fracture determination were the same in both groups. Overall, these data begin to add evidence that bisphosphonates may have benefits for some children with OI. Good comparative studies and the assessment of long term effects are still necessary to understand if these protocols will ultimately benefit some, or all, individuals with OI.

Collagen Mutations and Molecular Assembly

Almost 40 additional collagen mutations have been described in the last few months. Mutations that result in the substitution of glycine residues in the triple helical domain of the proα1(I) chain include Gly127Ser (OI type IV), Gly133Arg (OI type IV), Gly229Ser (OI type III), Gly526Ser (OI type III), Gly688Cys (OI type III) [334] and Gly238Cys (OI type IV). In the proα2(I) chain [335], newly identified mutations include Gly127Val (OI type IV), Gly184Asp (OI type IV), Gly196Ala (OI type IV), Gly208Arg (OI type IV), Gly247Pro (OI type III), Gly274Ser (OI type IV), Gly301Cys (OI type IV), Gly436Glu (OI type III), Gly442Glu (OI type IV), Gly481Arg (OI type IV), Gly511Ser (OI type IV), Gly538Arg (OI type IV), Gly574Asp (OI type III), and Gly586Arg (mild OI). Among these, the substitution of glycine by proline requires a two-nucleotide change (GG → CC), a rare mutational event. These mutations begin to provide a more highly saturated mutation map that will, ultimately, provide the basis for better modeling of the relationship between mutation and phenotype. That map, as can be seen from Figure 1, is still incomplete. Insertions and deletions of single amino acids and triplets of amino acids, or their multiples, appear to be somewhat more common than previously recognized, as a dozen such variants are included among the newly described mutations [334,336].

Mutations in the carboxyl-terminal domains of the type I procollagen chains have recently been described in a human family [337] and in a dog with osteogenesis imperfecta [338]. In the human family, a mild form of osteogenesis imperfecta was associated with a Cys1299Trp mutation that disrupted one of the intra-chain disulfide bonds in the carboxyl-terminal propeptide of the proα1(I) chain. The mutation allowed the slow assembly and secretion of overmodified, but stable, type I procollagen trimers. In the dog, a moderately severe form of osteogenesis imperfecta was associated with the formation of a premature termination codon that truncated the carboxyl-terminal propeptide of the proα2(I) chain. The excess proα1(I) chains appeared to form proα1(I) homotrimers. Few mutations in the carboxyl-terminal propeptide of the proα2(I) chain have been identified in humans.

N.B. With the permission of the authors, the editors have added as Fig. 18, a number of photographs that they felt might be of some clinical interest.

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ELECTRONIC DATABASE

Database of Human Type I and Type III Collagen Mutations (http://www.le.ac.uk/genetics/collagen/index.html).

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Chapter 9

The Ehlers-Danlos Syndrome

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SUMMARY

- **The Ehlers-Danlos syndrome (EDS) is a clinically and genetically heterogeneous group of connective tissue disorders affecting mainly skin, joints, and ligaments but in some cases also arteries, intestine, and the gravid uterus. Its hallmarks are hyperelasticity of the skin, hypermobility of the joints, and tissue fragility.**
- **The classical type of EDS (EDS I and II) is the most common form and is characterized by marked skin hyperextensibility and fragility and by joint hypermobility. The disorder is most often inherited as an autosomal dominant trait, but there is considerable locus heterogeneity.**
- **The hypermobile type of EDS (EDS III) is characterized by marked joint hypermobility, moderate skin involvement, and an absence of tissue fragility. It is inherited as an autosomal dominant trait; the underlying defect is unknown.**
- **The vascular type of EDS (EDS IV) is the most severe form of the disorder, with a markedly reduced life span due to spontaneous rupture of internal organs such as arteries and intestine. It is inherited as an autosomal dominant trait and caused by mutations in the *COL3A1* gene coding for collagen III. Unlike in the classical type, the skin is not hyperelastic but thin, translucent, and fragile, joint hypermobility is restricted to the minor joints, and affected subjects may have a characteristic facial appearance.**
- **The kyphoscoliotic type of EDS (EDS VI) is characterized, in addition to the features of the classical type, by severe muscular hypotonia after birth, progressive kyphoscoliosis, a Marfanoid habitus, osteopenia, and occasionally rupture of the eye globe and great arteries. It is inherited as an autosomal recessive trait and caused by deficiency of collagen lysyl hydroxylase activity (EDS VIA). A subgroup of patients with similar clinical features does seem to exist, however, in whom lysyl hydroxylase activity appears to be normal (EDS VIB).**
- **The arthrochalasic type of EDS (EDS VIIA and VIIB) is characterized by congenital bilateral hip dislocation, severe generalized joint hypermobility, moderate skin involvement, and osteopenia. It is inherited as an autosomal dominant trait and caused by mutations leading to the loss of the amino-terminal telopeptide of one or other of the two distinct α-chains of the heterotrimeric collagen I molecule.**
- **The dermatosparactic type of EDS (EDS VIIC) is characterized by redundant and fragile skin, prominent herniae, joint laxity, and dysmorphic features. It is inherited as an autosomal recessive trait and caused by a deficiency of procollagen I N-terminal proteinase.**
- **Other classified forms of the EDS, the existence of which as separate entities is questionable, include X-linked EDS (EDS V), the periodontotic type of EDS (EDS VIII), and fibronectin-deficient EDS (EDS X). Further types of EDS include the progeroid form, unspecified types, and chance associations as confounding factors.**
- **Animal models — naturally occurring, transgenic, and experimentally induced — are useful models for the elucidation of physiological and pathophysiological processes.**

INTRODUCTION

The Ehlers-Danlos syndrome (EDS) is a heterogeneous group of heritable disorders of connective tissue characterized by articular hypermobility, skin hyperextensibility, and tissue fragility affecting skin, ligaments, joints, blood vessels, and internal organs. Its clinical and historical aspects have been reviewed in the classical monographs of Beighton [1] and McKusick [2]. In the preface to his book, Beighton [1] writes as follows:

> "Until recent years, no fairground was complete without an 'Elastic Man'. Audiences would be amazed by his ability to stretch his skin for a prodigious distance, before allowing it to snap back into place. He often had hypermobile joints, and would happily place his left great toe in his right ear, or his feet in his trouser pockets.
>
> As medicine became increasingly scientific, the appellation 'Ehlers-Danlos syndrome' was used and he is now more likely to be encountered in a clinical meeting than in a circus side show."

Connective Tissue and Its Heritable Disorders, pages 431–523

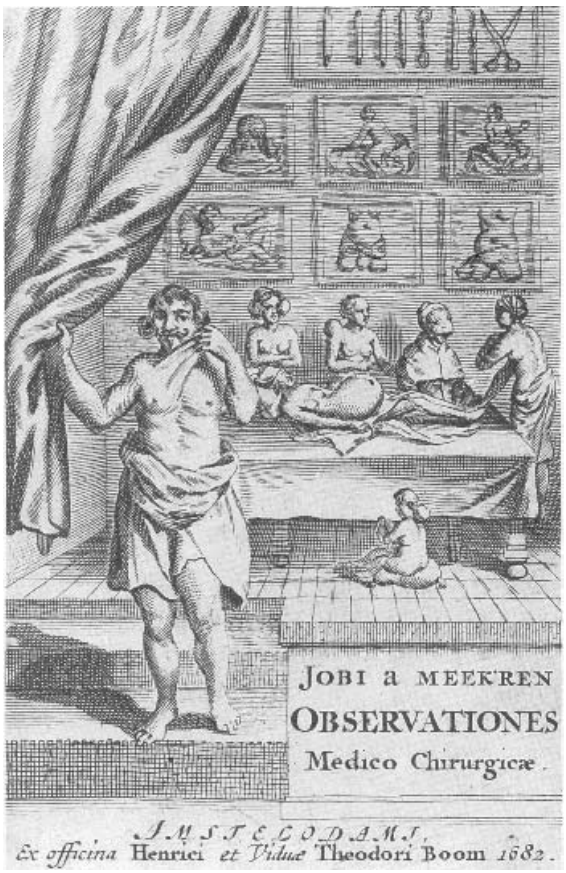

Figure 1. First description and picture of an individual with EDS published by Job Janszoon van Meek'ren. **Foreground**: Spaniard with hyperextensible skin. **Background**: On the table, a woman with a distended abdomen because of ascites and an ovarian tumor; she was the wife of Govert Flinck, a famous Dutch painter, and her case was also described in the same book [9]. Van Meek'ren was a notable surgeon of Amsterdam, who was a pupil of Tulp, the original of Rembrandt's ''Anatomist'' [12]. His observation made in 1657 was first reported posthumously in ''Belgian'' in 1668 [9] and translated into German in 1675 [10]. Below follows a translation from the German by Michael Roth and Ursula Zeller von Murg of Zürich. The original Belgian report was also translated into Latin in 1682 [11], and an English translation of this last is given in McKusick [2].

''Of a Soft-Skinned Spaniard''

''Out of pressing needs and unforgettable sufferings our ancestors raised us, as it were, from the cradle with the belief that there is no wilder, more merciless and cruel people to be found in the world than the Spanish. As the history books, especially the chroniclers of the Dutch wars and the American barbarities to excess prove.[1] In spite of this we must confess that we have not seen a softer or more lithe Spaniard than Gregorius Albes, begotten by Spanish parents and on a Canary Island born. However, in his skin alone was he such. We saw him together with the famous professors, Johann von Horne, Francisco Sylvio, Guil. Pisone, and Francisco von der Schaagen in the year 1657 in the large hospice. He was a young fellow, twenty-three years of age, healthy in body and build. In our presence he took with his left hand the skin from his right shoulder and pulled it to his mouth, like an archer pulls the string on a cross-bow. The skin, however, from the chin he pulled with both his hands into a point like a beard, to his breast, from whence he then pulled the self same skin over his head, covering his eyes in a manner such that we could no longer see them. Even more of a wonder was how his skin, when he let go of it, fell back immediately into its proper place in such a manner as if it had never been touched. In just such a way he pulled the skin from his right knee up and down about half an arm's length. And once he let go of it, a man could not notice that it had once been pulled up.

At the same time we were astonished to discover that the skin on his left shoulder and knee in no way let itself be pulled, as it was in these places so fixed and firm, it would have been impossible.

What however the causes of the soft parts as well as the firm parts were remains to us till this very hour unknown.''

Chapter 29 in ''Rare and Fantastical Observations of the Surgical and Healing Arts, as they were first made public five years ago, shortly after the passing away of the author, after much urging and desire to please the students of the Healing Arts; now for the advantage of High German speakers truly translated and printed. Throughout adorned with copper engravings and supplied with a complete register. Set and printed by Paul Fürstens, Art and Book dealer, Late Wittib and Successors, Nürnberg, An 1675'', pp 186–188. (Reproduced from [11] with permission of the British Library.)

Note that, most interestingly, there is no mention of joint involvement or scarring and that the left side is not involved.

[1] The author points out that Spaniards are the ''most cruel'' people in the world, a remark to be interpreted in the light of the fact that the Dutch had just undergone an 80-year war with Spain.

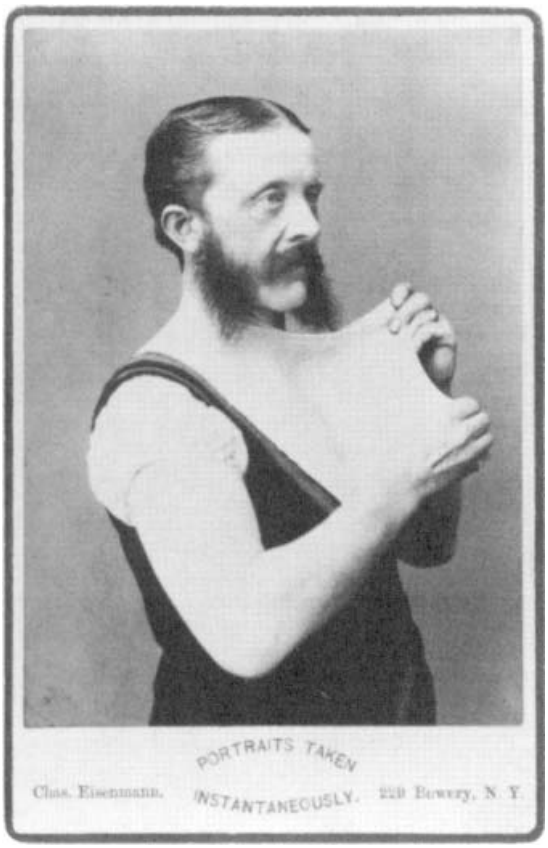

Figure 2. First known photographic documentation of a person with EDS. Felix Wehrle, the ''Elastic Skin Man,'' who, besides having the power to stretch his skin could readily bend his fingers backward and forward, was photographed by Charles Eisenmann[2] in about 1880 (Cabinet card photograph, private collection). The same person was photographed in 1888 in Budapest and his picture was published in 1896 [3]. Wehrle was apparently consigned to the ''museum circuit,'' where he did contortions along with skin stretching. His career seems to have been overshadowed by the more spectacular feats of James Morris, ''The India Rubber Man.'' An Eisenmann portrait in the files of the Museum of the City of New York shows that Morris was able to pull the skin of his throat up over his eyes, an achievement that won him long-standing contracts with various Barnum shows.

The changes in social structure and the advances in biomedical sciences have had as consequences that affected persons earlier enjoyed collectively as curiosities [3,4] are now regarded as individuals suffering from a disease and that the anecdotes and picturesque descriptions with their undoubtedly high didactic value (for examples, see [1]) have been replaced today by more scientific clinical, morphological, biochemical, and functional characterizations published in papers or monographs (for reviews, see [5–8]).

The first partial description of the syndrome was provided by Job Janszoon van Meek'ren (1611–1666), a surgeon from Amsterdam, who in 1657 described a 23-year-old Spaniard named Georg Albes in a report published posthumously in 1668 in ''Belgian'' [9], which was translated into German in 1675 [10] and Latin in 1682 [11], containing one and two etchings (Fig. 1), respectively. It is of note, however, that no mention was made of abnormal scarring or joint laxity and that the left side is not involved. Photography as a new method of clinical documentation started after 1850, and it was in about 1880 that the first known photograph of an individual with EDS was taken, the subject being a traveling showground performer (Fig. 2).

Merit for the classical description of the syndrome should be assigned to the Russian dermatologist A.N. Tschernogubow, who presented at the first meeting of the Moscow Dermatological and Venereological Society, November 13, 1891, a 17-year-old sporadic case with all the hallmarks of the syndrome [13] (translated by Denko [14]; abstracted by Lanz [15] and von Trautvetter [16][3]). He described the skin as pale, lusterless, velvety, thin, hyperextensible (''it is easily pulled away, far beyond normal limits, and rapidly like elastic it regains its normal position''), and friable, with scar formation in a ''strange fashion'' and with secondary wound dehiscence because all sutures cut through the skin; the joints had an extreme degree of mobility, and there was subluxation of a hip and an elbow; there were ''fleshy tumors'' mainly on protruding areas such as the elbows, and cysts on the buttocks and knees; upon palpating the skin, especially that of the extremities, there were round subcutaneous nodules, fine, small, quite hard, and painless, which on removal were found to be deposits of mineral salts; the patient was born feeble, had recurrent convulsions starting in infancy, began to walk only during his third year, and continued to fall frequently; he was rather intelligent, with a ''good head on his shoulders''; gentle natured, as a rule, with a ''good frame of mind and spirit.'' Tschernogubow then concluded that, in the future, ''there might be an opportunity to clarify the observed looseness of the connective tissue that impaired the generalized development of all connective tissue components... It is possible that the development of this looseness is due in part to a deficiency in the supporting structures with a resulting diminution in the ability of the skin to resist deformation.'' In the Russian literature, this condition is called today, quite appropriately indeed, ''Tschernogubow syndrome,'' but because the report was written in Russian, it was not known to most European and American dermatologists. Tschernogubow did not describe further patients (N.P. Bochkov, Moscow, personal communication, 1990).

In 1901, Edvard Ehlers (1868–1937) (Fig. 3), a dermatologist from Copenhagen, reported a patient with ''cutis laxa,'' a marked tendency to hemorrhages, and loose-jointedness [17]. Henri-Alexandre Danlos (1844–1912)

[2]Charles Eisenmann, ''the popular photographer'' as he called himself, was born in the then independent German State of Würtemberg in 1850 and moved to New York, where he opened his studio, in 1879. Eisenmann's interest in human grotesques was reflected in the world of second-string vaudevillians and first-rank freaks; he was the official photographer of the dime museum *demi-monde*. The term ''portraits taken instantaneously'' refers to the collodion process, which transformed the minutes of the daguerreotype exposure into approximately as many seconds and arose when early users of the wet plate system discovered that the process allowed them, for the first time, to record street scenes with arrested movement [4].

[3]Both abstracts are entitled ''cutis laxa'' instead of the original ''cutis laxae,'' and von Trautvetter [16] reported erroneously a 50-year-old woman who was, in fact, the healthy mother of the 17-year-old proband described by Tschernogubow, thereby giving the misleading impression to readers, including Beighton [1] and McKusick [2], that there were actually two different case reports.

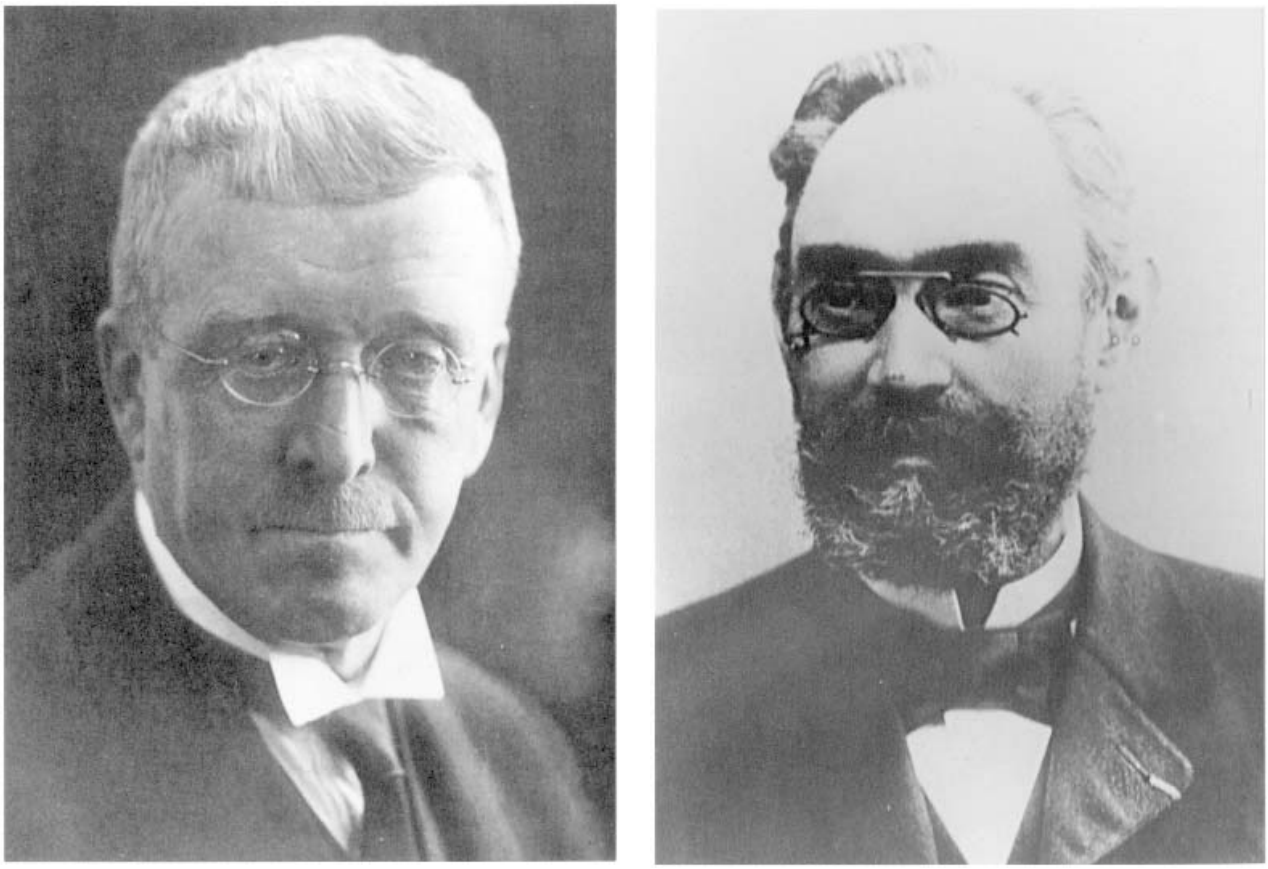

Figure 3. Portraits of Edvard Ehlers and Henri-Alexandre Danlos. Edvard Ehlers (1863–1937) (**left**) presented in 1899, at a meeting of the French Dermatological Society, a law student with lax skin, a tendency to hematoma of the skin, and generalized loose jointedness; he published this case in 1901 [17] but subsequently refrained from doing more work on the condition later bearing his name. Henri-Alexandre Danlos (1844–1912) (**right**) discussed in 1908, at a meeting of the French Dermatological Society, the nature of heaped-up lesions on the elbows and knees of a boy in whom a diagnosis of juvenile pseudodiabetic xanthoma had previously been made by his colleagues, Hallopeau and Macé de Lépinay. Danlos recognized that the skin of this patient was fragile and extensible, and he pointed out the similarity of these features to those of the patient presented by Danlos to the same society in 1899; he published the case the same year [18]. In 1900, Morris presented a similar case, a lad with "elastic" skin and numerous cutaneous nodules [19]. (Courtesy of Dr. Pierre Maroteaux, Paris.)

(Fig. 3), a dermatologist from Paris, described in 1908 another patient with hyperelastic, thin, and fragile skin and explained the molluscoid pseudotumors as chronic contusions of the vulnerable tissues [18]. The terminology became complex and, apart from general terms such as "dystrophia mesodermalis" and "fibrodysplasia elastica generalisata," many terms that were descriptive of various features of the syndrome were employed, such as "dermatorrhexis," "dermatolysis," "Gummihaut," "cutis pendula," "cutis hyperelastica," "chalasodermia," "human pretzel," and "India rubber man." Between 1932 and 1936, the condition received its eponymous title and (by this) achieved scientific respectability [20–23]. In 1936, Sack described a patient with excessive friability of the arteries and called the condition "status dysvascularis" [24]. Dominant inheritance was mentioned as early as 1888, and from then on occasionally [25–28]. In 1949, Johnson and Falls [29] demonstrated autosomal dominant inheritance on the basis of their study of an extensive kindred containing 32 affected persons over five generations. Six years later, Jansen [30] suggested that the defect affected the collagen "wickerwork," which he described as being excessively loose in this disorder.

In 1960, McKusick reported genetic heterogeneity of the EDS in the second edition of "Heritable Disorders of Connective Tissue" [2b]. In 1967, Barabas [31] delineated three clinical types, including the arterial-ecchymotic type, later also called the Sack-Barabas syndrome and now known as the vascular type of EDS, or EDS IV. One year later, Beighton [32] distinguished five clinical forms, and in 1972, McKusick listed seven types [2], including EDS VI, the ocular-scoliotic type, due to lysyl hydroxylase deficiency [33], EDS IV, the arterial-ecchymotic type, due to collagen III deficiency [34], and EDS VII, arthrochalasis multiplex congenita, which has turned out to be caused by a failure of removal of the amino-terminal globular propeptides of procollagen I [35,36,305].

The importance of identifying correctly the type of EDS with which a patient is affected cannot be stressed enough because the natural history and mode of inheritance differ among the types. Unfortunately, much of the older literature does not differentiate clearly between types, and the severe complications of EDS IV are often cited as characteristic of the syndrome as a whole, thereby creating unnecessary anxieties. In this chapter, we try to cover the EDS in a

comprehensive and authoritative way, to weigh the different aspects as objectively as possible, and to clarify earlier concepts and conclusions that today are no longer tenable. To give the reader an idea of how knowledge was gained, references are often cited in a historical sequence.

GENERAL ASPECTS OF THE EHLERS-DANLOS SYNDROME

Common Signs and Symptoms

The signs and symptoms listed in this section may be encountered in various of the different types of EDS, although to variable degrees, and are therefore described together (see Table 1).

Dermatological Features, Facies

The skin is of a white color and lusterless. It is thin, smooth, soft, and velvety, like "wet chamois leather," a "fine sponge," "the skin you love to touch," or "marshmallow," with a doughy feel. The thinness of the skin is best appreciated clinically on the dorsal aspect of the proximal phalange of the fourth finger. The face may be characteristic, with its pale, pastel-colored appearance resembling a portrait by Manet, with irregular scars over the forehead and chin, which are remnants from early childhood (Figs. 4b, 5b, 6a), epicanthic folds, or, later in life, telecanthus, secondary cutis laxa of the eyelid, a crooked nose with a soft cartilage, crowded teeth, and lopsided and floppy ears. In elderly patients, the forehead shows both horizontal and vertical creases (Fig. 6a), giving a peculiar reticular pattern.

Cutaneous hyperextensibility. This is a cardinal feature of EDS, except EDS IV. The skin, and also the mucosa, is hyperelastic[4]; that is, it extends easily and snaps back after release (unlike lax, redundant skin as in cutis laxa). It seems loosely attached to the subcutaneous tissues, and when traction is applied there is a sensation felt by the applier of the skin "coming away." This is particularly striking over areas such as the thenar, the upper part of the chest, and other parts of the body where the skin is usually tightly fixed (Figs. 2,4a, 25d). A "man who could hide behind his skin" is shown in Figure 1 ([11], from which the figure is reproduced).

Skin hyperelasticity should be tested at a neutral site, meaning one that is not subjected to mechanical forces or scarring, such as the volar surface of the forearm. Although in infants hyperelasticity of the skin is difficult to assess because of abundant subcutaneous fat, in the adult, with time, loose folds of skin may appear over the elbow joints like a dewlap (Fig. 4c); the palms may be peculiarly wrinkled (Fig. 25d), the skin of the hands resembles loose gloves, and the soles of the feet appear loose-fitting and resemble moccasins or oversized ankle socks (Figs. 5c–e). Cutaneous hyperextensibility varies with the site of the body, and simple clinical scores have been established to assess it [44,45]. Elderly individuals may also develop abnormally lax skin (acquired cutis laxa), such as of the eyelids (blepharodermatochalasis, see below). *In vivo* methods of measuring skin elasticity have been developed by Grahame and Holt [46], as discussed by Daly [47], and applied by Henry et al. [48]. In both normal individuals and affected persons, the module of elasticity increases with age, always being higher in females than in males, and clinical evidence of Langer's lines shows that skin is not mechanically isotropic [49]. Tensile testing shows this anisotropy to be related only to the magnitude of the initial large extension region and not to the low and final high stiffness [47].

Cutaneous fragility (dermatorrhexis). This is manifested by splitting of the dermis or mucosa following relatively minor trauma, which occurs mainly over pressure points (knees, elbows) and areas prone to trauma (shins, forehead, chin). The wounds often present a gaping, "fish-mouth" appearance with protruding subcutaneous fat lobules and usually bleed little because the edges retract due to the elasticity of the adjacent skin, thereby compressing the ends of the blood vessels. Stitches may hold poorly because the thread cuts through the skin, and dehiscence may occur; in addition, wound healing *per se* is delayed. Stretching of the scars after apparently successful primary wound healing is a characteristic of all EDS types; they become wide, thin, and shiny, with a "cigarette paper," papyraceous, or "burn scar"-like appearance (Figs. 4b, d; 5b, f; 25a, b, f). Due to repeated trauma, the scars often become darkly pigmented or violaceous and usually corrugated by fine wrinkles, and telangiectasia may form within their borders. Many lacerations occur during childhood when the child takes its first steps and then more frequently in boys than girls. Proneness to skin lacerations is later aggravated by the unstable joints and muscular hypotonia, which cause stumbling and falling. The tendency toward skin-splitting sometimes decreases as the patient grows older, but it is difficult to know whether this represents a change in the connective tissue itself or whether it is due to less frequent exposure to trauma when the patient reaches adult life. It has been shown that patients with the thinnest skin have the greatest tendency toward skin-splitting [50]. As a rule, the greater the degree of scarring, the more readily soft tissues inside the body will tear on operation.

Hematoma and bruising. Easy bruisability is common and is frequently the presenting complaint to the pediatrician. It is probably due to the friability of the perivascular connective tissue and the walls of the small blood vessels. Child abuse is often suspected (see legend to Fig. 7). Bleeding from the gums following brushing of the teeth and profuse bleeding after tooth extraction are frequent; gastrointestinal bleeding and hemoptysis, however, are rare (with the exception of EDS IV). The bleeding tendency may lead to an extensive search for a coagulopathy. The Rumpel-Leede test may be positive, indicating capillary fragility. Although a variety of plasma clotting factor or platelet abnormalities have been recognized as a result of a greater awareness of EDS, these are probably chance associations, and most patients will be normal in this regard (see EDS X).

Molluscoid pseudotumors and spheroids. The peculiar mechanical properties of the skin in EDS may lead to a

[4] "Elastic" is used synonymously with "stretchy" to indicate a rubberband-like property — that is, the ease with which a material is reversibly deformed — as opposed to the formal physical definition described by Hooke in 1670:

$$\Delta l = \frac{F \cdot l}{E \cdot q}; E = \frac{l \cdot F}{\Delta l \cdot q}$$

where Δ l is the change in length (l) of a material with cross section (q) when a certain force (F) is applied; E, the elastic modulus, is the constant, characteristic for the material. E is larger the less the material is deformed (e.g., steel) and is a measure of the resistance of a particular material against deforming forces. For a historical discussion about the difference between the terms "popular" and "physical" elasticity, see Cohn [43].

TABLE 1. The Ehlers-Danlos Syndrome

Nomenclature		Signs and Symptoms: Skin			Signs and Symptoms: Joint Laxity	Signs and Symptoms: Other Distinctive Features, Complications	Inheritance	Primary Defect	Relative Frequency	MIM No.
New*	Type	Hyperelastic	Fragile	Bruisable						
Classical type	I	+++	+++	++	+++	Vascular and intestinal complications occasionally	AD	Collagen V-defects, other defects	Common	130000
	II	++	++	+	++				Common	130010
	II/III					Normal wound healing	AR	Tenascin-X deficiency	Rare	
Hypermobile type	III	+	−	+	+++	Arthritis	AD	?	Common	130020
Vascular type	IV	−	++++	+++	+	Rupture of arteries, intestine, uterus; pneumothorax; acrogeria; periodontitis; thin skin with easily visible venous pattern; characteristic facial expression	AD	Abnormal and/or reduced type III collagen	Not so rare	130050 (225350) (225360)
Kyphoscoliotic type	VI A	+++	++	++	+++	Muscular hypotonia, kyphoscoliosis, osteoporosis, microcornea, ruptures of arteries and the eye globe	AR	Deficiency in lysyl hydroxylase (normal enzyme)	Rare	225400
	(VI B)									(229200)
Arthrochalasic type	VII A	++	+(+)	+	+++	Congenital hip luxations, osteoporosis, Wormian bones, fractures	AD	Missing N-telopeptide of α1(I) or α2(I) chains of collagen I	Rare	130060
	VII B	++	+(+)	+	++					(225410)
Dermatosparactic type	VII C	−	++++	+++	+	Skin doughy and lax	AR	Procollagen I N-proteinase-deficiency	Rare	225410
Other Types	(V)	++	+(+)	+	+		XL ?	?	Rarissim	(305200)
	(VIII)	+	++	++	++	Periodontal disease, early loss of teeth	AD	?	Rare	(130080)
	IX**	+/−	+	−	+	Skin lax rather than hyperelastic; bladder diverticula; osteoporosis, exostoses, ''occipital horns''; mental retardation	XL	Copper-transporting P-type ATPase↓	Rare	304150
	(X)	+	+	+	++	Platelet dysfunction, petechiae	?	Defect in fibronectin ?	Rarissim	(225310)
	XI**	−	−	−	++	Arthritis	AD	?	Not so rare	147900
Progeroid type							AR	Galactosyltransferase II↓	Rare	130070
Unspecified type							AR/AD			130090
Unspecified type										225320

MIM numbers [37] refer to the predominant form of each type for which the inheritance is given; MIM numbers in parentheses refer to less common variants, the existence or inheritance of which may be questionable. Inheritance: AD, autosomal dominant; AR, autosomal recessive; XL, X-linked.

*The new nomenclature is defined in [39]; see also Footnote 5, below.

**EDS IX, originally called X-linked cutis laxa, and EDS XI are included for historical completeness but, as mentioned in the text (see also [38,39]), have been reclassified as the occipital horn syndrome, a disorder of copper transport (see Chapter 14, this volume), and the familial joint hypermobility syndrome, a disorder of unknown cause, respectively. The existence of EDS V, VIII, and X as separate entities is questionable (see the text), as indicated by parentheses. For a description of EDS VIB, which clinically resembles EDS VIA but is characterized by normal lysyl hydroxylase activity, see the text.

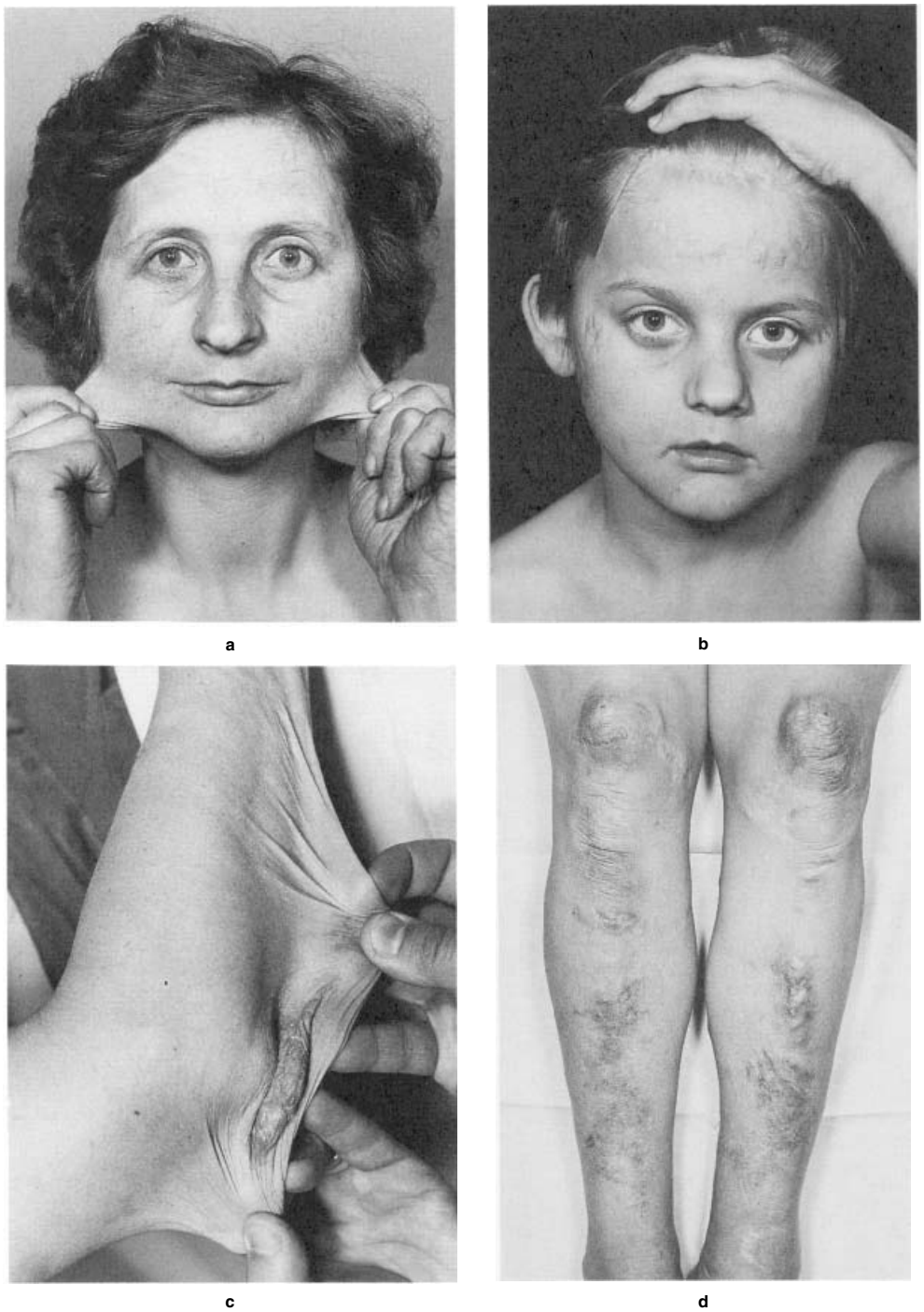

Figure 4. Classical type of EDS (EDS I). A.V. (4 Jul 1933) and her son U.V. (29 Dec 1964) are two of four affected members of a three-generation Swiss pedigree who were nicknamed ''those with the worn-out skin.'' Ultrastructural analysis of the skin of U.V. and A.V. has been reported (patients 1 and 2 in Vogel et al. [40]). (**a**) Hyperextensible skin, moderate scar formation on the forehead, and discrete telecanthus in A.V. (43 years). (**b**) Extensive atrophic broad scars on the forehead and over the nose and cheekbones, which are remnants from early childhood (U.V., 12 years). (**c**) Hyperelasticity and loose folds of skin resembling a dewlap, and a soft, fleshy molluscoid pseudotumor over the elbow (A.V., 43 years). (**d**) Atrophic and hypertrophic hemosiderotic scars in the doughy-feeling skin over the knees and shins (A.V., 43 years).

As a schoolgirl, A.V. had recurrent posterior luxations of the left tibia, the last time being at the age of 16 years. Deliveries of her first two, unaffected children were 2 and 7 weeks before term, unexpectedly and rapidly on the couch at home. Her third child, U.V., and her fourth, unaffected child were born at term. She has had no striae gravidarum. At age 42 years, she had a hysterectomy because of prolapse of the uterus with urinary incontinence; postoperative wound healing was normal.

Follow-up at age 58 showed that this intelligent and witty person had had few further complications; she commented that even her ''loose mouth'' was firmer than her connective tissue.

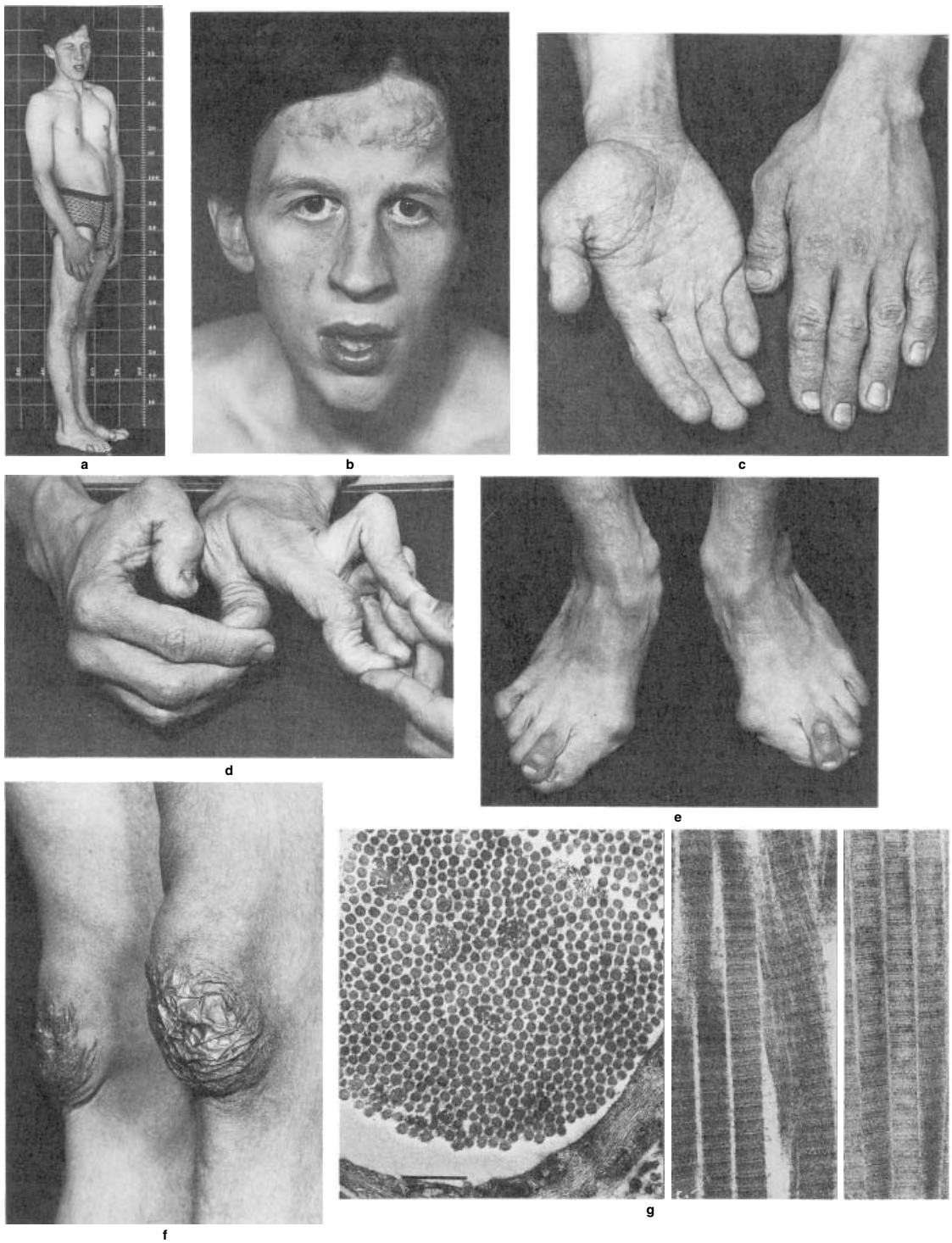

Figure 5. Classical type of EDS (EDS I). B.S. (5 May 1955), at the time of the photographs a 21-year-old man, mentally retarded and institutionalized; nothing is known about his perinatal history; his father is also affected. A heterozygous mutation in *COL5A1* (IVS 28 + 1G > A) predicts a premature stop codon in the mRNA after the insertion of 178 bp of intronic sequence (see ''EDS43'' in [41]). (**a**) Marked pectus excavatum and moderate kyphoscoliosis. (**b**) Atrophic and hypertrophic scars on the forehead. (**c**) The skin over the hands is abnormally wrinkled and redundant, resembling a pair of oversized gloves. (**d**) Hyperlaxity of the small joints of the fingers. (**e**) Dynamic flat foot, hallux deformity, and formation of pseudotumors over pressure points. (**f**) Large, papyraceous, and hemosiderotic scars over both knees. (**g**) Electron microscopic pictures of the dermis of B.S., showing large composite fibrils with an irregular contour and an abnormally wide range of diameters of the round collagen fibrils in cross section (left), and a spiral-like appearance of the composite fibrils in longitudinal section (middle); for comparison, a site-matched skin from an age and sex-matched control is included (right). (Case 4 in Vogel et al. [40] copyright by U.S.-Canadian Academy of Pathology Inc., reprinted with permission; a–f reprinted with permission from Steinmann [42].)

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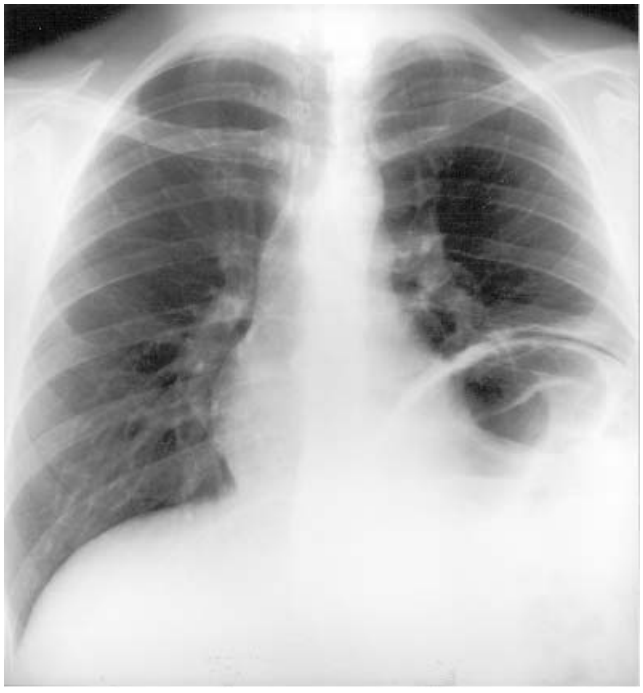

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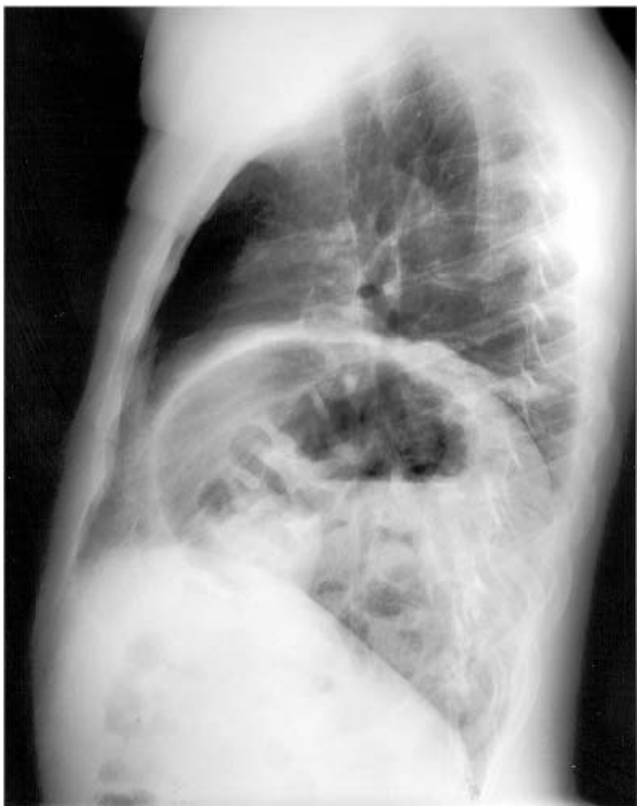

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Figure 6. Classical type of EDS (EDS II). (**a**) A three-generation family with EDS II. The grandmother is 55 years old, the father (E.C., born 29 Jan 1959) 32, the girl 9 (to the left), and the boy 6. Note the multiple scars on the forehead and the horizontal and vertical creases on the grandmother's. (**b**) Anteroposterior and lateral (**c**) radiographic views of the thorax of E.C. demonstrate an elevated diaphragm (relaxatio diaphragmae) on the left side. After a trivial accident with his motorcycle at age 35, E.C. complained of chest pain, and upon the radiological finding of an elevated diaphragm, rupture of the diaphragm with eventration of the stomach or paralysis of the phrenic nerve were considered. A diagnostic thoracotomy revealed relaxatio diaphragmae due to weak tendinous structures. One year later, he rapidly became anemic and had profuse melena due to a Dieulafoy ulcer, which necessitated ligation of the enlarged artery below the muscularis mucosae.

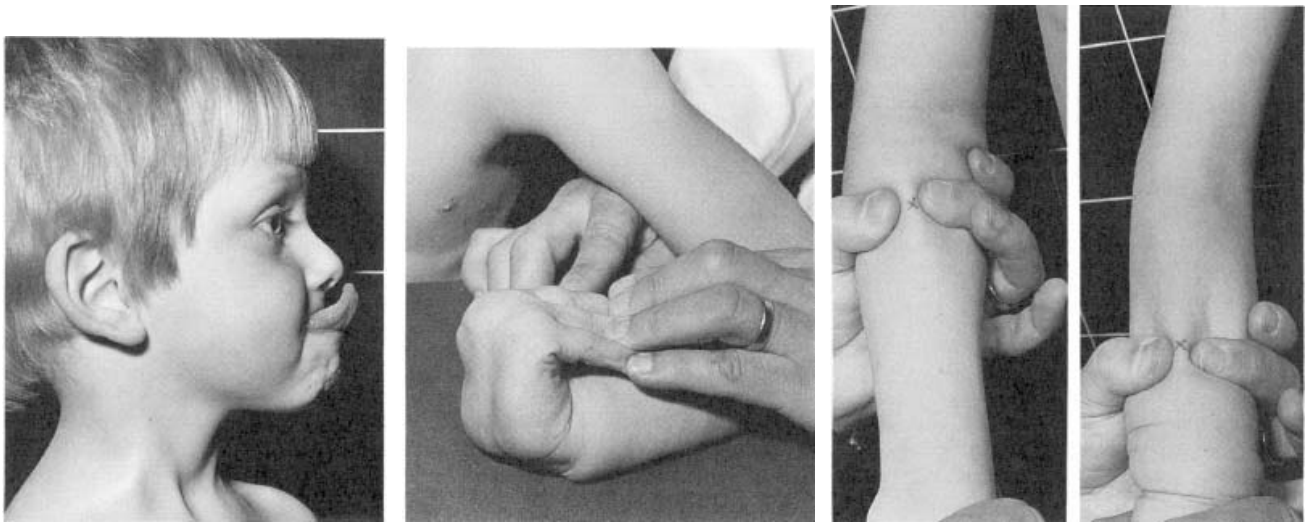


Figure 7. Classical type of EDS (EDS II). (**a**) Positive Gorlin sign (i.e., the ability of a patient to extend the tongue to the tip of the nose). (**b**) Hyperextensibility of the fingers and hands. (**c**) The skin can be displaced up (**left**) and down (**right**) over the subcutaneous fat of the forearm to a great degree. F.K. (20 Jan 1975) was hospitalized at age 2 years and 4 months because of suspected child abuse presenting with multiple hematoma and hemarthros. The diagnosis of EDS was only made at the age of 5 years (Figs. a–c). He is a sporadic case.

Because of recurrent luxations of the right shoulder while exercising, and especially during sleep, and the right patella, he was operated on successfully at the ages of 10 and 12 years, respectively. He luxated his left knee numerous times between the ages of 10 and 12 years but has not done so since. His skin feels doughy but is not excessively fragile.

Suspected child abuse, late diagnosis in a sporadic case, and the difficulty of classifying a patient into a defined group are characteristic of the situation with many individuals with EDS. ((**a**) reprinted with permission from Steinmann [41]).

variety of secondary lesions that develop with time in areas exposed to frequent microtrauma and pressure. Molluscoid pseudotumors are fleshy, heaped-up lesions associated with scars over pressure points such as the elbows (Figs. 4c, 25c) and knees, which usually appear after entering kindergarten (they were already described as features by Tschernogubow in 1891 [13] and Danlos in 1908 [18]). Redundant knuckle pads may develop over deformed and hyperflexed toes. These probably develop through frequent microtrauma, bleeding, and fibrotic scarring and may spontaneously rupture and discharge necrotic material. Spheroids (or spherulae) are small, cyst-like, hard shot-like nodules, freely movable in the subcutis over the bony prominences of the legs and arms. They occur in about one-third of patients [51], are numerous, and feel like hard grains of rice or small pieces of shot. On x-rays, they present an outer calcified layer with a translucent core. In contrast, phleboliths, although presenting a similar appearance, usually are not superficial and are rarely as numerous as spheroids, while calcified cisticerci taper in shape and are found in muscles rather than subcutaneous tissues [51,52]. The spheroids represent subcutaneous fat lobules that have lost their blood supply and then become fibrosed and calcified [53]. Alternatively, they may present as "subcutaneous mobile encapsulated lipoma" with or without tenderness [54].

Other dermatological features. These comprise hyperkeratosis follicularis; piezogenic papules, which are small, painful, reversible herniations of underlying adipose tissue lobules through the fascia into the dermis, such as on the medial and lateral aspects of the feet upon standing (Fig. 8) [55]; elastosis perforans serpiginosa (Miescher elastoma, [56]; MIM 130100) (see EDS IV), not to be confused with mycosis; acrocyanosis and chilblains; and a conspicuous lack of striae gravidarum, which are focal tears in the dermis perpendicular to the direction of stress without disruption of the overlying epidermis.

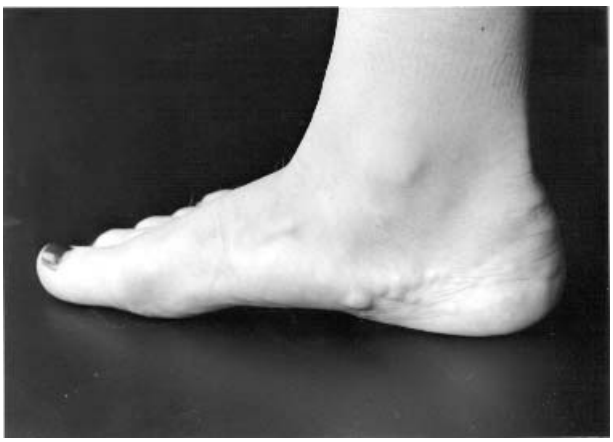


Figure 8. Classical type of EDS (EDS II). Piezogenic papules in a 20-year-old woman, M.M. (21 May 1973). These are small herniations of adipose tissue lobules through the fascia on the medial or lateral aspect of the foot upon standing, which may be painful and require support or special shoes.

Orthopedic Aspects

Significant orthopedic abnormalities are, in decreasing order of frequency: pes planus, joint dislocations, spinal deformity, joint effusions, thoracic cage deformity, osteoarthritis, talipes, and congenital hip dislocation [57].

Hyperextensibility or hyperlaxity of the joints. This is also frequently called "hypermobility" of the joints and is a result of laxity of the joint capsules, ligaments, tendons, and possibly muscular hypotonia. It may be a source of enjoyment to patients to show their double-jointedness, and in the past certain individuals amused people as contortionists in sideshows (for anecdotes, see [1,2]). On the other hand, it may cause the patient considerable discomfort in walking, writing, or performing other skilled functions. Questions such as "Are you double-jointed?" and "Can you do tricks with your fingers?" may elicit a remarkable display of maneuvers from a patient with EDS, such as finger contortions, placing the ankles behind the neck, or placing the head between the knees while bending backward. As a rule, joint laxity is generalized, affecting both large and small joints (with the exception of EDS IV), and is usually noted when the child starts to walk. It also depends on age, sex, and race, and on the dominant side of the body, especially with regard to the hands, and increases during pregnancy and decreases with the passage of time [58]. Several grading systems for the objective semiquantification of joint hypermobility using a simple score have been proposed [39,58–60], among which the "Beighton score" [58] has become the most widely accepted screen for detecting generalized hypermobility and correlates well with more quantitative, instrument-dependent methods (Fig. 9).

Ligamentous laxity may be observed by pulling on the distal phalanx of a finger, which results in a considerable lengthening of the finger ("telescoping"), with radiological widening of the joint spaces [61]. Dynamic swan neck deformity is occasionally observed.

Dislocations. Occasional or habitual dislocation of the shoulder, patella, digits and thumbs, hip, radii, and clavicles is common [62], and its frequency is proportional to the degree of joint laxity, although in some persons a surprising range of joint movement can occur without any problem. Dislocations are usually resolved spontaneously and are easily reduced by the patient. Dislocation of the shoulder may follow a simple everyday movement, such as putting on a coat or raising an arm during a school class. Dislocation of the interphalangeal joints of the thumb leads to difficulty in using a pinch grip (Fig. 20).

Joint effusions. These are common and may be persistent or recurrent. They are associated with activity, affect mainly joints of weight-bearing parts (in decreasing order: knees,

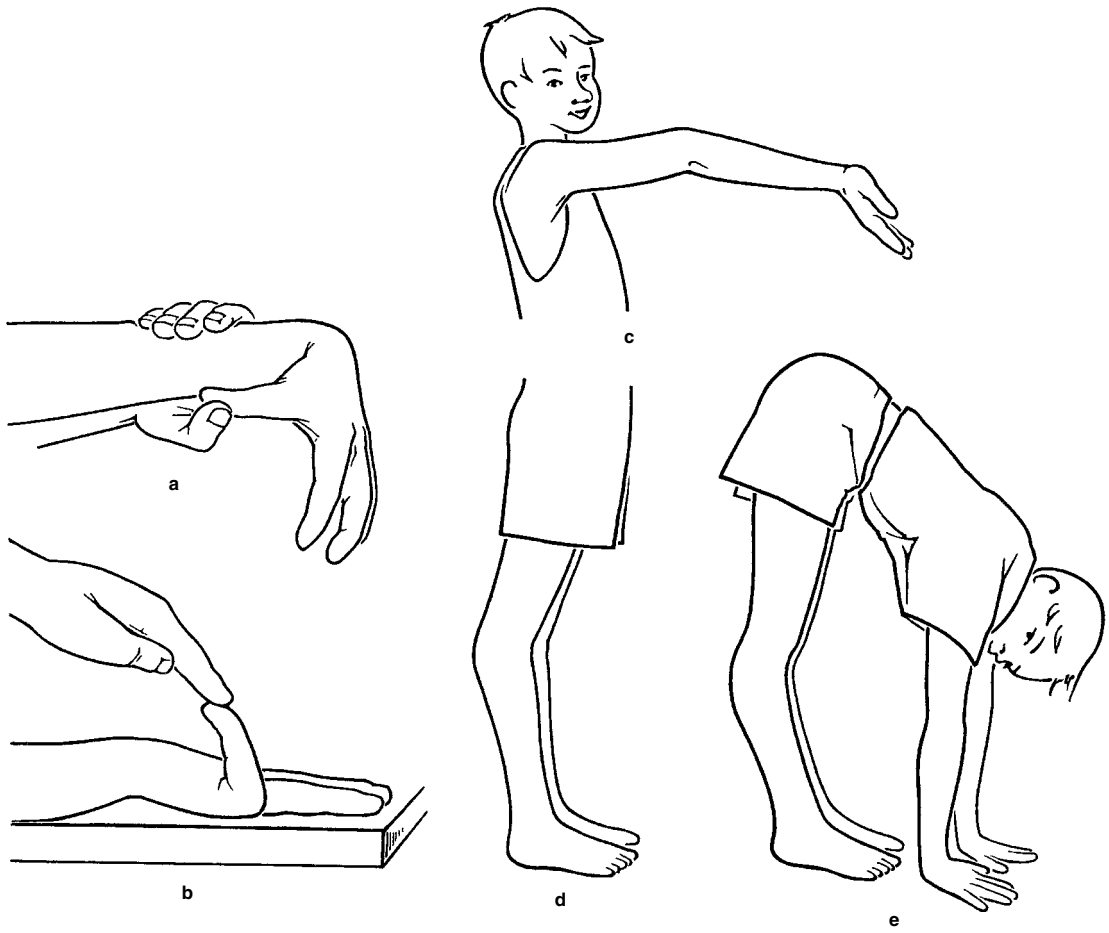


Figure 9. The "Beighton-score" to assess joint hypermobility. (**a**) Passive apposition of the thumb to the flexor aspect of the forearm; one point for each hand. (**b**) Passive dorsiflexion of the little finger beyond 90°; one point for each hand. (**c**) Hyperextension of the elbow beyond 10°; one point for each elbow. (**d**) Hyperextension of the knees beyond 10°; one point for each knee. (**e**) Forward flexion of the trunk with the knees fully extended so that the palms of the hand rest flat on the floor; one point.

A score of 5/9 or greater defines joint hypermobility [58]. (Figure drawn by Susanne Staubli, University Children's Hospital, Zürich.)

ankles, elbows, and also digits), and commonly appear at the end of the day. The tendency for joint effusions to develop is again proportional to the degree of joint laxity. Hemarthrosis may occur, mainly in EDS IV.

Joint instability. Joint instability results from lax ligaments and poor muscle tone. Children tend to walk late and fall easily because of the difficulty in controlling their unstable limbs. Unstable ankles and knees frequently need supportive bandages. The gait is typical: the feet are placed firmly and flatly on the ground, and the hips are hyperextended during weight-bearing to counteract the genu recurvatum. Running or wearing high heels may be impossible. The handshake may be characteristic and sometimes allows a diagnosis at the first encounter; the musculoskeletal structure of the hands seems to collapse on pressure, and the hand feels "like a bag of little bones." Trapeziometacarpal abnormalities lead to instability of thumbs and fingers and hence difficulty in picking up large or heavy objects with one hand or unscrewing bottle tops [63]. One of our patients never wanted to wash dishes for friends because she often crashed the plates — a not unprecedented excuse also for normal individuals — and, like this, other aspects of clumsiness in EDS may be explained, at least in part, by disturbed proprioception. Spinal deformity is usually due to strains and confined to thoracolumbar kyphoscoliosis, which progresses with age (Fig. 29e). Chest deformity such as pectus excavatum or carinatum does occur (Fig. 5a). Spondylolisthesis is not uncommon (see legend to Fig. 11). Foot deformities are the most prevalent signs: congenital clubfoot due to intrauterine malposition, progressive static and dynamic pes planus, talipes equinovarus, and hallux valgus (Figs. 5e, 23d, 25 g, 29c, 30d).

Osteoarthritis. Osteoarthritis as a consequence of joint instability is a major problem. In one study, 20 out of 100 patients were affected, or as many as 16 out of 22 patients over 40 years of age [57], the main complaints being the knees, hands, ankles, general joints, and shoulder. Here we might cite Beighton's patient, who said "When I was a baby they called me a floppy infant, and now the doctors tell me that I am a loose woman" [62], and might add that she may soon be a stiff elderly lady due to osteoarthritis.

Bursae. Over the olecranon and prepatellar region, enlarged bursae are often encountered, and these have to be distinguished from hematoma or molluscoid pseudotumors, which occur at the same sites. The excision of these bursae usually yields reasonably satisfactory results [1].

Bones. Bone involvement was not considered a feature of EDS until it was recognized that osteopenia is a constant feature of EDS VI, VIIA, VIIB, and VIIC, and a propensity to fractures has been reported in the latter group (see in EDS VII). In a case-control design study of 23 EDS individuals (10 with classical EDS and 13 with EDS III), Dolan et al. [64] showed that EDS subjects have a previously unrecognized tendency to fracture characterized by a low bone mass and abnormal bone structure, and concluded that this finding is likely to be multifactorial, with an inherited structural element accentuated by reduced mobility and exercise, and the possibility of a proprioceptive defect of the hypermobile joints. Carbone et al. [65] come to the conclusion that their 23 cases with EDS III had a significantly decreased bone mineral density at the femoral neck compared with controls but that this difference disappeared after adjustment for body height, weight, and physical activity levels.

Chronic joint and limb pain. This is a common complaint, but skeletal radiographs are normal. Frequently, it is difficult to establish the precise anatomical localization of the pain [39]. Osteoarthritis is more pronounced and pain is worse in the nondominant hand. Pain is worse during pregnancy because there is normally an increase in joint mobility in this state. Pain, swelling, dislocation of joints, a history of surgery of the joints, and impaired ambulation seem to be more frequent and more pronounced in patients with EDS III than in those with EDS I, II, or IV (see below, the hypermobile type of EDS [66]).

Dural ectasia. Enlargement of the spinal canal, with or without clinical complaints such as weakness in the lower extremities and abnormal gait, has occasionally been reported [67,68]. Dural ectasia and spinal canal widening seem to be more prevalent and pronounced in Marfan syndrome than in EDS, and their extents were found in two small series of ill-defined patients to be independent of age, sex, or symptoms, such as low back pain or sciatica [69]. Dural ectasia seems to be a major diagnostic criterion in Marfan syndrome [70].

Pregnancy, Obstetric, and Gynecological Features

The pregnancy of a woman with EDS bears risks for the newborn as well as the woman. During pregnancy, joint laxity may increase, but women enjoy the absence of striae gravidarum. Varicose veins in the legs and varicosity of the vulva may become prominent. Cervical insufficiency may lead to miscarriage or premature birth. Gross abdominal hernia and spinal deformity with backache may develop.

Fetal complications include prenatal rupture of the membranes (if the fetus is affected) and prematurity. Out of 18 children with nonspecified EDS, 14 were premature, and 13 of these had premature rupture of the membranes; in contrast, normal siblings were never premature, demonstrating that the fetal membranes are affected to the same extent as the fetus [71]. Breech presentation is more frequent if the baby is affected, and therefore hypotonic, and may lead to dislocation of the hips or to arms with injury of the plexus brachialis (Erb palsy).

Delivery may be unusually smooth or even precipitous (see legend to Fig. 4); however, severe pre- or post-partum hemorrhage may occur through perineal tears, the extension of episiotomic incisions, tearing by forceps, or prolapse of the uterus and bladder, all of which occur mainly in EDS I and IV. Rupture of the uterus and large arteries is a typical complication of EDS IV. The pubic symphysis may distract and cause pain for several months. Dehiscence of sutured incisions of skin, mucosa, or the uterine wall is frequent in EDS.

Dyspareunia and sexual dysfunction are occasional complaints in the classical and other types of EDS [72,73] and result from tissue fragility leading to small tears and recurrent infection in the vaginal mucosa and the skin of the external genitalia. Coital vulval laceration has been described occasionally, is possibly underreported for various reasons, and may be erroneously considered to be due to rape [74].

Gastrointestinal Complications

Gastrointestinal manifestations in the EDS can be subdivided into those that are a consequence of tissue extensibility and those that result from tissue fragility (see EDS IV). They include gastric, duodenal, and colonic diverticula, visceroptosis, gastric atony, megaesophagus, and megacolon. Constipation is a common complaint and probably results from flaccidity of the large bowel. Gastroesophageal reflux and irritable bowel syndrome are common complications of classical and hypermobile

EDS [75]. Inguinal and umbilical herniae are frequent and may recur after surgical correction. Femoral, incisional, hiatal, and diaphragmatic herniae, relaxation of the diaphragm (Fig. 6b), and even its eventration have been reported. Gastric, duodenal, jejunal, and colonic diverticula may lead to bleeding or perforation [76–78]. Recurrent rectal prolapse usually resolves before the age of 4–5 years ([79]; own observations).

Neuromuscular and Psychological Features

Primary muscular hypotonia in the newborn, and especially the premature, is frequent and may be so severe, especially in EDS VI, that affected infants cannot be breast-fed and need teats with especially large holes. Neuromuscular disease is often suspected, especially when clubfoot or hip dislocation coexist. A complete neuromuscular workup is usually performed in such "floppy infants," with unrevealing results. Hypotonia is also considered responsible for the increased frequency of breech presentation, which, together with the laxity of ligaments and joints, favors Erb palsy and congenital hip dislocation. In children, joint hypermobility and hypotonia may cause delayed motor development, problems with ambulation (delayed walking, frequent stumbling), and mild motor disturbance ("clumsiness"). Consequently, hypotonia may be maintained through the poor development of muscles resulting from an avoidance of exercise and activity because of the laxity and instability of the joints.

Fatigue is a frequent complaint. Muscle cramp in the calves, especially at night, is common in children but gradually disappears. Histology reveals that the connective tissue in the muscles is very sparse, so that the muscle bundles are hardly held together [1]; it may be speculated that the muscle fibers might lose their parallel orientation when they contract, thus decreasing mechanical efficiency. That dysfunction of the Golgi corpuscles embedded in the loose tendons may play a role in determining hypotonia is another possibility. Impaired proprioception has been documented in the hypermobility syndrome (MIM 147900), but its underlying mechanism remains open [80].

Genuine intelligence is normal; however, prematurity predisposes to birth trauma and complications, such as intracranial hemorrhage, respiratory distress syndrome, or sepsis, which may lead to epilepsy, as observed also in the patient described by Tschernogubow in 1891 [13]; epilepsy, however, has occasionally been reported in EDS [81]. The social consequences of disfiguring scarring, particularly of the face, and musculo-skeletal deformity may lead to personality problems and psychosocial distress, which is also common in chronic illnesses in general [73]. As a rule, there are many more affected women than men in EDS support groups (as is the case for other support groups as well). This may reflect a difference between the sexes in coping with the handicap rather than any true difference in clinical severity; however, this fact is important for the critical evaluation of studies based on circular letters, which may be skewed by selection bias [66,82]. Living with fear, living with pain, feeling stigmatized, and experiences of nonaffirmation in health care are identified as conditions leading to limited self-actualization in all areas of daily life [83].

Nerve compression and traction of the brachial and lumbar plexuses through hematoma and luxations at birth or later have occasionally been reported ([1,84–87]; H.A. with EDS VI, case 19 in legend to Figs. 22 and 23c). Occasionally, patients complain of cutaneous hyperesthesia/hyperalgesia when friction is applied to the skin, such as while drying with a towel or during the measurement of blood pressure [88]. Some patients report insufficient analgesia during minor surgery (e.g., dental and oral surgery) and may be characterized as hysterics [89,90]; it has been shown that the lack of effectiveness of local anaesthetic solutions is not due to their rapid dispersal through the loose connective tissues and therefore is unlikely to be compensated for by simply increasing the amount used [91]. An ill-defined polyneuropathy in two siblings of consanguineous parents together with typical signs of EDS VI has been described [92].

By far the most severe neurological complications are those due to intracerebral hemorrhages, such as transient aphasia or amaurosis, and hemiplegia or fatal stroke-like events caused by multiple arterial aneurysms or arteriovenous fistulae [93,94], which are typical findings of EDS IV.

Cardiovascular Features

Structural cardiac malformations are rare and are probably rather chance associations (for a review, see [95]). Mitral valve prolapse and, less frequently, tricuspid valve prolapse are due to redundant chordae tendineae and valve cusps [96]. However, stringent criteria should be used for the diagnosis of mitral valve prolapse [97]. Mitral valve prolapse and proximal aortic dilatation should be diagnosed by echocardiography, computed tomography, or magnetic resonance imaging. Mitral valve prolapse is a common manifestation, and aortic root dilatation may not be as uncommon as previously thought [98], although Dolan et al. [99] reported that none of their 12 patients with EDS I and II or 18 patients with EDS III had mean aortic diameters outside the normal range. In a small proportion of patients with EDS, aortic dilatation may be progressive [96]. Dilatation of the aortic root should be diagnosed when the maximum diameter at the sinus of Valsalva exceeds the upper normal limits for age and body size [100,101]; in such a case, annuloaortic ectasia needs to be considered in the differential diagnosis. Tortuosity of the aorta and its major thoracic divisions, including the coronary arteries, and peripheral pulmonary stenoses [102], aneurysm of the sinus Valsalvae [103,104], aortic root dilatation, and dissection of the aorta, sometimes familial, have been observed ([2c,105,106], own observation). Spontaneous rupture of large arteries [31,107,108] and intracranial aneurysms and arteriovenous fistulae [93,94] are all typical of EDS IV, and to a lesser degree of EDS VI, but may also occur in EDS I, sometimes even in persons with minimal external findings [109].

Acrocyanosis is a common complaint. The association of EDS II and III with orthostatic intolerance and chronic fatigue syndrome has been reported and tentatively explained by the connective tissue abnormality leading to excessive venous pooling and an exaggerated hemodynamic response [110].

Ophthalmological Aspects

It is not surprising that such a complex and delicate organ as the eye should be affected in many different ways, as tabulated by Pemberton et al. [111]. Extraocular signs are not uncommon. In a series of 100 affected patients, 27 had epicanthic folds (which lessen or disappear with age, or change to telecanthus, giving the impression of widely spaced eyes), seven had blueness of the sclerae, seven had strabismus (probably due to laxity of the tendons of the extrinsic muscles of the eyes), and eight had myopia, while redundant skin on the upper eyelids (blepharodermatochalasis, not to be confused with acquired or inherited blepharochalasis, in

which the whole eyelid is lax [112]; MIM 109900, MIM 110000), which makes putting on mascara difficult, and ease of eversion of the upper lid (Méténier's sign) were frequently encountered; none of the patients in this series, however, had any serious ophthalmological lesion [113]. Ocular signs are not frequent and include keratoconus (forward bulging and thinning of the central part of the cornea, which is a rare finding [114]; however, see also [115]); megalocornea; microcornea, which occurs mainly in EDS VI but occasionally also in EDS I (see [116]); myopia, due to distortion of the eye-globe or, very rarely, to subluxation of the lens; brittleness of the sclera and cornea; angioid streaks; retinal detachment; and retinitis proliferans due to hemorrhage. Eye fatigue may be a result of inappropriate eye movements because of increased concentration, tension, general fatigue, immobilization, or pain, such as a stiff neck. It is of interest that in the majority of individuals with sight-threatening lesions, autosomal recessive inheritance is likely (see EDS VI). Pulsating exophthalmos in EDS IV is due to retrobulbar arteriovenous fistula. It is of note that vision is not impaired by any corneal deficiency in patients with the classical type of EDS resulting from mutations in the collagen V genes. Since abnormal cauliflower-like collagen fibrils are observed in dermis from these patients (see classical type of EDS below) and in dermis and cornea of pN-knockout mice (see "Animal Models and Lathyrism" below), it is remarkable that the cornea, an organ rich in collagen V, seems to be normally translucent in these patients (see also Chapter 1, Part V, this volume).

Additional Features

Hemoptysis, hematothorax, and spontaneous and recurrent (hemato-)pneumothoraces, with or without mediastinal and subcutaneous emphysema, are typical of EDS IV [117]. Tracheobronchiomegaly (MIM 275300) has been reported [118,119].

Anatomical abnormalities of the urinary tract, such as urinary bladder diverticula, are common and usually asymptomatic. Cuckow et al. [120] reviewed 24 cases of bladder diverticula, all observed in males aged between $1\frac{1}{2}$ and 49 years, with 80% presenting before 16 years (for a further review, see [121]). Vesico-ureteral reflux may lead to recurrent urinary tract infection and renal insufficiency [116]. Multiple and large bladder diverticula are especially typical of the cutis laxa syndromes and the Menkes/occipital horn syndrome (EDS IX) (Chapters 10 and 14, respectively, this volume).

Oral aspects of the EDS have been described by Barabas and Barabas [122] (see also "Recent Developments"). The oral mucosa is fragile, easily bruisable, and often presents hemorrhagic blisters; bleeding from the gums is a common complaint. The gingival tissues are more liable to injury, leading to periodontal disease with early loss of teeth. The teeth are often crowded but are otherwise normal [1]; hypoplastic areas of enamel, the formation of pathologic dentin, and teeth with high cusps and deep occlusal fissures, stunted and deformed roots, and large pulp stones have been reported [8,123]. These manifestations, however, are not clearly associated with a particular type of EDS. Histological abnormalities include changes in the amelo-dentinal and cemento-dentinal junctional areas, irregular and ill-formed secondary dentinal tubules, vascular dentinal inclusions, and fibrinoid gingival deposits. Chronic habitual luxation and arthrosis of the temporo-mandibular joint may occur but rarely needs condylectomy [124]. The Gorlin sign (i.e., the ability of the patient to extend the tongue to the tip of the nose; Fig. 7a) is rather more astonishing or amusing than specific, 50% of EDS patients having this ability compared with 10% of controls [8]. A high prevalence of speech and swallowing difficulties has been reported [125]. In some individuals, inappropriate use of the voice, such as by shouting, may cause recurrent aphonia for which systematic voice training may be needed.

Radiological aspects of EDS have been reviewed (see above and [51,61,126,127]) and include telescoping fingers, calcified spheroids, and spondylolisthesis. Osteoporosis is a prominent feature of EDS VI and to a lesser degree also of EDS VII; exostoses may be observed in occipital horn syndrome (EDS IX) and EDS VII, acroosteolysis in EDS IV, and Wormian bones in EDS VII (see "EDS VII").

The fragility of the skin and most organs (except the skeletal system) may come as a bad surprise for the unaware surgeon at operation and may be remarked upon by the pathologist during subsequent autopsy. The friability of organs has frequently been described as being like "wet blotting paper" and making surgical incisions as "like cutting with a knife through butter" or through "cold porridge."

Diagnostic Approach to the Patient

As with most other genetic disorders, the first step in making the diagnosis of EDS is clinical awareness. The possibility of EDS will be brought to mind by complaints and physical findings such as hyperextensible joints, hyperelastic skin, and abnormal wound healing, or by personal history such as easy bruisability, habitual luxations, joint effusions, and recurrent herniae. The next step then consists of looking for elements that can reinforce the diagnostic suspicion, positive family history, and a more detailed personal history with regard to possible connective tissue disease such as prematurity, breech presentation, hypotonia, and delayed motor development. The importance of comprehensive anamnestic and clinical assessment of individuals cannot be overemphasized.

If the suspicion of a connective tissue disorder can be substantiated, an attempt should be made to understand whether it is a "genuine" connective tissue disorder such as the EDS or whether it is part of a "broader" genetic disorder or syndrome. Although exceptions exist, the presence of significant mental retardation and/or overt dysmorphic features argues against EDS. If clinical and anamnestic evidence points to an isolated connective tissue disorder such as EDS, consultation of textbooks, experts, and synoptic tables (see Table 1) may help in establishing which EDS type is more probable. This is crucial for further diagnostic investigations, such as skin ultrastructure and/or fibroblast culture and/or urinary cross-link products.

The clinical assessment of individuals with hyperextensible and thin skin and/or with lax joints needs some clinical experience, not so much in the case of patients with marked findings but rather in those with moderate findings; clinical experience is required especially in the latter group because the findings are graded according to age, anatomical location, sex, and race, as they are in normal individuals. Skin hyperelasticity [44,45] and thickness, and joint hyperextensibility [59,60,62], can be assessed semiquantitatively with scoring systems or with physical devices (see also Fig. 9). Unless diagnostic findings such as congenital bilateral hip dislocation and generalized joint laxity are present (EDS VII), the diagnosis of EDS is difficult in neonates and infants because the adipose tissue is normally abundant in healthy subjects and masks

hyperelasticity of the skin, because muscular hypotonia is frequent and unspecific, and because bruising and splitting of the skin do not occur until the child begins to walk and fall. In the adult individual, the diagnosis of EDS is usually made easily and can sometimes be made instantly — the characteristic handshake upon the first encounter in the office or at a cocktail party may be diagnostic; the velvety touch of the dorsal skin on a darkened dance floor may immediately enlighten the clinical geneticist.

Since all the signs are graded and age-, sex-, and race-related, semiquantitative assessments of the extensibility of the skin [44,45] and joints [59,60,62] must be compared by the same observer with the appropriate controls, and because these parameters also vary with anatomical location, identical sites should be chosen for better comparison, such as the skin over the fourth metacarpal, the thenar, the dorsum of the wrist, the neck, and so forth.

The diagnosis of EDS is difficult in newborns or infants because the laxity of the joints may be indistinguishable from that of infants with unrelated muscular hypotonia, hyperelasticity of the skin may be masked by the abundant subcutaneous tissue, and other skin changes are not easily assessed because the bruising and skin-splitting tendency do not usually become apparent until the child begins to walk and fall. In older persons, the skin is more redundant and somewhat lax, but atrophic, hemosiderotic scars easily distinguish the condition from cutis laxa.

Classification, Nomenclature, and Relative Frequency

The classification of EDS is first made on clinical grounds and substantiated by consideration of the most likely inheritance pattern and by biochemical and molecular analysis when possible. This is reflected in the historical development of EDS classification, which comprised three types in 1967 [31], five types in 1968 [32], and seven types in 1972 [2]. The formerly most generally used classification, which comprises ten or more types, is based on a combination of clinical, genetic, and biochemical criteria (Table 1) [37,38].

Over time, it became apparent that the diagnostic criteria established and published in 1988 [38] do not adequately discriminate between the different types of EDS or between EDS and other phenotypically related conditions. In addition, elucidation of the molecular basis of several types of EDS has added a new dimension to the characterization of this group of disorders. A revision of the classification of EDS, the "Villefranche Nosology, 1997," has thus been proposed, based primarily on the cause of each type [39][5]. Major and minor diagnostic criteria are defined for each type and complemented wherever possible with laboratory findings. This simplified classification will aid in accurate diagnosis of the types of EDS, thereby facilitating development and improvement in the following aspects: (1) diagnostic uniformity for clinical and research purposes, (2) natural history, (3) management, (4) genetic counseling, and (5) identification of potential areas of research [39].

In this revised Villefranche nosology [39], a simplified classification of EDS into six major types is proposed (Table 1). The guiding principle in formulating the classification was its usefulness to the "generalist." For each type, major and minor diagnostic criteria are defined. A major criterion has high diagnostic specificity because it is infrequent in other conditions and in the general population. The presence of one or more major criteria is either necessary for clinical diagnosis or highly indicative, warranting laboratory confirmation whenever possible. A minor criterion is a sign of lesser diagnostic specificity; the presence of one or more minor criteria contributes to the diagnosis of a specific type of EDS. However, in the absence of major criteria, minor criteria are not sufficient in themselves to establish the diagnosis. Their presence alone might be more suggestive of other EDS-like conditions, the nature of which will be elucidated when their molecular basis becomes known [39].

In this chapter, both nosologies are used interchangeably — the traditional one, using Roman numerals I to X, as well as the newly designated types — for reasons of convenience and completeness.

In many cases (20–40% as estimated by Hollister [128]), however, an unambiguous classification is not possible because there is a phenotypic continuum and, especially in children, the appearance of recognizable symptoms is age-dependent.

The relative frequencies of the different EDS types are not known precisely. In 1966, during a survey in southern England, Beighton examined a unique series of 100 patients with EDS. Seventy-one of them were members of 50 separate families, while 29 (17 males and 12 females) were sporadic cases [1]. A follow-up of eight of the 17 sporadic males revealed that three had produced children with EDS I, two had normal offspring, and three had no children [129]. Among the 50 families, EDS I had occurred in 22, EDS II in 17, EDS III in six, EDS IV in three, and EDS V in two. Although these figures are quite realistic in genetic terms, they are prone to inherent ascertainment bias, the more severely affected patients being most likely to have been diagnosed and hence reported in the literature, whereas patients with milder symptoms (EDS II) are more likely to have been able to escape medical attention and malpractice; on the other hand, severe cases leading to early death (EDS IV) are frequently missed, especially when sporadic. Individuals at both ends of the spectrum of severity may therefore be underrepresented.

Differences between individuals with the same type of EDS may reflect inter- or intra-familial variability or genetic heterogeneity (see EDS I, II, IV, VI, VII, IX). The definition of a genotype may then help to describe better the phenotypic spectrum. The Villefranche nosology will doubtless have to be revised and extended in the future as distinct, specific clinical signs and molecular defects are recognized and as genotype-phenotype correlations become clearer.

Prevalence and Epidemiology

There are no well-founded figures for the prevalence of EDS. While Beighton [1] gives a figure of 1:156,000 for southern England, McKusick states that EDS is one of the more frequent heritable disorders of connective tissue [2]. It seems likely that only a fraction of patients are ever diagnosed, owing to the lack of symptoms alarming to both patient and doctor and to the fact that single symptoms, such as habitual luxation of the patella, easy bruisability, or recurrent inguinal hernia, may not be considered as part of one generalized disorder. With increased medical awareness, however, the presumed rarity seems likely to disappear. At the other end of the spectrum, Holzberg et al. [45] claim a frequency of 9% in a predominantly black population. The aggregate frequency of EDS may be about 1:5,000 births,

[5]In this chapter, we designate the different types of EDS with adjectives throughout rather than a mixture of nouns and adjectives as given in [39].

with no racial or ethnic predisposition. The syndrome has been observed all over the world [1,2].

THE CLASSICAL TYPE OF EDS — EDS TYPE I (MIM 130000) AND EDS TYPE II (MIM 130010)

Diagnostic Criteria

These two types of EDS are described together because they differ only in their degree of involvement and occasionally are allelic. They comprise about 90% of all cases of EDS and correspond to the first historical descriptions, hence the term "classical." Locus heterogeneity has been demonstrated. The classical type of EDS is inherited as an autosomal dominant trait and characterized as follows [39]:

- Major diagnostic criteria
  - Skin hyperextensibility
  - Widened atrophic scarring (manifestation of tissue fragility)
  - Joint hypermobility
- Minor diagnostic criteria
  - Smooth, velvety skin
  - Molluscoid pseudotumors
  - Subcutaneous spheroids
  - Complications of joint hypermobility (e.g., sprains, dislocations, subluxations, pes planus)
  - Muscular hypotonia, delayed gross motor development
  - Easy bruisability
  - Manifestations of tissue extensibility and fragility (e.g., hiatal hernia, anal prolapse in childhood, cervical insufficiency)
  - Surgical complications (postoperative herniae)
  - Positive family history

Severity, Special Signs, Features, and Complications

EDS I (MIM 130000). In EDS I, skin involvement is marked and joint laxity is generalized and gross, with musculoskeletal deformity and diverse orthopedic complications. This form is also called the "*gravis type*" (severe type) according to an earlier nomenclature. Its frequency has been estimated to be 1:20,000 [5]. Prematurity occurs in ~50% of cases. It is in this severe (gravis) group, in addition to EDS IV, that internal complications such as aortic and bowel rupture may occasionally occur.

EDS II (MIM 130010). EDS II is probably the most prevalent type of EDS. It has all the stigmata of EDS I to a minor degree, and some patients may easily remain undiagnosed. This form is also called the "*mitis type*" (mild type) according to an earlier nomenclature. Joint laxity is limited and may be confined to the hands and feet. Skin involvement is less evident. Prematurity does not occur more frequently than normal. Mitral valve prolapse is rare.

The "late onset of EDS" [130,131] may represent mild conditions that become manifest only in adulthood; other cases may be acquired, especially when the symptoms appear late in life and are restricted to skin in a particular area [132].

Genetics

The first pedigrees compatible with autosomal dominant inheritance were published as early as 1888 [25–28,133]. In 1949, Johnson and Falls [29] demonstrated autosomal dominant inheritance on the basis of an extensive kindred containing 32 affected persons (21 males and 11 females) over five generations. Among them, a consanguineous couple of affected cousins had eight children, three of whom were unaffected, three of whom had the same stigmata as their parents, and two of whom had the condition to a very severe degree. The authors concluded that the latter two may have been homozygous in which case all of their children should have been affected; unfortunately, no follow-up is available. The heterozygous mother also had four miscarriages, which, according to the authors, might also have represented homozygous, severely affected fetuses or might have been due to cervical insufficiency, which, as we now know, is quite typical of EDS. A severe, presumably homozygous form of EDS has also been observed in a highly inbred kindred with EDS type I [134]. Jansen [135] reviewed the literature and came to the same conclusion of autosomal dominant inheritance. Parental gonadal mosaicism may explain why neither parent of the two affected sisters, cases 6 and 7 in [40], has the syndrome.

In Beighton's series of 100 patients with EDS, 63 were from 20 families and inherited the condition as an autosomal dominant trait [1,136]. The 29 sporadic cases were clinically indistinguishable from the familial cases, and no parental age effect was evident, paternal age being 30.7 versus 29.3 years and maternal age 29.3 versus 28.4 years; birth rank did not deviate from normal.

Segmental cases, confined to one or more body segments, were already described by van Meek'ren [9] (Fig. 1) and Du Bois [137] and have subsequently been reported by Beighton [1] and Cullen [132]. These may represent somatic mutations similar to the reported case of asymmetric Marfan syndrome [138]. However, no mention is made of articular involvement in these cases.

Interfamilial variability may be due to genetic or locus heterogeneity, and intrafamilial variability may be due to differences in genetic background (i.e., differences in the composition of other connective tissue components inherited in a multifactorial way), age dependence, and, apparently, differences in the assessment of clinical severity. Allelism in EDS I and II has been demonstrated [139,140].

Basic Defect(s) in EDS I/II

In EDS types I and II, the basic defects were unknown until recently and are heterogeneous. In 1955, Jansen [30] described a loose, disorderly arrangement of apparently normal collagen fibers and postulated that the collagen fibrils might be abnormally cross-linked in such a way that the interlacing network of connective tissue was deficient. This theory would account for the changes in the physical properties of the skin in the classical form of EDS and for the lack of specific histological abnormalities. In the past, urinary excretion of hydroxyproline, uronic acid, mucopolysaccharides (glycosaminoglycans), and amino acids, serum levels of elastase inhibitor, and amino acid profiles of skin and elastin in skin were measured (for references, see Beighton [1]), but no consistent abnormality has been described. An abnormal cross-link profile was observed in the dermis of one patient, but no clinical data were given [141].

Occasionally, chromosomal anomalies have been found in EDS, most of which have been considered insignificant [142,143]. Scarbrough et al. [144] speculated that genes located in the area of breakpoint 6q27 or 13q11 may be responsible for EDS II. Linkage analysis has excluded changes in the genes for collagens I, II, and III as being responsible for the classical type of EDS [145–147].

Mutations in the Collagen V Genes

Evidence for *COL5A1* on chromosome 9q34 and *COL5A2* on chromosome 2q24.3-q31 as candidate genes for EDS I and II came from the following studies, which indicated the role of collagen V in regulating the formation of heterotypic fibrils in the extracellular matrix. First, the high concentration of collagen V in the chick corneal stroma is one important factor responsible for the small, uniform collagen fibrillar diameter observed in this tissue in contrast to other collagen I-containing tissues with larger diameter fibrils, as shown by *in vitro* collagen fibrillogenesis [148]. $[\alpha1(V)]_2\alpha2(V)$ heterotrimers are localized within the mature heterotypic fibrils, with their N-telopeptides exposed at the fibril surface, and modulate fibril diameter by a steric or electrostatic mechanism [149]. Second, genetically altered homozygous mice lacking the exon 6-encoded N-terminal telopeptide of the α2(V) chain demonstrated collagen fibrils displaying abnormal diameter, contour, and packing in skin and, especially, cornea, and presented with EDS-like features (see "Animal Models and Lathyrism" below; [150]).

Linkage studies in man strengthened this concept [139,140,151], which has now been proven by the direct demonstration of structural mutations in *COL5A1* [116,152–155] and *COL5A2* [156,157], which exert a dominant negative effect. In one patient, however, EDS I was found due to haploinsufficiency of collagen V caused by a translocation that disrupted one *COL5A1* allele [158]. It must be stressed, however, that mutations in *COL5A1* and *COL5A2* account for only a minor fraction of EDS-causing mutations because evidence for a collagen V defect was found in only six patients in a series of 35 patients/families [159].

Protein chemical demonstration of abnormal α1(V) or α2(V) chains produced in fibroblast cultures is, unfortunately, rather ineffective. For documented mutations in *COL5A1* and *COL5A2*, gel electrophoretic changes have been reported only rarely [116,152,154,157,159a]; these include faint doublets, reduced mobility of the chains, or decreased amounts of collagen V. This last is not unexpected, in any case, given the low amount of collagen V deposited in the cell layer and the variability in the relative amounts of collagens I, III, and V deposited from experiment to experiment. In the previously mentioned series of 35 patients/families, only two showed abnormal collagen V profiles, fewer than the six detected as having *COL5A1* and *COL5A2* mutations by molecular means [159]. Despite linkage, cDNA abnormalities of *COL5A2* and *COL5A1* were found in only four and eight, respectively, among 28 families, and the different authors concluded that the classical type of EDS is mainly due to haploinsufficiency caused by nonsense mutations [41,157,159a,160] in one-third of the cases of *COL5A1* [41,160].

Intrafamilial clinical variability in a three-generation family with the classical type of EDS has been explained by the finding that the more severely affected propositus was a compound heterozygote carrying the disease-causing mutation Gly1489Glu and an additional, putative disease-modifying substitution Gly530Ser in *COL5A1* (Fig. 10; [116]). The hypothesis that the heterozygous Gly530Ser substitution is indeed disease-modifying seems to be strengthened by the observation that the same substitution in the homozygous state appears to be disease-causing. Giunta et al. found that a Turkish boy with classical EDS born to consanguineous parents was homozygous for the Gly530Ser substitution and had no further mutations in the *COL5A1* and *COL5A2* genes, and linkage to the genes coding for

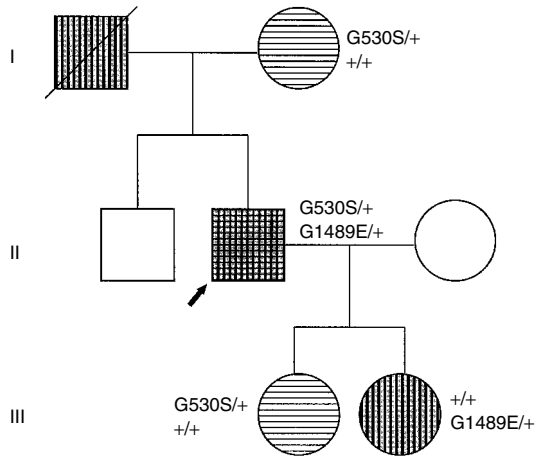


Figure 10. Intrafamilial variability in the classical type of EDS (EDS I) due to compound heterozygosity for the *COL5A1* genes. In each affected individual in family K, the genotype for the disease-causing mutation in exon 58 of *COL5A1* (Gly1489Glu) is represented by bold vertical lines and the disease-modifying substitution in exon 13 (Gly530Ser) by horizontal lines. The compound heterozygous propositus G.K., indicated by the arrow, is the most severely affected individual among the three with classical EDS. (Taken and corrected from Giunta and Steinmann [116] with permission.)

the α3(V) chain, decorin, thrombospondin 2, and tenascin-X was excluded by homozygosity mapping [161].

Because the α3(V) chain is primarily expressed in the epimysial sheaths of developing muscles, in nascent ligaments to forming bones, and in joints [162], *COL5A3* is a candidate locus for at least some cases of classical EDS in which *COL5A1* and *COL5A2* have been excluded and for at least some cases of the hypermobile type of EDS (see below).

Tenascin-X Deficiency

The tenascins (see Chapter 5, this volume) constitute a family of large extracellular matrix proteins. Although they have been implicated in several cellular processes, such as cell adhesion to collagen, no function has been clearly established for any of them. Tenascin-X (TN-X; MIM 600261) is a collagen fibril-associated protein that is highly expressed in dermis, tendon, and ligaments and in the connective tissue of striated muscle. The human *TNXA* gene is on chromosome 6p21.3 at the MHC class III locus, and its 3′ end overlaps the steroid 21-hydroxylase gene on the opposite strand of DNA. In 1997, Burch et al. [163] described a new contiguous gene syndrome, involving the *CYP21B* and *TNXA* genes, that resulted in 21-hydroxylase deficiency combined with a connective tissue disorder resembling EDS II, which is inherited in a presumed recessive fashion.

The proband was a 26-year-old man in whom a diagnosis of severe salt-wasting congenital adrenal hypoplasia due to 21-hydroxylase deficiency had been made at $1\frac{1}{2}$ weeks of age. He complained of a 2–3 year history of worsening joint pain and easy bruisability of many years duration. His history was significant for the release of a trigger finger at age 10, the repair of testicular torsion at age 15, and bronchiectasis confirmed radiographically at age 23. He had always had slow healing of wounds. Physical findings included micrognathia; a high, narrow palate; patellar chondromalacia; talipes planus; hypermobile joints of the fingers, wrists, and knees; a soft, doughy, hyperextensible

skin; and vascular fragility as evidenced by multiple small bruises and ecchymoses. The only family history of connective tissue disease was in his father, who also had hyperextensible finger joints but was otherwise normal [163].

Unusual for EDS II were the ultrastructural findings in his skin [163] but not in skin of further TN-X-deficient patients (see below). The collagen fibrils were not irregular in contour, had a diameter somewhat smaller than normal, and were reduced in number in the reticular dermis, where tenascin-X is mainly expressed. Also, there were abnormal elastin bodies beneath the dermo-epidermal junction. Capillaries in the papillary dermis were prominent and demonstrated increased perivascular matrix, and cutaneous nerves showed abnormal packing of the lamellae of myelin sheaths.

Biochemical and molecular studies of the patient [163] showed that he had a *TNXA*-null phenotype: no mRNA for tenascin-X was detectable, and tenascin-X was absent from skin and fibroblasts. He, as well as his father and one of his two sisters, carried a novel 30 kb deletion arising from the recombination of *TNXA* and its partial duplicate gene, *XA*, precluding TN-X transcription. The nature of his second maternal *TNXA*-null allele remains unresolved.

The patient's distinct histopathologic status suggests a novel mechanism of disease. Hence, TN-X may regulate the macromolecular organization and distribution of matrix elements such as fibronectin, collagen, and elastin; it could interact with cell receptors, modulating the cellular deposition of matrix; it may cross-link matrix fibers or proteoglycans, thereby strengthening the matrix; or, alternatively, it may play a primary structural role in skin, joints, and vascular connective tissues [164]. Definition of the precise role of TN-X in the EDS phenotype will require the identification of other TN-X-deficient patients and *Tnx* knockout mice, as well as the further study of this complex protein.

A quite similar but unrelated patient has been identified [165], who, however, does not present the same morphological findings (L. Smith, personal communication, 1999). A further patient, heterozygous for the *CYP21/TNXA* deletion with an unidentified inactivating mutation on the second *TNXA* allele, and three patients with isolated such mutations have been reported [166], but it remains unclear whether EDS resulted from TN-X deficiency alone and what proportion of patients with EDS might be accounted for by *TNXA* mutations [167].

The same group identified a 140 kDa carboxyl-terminal fragment of TN-X in serum of normals by western blotting, which facilitated the large-scale screening of 151 patients with EDS (35 classical, 87 hypermobile, 1 vascular, and 28 unclassified types of EDS). Among these, TN-X was absent in five individuals with unclassified EDS in each of whom the mutation was identified [167,168]; examination of TN-X-deficient fibroblasts confirmed the failure of TN-X synthesis and showed abnormal deposition of collagen I into matrix *in vitro*. The phenotype of the five patients is characterized by joint laxity, hyperelasticity of the skin, easy bruising, and absence of atrophic scarring. Ultrastructural examinations of the dermis did not show the typical cauliflower collagen fibrils observed in dermis of individuals with EDS caused by mutations of the genes coding for collagen V, nor was the density of the collagen fibrils reduced as observed in the tenascin-X knockout mouse (see "Animal Models and Lathyrism" below). Obligate heterozygotes had approximately 50% of normal TN-X levels in serum and conditioned culture medium.

Mutations in Collagen I

α2(I)-deficient collagen I. Patients with a total α2(I) deficiency are extremely rare — only four have been described so far — and they show markedly different clinical phenotypes. Sasaki et al. [169] described a 30-year-old man, born to parents who were second cousins, with hypermobility of the joints and hyperextensibility of the skin, who was operated on because of severe aortic regurgitation. Hata et al. [170] reported a 35-year-old woman with EDS II (hypermobility of the joints, hyperextensibility of the skin, with easy bruising and a tendency toward scar formation) with slightly blue sclerae and mitral valve regurgitation. Her unrelated parents and her son are healthy. At age 42, she was operated on for mitral valve replacement; by electron microscopy, dermal collagen fibrils were more variable than normal in diameter and contour [171]. Nicholls et al. [172] described a 9-year-old girl with normal height, generalized joint laxity, pes planus, and valgus heels leading to a secondary shortening of the Achilles tendon. Her skin was normal, her sclerae were pale blue, and her teeth were without signs of dentinogenesis imperfecta. She was born prematurely. There was a history of recurrent patellar dislocations and fractures of the skull, clavicle, fingers, and a toe. Her parents were consanguineous. Skin fibroblasts from the three patients synthesized no proα2(I) chains, produced only half the normal amount of collagen, and degraded newly formed collagen intracellularly to a greater extent than normal. In the first and third cases, α1(I) chains were reported to be overmodified (i.e., to show excessive post-translational modification of lysyl and hydroxylysyl residues), apparently as a result of slowed helix formation of the α1(I) homotrimer [169]. In the first two cases, mRNA for the proα2(I) chain was present at less than 10% of the normal amount [170,173] and was interpreted as being unstable because the rate of transcription was normal [173]. Neither stimulation of transcription of the *COL1A2* gene nor increase in the level of mRNA was observed after activation by ascorbic acid 2-phosphate, in contrast to the situation with *COL1A1* in the patient and both genes in controls. From Southern blot analyses, it was further concluded that the patient was homozygous for a functionally defective *COL1A2* gene [174]. The third case had a homozygous substitution in IVS46+2T>C of *COL1A2*, which leads to the use of a cryptic donor splice site 17 bp upstream of the normal splice site; as a result of this, the last 17 bp of exon 46 are deleted and the resultant frame shift introduces a new termination codon just three codons further downstream.

The three patients have normal bones, in striking contrast to the patient with severe osteogenesis imperfecta type III in whom a homozygous 4 bp deletion in the C-propeptide of the proα2(I) chain prevents these chains from assembling and being incorporated into normal heterotrimers [175]. Although studies on collagen extracted from skin and bone have not been performed in either of the patients, the discrepancy may well be explained by tissue-specific differences in expression of the proα2(I) chain. (Similar tissue-specific differences in collagen gene expression have been documented in fibroblasts, odontoblasts, and osteoblasts derived from the Mov-13 mouse [176,177]). Given the relatively benign EDS phenotype without bone involvement on the one hand [169] and the severe OI phenotype on the other [175], we speculated that the EDS patient may have lacked a putative tissue-specific transcription factor in skin, ligaments, and aorta, transmitted as an autosomal

recessive trait, but that a different transcription factor was present in bone and thus allowed the production of normal heterotrimeric collagen I and the formation of normal bones. However, polyethyleneglycol-induced hybridization of the EDS and the OI fibroblasts failed to result in complementation of the α2(I)-deficient cell strains in heterokaryons (C. Giunta and B. Steinmann, unpublished experiments).

Mutations in the *COL1A1* and *COL1A2* genes. Different mutations in three unrelated families led to skipping of exon 9 in *COL1A2* (A. Nichols, personal communication, 1999), and in two sporadic cases substitution of the conserved arginine in exon 14 led to an Arg134Cys substitution in the α1(I) chain, which also resulted in the classical type of EDS [178].

Other Candidate Genes

Decorin and lumican, as well as thrombospondin-2, play an important role in collagen fibrillogenesis. Targeted disruption of the decorin gene led to viable homozygous mice whose skin was fragile, with a markedly reduced tensile strength. Ultrastructural analysis revealed abnormal collagen morphology in skin and tendon, with coarser and irregular fibril outlines [179]. Mice homozygous for null mutations in lumican [180] and thrombospondin-2 [181] displayed skin laxity and fragility similar to that in EDS (see "Animal Models and Lathyrism" below). Thus, decorin, lumican, and thrombospondin-2 are candidate genes for human EDS.

Physical and Morphologic Properties of Connective Tissue and Pathogenesis of the Disorder

Skin elasticity. In normal individuals, elasticity has been assessed using a "pinchmeter" by determining the ease with which a fold of skin from the dorsum of the wrist could be raised by a spring-loaded caliper, or by a suction cup with increasing negative pressure, and recording the resulting distortion [46]. Using the latter method, it was determined that the modulus of elasticity is significantly higher in female than in male control individuals [50] and that it rises progressively with age in both sexes [46]. Mechanical properties of skin have also been evaluated in 17 children aged 3–10 years with EDS I, II, or III and compared with normal values from 63 healthy age-matched children; prominent increases in skin extensibility and elasticity were the most distinctive and diagnostic features, being the greatest in EDS I and the least in EDS III [48].

Stress-strain analysis carried out on skin from EDS patients has shown that the initial lag phase is prolonged and followed by a phase in which the stress-strain relationship is linear and parallel to that of the control tissue [50,182]; that is, the fibrils must be cross-linked and virtually inextensible at this stage. The long lag phase is therefore not due to an absence of cross-links within the fibrils but to an abnormal packing of these fibrils into tight bundles. This interpretation is supported by observations made by scanning electron microscopy that the fiber bundles are less tightly packed than normal [183]. Under tension, considerable extension will be required before the majority of the bundles are aligned in the direction of the tension, at which point they will be tightly packed and resist further extension. If a defect in cross-linking had been present, such as that which occurs in lathyrism or in dermatosparaxis in cattle, continuous extension of the fiber would have occurred as the fibrils slipped past one another, up to the point of rupture. In such conditions, no restoring force would be present, and the effect would not be reversible, whereas in EDS subjects the skin returns to its normal position after hyperextension [183]. These studies have provided support for the previously unsubstantiated proposal by Jansen [30] that skin hyperextensibility in EDS is due to a defective "wickerwork" of apparently structurally normal collagen fiber bundles.

In vitro studies of skin have shown that its tensile strength depends on its water content, the texture of the dermis, and the strength of each single collagenous fiber, and that it increases with age. In a 35-year-old patient with EDS, the tensile strength of the skin was 0.34 kg/mm^2 versus 1.61 ± 0.08 in controls [184], whereas the difference in tensile strengths of the tendons was less marked, namely 4.3 kg/mm^2 versus 9.0 in controls [185].

Skin thickness. Skin thickness can be assessed *in vivo* by the Harpenden caliper [50,186–188], radiographically [189], or by ultrasound [188]; all three methods give quite similar results. There is a direct relationship between dermal thickness measured *in vivo* or histologically and the content of collagen; skin collagen in normal individuals decreases linearly with age by about 1% per year throughout adult life and is less in females at all ages [190]. The skin of 14 patients with EDS studied was thinner than that of controls (0.8 mm versus 1.15 mm), and there was a significant inverse relationship between thickness and the tendency toward skin-splitting as evidenced by the degree of scarring [50].

Morphology of skin. For a historical review of the morphology of the skin, molluscoid pseudotumors, spheroids, and tendons, and of the cardiovascular, gastrointestinal, and pulmonary systems in EDS, see references in Beighton [1] and Wechsler and Fisher [191].

Light and electron microscopic studies of skin from patients with inherited connective tissue disorders are excellently reviewed by Holbrook and Byers [192]. Their generalizations indicate that mutations rarely affect only a single aspect of macromolecular function and that, because of the interactions of matrix components in this complex organ, they often disturb the organization of the entire dermis. Because similar structural abnormalities may result from different molecular defects, most morphological findings are not specific and are often subtle due to a limited repertoire of the dermis for changes. Structural alterations of matrix components include collagen fibrils that are excessively large and irregular in cross-sectional appearance, which have been called composite fibrils or flower or cauliflower figures, and mixed populations of large- and small-diameter fibrils; these changes were first recognized in individuals with EDS I by Vogel et al. [40] (Fig. 5g). Although structural alterations in connective tissue fibers are rarely specific for a given disease, there are characteristic patterns of structural changes in the matrix that may be used to confirm a diagnosis. Hausser and Anton-Lamprecht [193] found a certain correlation between the ultrastructural changes and the severity of the phenotype. In EDS I, ultrastructurally abnormal fibrils were present immediately below the dermo-epidermal junction and, in contrast to EDS II and III, there were almost no normal fibrils. In EDS II, the upper dermis contained only a few abnormal fibrils, in contrast to the middle and deep dermis, which had many abnormal fibrils. In EDS III, on the other hand, isolated smaller collagen "flowers" were only present in the reticular dermis. Such a morphologic gradient, however, needs to be confirmed by genetic analysis.

What is intriguing to us is the fact that the morphology of the collagen fibrils is so patchy and heterogeneous. Why are the cauliflower fibrils sparse and scattered widely apart, and why are there normal-appearing fibrils at all instead of a homogeneous distribution of abnormality? Is haploinsufficiency for collagen V not operative in all sites due to local differences? In this context, it is of note that vision seems not to be impaired by any alteration in translucency of the cornea, an organ rich in collagen V, because abnormal cauliflower-like collagen fibrils are observed in dermis and cornea of pN-knockout mice (see "Animal Models and Lathyrism" below and Chapter 1, Part V, this volume); ultrastructural studies on the cornea of patients with the classical type of EDS have not been reported.

Elastic arteries. The mechanical properties of elastic arteries have been measured by noninvasive monitoring of the pulsatile changes in diameter of the distal abdominal aorta and the common carotid artery by the use of an electronic echo-tracking instrument; however, the diameter and stiffness in several EDS I and II patients were not different from those in the reference population, and the study was unable to demonstrate any alteration in wall mechanics as a sign of disturbed vessel wall integrity [194].

In conclusion, the whole spectrum of tactile, mechanical (log phase), histological and ultrastructural (cauliflower collagen fibrils), and biochemical changes (decreased collagen and increased elastin content) are not yet well-understood.

Diagnosis and Differential Diagnosis

For most practical purposes, diagnosis remains syndromic thus far (see Table I). For specific practical or research purposes, laboratory investigations include a skin biopsy for the following analyses: (1) Ultrastructure quite often may suggest disturbed collagen fibrillogenesis [40,192,193], as in most cases with mutations in the collagen V genes, or other causes such as decorin deficiency, which has not yet been observed in man (see "Animal Models and Lathyrism" below). (2) Analysis of collagens produced by cultured fibroblasts may indicate abnormal collagen V, although this is not a sensitive assay, or, exceptionally, may reveal α2(I)-deficient collagen. Frozen cells may be useful for later studies. (3) A piece of skin should be stored frozen for immediate or later studies. (4) In large pedigrees, linkage analyses should be done to include or exclude known genes responsible for EDS or to map candidate genes.

Joint laxity may be found in the Marfan syndrome (MIM 154700), the Marfanoid hypermobility syndrome (MIM 154750), the familial articular hypermobility syndrome(s) (MIM 147900; the distinction between this syndrome and EDS III may be artificial), the Larsen syndrome (MIM 150250, MIM 245600), certain forms of osteogenesis imperfecta, and muscular hypotonia of various causes. Contortionists may achieve extraordinary hypermobility of the large joints by training. Stickler's hereditary arthroophthalmopathy (MIM 108300), the osteoporosis-pseudoglioma syndrome (MIM 259770), pseudoachondroplasia (MIM 177150, MIM 264150), Morquio syndrome (MIM 253000, MIM 253010), spondyloepimetaphyseal dysplasia with joint laxity (MIM 271640), Desbuquois syndrome (MIM 221880), and hyperlysinuria (MIM 238700) share with EDS hyperextensibility of the joints but are readily differentiated by other distinct features. Ligamentous laxity is also a feature of many other genetic disorders, such as the fragile X-syndrome (MIM 309550), trisomy 8-mosaic syndrome, and multiple endocrine neoplasia type 2b (MIM 162300). Joint laxity and soft skin are frequent in the renal-coloboma syndrome (MIM 120330) and the Langer-Giedion syndrome (MIM 150230). Hyperelasticity of the skin and joint laxity together with mental retardation and cataracts have been described in siblings with an autosomal recessive disorder due to impaired proline synthesis (MIM 138250) [195,196]. Skin and joint laxity has been reported together with lengthening and tortuosity of systemic, pulmonary, and coronary vessels and an elongated facies [197]. In the fetal anticonvulsant syndrome, joint laxity is a frequent finding, involves all sizes of joints, and is part of a generalized connective tissue disorder [198].

Hyperelastic skin can be found in the Noonan syndrome (MIM 163950) and should be distinguished from that observed in cutis laxa syndromes (MIM 123700, MIM 219100, MIM 219150), the De Barsy syndrome (MIM 219200, MIM 304150), gerodermia osteodysplastica hereditaria (MIM 231070), and Menkes disease (MIM 309400), in all of which the lax redundant skin, which is not fragile, hangs in loose folds and, although it may be stretched, will return only very slowly to its former position. Such patients have a "bloodhound facies" and look considerably older than they are. In the autosomal recessively inherited wrinkly skin syndrome (MIM 278250), the skin is wrinkled over the dorsum of the hands and feet, and the palms and soles, and the abdomen [199] (see also Chapter 10, this volume).

Increased skin fragility is found in osteogenesis imperfecta and in senile people, and may be mimicked by self-affliction by retarded or hysterical persons (Münchhausen syndrome). Excessive bruising is found in thrombo- and coagulopathies (see EDS IV) and in nonaccidental injury ("battered child") (see legend to Fig. 7).

Cardiovascular anomalies similar to those in EDS I may be found in familial mitral valve prolapse (MIM 157700) and annuloaortic ectasia Erdheim (MIM 132900), and are frequently associated with adult polycystic kidney disease (MIM 173900) [200], sometimes together with a Marfanoid habitus [201]. Dilatation of the aorta is typical of the Marfan syndrome (MIM 154700), aortic arch arteritis Takayasu, and other forms of arteritis and aortic malformations. For multiple arterial aneurysms, see EDS IV.

Musculoskeletal deformities, especially in EDS VI (Figs. 24,25), may resemble those in the Marfan syndrome.

Keratoconus was found in 22 of 44 patients with hypermobility, mainly of the fingers and thumbs, but without hyperelasticity or fragility of the skin; this condition is compatible with autosomal dominant inheritance and, according to the author in question, with EDS II [115].

Management and Genetic Counseling of EDS I and II

Because causal therapies are not available, medical intervention is limited to symptomatic therapy, prophylactic measures, and counseling.

Medical Therapy

Ascorbic acid, a cofactor of prolyl and lysyl hydroxylases, has been given to some patients with EDS but in spite of anecdotal reports of a beneficial effect does not change the basic clinical picture. The same holds true for zinc therapy [202,203]. Mild antirheumatic drugs may be indicated in patients with articular pain. Patients with mitral valve regurgitation require antibiotic prophylaxis for bacterial endocarditis and should carry a medical identification card with the appropriate instructions. The bleeding tendency is thought to be caused primarily by tissue

and capillary fragility rather than an intrinsic platelet or plasma defect. DDAVP (vasopressin) has been successfully used to reduce blood loss in patients without connective tissue disorders undergoing heart surgery and may be useful in EDS patients with chronic bruising and epistaxis, or perioperatively (e.g., for tooth extraction), in whom bleeding time is normalized by DDAVP (P. Tuchschmid and B. Steinmann, unpublished, 1996; [204]).

Physical Therapy, Orthopedics, and Prevention

A physiotherapeutic program is important in children with hypotonia and delayed motor development. In milder cases, light, non-weight-bearing muscular exercise (e.g., swimming) is useful to correct hypotonia and promote development of the musculature and muscular coordination, thus stabilizing the loose joints; gymnastics and sports with heavy joint strain are to be discouraged. Sports instructors should be informed about those with EDS. High-impact activities and hyperextension of all joints are to be avoided. If there is significant joint laxity and hyperextension at the interphalangeal joints, the occupational therapist should evaluate patients for ring splints. Bracing of the lower limb is indicated when joint instability and hypotonia prevent walking and physiotherapy has not been effective; ultimately, surgery is indicated when other means have failed. Special shoes may be required by patients with flat feet, other foot deformities, or painful piezogenic papules. Osteopathy and relaxation for pain control and management may be helpful.

Surgery

Because of tissue fragility, any type of surgery may be more difficult in patients with EDS, and it is not unusual for the original problem (e.g., herniae) to recur after intervention. Surgery may be indicated for fixation of a particularly unstable or habitually luxated joint, for the correction of dislocated hips (see EDS VII), for the repair of herniae or diverticula, and for scoliosis or cardiovascular problems [205]. Wounds should be closed without tension, preferably in two layers. Deep stitches should be applied generously and cutaneous sutures left in place for twice as long as usual. Long-lasting cutaneous analgesia may be difficult to obtain [89]. Tooth extraction may be difficult because of dilated irregular and poorly formed terminal roots, and prolonged post-extraction hemorrhage should be anticipated because of the poor vascular retraction. From a retrospective study, based on the patients' reports of their own experiences after surgical procedures to the shoulder, the elbow, the knee, or the ankle for pain, instability, poor range of motion, or a combination thereof, Weinberg et al. [206] conclude that surgical complications are common in EDS.

Prophylaxis of Skin Changes

Young children with pronounced skin fragility should wear protection in the form of athletes' pads or bandages over the forehead, knees, and shins in order to avoid skin tears. Later, the child may learn to avoid violent sports. When skin tears do occur, irregularly frayed wound margins should be excised and precisely adapted to allow (rapid) healing without dystrophic scarring, which is especially important in the case of facial wounds. Numerous fine, atraumatic stitches should be used and left in place for twice as long as usual. Additional fixation of the adjacent skin with adhesive tape is very helpful in preventing stretching of the scar; however, removal of adhesive material has to be done carefully to avoid secondary dehiscence. Hyperpigmentation and premature aging of both the scars and normal skin are of concern to many patients.

Other

The majority of patients with EDS learn to cope with their disease and eventually adapt well to daily life. However, some patients are troubled by hypotonia and joint instability, and many by chronic joint pain. These handicaps should be recognized and discussed, and lifestyle and professional choices should be adapted accordingly; strenuous physical activity should be avoided. Behavioral and psychological therapy may be indicated. Patients may gain valuable up-to-date information, confidence, emotional support to accept and cope with their handicap, and much practical advice from meeting other patients who are members of self-help organizations (see "Patient Support Groups" below).

Genetic Counseling

EDS types I and II are inherited as autosomal dominant traits. Affected persons have a 50% risk of transmitting the disorder. Affected first-degree family members should be examined to obtain an estimate of intrafamilial variability and thus the phenotypic range to be expected should the child be affected. Prenatal diagnosis has not been attempted.

THE HYPERMOBILE TYPE OF EDS — EDS TYPE III (MIM 130020)

Diagnostic Criteria

The hypermobile type of EDS is inherited as an autosomal dominant trait and characterized as follows [39]:

- Major diagnostic criteria
  - Skin involvement (hyperextensibility and/or smooth, velvety skin, absence of tissue fragility)
  - Generalized joint hypermobility
- Minor diagnostic criteria
  - Recurring joint dislocations
  - Chronic joint/limb pain
  - Positive family history

EDS III (hypermobile type) (MIM 130020) is characterized by quite severe, generalized joint laxity (Fig. 11) and the sequelae thereof, such as dislocations, effusions, and precocious arthritis. Certain joints, such as the shoulder, patella, and temporo-mandibular joints, dislocate frequently. Pain, swelling, dislocation of joints, a history of surgery of the joints, and impaired ambulation were reported by questionnaire during a National Ehlers-Danlos Syndrome Foundation Conference to be more frequent and pronounced by patients with EDS III than by patients with EDS I, II, or IV; the authors came to the conclusion that EDS III is the most debilitating form with respect to musculoskeletal function, although the data reflected an inherent bias because the patients were voluntary attendees at the educational symposium [66]. Skin hyperextensibility is variable; because tissue fragility is absent, the presence of atrophic scars, spheroids, or molluscoid pseudotumors with joint hypermobility suggests diagnosis of the classical type. The frequency of prematurity is not increased. Mitral valve prolapse is more prevalent than normal.

Musculoskeletal pain in EDS III is early in onset, chronic, and sometimes debilitating [207]. Its anatomical distribution is wide, and tender points can sometimes be elicited (a tender point is defined as an area that, when palpated with the thumb or two or three fingers, will be painful at a pressure of 4 kg or less [208]). In rheumatology practice, large numbers of patients present with generalized joint hypermobility.

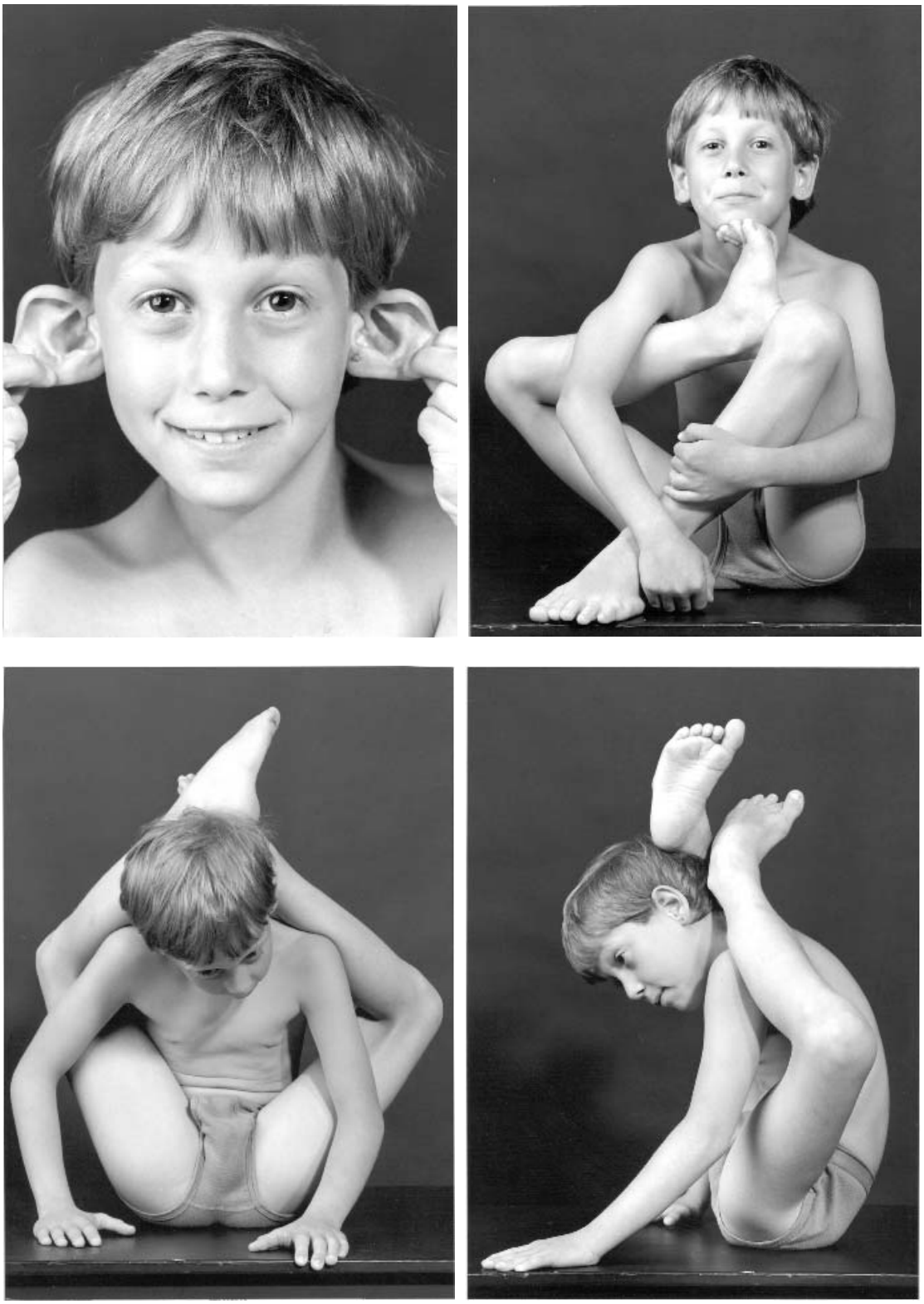


Figure 11. Hypermobile type of EDS (EDS III). General joint hypermobility in a 10-year-old boy (A.T., 18 Apr 1981) with hyperelastic, smooth, velvety skin, which is not fragile and has no abnormal scars, and hyperelastic, floppy ears.

He is a sporadic case and had several episodes of rectal prolapse starting at age 2.5 years, which was operated on; rectal prolapse has not recurred but it remains open if this is due to the operative fixation or simply represents the natural history. Because of chronic back pain, spondylolisthesis L5/S1 was operated on by dorsal spondylodesis, and an indwelling urinary catheter was inserted, which led to urethral strictures needing several bougienages. He suffered from several hemorrhages into the knee joints and complained of chronic fatigue.

For clinical research purposes, it is important to distinguish these individuals from those affected with the hypermobile type of EDS. There is considerable debate as to the causal interrelationship, if any, between the two phenotypes.

EDS III is similar to, although by no means as severe as, EDS VIIA and VIIB, but congenital hip dislocation does not usually occur. Furthermore, a separate autosomal dominant condition, familial articular hypermobility syndrome, the former EDS XI (MIM 147900; Table 1), which is characterized by severe joint laxity, occasional congenital hip dislocation, but no skin changes, has to be distinguished. Whether the familial articular hypermobility syndrome represents the upper end of the normal spectrum of variation of joint mobility or reflects a mild connective tissue disorder remains open, and in the absence of biochemical and genetic markers, the nosologic relationship between it and EDS III remains unclear.

Surprisingly, Narcisi et al. [209] described a family in which a point mutation in the *COL3A1* gene (Gly637Ser) was associated with a phenotype they described as EDS type III, a condition that is unlikely to be confused with EDS IV; it may well be that individuals of this family will have a later onset of significant symptoms of EDS IV because at description most were well below the age at which major complications would have been expected. Only the first affected individual was over 60 years of age, and mosaicism for the mutation was not excluded.

COL5A3 is a candidate gene for at least some cases of the hypermobile type of EDS (and some cases of classical EDS in which *COL5A1* and *COL5A2* have been excluded, see above) because the α3(V) chain is primarily expressed in the epimysial sheaths of developing muscles, in nascent ligaments to forming bones, and in joints [162].

To control the pain, which is sometimes considerable in this type of EDS, and the consequent impairment of well-being, behavioral, physical, medical, and psychological therapy may be indicated (see classical type of EDS above).

VASCULAR TYPE OF EDS — EDS TYPE IV (ARTERIAL-ECCHYMOTIC TYPE OF SACK-BARABAS) (MIM 130050)

Diagnostic Criteria

The vascular type of EDS is inherited as an autosomal dominant trait and is caused by structural defects in the proα1(III) chain of collagen III encoded by *COL3A1*. It has the worst prognosis and is characterized as follows [39]:

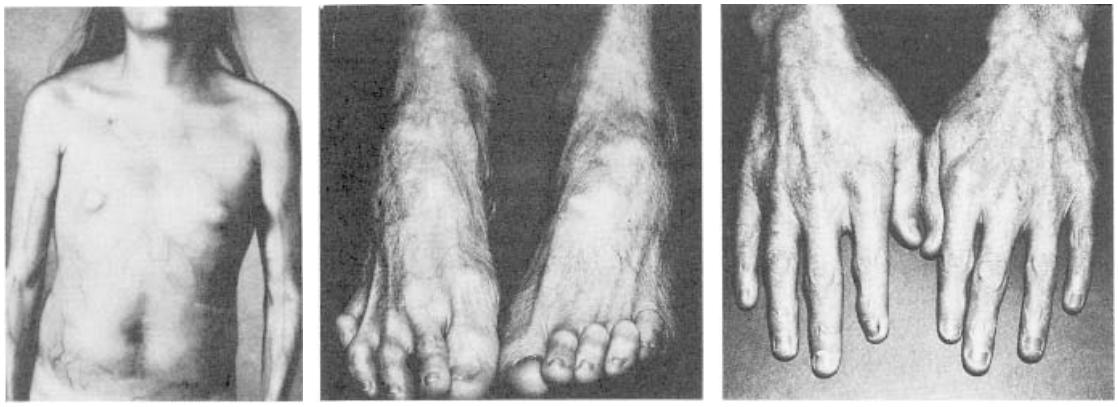
- Major diagnostic criteria
  - Thin, translucent skin
  - Arterial/intestinal/uterine fragility or rupture
  - Extensive bruising
  - Characteristic facial appearance (Fig. 13)
- Minor diagnostic criteria
  - Acrogeria
  - Hypermobility of small joints
  - Tendon and muscle rupture
  - Talipes equinovarus (clubfoot)
  - Early-onset varicose veins
  - Arteriovenous, carotid-cavernous sinus fistula
  - Pneumothorax/pneumohematothorax
  - Gingival recession
  - Positive family history, sudden death in (a) close relative(s)

Note: The presence of any two or more of the major criteria is highly indicative of the diagnosis, and laboratory testing is strongly recommended.

Historical Introduction

EDS IV is a life-threatening and disabling disease, "dramatic, deceptive and deadly" [210]. In 1967, Barabas [31] pointed out that the extensive tendency toward bruising and bleeding and the extreme arterial fragility distinguished this variant from other forms of EDS. Sack [24], and probably others, had described the same entity many years before. Affected individuals have only slight joint hypermobility, usually limited to the digits, and skin hyperextensibility is minimal or absent [142], hence the designation ecchymotic or arterial type (or Sack's type) [1,2,211], now the vascular type of EDS [39]. It is unfortunate that the complications typical of EDS IV are sometimes cited in texts as characteristic of other types of EDS, thus creating unnecessary anxieties.

EDS IV was the second of the disorders of collagen to gain a biochemical identity. It was recognized that "patients with Ehlers-Danlos syndrome type IV lack type III collagen" [34], a description still confusing to clinicians less familiar with the terminology. The possibility that the manifestations of EDS IV could be caused by an absence of collagen III was first raised by Pope et al. [34]. They found that in the aorta and skin of their patient P.P. (Figs. 12b and 13a; also Fig. 6–10 in [2]), the total amount of collagen was markedly decreased and neither collagen III nor its CNBr-derived peptides were detectable. Fibroblasts grown from this and four additional patients did not secrete medium proteins that eluted on DEAE-cellulose column chromatography in the region expected for procollagen III. Furthermore, cells in culture from two of the five patients failed to stain by immunofluorescence with antibodies specific for procollagen III [212]. These authors concluded, wrongly as it happens (see below), that the defect was absent synthesis of procollagen III rather than a post-translational abnormality of procollagen secretion. Because clonal cells from dermatosparactic animals produce both collagens I and III [231], and because normal fibroblasts react with antibodies to both procollagens I and III, it was considered unlikely that there was a deficiency of a specific cell type synthesizing procollagen III [212]. Studying another patient by more sensitive methods, Byers et al. [232] found that the amount of procollagen III in fibroblast culture medium was indeed reduced to 10–15% of that in controls but that it was retained within the cells both *in vitro* and *in vivo* [233]. The finding that the stored collagen III was of higher molecular weight than expected suggested to them that the procollagen might have extensive post-translational modifications, possibly secondary to some structural alteration of the proα1(III) chains. They further proposed that the relatively low amount (10%) of normal collagen III secreted would be approximately that predicted if only one allele at a single locus were normal: because procollagen is a homopolymer containing three identical chains, only one-eighth of the assembled molecules would be normal if the molecules were assembled randomly from a pool containing equal numbers of normal and mutant chains. The seven-eighths of abnormal collagen III molecules might then be retained and/or degraded intracellularly. Finally, it could be predicted that such an abnormality would be inherited as an autosomal dominant trait [232]. Their concept of heterozygous mutations leading to structural defects of collagen α-chains and instability and/or nonsecretion and degradation of the abnormal



a b

Figure 12. Vascular type of EDS (EDS IV). (**a**) Thin, translucent skin with an easily visible venous pattern in E.A., born 1949 (Johns Hopkins Hospital #1287186), at 12 years of age. She also had acrogeria and acrocyanosis with atrophy of the fingertips and toe tips.

She was the second child born to healthy parents at term; her four brothers were unaffected. Labor was induced because of ruptured membranes before the onset of uterine contractions. Birth weight was low (1.8 kg), in contrast to that of her brothers, which ranged from 3.2 to 3.6 kg. She had pyloric stenosis operated on at 7 weeks, easy bruisability and skin fragility from infancy, splenic rupture at age 12 years, intramural hematoma of the colon at 17 years, and bleeding into the peritoneal cavity at 19 years. At age 25 years, she had recurrence of colonic hematoma, which required surgical intervention. She had extensive postoperative bleeding into the rectus muscle sheet and the abdominal cavity, was reoperated on twice, and died in shock ([2], and follow-up by B. Steinmann, 1978). (**b**) Acrogeria in P.P. (born 11 Nov 1957, died 17 Nov 1973) at age 14 years; see also Fig. 13a. (For references, figures, and cell strains see [34,212–220]; Fig. 6-10 in [2]; CRL 1145, and CRL 1243).[6] ((**a**) is (Fig. 6-21 in McKusick [2], reprinted with permission.)

procollagens — a process that later became known by the somewhat misleading term "protein suicide" coined and propagated by Prockop [234] — proved not only to be correct but crucial also for the understanding of mutations of procollagens I, II, IV, V, VI, VII, IX, X, XI, XVII and XVIII, leading to osteogenesis imperfecta and various other connective tissue disorders described in this volume.

Clinical Findings, Natural History, and Prevalence

Unlike in other types of EDS, affected individuals have nonhyperelastic, thin, translucent skin, through which the venous pattern over the chest, abdomen, and extremities is readily visible (Fig. 12a). Bruising is marked and wound healing delayed. Joint hypermobility is minimal and limited to the small joints of the hands and feet, although shoulder dislocation may occur. The skin covering the hands and feet may be extremely thin, finely wrinkled, and look "older" (so-called acrogeria — see Fig. 12b; some individuals with acrogeria in the older literature [235–239] probably had EDS IV). In contrast, tightness of the skin over the face may result in a "younger" appearance, not unlike after a face-lifting procedure (Fig. 13). The nose is thin, delicate, and pinched, the lips are thin, the cheeks are hollow, and the eyes appear prominent and staring because of a paucity of adipose tissue; the pinnae of the ears are firm and tight and frequently without a free ear lobule (Fig. 13h); there is a tendency to alopecia. The facies is therefore often quite characteristic (Fig. 13a–g) [220–222].

Congenital club foot and inguinal hernia are common, as are keratoconus [240], periodontal disease, and venous varicosity and thrombophlebitis. Elastosis perforans serpiginosa ([241–243]; MIM 130100) is not uncommon in EDS IV and presents as a circular rash with raised rough edges and a clear center, sited over the neck and flexures. This is the result of fragmented elastic fibers that are extruded through the epidermis.

Keloid formation (Fig. 14a) [246], Raynaud phenomenon, acroosteolysis [222,247,248], and skull defects [249] have been reported. Adult patients have been subdivided into ecchymotic and acrogeric varieties [250]. This distinction is more confusing than helpful because all patients with EDS IV tend to have ecchymoses, whereas only a proportion will show the additional feature of overt acrogeric changes of hands and feet. In children, even if at risk, it is often difficult to make the diagnosis on clinical grounds alone unless bruising is severe or the venous pattern particularly noticeable [222]. Although mitral valve prolapse was observed in most affected individuals from a large pedigree with EDS IV [251], it does not appear to be a consistent finding.

The hallmarks of EDS IV are the severe, life-threatening internal complications, that usually occur after puberty and include spontaneous rupture of the arteries, the colon, and the gravid uterus, and (hemato-)pneumothorax [252]. There does not appear to be any familial predisposition for a certain type of complication because different catastrophic events can occur in different individuals within one large family (Fig. 18) or sequentially in one person [210,253].

Arterial complications have been collated in a large collective review of literature published over 20 years, from 1975 to 1995, in which only patients for whom biochemical confirmation was available are included; not considered were intracranial vascular complications [254]. The 45 vascular

[6]Cell strains detailing the cells studied in the original papers are given in the 1st edition of this volume; these cells, designated CRL, are commercially available from: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

[7]The known tendency toward keloid formation in patients with EDS IV is somewhat at odds with the observation that keloid tissue in other subjects contains 32% collagen III relative to collagen I, as compared with 21% collagen III present in dermis of normal skin [245].

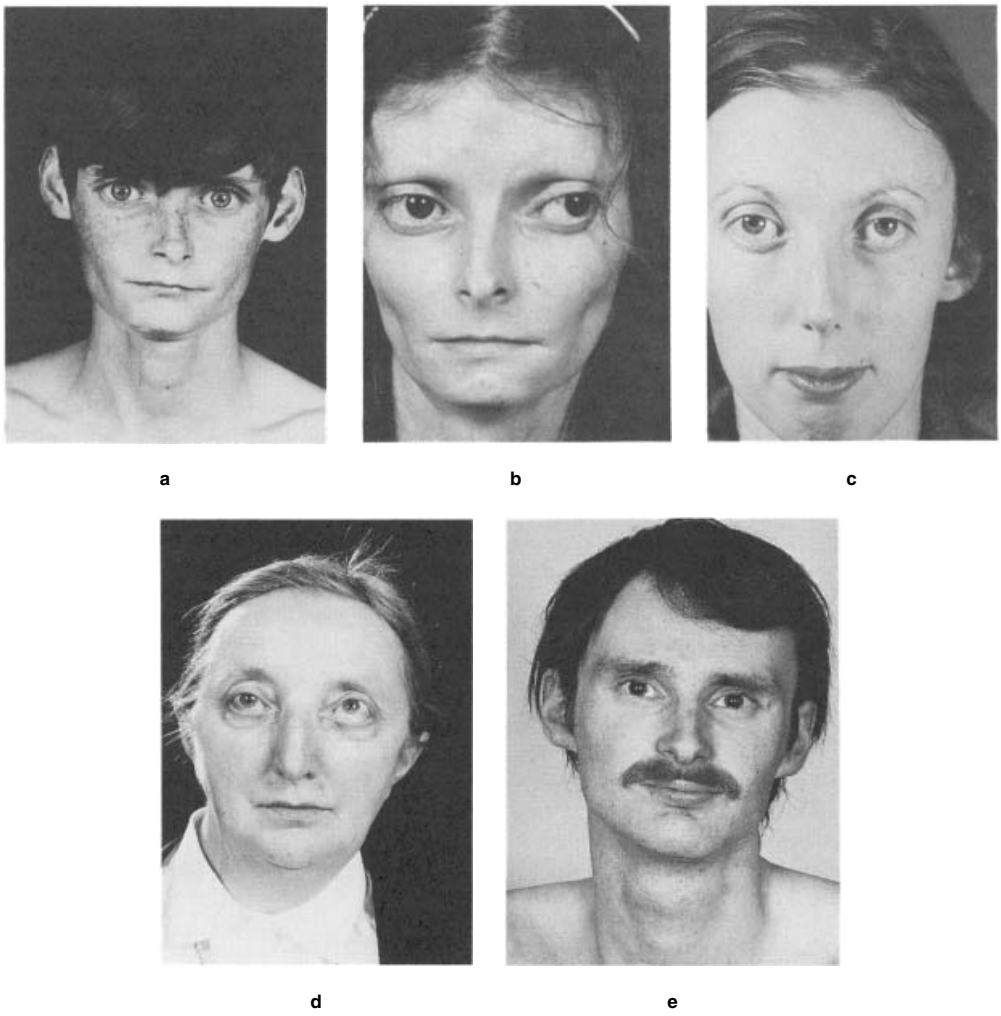


Figure 13. Facial appearance in the vascular type of EDS (EDS IV). The facies in EDS IV patients is often quite typical (**a**, **b**, **f**), with a thin, delicate, and pinched nose; thin lips; tight skin; hollow cheeks and prominent and staring eyes because of a paucity of adipose tissue; and tight, firm, lobeless ears. In some patients (**e**), however, the facial characteristics are less apparent. For further illustrations, see [220]. (**a**) P.P. at age 14 years (see also Fig. 12b). His molecular defect is a Gly1021Arg substitution in the α1(III) chain [220]. (**b**) and (**c**). The defects are a Gly1021Glu substitution and skipping of exon 43, respectively [120]. (**d**) M.D. (2 Jan 1933) at age 55 years. Her molecular defect is a Gly910Val substitution (for further references, see [223–225]). (**e**) D.S. (born 27 May 1964, died 27 Sep 1990) with a multiexon deletion [226,227] at age 22 years (for further references, see [213,219,228,229]). (**f**) Baby, childhood, and adult facies of a woman with EDS IV who died at age 33 years from a rupture of the splenic artery. The authors claim that ''the diagnosis is unmistakable in all three pictures''; although we do agree that the adult face is typical, the two younger facies would be quite inconspicuous to a pediatrician. This demonstrates that in children it is often difficult to suspect the diagnosis on clinical grounds unless bruising is severe and/or the venous pattern particularly noticeable, or unless there is a positive family history. Her molecular defect is a Gly1003Asp substitution [220]. (**g**) R.I. (11 Feb 1976) at age 18 years. He was born to healthy unrelated young parents after premature rupture of the membranes three weeks before term. At three months, bilateral inguinal herniae were operated on and his skin was noted to be unusually fragile with an easily visible venous pattern. Subcutaneous fat was sparse and, according to his mother, he never ''looked like a baby.'' At 5 months, hydrocephalus due to chronic subdural hematoma was diagnosed, and it was drained at 9 months. Easy bruising was a continuous complaint. At age 17 years, after an excess of alcohol, vomiting provoked pneumothorax with subcutaneous emphysema and rupture of the lower esophagus (Boerhaave syndrome) with consequent mediastinitis and chronic pericarditis responsible for heart failure. Dilatation of the esophageal stricture — before the diagnosis was appreciated — led to pneumomediastinum and massive symptomatic pneumoperitoneum that required needle aspiration. Over a year later, he had recurrent left inguinal hernia repaired, an operation that was complicated by severe intraoperative hemorrhage from extremely dilated, tortuous, and fragile veins at the level of an almost nonexistent fascia transversalis. He developed pterygium-like contractures of the knees, which made ambulation difficult. His molecular defect was a single nucleotide exchange (IVS42+5G>A), which led to an insertion of ten amino acids. At age 23 years, he died the victim of a tragic car accident [230]. (**h**) Ear of L.R. at age 62 years, which is poorly modeled, lobeless, and has a very tight feel. Her molecular defect is a G to A transition at nucleotide 730, which leads to a Gly43Asp substitution [230] (see also legend to Fig. 20). ((**a**) reprinted from Pope et al. [221] with permission; (**b**) and (**c**) reprinted from Pope et al. [222] with permission; (**d**) and (**e**) reprinted from Steinmann [42] with permission; (**f**) reprinted from Pope et al. [221] with permission.)

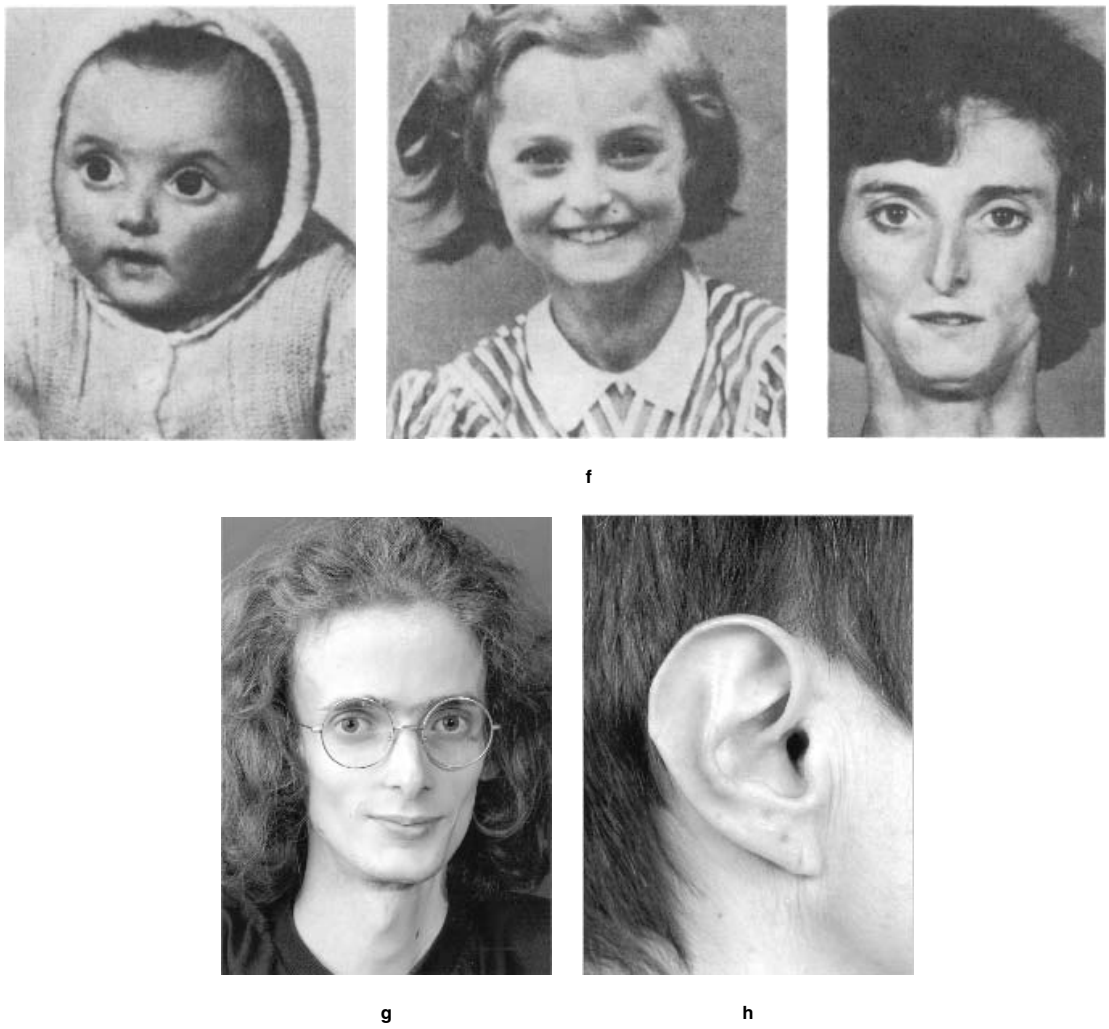


Figure 13. (*Continued*)

complications reported comprise 22 spontaneous hemorrhages, 17 aneurysms, five arterial dissections, and one arteriovenous fistula. The patients, 20 males and 20 females, had a mean age of 27.2 ± 10.9 years (range 11–63 years).

Bleeding seems to be more frequent in young males [255,256] and in patients during the postoperative period, and may occur at virtually every possible site and lead to symptoms such as sudden death, cerebral stroke, hemoptysis, hematemesis [244,253,257], renal colic and hematuria (see legend to Fig. 20) [219], acute abdomen, respiratory distress, retroperitoneal bleeding, and muscle swelling and shock. The most common locations of arterial bleeding are in the abdominal cavity and involve the small arteries rather than the aorta itself (Fig.15). In some individuals, there is evidence of aneurysmal dilatation and dissection or of arterio-venous fistulae, whereas in others, slit-like defects detectable at autopsy may occur in arteries that appear normal by angiography. In a review of the literature, Bergqvist found 112 cases in which vascular complications had been reported, and aortic dissection had occurred in 12 of these [261]. Acute myocardial infarction is a rare complication, reported in only eight cases, and is due to coronary dissection or rupture (see [262]).

Tortuosity of arteries has also been documented. Arterial rupture accounts for most deaths in EDS IV because it is frequent, the hemorrhage is rapid, and repair, even when timely, is difficult due to the marked friability of the tissues [255]. Carotid-cavernous sinus fistula formation and resultant exophthalmos has been described in several individuals [72,93,94,263–267]. Of 212 patients with carotid-cavernous fistulae treated by Halbach et al. [268], four had both EDS IV and spontaneous onset of their fistulae. Out of 202 individuals from 121 families with EDS IV proven by biochemical or molecular studies, 19 had cerebrovascular complications at a mean age of 28.3 years (range 17–48), of whom four died [269].

EDS IV has been proposed as a model of more common forms of aortic and intracranial aneurysms which often cluster in families, but, to date, most studies have excluded the *COL3A1* gene as the locus for such mutations in the absence of some findings of EDS IV [270–272]. However, there is evidence that a small number of cases of familial aortic aneurysm may be caused by collagen III deficiency [273–276] in the absence of significant skin changes. However, it must be noted that familial aortic dissection has been reported in EDS I as well [2]. The first

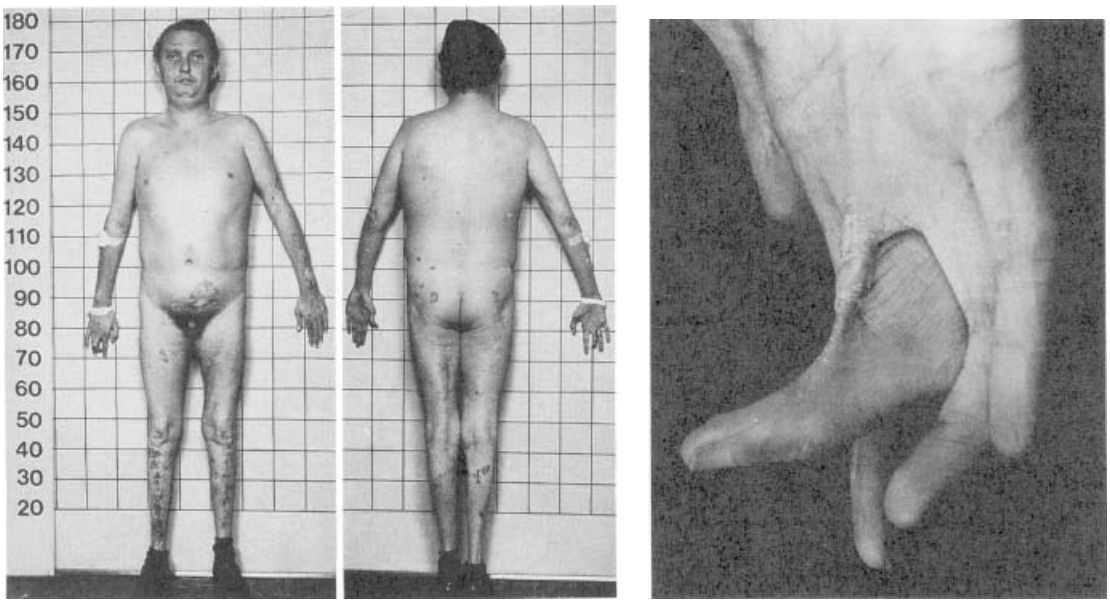


Figure 14. Vascular type of EDS (EDS IV). C.E. (3 Jul 1946, died ~35 years), at age 27 years showed atrophic scars on the shins, keloid formation[7] on the abdomen over the symphysis, numerous scars on the forehead and over the chin (**a**), and pterygium-like cutaneous flexion contractures of the thumb and the third finger of the right hand (**b**). For further references, see [34,212–214,216–219,244] and CRL 1299 and CRL 1409[6]. His molecular defect was a single nucleotide substitution (IVS25+5G>T) leading to skipping of exon 26 [227]. Sporadic case, bilateral clubfoot at birth; easy bruising since boyhood, several episodes of hemorrhage. (Reprinted from Steinmann [42] with permission.)

demonstration of microangiopathy in EDS IV is presented in Figure 16.

Rupture of the colon, usually in the sigmoid region, is the most common of the bowel problems and occurs at sites where the bowel surface appears normal. Constipation seems to play an important role in the pathogenesis of colonic perforation [279]. In a literature review spanning 20 years (see above), 41 colonic perforations among 44 gastrointestinal complications were cited: 33 of the sigmoid, four of the descending, two of the ascending, and one of the transverse colon, and one of the rectum. The mean age of patients with gastrointestinal complications was 31.5 ± 16.9 years (range 7–66 years), and the sample included 17 males and 13 females [254]. The small intestine rarely ruptures (for exceptions, see [279,280]), but intramural hemorrhage may lead to recurrent abdominal pain. Spontaneous esophageal rupture after vomiting — Boerhaave syndrome — has been observed only exceptionally [230,281,282].

Complications in late pregnancy, or during or after delivery, are not so rare and include vascular, intestinal, or uterine rupture (Fig. 17), vaginal lacerations, prolapse of uterus and bladder, and premature delivery because of cervical insufficiency or fragility of membranes [1,241,283–288]. In the largest survey of "classical" EDS IV individuals, pregnancy-related complications led to death in 9–15% of women who became pregnant [256].

Pulmonary complications may arise either from a primary defect in the lung parenchyma or from primary intrathoracic vascular rupture. Lung disease has been reviewed by Dowton et al. [289], who concluded that no death has been recorded as solely due to pulmonary manifestations of EDS IV.

Liver rupture is rare. A report describes the spontaneous rupture of a transplanted donor liver, which was most probably derived from an unrecognized individual with EDS IV [290]. After completing the caval and portal anastomoses, the liver was revascularized; within seconds, the donor liver developed multiple large subcapsular hematoma that spontaneously ruptured with extrusion of liver parenchyma. Despite efforts to obtain hemostasis, the liver continued to fragment, requiring hepatectomy (later retransplantation with another liver was successful). The donor was a 38-year-old woman with brain death secondary to subarachnoid hemorrhage. During collection of several internal organs, the right renal hilum was torn during removal of the kidney, and it was noticed that the heart valves were fragile and had to be discarded. Little was known about her personal and familial history except that her cousin had died from rupture of a visceral aneurysm. Attempts to culture fibroblasts failed, but electron micrographs showed that collagen fibrils from small hepatic arteries were smaller than normal in diameter and irregularly packed [290].

Descriptions of tissues as "fragile like wet blotting paper" are not uncommon in surgical and autopsy reports [2,291]. In the father of the propositus D.S. (Figs. 13e and 16), open chest cardiac massage resulted in avulsion of the heart from the superior vena cava and death [226]. For a similar case, see Krog et al. [292]. In two patients with shoulder dislocation, closed reduction and surgery resulted in the rupture of the brachial plexus (see also legend to Fig. 15) and rupture of both brachial plexus and brachial artery, respectively [85]. The degree of tissue friability differs among individuals and even in the same individual with aging [210]. An unusual combination of multiple aneurysms, a hepatic artery to portal vein fistula, and diverticula of the biliary passages, the sigmoid colon, and bladder has been reported in a patient possibly affected by EDS IV [293].

Natural history. In the largest survey [256] available of patients with "classical" EDS IV (comprising 220 index

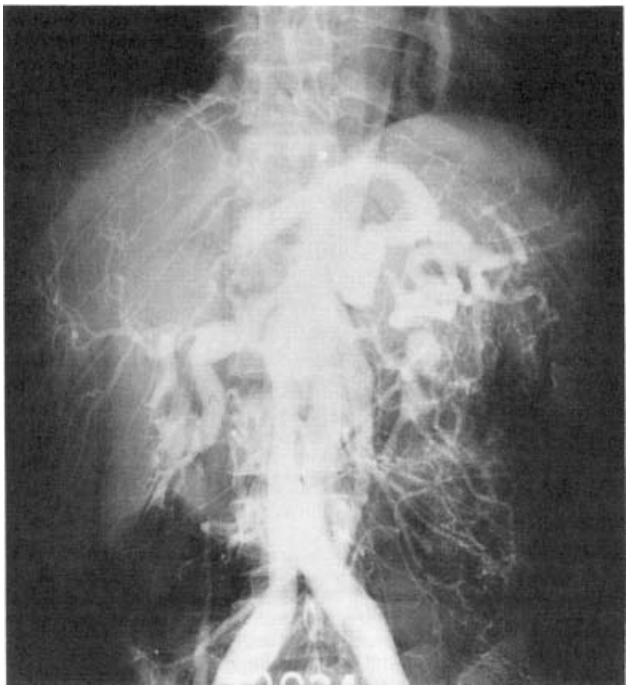


Figure 15. Diffuse dilatations of abdominal arteries and dissecting aortic aneurysms in vascular type of EDS (EDS IV). Y.P. (2 Dec 1941), mother of F.P. (see below), spent a normal childhood and adolescence and practiced many sports, including judo and horseback riding. Two deliveries at ages 24 and 29 years were uneventful. She always had skin of ''bad quality,'' a tendency to keloids and scar dehiscence; alopecia started at age 30 years. She had bilateral club feet, as has her daughter, needed early dental prostheses, and had hyperextensible elbow, knee, and foot joints (requiring arthrodesis), and recurrent luxations of both shoulders. Surgery on the left shoulder resulted in rupture of the brachial plexus at age 37 years.

At age $37\frac{1}{2}$ years, because of suspected inguinal adenopathy and deep venous thrombosis on the left side, she was given antibiotics, anticoagulants (Marcumar®), and anti-inflammatories (Tanderil®). Shortly thereafter, she was in deep shock, with bilateral hematothorax and bleeding in the abdominal cavity, the retroperitoneum, and the subpericardial and hepatic subcapsular spaces. After resuscitation, angiography was done (the diagnosis was still unknown at that time) disclosing diffuse dilatations of abdominal arteries and dissecting aortic aneurysms.

At age 42, she had a spontaneous rupture of the right posterior tibial artery, which was diagnosed by arteriography of the right femoral artery performed in a peripheral hospital. Because of the fragility of the artery, a repair with sutures was impossible and a simple ligation was performed; nevertheless, the leg remained viable [258]. She also had vertigo due to a vertebro-basilar syndrome; radiographically, both vertebral arteries were markedly calcified. Four years later, she suddenly experienced a heavy pain in the right groin and died in shock two hours later. Autopsy was not performed (own observation).

Her daughter, F.P. (21 Aug 1965), was shown to have a Gly595Cys substitution, which resulted in the formation of unusual trimers that contained, in addition to the normal disulfide bonds at the C-terminus, two proα1(III) chains which were disulfide bonded in the middle of the chains and, probably for ill-understood conformational reasons, migrated faster than the regular normal homotrimers [259]. By direct collagen analysis of chorionic villus biopsies, two prenatal diagnoses predicted normal fetuses, and she delivered vaginally and without further complications two babies who were confirmed to be normal [260].

Comments: (1) The first hemorrhagic event in Y.P. occurred shortly after beginning therapy with anticoagulants, nonsteroidal anti-inflammatory drugs, and penicillin. These drugs are likely to have promoted the hemorrhage and are contraindicated in EDS IV. (2) Although the second arteriography was uneventful, the lethal hemorrhagic event 4 years later could have been promoted by this intervention. (3) The general risk of arteriography was recognized in the university hospital but was not conveyed to the peripheral hospital; therefore, this type of information should be marked on a medical identification paper. (4) The risk of injuring the plexus brachialis while operating on the shoulder should also be noted.

patients with biochemically confirmed disease and 199 of their affected relatives), complications were rare in childhood; 25% of the index patients had a first complication by the age of 20 years, and more than 80% had had at least one complication by the age of 40 years. The calculated median survival was 48 years (range 6–73) and did not differ between men and women. The most frequent complications were, in decreasing order, arterial rupture (79%), organ rupture (uterus, heart, liver, spleen) (10%), and gastrointestinal rupture (8%). Bowel rupture was often amenable to surgical treatment and thus rarely fatal, while arterial or organ rupture was associated with higher mortality with or without surgical intervention. The different types of complications were not associated with specific mutations in *COL3A1* (Figs. 18 and 19).The relative frequencies of arterial and gastrointestinal complications were similar for the first and second complications; thus, the nature of the first complication does not predetermine the nature of the following complications. We have documented the condition in a woman, L.R., with a Gly43Asp substitution (Figs. 13 h and 20) [230], who reached the age of at least 62 years in spite of numerous complications, whereas another report describes sudden infant death [296]. Gilchrist et al. [297] described a large kindred with a low risk of pregnancy complications and

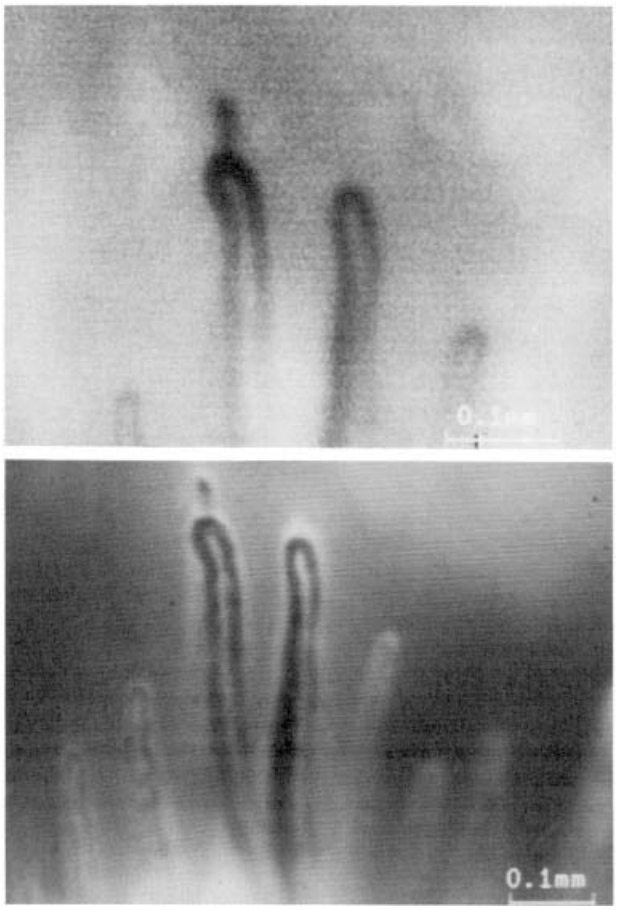


Figure 16. Microangiopathy in vascular type of EDS (EDS IV). In patient D.S. (see also Fig. 13e), intravital fluorescence microscopy of the nailfold was carried out with a fluorescence videomicroscopy system [277]. Before the application of dye, the morphology was examined under incident white light (**top**). Transcapillary diffusion was studied after i.v. bolus injection of sodium fluorescein (**bottom**), and the diameter of the capillaries of the arteriolar and venular limbs was measured after i.v. injection of indocyanin green (Cardiogreen®).

The microangiopathy consisted of multiple microaneurysms located at the apices of the capillary loops, which had a smaller neck and a larger head (indocyanin green); enlargement of the apical, arteriolar, and venular limbs of several loops; presence of microbleedings; and increased transcapillary diffusion of sodium fluorescein. Similar results were obtained in F.P. (legend to Fig. 15) [278].

The demonstration of microangiopathy in EDS IV indicates that the disease is not restricted to large vessels and thus points to a role of collagen III in the function of the capillary vessels.

unexpected longevity in some affected relatives; no deaths and few significant complications occurred among the eight affected women over a total of 30 full-term deliveries. For a family with a *COL3A1* mutation and apparent EDS III [209], see above.

The prevalence of EDS IV is not so low and is currently estimated to be 1:50,000. This estimate suggests that the frequency of mutations in the *COL3A1* gene is about the same as that of mutations in the collagen I genes that result in osteogenesis imperfecta, that the pleiotropic effects of the mutations obscure the diagnosis, and that, most unfortunately, knowledge about the disorder is still not widespread.

Defect and Pathogenesis

EDS IV is due to mutations in the *COL3A1* gene, located at 2q31-q32, which contains 51 exons distributed over 44 kb. The gene encodes a protein of 1,467 amino acids, of which 1,029 are located within the central triple-helical domain. The triple-helical domain is encoded by portions of 44 exons, of which 42 are cassettes that begin with a glycine codon and end with a Y-position codon, so that deletion of a single exon would result in an in-frame but shortened protein. The majority of published mutations in *COL3A1* result in substitutions of single glycine residues within the triple-helical domain, approximately one-third result in exon skipping, and a small number are larger genomic deletions [220,256,298] (for a list of over 200 mutations and numerous polymorphisms in *COL3A1*, see the Web site http://www.le.ac.uk/genetics/collagen/col3a1.html — see "Appendix", below).

Because procollagen III is a homotrimer, the synthesis of an equal number of normal and mutant α1(III) chains results in 7/8 of the collagen III molecules produced being abnormal and containing one, two, or three mutant chains. The abnormal collagen III leads to a quantitative deficiency of collagen III and sometimes, more deleteriously, to disturbed fibril formation by the remaining normal collagen III molecules. Therefore, tissues normally rich in collagen III, such as skin, blood vessels, and internal organs, are affected, in contrast to bone and cartilage, for example, which lack collagen III. In all cases studied at the molecular level, and in many less well-characterized patients, structural defects in collagen III lead to impaired secretion, intracellular storage and degradation, or lower stability of the secreted molecules, or both.

Different types of mutation most certainly have different consequences.

(**1**) In the case of glycine substitutions, triple-helix formation of molecules containing one, two, or three mutant α1(III) chains seems to be delayed for sterical reasons, as shown directly for abnormal collagen I in fibroblasts from patients with osteogenesis imperfecta [299] and suspected for collagen III in cells from EDS IV patients [300]. As a result, abnormal collagen III is overmodified and secreted only slowly or not at all and is degraded intracellularly to a significant extent [301,302].

(**2**) In the case of mutations leading to exon skipping, the abnormal molecules contain one, two, or three shortened chains, there is efficient secretion only of normal homotrimers and abnormal, shortened homotrimers, which are deposited efficiently into the extracellular matrix. Among individuals with exon skipping due to a single base substitution, Schwarze et al. [303] identified only two among 28 in whom the splice-acceptor site was affected, Pope et al. [220] two among five, and Giunta and Steinmann [230] one among four. The underrepresentation of splice-acceptor site mutations suggests to the current authors that the usual consequence of such mutations is the use of an alternative acceptor site that creates a null allele with a premature termination codon and thus leads to a 50% reduction in normal collagen III and a phenotype that may escape medical attention (see "Recent Developments").

(**3**) Splicing efficiency has been shown to be temperature-dependent in one patient, C.E., in whom a G to T transversion at position +5 of intron 25 resulted in skipping

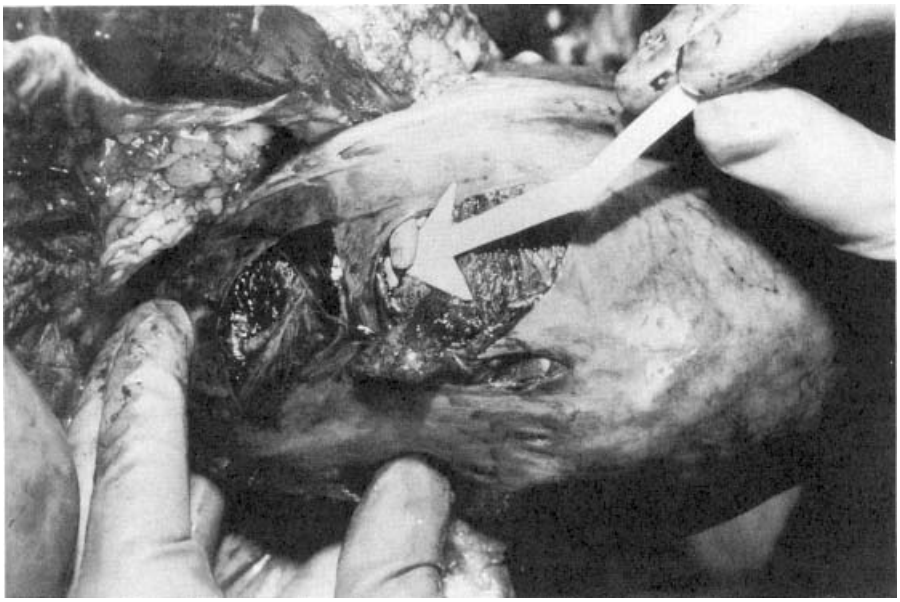


Figure 17. Rupture of the gravid uterus in vascular type of EDS (EDS IV). N.K., with a negative family history of EDS IV, had suffered spontaneous rupture of her sigmoid colon after an 8 week gestation at age 31 years; a therapeutic abortion was performed at the time of operation. Two years later, she was readmitted at 28 weeks gestation in premature labor and died shortly thereafter. At necropsy, the tissues were unusually easy to dissect. The thoracic aorta was torn transversally above and below a large dissection. The uterus had a complete, irregular 4 cm tear of the myometrium. The arrow points to fetal fingers stretching out of the torn uterus. It is of note that death may also occur several days or weeks postpartum [230]. (Reprinted from Rudd et al. [283] with permission.)

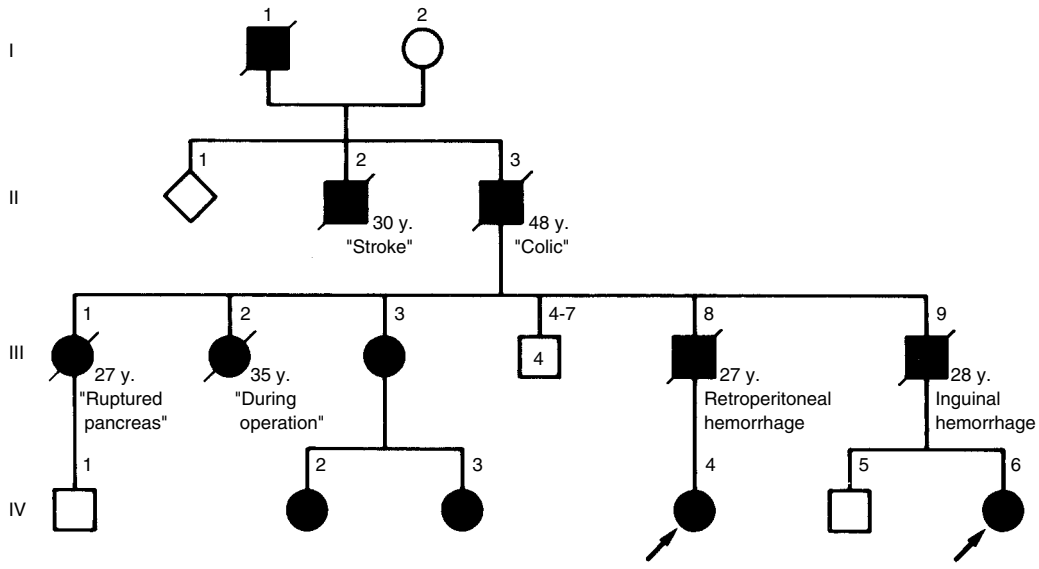


Figure 18. Pedigree of a family with vascular type of EDS (EDS IV). The index cases (**arrows**) came to medical attention in childhood because of easy bruising; the clinical diagnosis of EDS IV had earlier been made in subject III-9, who presented with recurrent bacterial meningitis due to bony defects in the skull [249]. The pedigree illustrates the manifold causes of death in EDS IV at various ages. It is also noteworthy that III-1 and III-3 brought pregnancies to term without major complications. The unaffected wife of individual II-3 could readily tell who among her nine offspring had the disease.

The molecular defect in this family consists of a Gly1003Asp substitution, which leads to a markedly reduced secretion of collagen III with intracellular retention and overmodification [259]; serum collagen III aminopropeptide is low in the two index patients [228]; for morphological studies, see [294,295].

This family originates from a small village in southern Italy where it was known as ''the family with the thin skin.'' The social implications of the disease are illustrated by this nickname and by the fact that the father of two young women tried (in vain) to dissuade them from marrying two brothers from the family (III-8 and III-9) because it was known that ''something was wrong'' with this family.

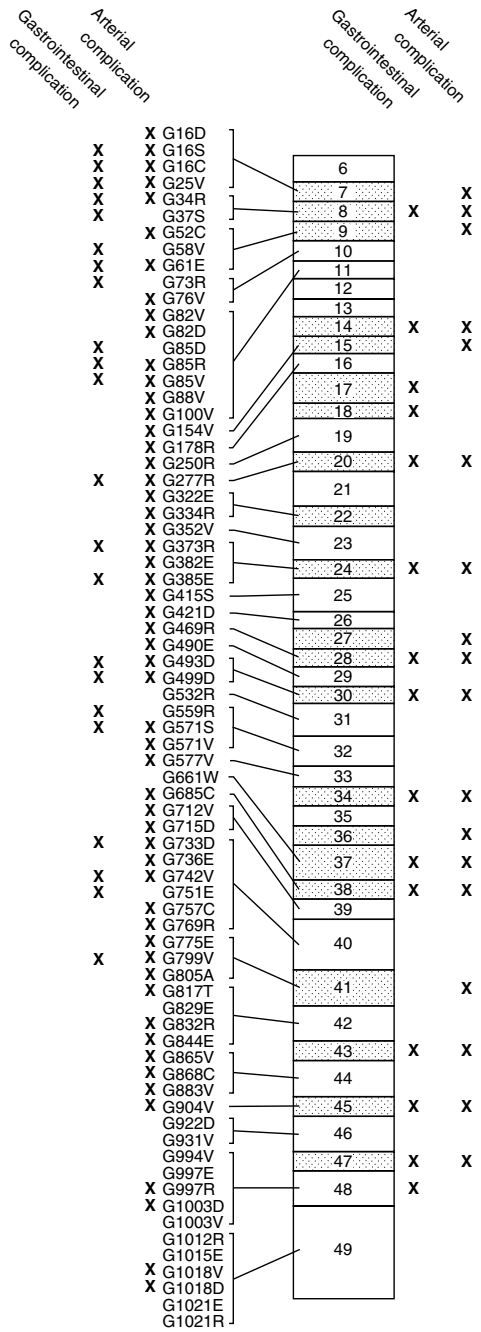


Figure 19. Lack of relationship between the nature and location of mutations in *COL3A1* and the types of complications in the vascular type of EDS (EDS IV). Causative mutations in the *COL3A1* gene were identified in 135 index patients, among whom there were 73 different mutations in 85 index patients that resulted in the substitution of some other amino acid for glycine (G) within the triple helix. Mutations in 41 patients led to skipping of a single exon (indicated by the stippling). The remaining mutations were more complex. The presence of an X at the site of a mutation indicates that one or more patients with that mutation had a complication of the indicated type. The amino acids are numbered from the first glycine of the major triple helix, which is residue 168 of the preproα1(III) chain. A denotes alanine; C, cysteine; D, aspartic acid; E, glutamic acid; R, arginine; S, serine; V, valine; W, tryptophan; and T, threonine. (Reprinted from [256] with permission.)

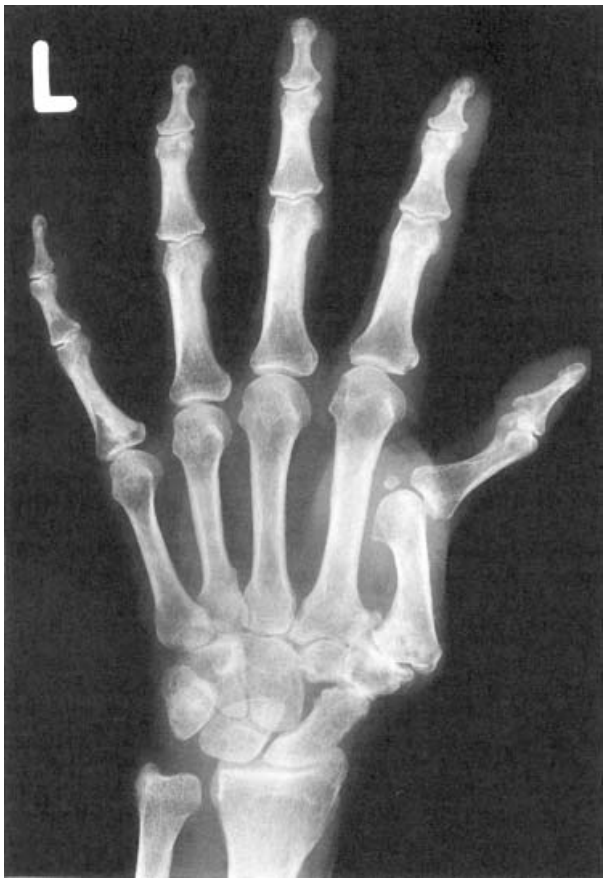


Figure 20. Vascular type of EDS (EDS IV). The x-ray of the left hand of L.R. (8 Jul 1928), at age 62 years, shows a chronically dislocated carpo-metacarpal joint of the thumb, marked arthritic degeneration of all joints, and a lack of adipose tissue.

Since the age of 50 years, recurrent luxations of the first metacarpal joint and arthritis of the trapezio-metacarpal joints had precluded a pinch grip, the picking up of large or heavy objects, and unscrewing bottle tops.

The patient was a sporadic case, born at term with a birth weight of 1.8 kg. Since early childhood she had suffered from easy bruisability, skin fragility, and mild joint laxity. She had had strabismus, hemopneumothorax, and hip dislocation after a car accident at age 29 years, the uneventful delivery of a healthy girl, an episode of ''nephrolithiasis'' due to a dissecting renal artery at age 45 years, several stroke-like episodes, and diverticulosis of the large intestine. Now she presented with a younger-looking face, often being asked if she had had a face-lift, with tight and lobeless ears (Fig. 13h); her skin was thin, and she had acrogeric hands and feet and suffered from the Raynaud phenomenon (for references and cell strains, see [213,214,217–219,228,230] and CRL 1384 and CRL 1397)[6]. Her condition was due to a Gly43Asp substitution [230].

of exon 25, which was almost corrected when his cells were incubated at a lower temperature [244]. The fact that his cells produced more collagen III at a temperature lower than 37°C is therefore due to a combination of reduced thermal stress on helix formation (see below), and hence rescue of mutant collagen [213], and a decrease in exon skipping [244]. An inverse temperature-splicing relationship has also been reported in a further patient [304]. (Another case of temperature-dependent splicing has been described in a patient with EDS VIIB [36,305].)

(4) Larger in-frame deletions may also lead to the secretion of two main populations of collagen III molecules, normal-sized and shortened ones. Patient B.H. had a 9.0 kb deletion spanning from intron 33 to exon 48 of *COL3A1*, and half of her α1(III) chains lacked the sequence corresponding to residues 586–999 [219,306,307]. Procollagen III molecules composed of either three normal length or three shortened chains were thermally stable and efficiently secreted, whereas those containing one or two shortened chains were unstable and completely excluded from secretion. Failure to secrete unstable molecules, and a possibly residual functional role of the stable "minicollagen III," which lacks the collagenase cleavage site, may explain the milder phenotype of this patient [306,307] compared with another EDS IV patient, D.S., who bore a similarly sized deletion toward the amino-terminal end of the α1(III) chain [226,227]. However, this concept of a possible role of the shortened homotrimeric collagen III *in vivo* may be challenged by the report of a 14-year-old sporadic patient [308] whose mutant collagen III lacked exon 41 and hence also the collagenase cleavage site; his mutant homotrimeric collagen III was efficiently secreted, not cleaved by collagenase, and had normal thermal stability. In the small skin biopsy specimen available, there was only 11% of the normal amount of collagen III; however, there was no evidence that the mutant homotrimers, which would be resistant to mammalian collagenase digestion, had accumulated in the dermis [308]. In another cell strain showing skipping of exon 17, Chiodo et al. [309] showed that the mutant shortened homotrimers were secreted normally and had a normal thermal stability but were not incorporated into the extracellular matrix of an *in vitro* model of dermis.

The stability of the triple helix of procollagen III in EDS IV is reduced as judged by its lower melting temperature when probed with proteinases. Approximately one-eighth of procollagen III molecules, composed of normal α1(III) chains, are secreted normally, which indicates that there is no defect in any putative secretory mechanism for procollagen III. Incubation of cultured fibroblasts at 30 °C decreases the thermal stress on helix formation and can increase the secretion of molecules that contain the abnormal chain for some, but not all, mutations [213]. The possibility that the presence of acrogeria correlates with the secretion of a thermally unstable collagen III, the extremities having a lower temperature than the core of the body, is intriguing but is at variance with findings in fibroblasts from the "acrogeric" patient, P.P. (Figs. 12b and 13a), from which no secretion of such unstable procollagen III could be demonstrated. One mutant cell strain did secrete abnormal collagen III almost as efficiently as normal [214]; however, it was extremely sensitive to proteases due to its Gly790Ser substitution (for further references concerning this case, see [228,310,311]).

Morphologic and Functional Aspects

Morphologic aspects. There have been only a few autopsies in which EDS IV-specific changes have been studied. Collagen III deficiency is most evident in the connective tissue scaffold of the blood vessel walls, dermis, intestine, lungs, and liver, and even in intervertebral disc tissue, where normally only little collagen III is present. The total amount of collagen extractable from lung and liver expressed per gram dry weight is similar in patients and controls, whereas the fraction of collagen III in pepsin-solubilized material is lowered to 3–4% of total collagen as compared with ~20% in control tissues [252,312,313]; in uterine leiomyoma tissue from an EDS IV patient, collagen III as determined by CNBr digestion was ~25% of that of controls [314]. Immunofluorescent staining of retained procollagen III with anti-procollagen III antibodies has been demonstrable in most fibroblasts [232,315], except in a few earlier reports [212,316]. In one case, C.E., the failure to stain [212] is clearly at variance with the biochemical evidence of retention of procollagen III by his fibroblasts [213].

Although collagen III constitutes only 10–22% of the total collagen in normal skin [317,318], at all levels of the dermis [319], its reduction in patients with EDS IV has a dramatic effect on both the thickness and architecture of their skin. In some patients, the skin measures only a quarter of the normal thickness and the reticular dermis is nearly identical in architecture to normal papillary dermis [233]. Collagen fiber bundles are small, and fibril diameters are either uniformly small or show marked variation. Elastic fibers are relatively abundant because of the decreased amount of collagen in the dermis. Because in the skin of normal fetuses below 20 weeks of gestation collagen III is the major collagenous protein present [317], it seems likely that it is important for the formation of a normal scaffold that can be elaborated upon by the subsequent synthetic activity of dermal fibroblasts and that its deficiency leads to the abnormal tissue structure observed in affected individuals. This notion is supported by the finding that all collagen fibrils in skin contain both collagens I and III, so-called heterotypic collagen fibrils [320]. The solubility of collagen in the lungs is markedly increased, which might also reflect an altered fibrillar organization resulting from a decreased content of collagen III [252].

Laurent and Agache [321] performed an early ultrastructural study demonstrating an engorged rough endoplasmic reticulum in fibroblasts from the dermis of an affected individual and suggested defective secretion of an unknown substance. This observation was crucial for the concept of Byers et al. [232,233,246] of impaired secretion of structurally abnormal procollagen III. Since then, dilatation of the endoplasmic reticulum in fibroblastic cells from skin [226,233,294,295,322] as well as in the lung [252] has been demonstrated in numerous other reports. Smith et al. [323] studied skin from 22 individuals with EDS IV in whom the *COL3A1* mutation had been identified. Dermal thickness ranged from 0.66 to 1.54 mm in affected skin compared to a mean of 1.25 ± 0.25 mm in 14 controls, elastic fibers were proportionally increased in relation to collagen fibers, and the collagen fibers themselves were finer and more loosely organized, as observed by scanning electron microscopy. The study suggested that different mutations in the *COL3A1* gene may have different effects on such parameters as secretion, fibrillogenesis, and skin architecture, depending on the nature and location of the sequence changes they induce. Substitutions for glycine and exon-skipping mutations at the C-terminal end of the triple helix led to very marked intracellular accumulation of mutant collagen III in the rough endoplasmic reticulum and to a considerably smaller than normal collagen fibril diameter (65–80 nm versus 92 ± 7.5, range 95–110 nm, respectively), as had been observed previously [193]. In contrast, mutations near the N-terminal end were associated with a more variable fibril diameter (85–120 nm), and there was less evidence of intracellular retention of abnormal collagen III [323]. The cross-sectional shape of fibrils often deviated from the regular rounded profiles of controls, but the changes were relatively subtle and different from the composite fibrils found in EDS I and

II [40,192,193]. In postcapillary venules in EDS IV skin, there was increased perivascular matrix and pericytes had dilated rough endoplasmic reticulum [323]. Why smooth muscle and endothelial cells, which also normally synthesize collagen III, do not have a similarly altered rough endoplasmic reticulum in the same patients is not clear [233,323]. Several features of EDS IV, namely the characteristic facial appearance, the frequently observed low birth weight [221], and clubfoot, are suggestive of a primary, morphogenetic defect. If one considers the preponderance of collagen III in the embryo, it is surprising that even more severe defects are not observed.

Because arterial ruptures are the most severe complications in EDS IV, arterial morphology is of interest. Arterial vessels may have a small bore and a thin wall, and their total collagen content is markedly reduced [34,324,325]. The adventitia is thin, and in the media collagen fibrils are diminished in number, while elastic fibrils are irregular, fragmented, and accumulated. The elastin appears accordion-pleated with a high waviness index, this being the ratio of the length of elastin in the internal elastic lamina to its circumference [326]. The average collagen fibril cross-sectional area was decreased in the media of all arteries and in the adventitia and intima of some arteries, whereas it was increased throughout the vena cava [325]. Immunofluorescence studies on tissues from patients whose fibroblasts secreted only 10% of the normal amount of collagen III did not reveal an abnormal distribution of collagen III, nor was the intensity of staining indicative of the amount of material deposited in the tissues of L.R. and C.E. [327] (K. von der Mark and B. Steinmann, unpublished observations, 1985). There have been only a few studies of the architecture of the gastrointestinal tract or of most other organs. Collins et al. [328] reported that even in areas away from rupture sites, the bowel wall was thin because of diminished submucosa and muscularis propria; similarly, the walls of the blood vessels in bowel submucosa and elsewhere in the abdomen varied in thickness and contained quite striking frayed and fragmented elastic tissue fibers.

Functional aspects. Only a few studies have been devoted to functional aspects of tissues from EDS IV patients. Nemetschek et al. [329] have shown by electron microscopy that the elastic lamellae of the media of an affected aorta seem to be rather isolated from the surrounding collagen fibrils, in contrast to control specimens, in which they are in close contact with the collagen fibrils. Because stress-strain curves show an increased stiffness of the affected arterial wall, the authors speculated that collagen III is involved in the optimal integration or anchoring of elastin in the arterial wall. In contrast, Østergaard and Oxlund [330] found an increase in the extensibility of the middle cerebral artery of patients not specifically classified as having EDS IV but dying from the rupture of intracranial saccular aneurysms at stress values between 100 and 200 mm Hg, but no such extensibility was found in brachial arteries of the same patients despite a deficiency of collagen III. Holzschuh et al. [331] determined by Doppler sonography the pulsatility index (i.e., the difference in flow velocity between systole and diastole compared with an overall mean flow velocity throughout the cardiac cycle) in the cerebral arteries of a 34-year-old woman and showed that it was lower than that in controls; they concluded that the elasticity was increased, which may favor the formation of aneurysms.

Abnormal myoelectric activity was measured *in vitro* in sigmoid and descending colon from an affected individual with colonic perforation, which suggested to the investigators concerned a possible link between abnormal myogenic activity and colonic perforation [332].

Genotype-Phenotype Correlation

At present, there is no clear correlation between the nature and location of mutations within the *COL3A1* gene and the clinical features of EDS IV. The most likely explanation of failure to find such a correlation is that the criteria for inclusion in studies require that most of the clinical findings be met. Thus, the range of phenotypic variation is likely to be small and may be limited to minor features such as acrogeria.

The phenotype to be expected for *COL3A1* null alleles is uncertain. In a small number of families, the synthesis of procollagen III is reduced, perhaps to only about half the normal level, and there is no evidence of intracellular storage. Such a biochemical phenotype would be expected to result in a milder clinical phenotype than classical EDS IV [333]. If these preliminary studies are confirmed, this type of defect would establish a distinct subclass analogous to mild osteogenesis imperfecta (OI type I, see Chapter 8, this volume). The negative results of two large studies of individuals with abdominal aortic aneurysms [271] and cerebral aneurysms [272] make it unlikely that *COL3A1* null alleles are frequent causes of these conditions. Also, heterozygous mice with a *COL3A1* null allele generated by targeted gene inactivation appear phenotypically normal [334] (see "Animal Models and Lathyrism" and below). Given that null alleles of the *COL1A1* gene result in the mildest form of osteogenesis imperfecta [335], type I, milder or late-onset visceral involvement may yet be a candidate phenotype for the *COL3A1* null allele phenotype. It will be interesting to see whether the so far underrepresented mutations that affect the splice-acceptor site, supposedly leading to null alleles [220,230,303], will be detected (see above); such an approach will have to be made by direct molecular genetic means because the ability to discern heterozygous null cells by protein assay is limited (see "Diagnosis"). A putative "mild" *COL3A1* mutation (Gly637Ser) has been associated with an EDS type III-like phenotype without arterial disease in a single family, but the phenotypic characterization of this family was incomplete [209] (see "Recent Developments").

Genetics

EDS IV is a dominantly inherited disorder, with a significant proportion (~50%) of cases representing new mutations [215]. As a rule, each patient or family has its own specific mutation. No paternal age effect has been demonstrated. Early linkage analysis studies showed cosegregation of the phenotype with *COL3A1* alleles [336,337].

Parental mosaicism has been documented in several instances. A clinically healthy father with an affected daughter was shown to be mosaic for a 2 kb deletion in *COL3A1*, which was present in only 10% of his leukocytes but 40% of his fibroblasts [280]. This difference in proportions of normal and mutant alleles between white cells and skin fibroblasts could have resulted from a difference in the allocation of cells early in embryogenesis or, alternatively, may reflect sampling from a clonally derived region in skin enriched for cells containing the mutant allele. If progenitor cells that contained the mutant allele were not selected against, and if they divided at the same rate as normal cells, then the proportion of cells with mutant alleles in blood indicates that the mutation occurred prior to lineage determination. Further cases of mosaicism

have also been documented [310,338,339] or have to be presumed or postulated because of the oligosymptomatic founder [209,230].

Autosomal recessive inheritance has never been demonstrated. On the basis of a study of two cases in which the putatively heterozygous parents were found to have apparently decreased levels of collagen III in skin compared with controls, and whose fibroblasts in culture seemed to secrete lower than normal quantities of procollagen III, it was suggested that the mode of inheritance of EDS IV was autosomal recessive [216]. This proposal is, however, invalid because it relied on the use of DEAE-cellulose column chromatography, which is markedly insensitive as a quantitative technique because of incomplete recoveries. More recent reinvestigation by SDS-polyacrylamide gel electrophoresis has disclosed that the cells of the first case, P.P., secrete approximately 10–15% of the normal amount of procollagen III and that small amounts of heavily overmodified procollagen III are retained within the cells. Normal amounts of mRNA with normal translation activity are detectable [217,226]. The demonstration of a heterozygous Gly1021Arg substitution in this case ends this debate definitively [220]. The second case, C.E., has since been defined as heterozygous for a point mutation leading to exon skipping [244]. The existence of a recessively inherited variant of EDS IV remains to be proven and, if such were confirmed, would appear to be unrelated to defects in collagen III [340].

The division originally proposed by Byers et al. [246] into subtypes A, B, C, and D is no longer meaningful because distinct biochemical and ultrastructural phenotypes can arise from different mutations at the *COL3A1* locus.

Diagnosis

This type of EDS has the worst prognosis, and biochemical confirmation should be sought, although analysis is labor-intensive and expensive. For this reason, careful examination of the patient is mandatory. The diagnosis is made by demonstration of structurally abnormal collagen III and/or direct mutation analysis (see "Recent Developments").

(1) Structurally abnormal collagen III produced by ascorbate-stimulated cultured skin fibroblasts or cells derived from noncutaneous tissues, such as artery, vein, or peritoneum [225], leads first to decreased overall production, defective secretion into the culture medium, and retention within the cells; second, to post-translational overmodification as judged by a slower electrophoretic mobility on SDS-polyacrylamide gels, especially of the portion retained within the cells; and, third, to lowered thermal stability and abnormal sensitivity toward proteases (Fig. 21). Procollagens or pepsin-treated collagens from culture medium and cell layers are best analyzed separately after radiolabeling the cells at 30 °C in addition to the normal temperature of 37 °C [213]. Culture conditions should also be standardized because cell density influences the ratio of collagen III to collagen I synthesized [218,342]. Because an apparent reduction of collagen III may occasionally be found in control fibroblasts, the demonstration of collagen III with abnormal stability or electrophoretic mobility is a better diagnostic criterion than quantitative measurements. Collagen III produced by fibroblasts from one large family with EDS IV was originally reported to be normal [336]; however, a more extensive analysis of the CNBr-derived peptides of collagen III revealed the abnormal migration of peptide α1(III)CB5 [343] due to a 27 bp deletion in exon 37 [344]. It should again be stressed that heterozygotes for a null allele would most probably be missed by this approach and that this may be one reason why such individuals have never been diagnosed; of course, an alternative explanation is that such individuals do not present an abnormal phenotype and thus escape medical attention (see "Recent Developments").

(2) Searching for a reduced amount of collagen in pepsin or cyanogen bromide extracts of skin is not recommended because of the excessive amounts of material required and the unreliability of results with regard to quantitative changes.

(3) Many cases of EDS IV can be detected by quantitative radioimmunoassay of the serum level of the procollagen III aminopropeptide (PIIINP), which is released during the conversion of procollagen to collagen. Indeed, the amount of procollagen III aminopropeptide is low in a major subset of patients with EDS IV whose fibroblasts secrete only little procollagen III [228]. However, this approach is experimental and not generally recommended because of biological variability, confounding concomitant conditions, and the analytical modification of the assay necessary for the detection of low serum procollagen III aminopropeptide levels [39,228]. Whether measurement of PIIINP in skin interstitial fluid (i.e., suction blister fluid), which is independent of its clearance by the liver, is superior remains to be proven [345]. Prolonged bleeding time has been used at the beginning of a study to ascertain family members [346], but this approach is to be discouraged because bleeding time is prolonged in most types of EDS and because it is an unreliable test.

(4) Direct molecular genetic analysis of the *COL3A1* gene will become faster and will provide a high analytical efficiency, especially when combined with the biochemical analyses obtained using cultured cells [230].

Differential Diagnosis

Excessive bruising is found in several coagulation disorders (hemophilias; von Willebrand disease [MIM 193400, MIM 277480]), platelet disorders (including leukemia), scurvy, and nonaccidental injury ("battered child") [347].

Poor wound healing is a feature of scurvy, dysfibrinogenemia (MIM 134820), and factor XIII deficiency (MIM 134570).

Ruptures of arteries occur in the Marfan syndrome (MIM 154700), arterial tortuosity (MIM 208050), isolated multiple cerebral aneurysms with or without polycystic kidney disease (MIM 173900) (for discussion, see Leblanc et al. [270]), arterial dissection with lentiginosis (MIM 600459), different forms of cerebral cavernous malformations (CCM1, CCM2, and CCM3; MIM 116860, MIM 603284, and MIM 603285, respectively), and hereditary cerebral hemorrhage with amyloidosis (MIM 105150) and have also been documented in mild osteogenesis imperfecta [348]. Spontaneous coronary artery dissection (MIM 122455), a rare event, occurs in relatively young persons, with a striking predilection for women. The association of lengthening and tortuosity of systemic, pulmonary, and coronary vessels together with skin and joint laxity and an elongated face seems to be an autosomal recessive disorder [197].

Recurrent pneumothoraces occur in the Marfan syndrome or as an isolated familial disorder (MIM 173600). Periodontal disease is also a major finding in the questionable entity EDS VIII [39]. Acrogeria of Gottron (MIM 201200) [349] and mandibuloacral dysplasia (MIM 248370) [350] are characterized by atrophic skin localized to hands and feet, but without translucent thin skin, mottled hyperpigmentation of

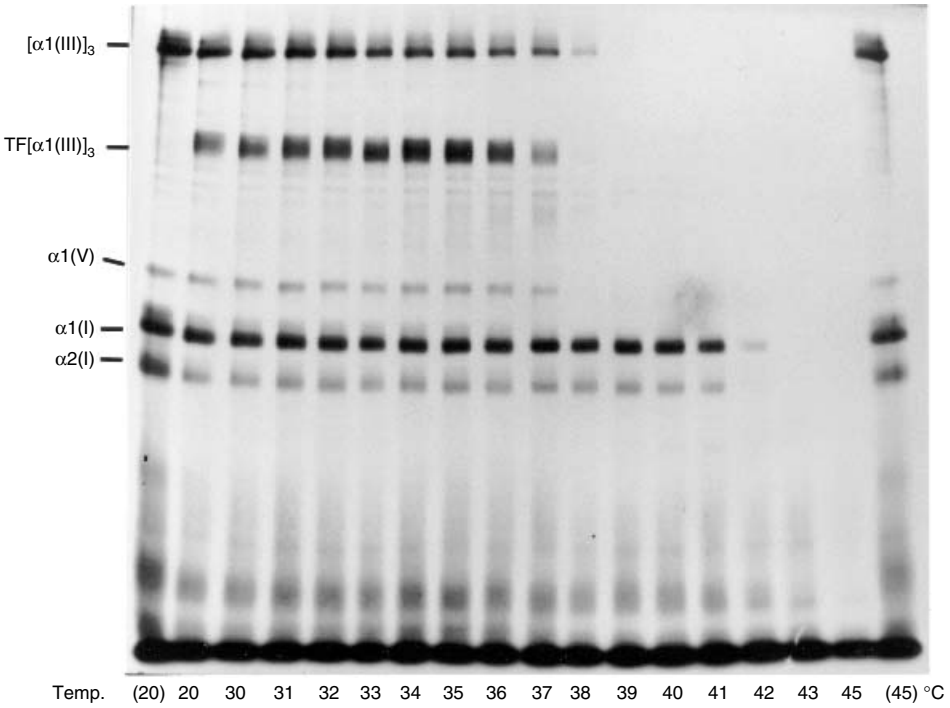


Figure 21. Abnormal thermal stability of collagen III in a patient with vascular type of EDS (EDS IV). Thermal stability of collagens produced at 37 °C in the cell layer of fibroblast cultures from Ch. W. Aliquots of pepsin-purified collagens were heated to the temperatures indicated, incubated with trypsin for 2 minutes, subjected to SDS-polyacrylamide gel electrophoresis, and visualized by fluorography (as a control, trypsin was omitted from the samples incubated at 20 °C and 45 °C, indicated by brackets). The results are summarized as follows.

First, the proportion of collagen III in relation to collagen I is increased, indicating cellular retention of abnormal collagen III molecules [341].

Second, the band representing collagen III is broadened as a result of overmodification caused by slower helix formation.

Third, incubation with the proteolytic enzyme produces a tryptic fragment (TF[α1(III)]$_3$) of abnormal molecules not seen in control preparations. This fragment loses helical conformation at approximately 37 °C. Moreover, [α1(III)]$_3$ trimers in this preparation are completely digested at 37–38 °C, whereas control [α1(III)]$_3$ trimers are resistant up to 39 °C (not shown).

Fourth, the thermal stability of collagen I (41.5 °C) and collagen V (37 °C) is identical to that in control preparations and serves as an internal control. (Reprinted from [259] with permission.)

the skin, thickened nails, micrognathia, and atrophic skin on the tip of the nose, there is no bruisability and no tendency to rupture of internal organs or vessels, and collagen III production is normal [351]. In the literature, the term acrogeria of Gottron has, unfortunately, often been used synonymously with EDS IV and *vice versa* [235,352,353]. Occasionally, patients with similar phenotypes, which may be dominantly or recessively (MIM 225350; MIM 225360) inherited, but with apparently normal collagen III metabolism, may be found ([215,340]; own observations).

Treatment and Management

Because causal therapies are not available, medical intervention is limited to symptomatic therapy, prophylactic measures, and counseling. Because of the danger of sudden arterial hemorrhage and bowel perforation, and the high risk associated with surgery, patients with EDS IV should carry a medical identification paper noting information about their diagnosis, possible complications, and blood group. General recommendations for anesthesia [265] and surgery [328] have been formulated; these include cross-matching of adequate amounts of blood, avoidance of intramuscular premedication, establishment of an adequate peripheral intravenous access and avoidance of arterial lines and central venous catheters if possible, control of hypertension, and gentle intubation maneuvers.

EDS IV represents a formidable challenge to the cardiovascular surgeon. The management of bleeding should be conservative as long as possible, especially when it is interstitial (muscular, retroperitoneal) [254,255,292]; bleeding in the abdominal cavity usually requires immediate transfusion and surgery. When operative therapy is required, minimal vessel dissection with balloon catheter or tourniquet occlusion should be performed, and vessel loops and vascular clamps, which frequently produce injuries that cannot be repaired, should be avoided. Standard anastomoses similarly often fail because of the poor tensile strength of the blood vessels. Primary arterial repair, if attempted, should be tensionless, using interrupted horizontal mattress sutures reinforced with pledgets. Vessel ligation with umbilical tapes, however, appears to be the safest operative therapy. Bypass grafting is then performed only if distal ischemia develops [254]. A review of the treatment of spontaneous carotid-cavernous fistula and outcome is given in [354].

Angiography should be avoided because of possible severe morbidity and frequent mortality [94,95,254,255,292] and replaced by ultrasonography [355] and/or subtraction angiography, although even this procedure is not free of risks [356].

The stripping of varicose veins may be difficult because they tear readily [312]. Surgical repair or embolization of carotid-cavernous fistula has been attempted with success in some patients [268,357].

Because of the high recurrence rate of distal colon perforation (15 cases out of 41 [254]), it is advisable to perform total colectomy and ileostomy when the first episode of perforation occurs [254,284].

Pregnancy in women with EDS IV should be closely monitored. Whether bed rest after 32 weeks of gestation and elective Cesarean section before incipient labor, to avoid the risks associated with vaginal delivery, should be recommended is still unclear [220,283,358]. It is worthy of note that sudden death may also occur several days postpartum. In the largest survey of "classical" EDS IV individuals, pregnancy-related complications led to death in 9–15% of women who became pregnant [256].

Because cerebrovascular complications occur in a minority of patients, screening for aneurysms is not warranted, and the risk of surgery would preclude intervention before the development of symptoms. Nevertheless, the occurrence of neurological symptoms, including headache, in individuals with EDS IV should alert the physician to exclude intracranial vascular pathology by noninvasive methods.

The management of hemopneumothoraces and thoracic cysts seems to be satisfactory using standard approaches [289].

Cough and constipation should be prevented by the timely use of antitussives and high-fiber diets and laxatives; enemas should not be given because colonic distention may result in perforation.

Many commonly used drugs interfere with platelet function, either alone (e.g., acetylsalicylic acid, nonsteroidal anti-inflammatory drugs) or in combination (penicillins and cephalosporins), and should be avoided or used with caution; paracetamol, pyrazolone derivatives, or synthetic opioids can be used instead. Anticoagulation therapy for venous thrombosis, or for the prevention of thrombosis during prolonged bed rest, should be avoided because it may lead to fatal bleeding (own observation; see legend to Fig. 15). In one patient, the administration of aspirin had to be discontinued because of a marked increase in spontaneous bruising [359].

Because atheromatous plaques, as observed in a patient described by Kontusaari et al. [275], may initiate arterial rupture, the prevention of atherosclerosis may be potentially beneficial.

Heavy physical exercise (isometric exercise, weightlifting), or procedures that lead to increased intrathoracic pressure such as blowing the alpenhorn or French horn [360], and contact sports are contraindicated. Psychological support may be important.

Genetic Counseling and Prenatal Diagnosis

In EDS IV, dominant inheritance should be assumed in sporadic cases also unless proven otherwise. When a low serum procollagen III aminopropeptide level or a defect in collagen III metabolism has been documented in the index case, this may be used for screening other family members [228], with the caveats outlined above. Linkage of EDS IV to *COL3A1*, direct demonstration of the gene defect, or protein studies on a chorionic villus biopsy are all methods of prenatal diagnosis. We have been able to rule out EDS IV in two pregnancies of one case (F.P.) by excluding the presence in a chorionic villus biopsy of a structurally abnormal collagen III previously observed in fibroblasts from the mother [260].

KYPHOSCOLIOTIC TYPE OF EDS — EDS TYPE VI (OCULAR-SCOLIOTIC TYPE) (MIM 225400)

Diagnostic Criteria

The kyphoscoliotic type of EDS is caused by a deficiency of lysyl hydroxylase 1 (PLOD1), a collagen-modifying enzyme, due to homozygosity or compound heterozygosity for a mutant *PLOD1* allele(s). It is characterized as follows [39]:

Major diagnostic criteria
- Severe muscular hypotonia at birth
- Generalized joint laxity
- Kyphoscoliosis at birth, which is progressive
- Scleral fragility and rupture of the ocular globe

Minor diagnostic criteria
- Tissue fragility, including atrophic scars
- Easy bruising
- Arterial rupture
- Marfanoid habitus
- Microcornea
- Radiologically considerable osteopenia
- Family history (i.e., affected sibs)

Note: The presence of three major criteria in an infant is suggestive of the diagnosis, and laboratory testing is warranted. In the majority of cases, the condition is caused by the enzyme deficiency and specified as EDS VIA, whereas a rarer, similar condition with normal lysyl hydroxylase activity is designated EDS VIB (see Table 1 and below), the biochemical/molecular basis of which is still unclear.

Historical Introduction

The existence of an autosomal recessively inherited, "ocular" form of EDS was suggested by Beighton in 1970 on the basis of case 3 in the legend to Fig. 22 (Fig. 6.16 in [2c]) because of the overall rarity of sight-threatening eye complications, which were, however, clustered in families with only sibs affected and/or with consanguineous parents [113]. Investigation of such a family led to the first definition of a chemical abnormality of collagen in man, a milestone achieved by Krane and his colleagues [33]. In 1970, they studied two sisters (cases 1 and 2 in the legend to Fig. 22) who, in addition to typical EDS, had marked muscular hypotonia, severe progressive kyphoscoliosis from birth, microcornea, and fragility of ocular tissues leading to rupture of the globe or retinal detachment. The mother ruptured her membranes while 3 months pregnant with the older child yet still managed to carry the pregnancy to term. The younger affected girl required enucleation following a relatively mild trauma to the eye. Biopsy specimens of skin from both children contained collagen that was normally soluble in nondenaturing solvents (such as dilute acid) but more soluble than normal in denaturing solvents (such as 4 M $CaCl_2$ or 9 M KSCN). In view of this, it was assumed that there was some defect in the intermolecular cross-linking of the dermal collagen. The most striking chemical abnormality, which came as a "surprise to the authors" (S. Krane, personal communication, 1991), was the decreased hydroxylysine content in skin (0.2 to 0.3 residues per 1,000 amino acid residues; 5–7% of normal). Because the hydroxylysine content of the skin of the clinically normal parents and older sister was normal, it was assumed that the disorder was inherited as an autosomal recessive trait

and was due to a reduced enzymatic hydroxylation of lysyl residues [33,362]. Indeed, when in a later study enzyme extract from the cells of case 3 was mixed with that from a control, the activities were essentially additive [374], an observation that favored the conclusion that a structural defect of the enzyme rather than a defect in the activation step or the presence of an inhibitor led to the deficient enzyme activity.

Because these patients had both features of the EDS and ocular abnormalities, but a distinct biochemical defect, it was suggested that they be classified as a new subtype, EDS VI, the ocular type [2], or the ocular-scoliotic type [37e], now the kyphoscoliotic type [39], of EDS because ocular signs, though dramatic, are less frequent features in larger series than was initially reported [39,363].

It remains to be explained how the chemical abnormality relates to the clinical manifestations because the presence of typical symptoms even in patients with an apparently normal or only somewhat decreased content of hydroxylysine in skin, and normal lysyl hydroxylase activity in fibroblasts ([88,92,409,410]; cases 4 and 5 in [383,411]), suggests a more complex relationship between the known molecular defects and clinical manifestations than has been thought up to now. As mentioned above, patients with a phenotype resembling that of the kyphoscoliotic type but with normal lysyl hydroxylase activity do exist, and these individuals are now classified as EDS VIB [37f].

Clinical Findings

The legend to Figure 22 lists the 57 reported and unreported patients with EDS VIA known to the authors, derived from 49 families, who have been described clinically and confirmed biochemically. Those newborns whose neonatal history is available were usually described as floppy, with a poor cry, difficulty in sucking, and delayed motor development (cases 1, 2, 5–8, 10, 12, 14–16, 18–20, 22–29, 51). Sometimes poor fetal movements were noted by the mothers. Because of the severe muscular hypotonia, many of the patients had undergone a neuromuscular workup. Leg cramps are a common complaint.

Kyphoscoliosis is often present at birth and is progressive and severe (cases 1–4, 7–14, 16, 18–20, 22–24, 33, 51) (Figs. 23a,b and 24). It is probably the result of muscular hypotonia together with ligamentous laxity because the vertebral bodies are structurally normal, and is often resistant to external bracing. Thoracic cage deformity and hypotonia

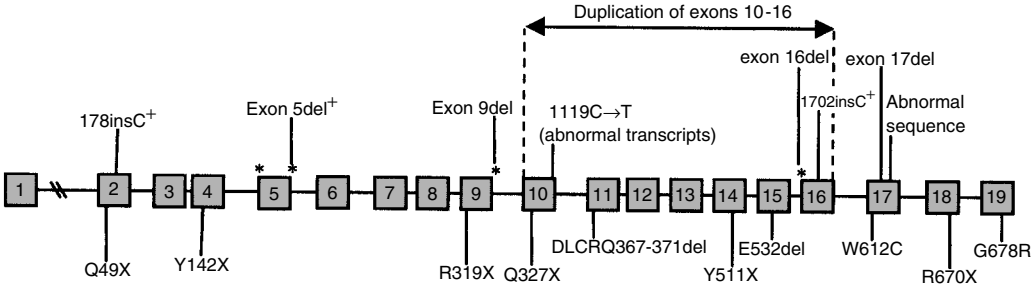


Figure 22. Structure of the *PLOD1* gene coding for lysyl hydroxylase 1 and mutations leading to the kyphoscoliotic type of EDS (EDS VI). The shaded boxes represent the 19 exons in the *PLOD1* gene joined by single lines indicating introns (not drawn to scale). The effects of point mutations and deletions on the amino acid sequence are shown below the line; above the line, the effects of other mutations are shown in the DNA: mutations that produce downstream preterm termination codons, PTCs, (+); splice-site mutations that result in exon skipping (*); as well as the common duplication of exons 10–16 (↔). (Adapted from [361] with permission.)

All biochemically proven patients known to the authors are listed below. The case numbers are followed by the mutation(s), sex, initials, and date of birth in parentheses, the ratio of urinary pyridinolines (LP/HP), and the references describing them, if the information is complete. Dup refers to the prevalent duplication of seven exons (exons 10–16) due to (a) homologous recombination event(s), Ter to a stop codon, PCT to preterm termination codon, and 0 to a functional null allele.

Cases 1 and 2, dup/dup, sisters (born 3 Dec 58 and 4 Jul 61) [33,362]; cases 7 and 8 in [363–373]; *cases 3 and 4*, dup/dup, sister and brother (J.L., 1924 and G.B., 1915), see Fig. 6-16 in [2], [37,111,113,373–378]; *case 5*, male [369,379,380]; *case 6*, male (J.D.H.), case 5 in [363,381,382]; *case 7*, del exon 16/del exon 17, male (9 Jan 76), case 1 in [383], [384–386]; *case 8*, male (18 years), case 2 in [383], [387–389]; *case 9*, male (33 years), case 3 in [383]; *case 10*, male (19 years), case 6 in [383]; *case 11*, del Glu532/Gly678Arg, male (A.T., 28 Jan 78), case 4 in [363], [390–393]; *case 12*, female (2 years) [392]; *case 13*, male (42 years), case 10 in [363], [426]; *cases 14–18*, cases 1,2, 3,6,9 in [363]; *cases 19 and 20*, Arg319Ter/Arg319Ter, sisters (H.A., 30 Jul 76 and N.A., 31 May 81), LP/HP 5.3 and 5.9 [394,395]; *case 21*, Tyr511Ter/del exon 5, male (SF 996, 3 Jul 89) [396,397]; *cases 22 and 23*, sisters (O.A., 29 Jun 84 and M.A., 16 Dec 94), LP/HP 6.3 and 5.9 [398]; *case 24*, dup/dup, male (A.R., 13 Mar 80), LP/HP 5.1 [373,399]; *case 25*, dup/dup, female (S.B., 12 Apr 77), LP/HP 5.3 [389,400,401]; *cases 26 and 27*, dup/?, brothers (A.B., 15 Sep 86 and H.B., 15 Oct 91), LH/HP 6.8 and 6.0 [373]; *cases 28 and 29*, dup/dup, brothers (T.D., 15 May 81 and A.D., 31 May 85), LP/HP 5.7 and 5.7 [373]; *case 30*, del exon 9/del exon 9, female (D.I., 18 Jan 90), LP/HP 4.3 [402]; *case 31*, dup/dup, male (A.R., 19 May 79) [401]; *case 32*, Trp612Cys/0, male (A.K., 14 Feb 80) [401]; *case 33*, male (T.O., 4 Oct 92), LP/HP 5.7 [own observation]; *case 34*, Tyr511Ter/Tyr511Ter, male (D.B. (1122), 34 years) [403]; *case 35*, D367LCRQdel/Gln49Ter, male (J.H., 3 Mar 84) [404]; *case 36*, D367LCRQdel/abnormal transcripts, female (C.C.) [404]; *case 37*, c.1702insC/c.1702insC, PTC in exon 16, female (K. (1072)) [405]; *case 38*, Tyr511Ter/Tyr511Ter, female (L.T., 17 May 70) [405]; *case 39*, Gln327Ter/Gln327Ter, female (A.C., 3 May 94) [405]; *case 40*, Tyr142Ter/del exon 14, female (A.F., 12 Aug 71) [405]; *case 41*, Arg670Ter/dup, male (M.C., 7 Feb 94) [405]; *case 42*, Tyr511Ter/Tyr511Ter?, male (J.H. (716)) [405]; *case 43*, IVS4-2A del/c.353insC, female (N.) [406]; *case 44*, Tyr511Ter/?, female [407]; *case 45*, female (D.A., 22 Apr 90), LP/HP 6.5 [own observation]; *case 46*, (A.A., 12 Mar 82), LP/HP 7.7 [own observation]; *case 47*, female (N.A-T., 31 Mar 95), LP/HP 6.9 [own observation]; *case 48*, female (Y.I., 10 Jun 93), LP/HP 7.8 [own observation]; *case 49*, male (J.S., 14 Jun 71), LP/HP 8.1 [own observation]; *Cases 50 and 51*, sisters (S.G., 14 Jun 70 and C.G., 23 May 65), LP/HP 4.6 and 7.8 [408]; *cases 52 and 53*, brother and sister (S.F., 12 Feb 80 and A.F., 28 Dec 81), LP/HP 3.9 and 4.1 [own observation]; *case 54*, female (F.Al-M., 9 Nov 00), LP/HP 8.1 [own observation]; *case 55*, female (K.K., 11 Oct 73), LP/HP 5.47 [own observation]; *case 56*, male (S.K., 20 Jul 93), LP/HP 3.5 [own observation]; *case 57*, male (D.D., 3 Oct 91), LP/HP 4.38 [own observation]; *case 58*, female (M.K., 9 Dec 95), LP/HP 3.4 [own observation].

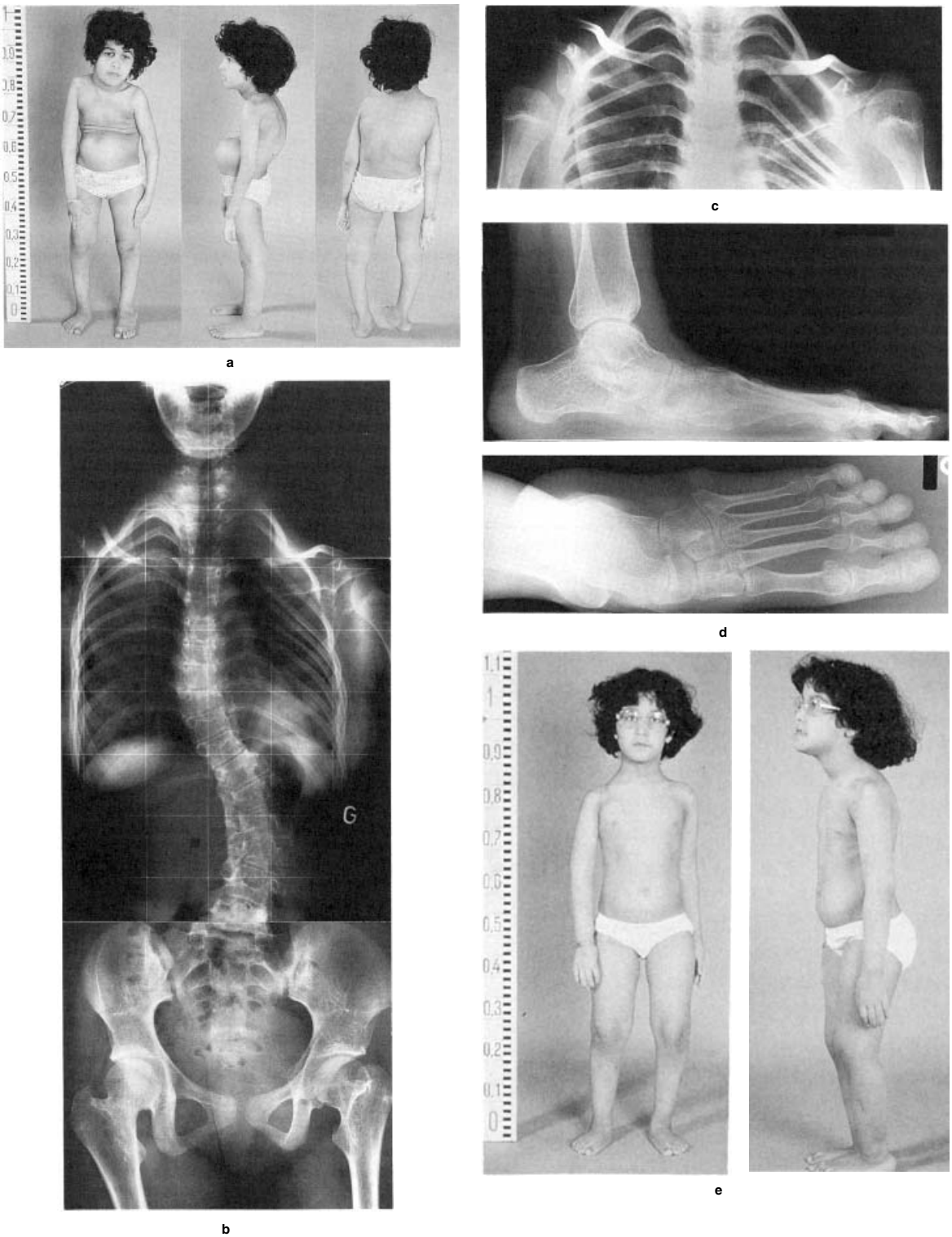


Figure 23. Kyphoscoliotic type of EDS (EDS VIA). H.A. (30 Jul 1976) and N.A. (31 May 1981) (cases 19 and 20 in the legend to Fig. 22) are the third and fifth children of healthy parents from Qatar who are first cousins. This family was the first in which the disorder was characterized at the molecular level [395]. (**a**) H.A. ($5\frac{1}{2}$ years old): severe kyphoscoliosis, dislocation of the right shoulder, flat feet in valgus position, microcornea. (**b**) X-rays of the spine 4 years after Harrington rodding and 3 years after removal of the rod because of its displacement due to premature loading (11 years). (**c**) X-ray of the shoulder showing dislocation of the right humerus (11 years). (**d**) X-ray of the foot showing marked osteoporosis and flat feet (11 years). Because of joint instability, arthrodesis of the lower ankle joint was required 2 years later. (**e**) N.A.: younger affected sibling who has a much milder phenotype, at age 5 years, shown for comparison, and thereby demonstrating marked intrafamilial variability. ((a), (e) reprinted from Steinmann [42] with permission.)

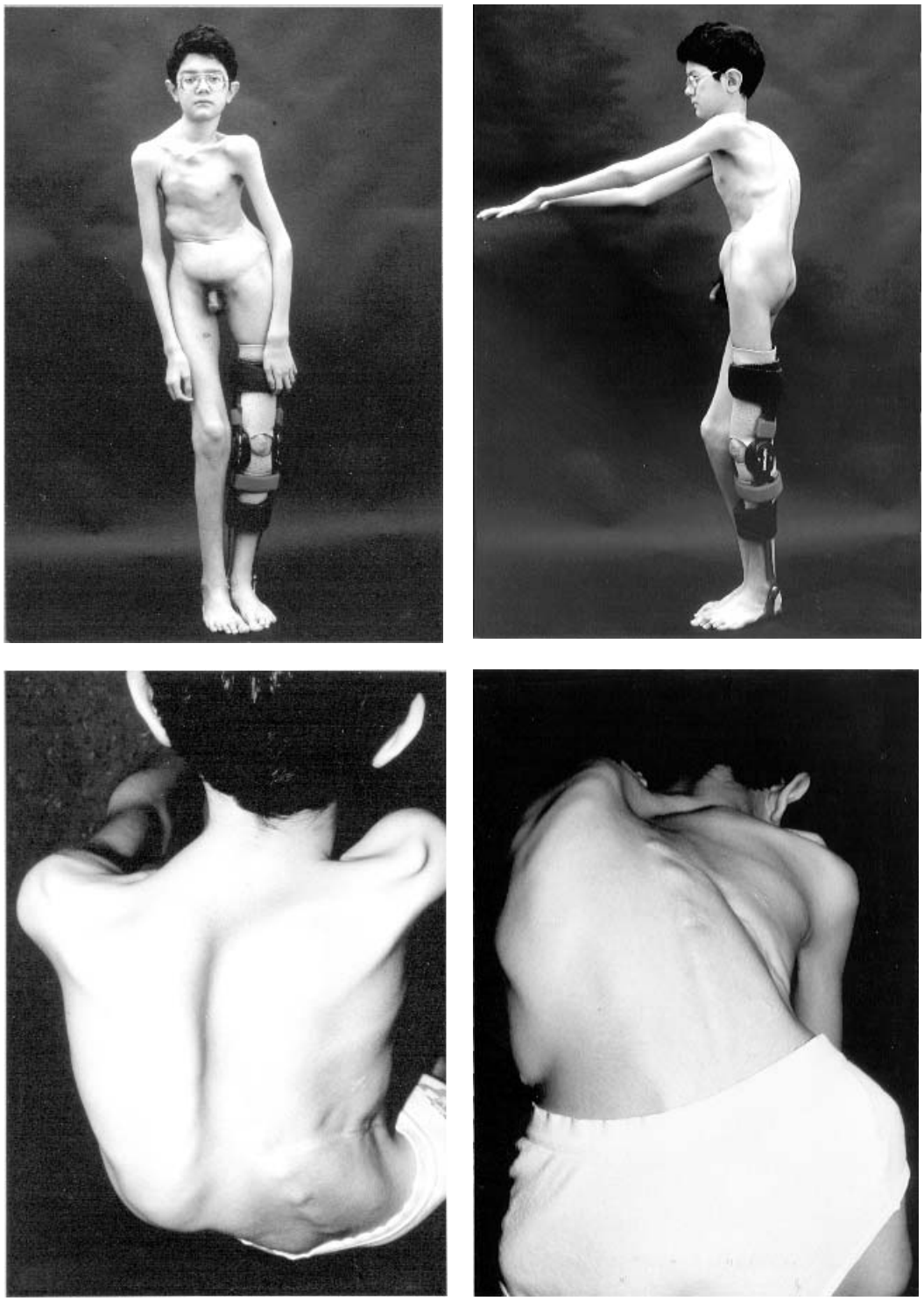


Figure 24. Kyphoscoliotic type of EDS (EDS VI). A.R., aged 13.3 years (case 24 in the legend to Fig. 22), with severe thoracic kyphoscoliosis and a Marfanoid habitus. He was only able to walk with the upper part of the body bent forward and preferred sitting in a wheelchair. He had microcorneae, brownish sclerae with decreased rigidity, and tortuous retinal arteries. Generalized joint hypermobility, habitual luxation of the shoulder, hypotrophic muscles, and marked skin hyperextensibility were present. There was mild aortic regurgitation, and lung function was severely restricted with a vital capacity of 30%. Only at age 13 years was EDS VI suspected and appropriate diagnostic procedures initiated.

He was born to consanguineous Iranian parents in the breech position. He was a weak baby and had difficulty in sucking. Treatment of the progressive kyphoscoliosis with a brace was not effective. Extensive and invasive neuromuscular workup remained noncontributory. During a ventral derotation spondylodesis from T11 to L5 at age 10.9 years, the inserted catheter led to an ectasia of an internal jugular vein, which was later resected. (Reprinted from Heim et al. [399] with permission.)

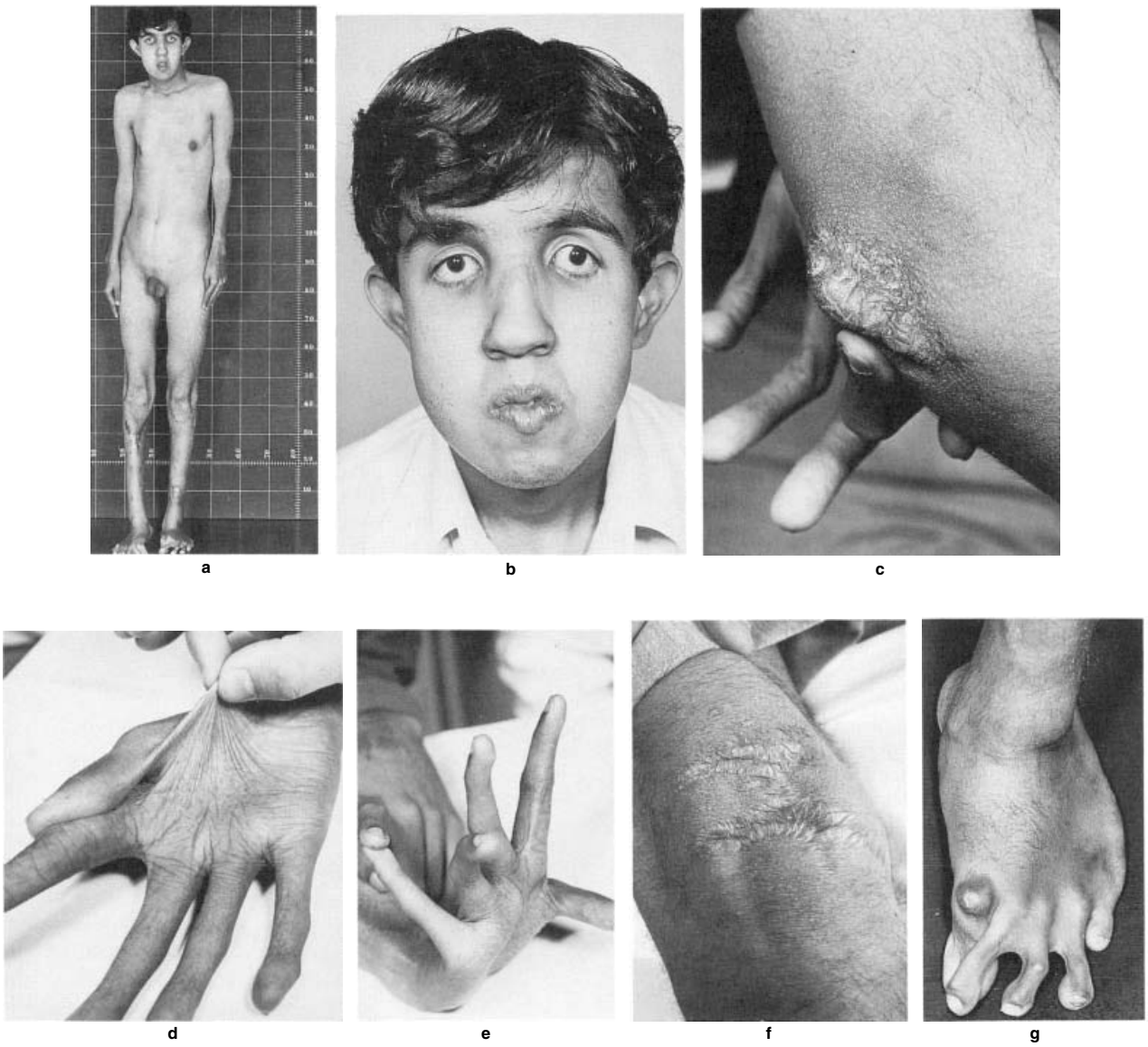


Figure 25. Kyphoscoliotic type of EDS (EDS VIB). A.S. (27 Apr 1956), 17 years old, the younger of two affected children born to healthy young parents from Pakistan who were first cousins. (**a**) Marfanoid habitus, moderate kyphoscoliosis and chest deformity, atrophic hemosiderotic scars, especially over the right shin, foot deformity, and mitral valve prolapse and regurgitation as judged by auscultation and echocardiography. His head circumference was normal (55 cm), and thus he differs from the cases with macrocephaly described by Cadle et al. [411] and Judisch et al. [409]. (**b**) Microcornea (9 mm in diameter), droopy eyelids, epicanthal folds, atrophic scars over the saddle of the nose, slightly lopsided and quite floppy ears. (**c**) Molluscoid pseudotumor over the elbow and spider-like fingers. (**d**) Hyperelasticity of the skin over the thenar and excessively wrinkled skin over the palm and fingers. (**e**) Hyperextensibility of the fingers. (**f**) Cigarette-paper-like scars over the knee. (**g**) Foot deformity and pseudotumor. Clinical follow-up: A.S. died at age 28 years of heart failure due to acute noncontrollable, bacterial endocarditis in his mother country (B. Steinmann, unpublished). (Reprinted from Steinmann et al. [88] with permission.)

lead to a decrease in pulmonary function and favor recurrent episodes of pneumonia, cardiac insufficiency, and early death (this occurred in case 4 at 54 years; in case 8 at 20 years; and in case 13 at 43 years). A Marfanoid habitus is quite common (Figs. 24, 25a). Osteoporosis seems to be a common finding but without any tendency toward fractures (Fig. 23d).

Ocular fragility leads to retinal detachment (case 3) and bleeding (case 3) and rupture of the globe, rather than of the cornea as in the brittle cornea syndrome, after minor trauma (cases 2–4, 9). Attempts to suture scleral tissue are often in vain (case 4). Microcornea is common (Fig. 25b) (cases 1–3, 6, 8, 10, 19, 20), the diameter in normal individuals being approximately 11.7 mm (range 11.0–12.5 mm) [412]; however, it also occurs in EDS I (as in the propositus and his affected daughter, Fig. 10). The sclerae are often bluish, in contrast to those in EDS I, II, and III, which are of normal hue.

Arterial rupture may be another prominent finding in EDS VI [363; personal observations]. Case 3 died at 51 years with symptoms typical of dissecting aneurysm of the

aorta; however, autopsy was not performed [37e]. Case 4 had a cerebrovascular accident in the distribution of the right middle cerebral artery at the age of 19 years [374]. Other impressive clinical descriptions of patients with classical features of EDS VI who were not studied biochemically have been reported in the older literature [2,119,413]. The natural history in relation to the vascular system is still not well-known. An ill-defined polyneuropathy in two siblings of consanguineous parents together with typical signs of EDS VI has been described [92].

To the authors' knowledge, affected females with proven EDS VI (see legend to Fig. 22) have not been reported to have given birth; in contrast, case 4 fathered four healthy children (see "Recent Developments").

Life span is markedly reduced because of arterial rupture and cardiopulmonary insufficiency due to severe kyphoscoliosis; there are, however, no precise data on mean age of survival (see above).

Obligate heterozygotes are clinically normal in our experience.

Macrocephaly has been reported in six patients with EDS VIB from three separate families and claimed to be a feature of a distinct syndrome [411] (R.G. Cadle, personal communication, 1990) (MIM 229200), but macrocephaly is clearly not present in other patients with EDS VIB (Fig. 25a) [88,440].

Interfamilial variability of EDS VI is quite considerable within EDS VIA, and even more so if EDS VIB, with its unknown genetic defect, is included. Intrafamilial variability as observed in the pairs of siblings (cases 1 and 2, 3 and 4, 19 and 20, 22 and 23, 26 and 27, 28 and 29, 50 and 51, 52 and 53; see legend to Fig. 22, Figs. 23a and 23e) further indicates that environmental factors and complications, such as neonatal brain hemorrhage in case 50 [408], and genetic factors other than mutations in the gene coding for lysyl hydroxylase are also important in determining clinical expression; the phenotype in case 51 is unusually mild [408].

Genetic Defect

The Gene, the Enzyme, and Physiology

Lysyl hydroxylase 1 (LH1) (EC 1.14.11.4; procollagen-lysine, 2-oxoglutarate 5-dioxygenase; PLOD1; MIM 153454) is a homodimer consisting of subunits with a molecular weight of about 85,000, each comprising 709 amino acid residues and a signal peptide of 18 amino acids. The C-terminal region is especially well-conserved, with a 139 amino acid region, residues 588–727, being 94% identical between the human and chick at the amino acid level, and with a 76 amino acid region coded for by exons 18 and 19, residues 639–715, being 99% identical [414,415]. By site-directed mutagenesis, it has been shown in expression systems that three residues, histidines 656 and 708 and aspartate 658, provide the three ligands required for the binding of Fe^{++} to a catalytic site, whereas the role of a third critical histidine (residue 706) remains to be established [416]. The enzyme appears to be a true resident of the endoplasmic reticulum despite the fact that it does not contain any known endoplasmic-reticulum–specific retention signals in its primary structure [417].

The ascorbate-dependent enzyme requires α-ketoglutarate, Fe^{2+}, and O_2 as cosubstrates and cofactors [418] and catalyzes the formation of hydroxylysine in collagen chains and other proteins with collagen-like amino acid sequences by the hydroxylation of lysyl residues in Gly–X–Lys sequences (for a review, see [419]). The hydroxylysyl residues formed have two important properties: first, their hydroxyl groups serve as sites of attachment for a monosaccharide or a disaccharide — galactose or glucosylgalactose, respectively — the function of which is unknown so far, although they may play a role in recognizing and activating collagen receptors in the cell membrane [420]; second, they are essential for the stability of the intermolecular collagen cross-links that provide tensile strength to most soft tissues and bone.

Cross-linking involving lysyl and hydroxylysyl residues occurs after the processing of procollagen and secretion of the molecules into the extracellular space. The first step in cross-linking is the oxidation of specific lysyl and hydroxylysyl residues in the telopeptide regions at both ends of the collagen molecule to form aldehydes [421]. This reaction is catalyzed by the extracellular copper-dependent enzyme lysyl oxidase (protein lysine 6-oxidase, EC 1.4.3.13, MIM 153455). When hydroxylysyl residues are present in the telopeptides, the hydroxylysine-derived aldehyde, hydroxyallysine, reacts with a specific peptidyl lysyl or hydroxylysyl residue in the triple-helical domain of a neighboring molecule in a fibril to form a bifunctional reducible intermediate that matures into a trifunctional nonreducible pyridinium cross-link through the addition of a third residue of hydroxylysine [421]. Two forms of pyridinium cross-link have been identified: hydroxylysyl pyridinoline (HP, or "pyridinoline"), formed from three hydroxylysyl residues, and lysyl pyridinoline (LP, or "deoxypyridinoline"), formed from one lysyl and two hydroxylysyl residues. These pyridinolines are especially abundant in bone, cartilage, and dentin [422]. Both compounds are biologically and chemically stable and are excreted in urine in free and peptide-bound forms as products of collagen degradation. Their urinary excretion correlates with bone turnover in acquired and inherited disorders affecting bone (see Appendix II, this volume), whereas a highly increased ratio of LP to HP is diagnostic for EDS VI (see below).

Lysyl hydroxylase 1 is coded for by the gene *PLOD1* (MIM 153454) on chromosome 1p36.2→p36.3 [415], which contains 19 exons and a 5′-flanking region with characteristics shared by housekeeping genes; the constitutive expression of the gene in different tissues suggests that lysyl hydroxylase has an essential function [423]. From sequencing the introns, where many mutations and rearrangements have been found to be concentrated, Heikkinen et al. [424] demonstrated extensive homology of intron 9 and intron 16 resulting from five and eight Alu sequences, respectively, contained therein (see Fig. 22).

The Mutations in the Gene Coding for Lysyl Hydroxylase

The mutations so far elucidated in lysyl hydroxylase in EDS VIA are indicated in Figure 22. Of special interest is the high frequency of the large duplication of nucleotides 1176 to 1955 corresponding to amino acids 326 to 585 in the normal sequence [415]. The seven-exon duplication in *PLOD1* is 8.9 kb in size, is caused by an Alu–Alu recombination in introns 9 and 16 of the gene, does not disturb the splicing of introns, and leads to a duplication of amino acids 326–585 [415,424]. This duplication was found in 19% of mutant alleles among 35 EDS VI families, but haplotype analysis showed variations in the sequence of the DNA region surrounding the duplication, thereby excluding a founder effect [373] and suggesting that it must be a frequent, independent event.

Pathogenesis

EDS VIA was shown by Krane et al. [33] to be due to a marked deficiency of lysyl hydroxylase. Residual activity in cultured skin fibroblasts ranges from 2% to 50% of normal using radiolabeled chicken protocollagen (unhydroxylated procollagen) as a natural substrate. These figures have to be treated with caution because the results may vary considerably from assay to assay, and even more so from laboratory to laboratory, making direct comparison difficult (e.g., in cases 1 and 2, residual activity was variously 14% and 10% [33] and 14.6% and 26.6% [363], respectively, in the same patients). Only a few studies of the kinetic and physicochemical properties of crude enzyme preparations from patients and controls have been performed. In one study using cultured fibroblasts from the two siblings above (cases 1 and 2), a mutant enzyme had optimal activity at 30 °C, rather than 34 °C, as for the wild-type enzyme, and did not form high-molecular-weight aggregates in low-ionic-strength buffers as did the normal enzyme. It also exhibited a higher apparent K_m for ascorbate (20 μM) than the wild-type enzyme (4 μM), and its activity was stimulated threefold or more by prior dialysis against buffer solutions containing 10 mM dithiothreitol, but no such effect was observed with the normal enzyme [364]. These differences in kinetic properties suggest that there is not simply a deficiency of enzyme protein in affected individuals but that an enzyme protein is present with abnormal properties. Miller et al. [382], in their study of cultured fibroblasts from case 6, found that the mutant enzyme had the same K_m for ascorbate (0.1 mM) as the wild type but that its apparent V_{max} was reduced to 25% of the control figure.

As a consequence of the lysyl hydroxylase deficiency, the hydroxylysine content of collagen is diminished, with a concomitant increase in lysine, although to variable degrees in different tissues. The amount of hydroxylysine is only 5% of normal in skin [362], 14–28% in fascia [362], 55% in tendon [387], 10–20% or 43–100% in bone, depending on the mode and site of sampling [363,365,387,425], and ~90% of normal in cartilage [362]. The level of hydroxylation in the C1q component of complement was initially reported as being 77% of normal by Pinnell [366], but a later study found it to be normal [384]; in both studies, the C1q was functionally normal. The analysis of different collagen types extracted from various tissues of case 8 and their respective chains gave similar results [387]. The extent of lysyl hydroxylation of collagen I extracted from skin was 0%, from bone 17%, tendon 36%, kidney 71%, and from lung 73% of normal. Collagen III extracted from skin and lung contained 0% and 50%, respectively, of the normal content of hydroxylysine, whereas collagens II, IV, and V from cartilage, kidney, and bone, respectively, were almost normally hydroxylated [387]. The authors of this latter study concluded that the varying degree of impairment of the hydroxylation of lysine residues correlated well with the severity of the clinical manifestations. Furthermore, cyanogen bromide cleavage of dermal collagen revealed that the hydroxylysine content was not uniformly low in the peptides examined but rather that it was zero in some peptides containing normally hydroxylatable lysyl residues and measurable, although still reduced, in others. Just one possibility to explain this is that the altered enzyme protein acts on different lysyl residues to different extents as a function of the variations in surrounding amino acid sequence [367]. Cultured skin fibroblasts from patients with EDS VI produce collagens I and III (and V), which are hydroxylated to a greater extent (~50% of normal) than their dermal counterparts formed *in vivo* [368,375,383,385,426]; overhydroxylation of collagen produced in culture is also observed in control cells [368] and may indicate that the rate of polypeptide elongation and/or helix formation in culture is delayed, thus allowing more extensive hydroxylation of lysyl residues. It is also clear that the content of hydroxylysine glycosides in collagen is likely to be markedly reduced. The ratio of glucosylgalactosyl hydroxylysine to galactosyl hydroxylysine in diseased bone appears abnormally high (1.04 and 1.24 in two cases versus 0.39 and 0.47 in two controls) [365]. However, the function of the sugar residues normally linked to hydroxylysine is not known.

The considerable extent of hydroxylation of collagen lysine residues that occurs in certain tissues in EDS VI patients may also explain why the urinary excretion of hydroxylysyl glycosides was only moderately decreased (62% of normal) in the two patients studied in this regard [362].

The above-mentioned wide variability in the extent of tissue-specific post-translational modification could be explained by several possibilities or a combination thereof:

1. Differences in the residual activity of lysyl hydroxylase in various tissues, resulting from a relative increase of either synthesis or activation of proenzyme. Krane et al. [369] reported that the ratio of lysyl to prolyl hydroxylase in dermal cells was 8–10% but that it was as high as 46–49% in bone cells of case 2.
2. Tissue-specific differences in the rate of collagen synthesis and triple-helix formation in the presence of limiting lysyl hydroxylase activity, as discussed above for the *in vitro* model.
3. Different affinities of the mutant enzyme for different collagens. Risteli et al. [388] found that the residual activity of lysyl hydroxylase in EDS VI fibroblasts (case 8) was 10% using underhydroxylated chicken collagen I as substrate but that it was as high as 35% using collagen IV from mouse sarcoma. The previously mentioned observation that the underhydroxylation of lysyl residues of CNBr-derived peptides is not uniform [367] could also be explained in this way.
4. Tissue differences in critical cofactor concentrations.
5. The existence of multiple forms of lysyl hydroxylase. This possibility may be supported by the unpublished results that lysyl hydroxylase activity in platelets from the two original patients was normal (cited in [367]) and the observation that cultured osteoblasts express a relatively higher residual activity than fibroblasts [369]. The urinary excretion of pyridinolines (see below) strongly indicates the existence of a lysyl hydroxylase activity other than the "classic type" [427]. Whether the isoform lysyl hydroxylase 2 (LH2), with its two tissue-specific splice forms, LH2a and LH2b [428], encoded by *PLOD2* located at chromosome region 3q23-q24 (MIM 601865) [429], is responsible for a substantial amount of the hydroxylysine formed is not clear because its tissue distribution has not yet been sufficiently studied, although it has been suggested that PLOD2 is associated with lysine hydroxylation in the nontriple-helical domains of collagen I in bone [430]. The same uncertainty holds true for isoform 3, lysyl hydroxylase 3 (LH3), encoded by *PLOD3* (MIM 603066) at

chromosome region 7q36 [431,432] — which has interestingly, in addition to lysyl hydroxylase activity, collagen glucosyltransferase activity [433] — and the putative lysyl hydroxylase 4 (LH4); all four isozymes are highly homologous at the C-terminal end and other parts of the molecules [431,432] and seem to be derived from an ancestral gene by two duplication events [434]. No differences in catalytic properties are found between the recombinant lysyl hydroxylase 3 and 1 isoenzymes using an artificial substrate, but the data do not exclude the possibility that differences might exist with respect to the hydroxylation of different collagen types [432]. It is possible that a deficiency of lysyl hydroxylase 2 and 3 isoforms may lead to some other variant of EDS or to some other heritable connective tissue disorder such as Bruck syndrome (see Chapter 26, Part IV, this volume).

6. The questions of why mRNA levels for LH1 do not correlate with lysyl hydroxylase activity measured in cells (R. Myllylä, unpublished data, cited in [431]) and why removal of the most conserved portion of LH1 does not totally abolish the enzyme activity [395] remain open.

The pathophysiology of this disorder is becoming clearer. Whereas early studies found no pyridinolines in normal skin [422], Pasquali et al. [435] re-examined skin, the major organ affected in EDS VI, tendon, and bone from controls and patients; the total amount of pyridinolines in patients was only ~50% of normal in skin but normal in tendon and bone, although the ratio of LP to HP was increased in the three organs. Therefore, although a qualitative abnormality in pyridinoline cross-links occurs in all tissues, only the skin of patients with EDS VI has reduced amounts of total pyridinolines, with absent hydroxylysyl pyridinoline. In long-term cultures containing ascorbic acid of fibroblasts from patients with EDS VI, pyridinium cross-links, when present, had a greatly increased LP to HP ratio (6.39 ± 3.86 versus 0.22 ± 0.10 in controls), comparable to that determined in urine from patients and controls, respectively [436] (see below)[8].

It appears that the solubility of collagen in denaturing solvents is inversely proportional to its residual content of hydroxylysine. Dermal collagen with a low (5% of normal) content of hydroxylysine is excessively soluble in 4 M $CaCl_2$ or 9 M KCSN [362,374], whereas collagen from bone (10–20% of normal hydroxylysine content) and cartilage (90% of normal hydroxylysine content) are extractable in these solvents in only moderately increased or normal amounts, respectively [362]. Because of the Amadori rearrangement and the formation of ketoamines, cross-links derived from hydroxylysine aldehydes are chemically more stable *in vitro* than those derived from lysine aldehydes, but this does not necessarily imply greater stability *in vivo*. Eyre and Glimcher [365] found an abnormal profile of reducible cross-links in skin and bone collagens, but again it is not clear how this is related to the mechanical weakness of the connective tissues because the lysine aldehyde-derived cross-links should have made equally effective intermolecular bonds. Two nonreducible trifunctional collagen cross-links, lysyl pyridinoline and hydroxylysyl pyridinoline, based on a 3-hydroxypyridinium ring, are derived from hydroxylysine and its aldehyde. Indeed, Eyre [370] has found decreased levels of the pyridinoline cross-links in the annulus fibrosus of a patient with EDS VI and the presence of a newly observed, more basic hydroxypyridinium compound that could be a lysine analog of the normal compound. It was shown that the ratio of total lysyl to hydroxylysyl pyridinolines in bone of case 8 (legend to Fig. 22) was 11.9 and 12.5 instead of 0.13 and 0.25 as in controls [389,435].

In EDS VI patients, the skin is unusually thin. This most probably is due to a reduced amount of collagen relative to elastin because the ratio of hydroxyproline to desmosine is only 1/4 of normal [365]. Why the amount of dermal collagen is reduced in these patients remains speculative but could be explained by its higher solubility, which favors rapid turnover and degradation. Bones are more radiolucent; osteoporosis seems to be a direct consequence of the qualitative change in pyridinolines and their glycosides in an as yet undefined manner and may also be enhanced by epigenetic factors such as muscular hypotonia and poor physical activity rather than to the underlying molecular cause (cases 19, 20; Fig. 23d).

Finally, it remains to be emphasized that residual enzyme activity and dermal hydroxylysine content correlate poorly with the severity of the phenotype. This is best exemplified by certain patients with EDS VIB, such as those described by Steinmann et al. [88,379].

Genetics

EDS VIA is inherited as an autosomal recessive trait. This is supported by the occurrence of affected siblings from unaffected parents (legend to Fig. 22), by the occurrence of affected children from consanguineous parents (cases 7, 8, 13, 19 and 20, 22 and 23, 24, 28 and 29, 50 and 51) and by there being intermediate enzyme activity in obligate heterozygotes who are clinically normal. However, in some parents, heterozygosity cannot be established using the usual enzymatic assay (father of cases 1 and 2, parents of case 5, father of case 11). Therefore, the disorder in the corresponding patients was thought to be most probably due to compound heterozygosity because it is the rule rather than the exception in very rare autosomal recessive disorders when there is no parental consanguinity, until it was discovered that the Alu–Alu repeats lead to frequent duplication events (legend to Fig. 22). The autosomal recessive mode of inheritance of EDS VIB is based only on formal genetic considerations [88,92,409,410] cases 4 and 5 in [383,411,440].

Diagnosis

The clinical diagnosis is still generally suspected very late and only after a negative workup of neuromuscular disorders [395,398,399]. This is mainly due to the rarity of the disease and its unfamiliarity to most physicians.

[8]Until recently, it was not known how pyridinoline cross-links are formed in cases such as cases 19 and 20 with proven absent activity of the "classical" lysyl hydroxylase [427]. Indeed, not only is lysyl pyridinoline formed — in excessive amounts, apparently in proportion to the decrease in hydroxylysyl pyridinoline — which requires hydroxylysine in telopeptides, but also some hydroxylysyl pyridinoline, which requires hydroxylysine within the triple helix, is formed as well. The most plausible explanation for pyridinoline formation is the existence of the predicted N-telopeptide-specific lysyl hydroxylase [437,438], which, however, has been shown to be bone-specific [430,439]. Furthermore, such an observation implies an alternative source of hydroxylase activity for at least some triple-helical region lysyl residues in collagen as well as the telopeptide hydroxylase activity [427] (for details regarding lysyl hydroxylase isoforms, see the text).

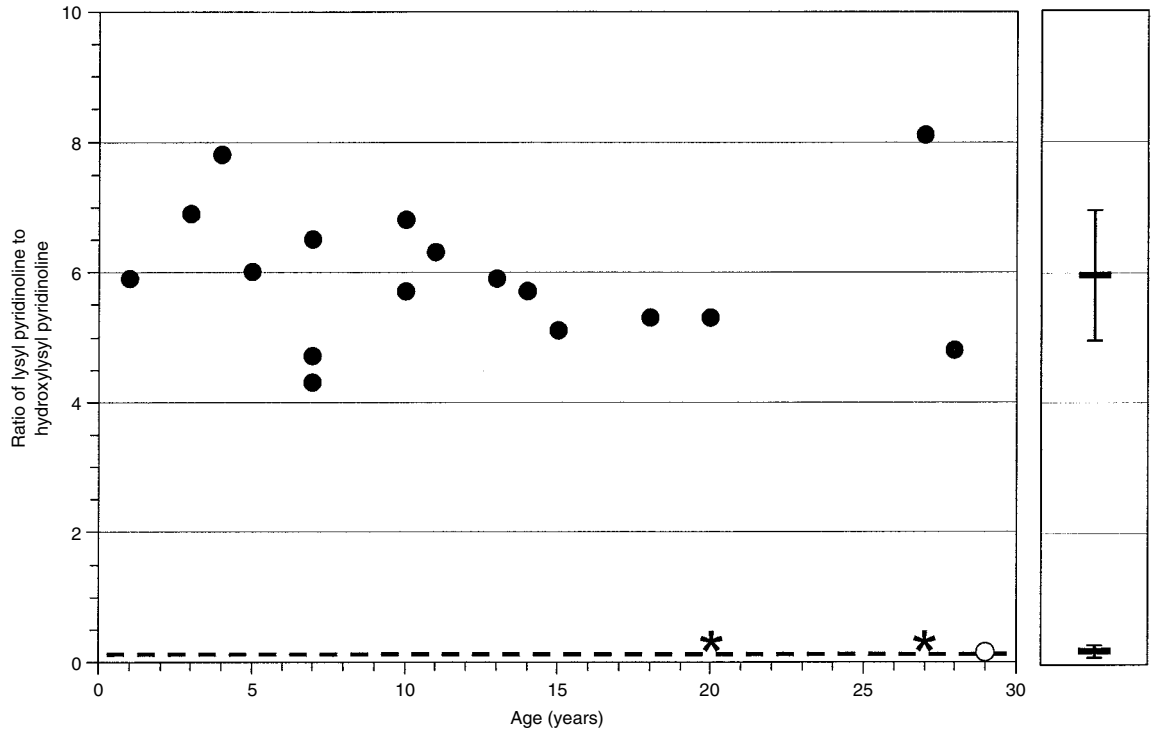


Figure 26. Urinary pyridinolines in patients with kyphoscoliotic type of EDS (EDS VI) and controls. The ratios of urinary total lysyl pyridinoline (LP) to total hydroxylysyl pyridinoline (HP) are given for each of 17 individuals with EDS VIA (•) as a function of age and compared with the mean of those of controls (▬ ▬) (**left panel**). Means of both groups of all ages are shown in the **right panel** and are 5.97 ± 0.99 (±1 SD, range 4.3–8.1) for EDS VIA and 0.2 ± 0.1 (±1 SD, range 0.1–0.4) for controls. The intraindividual variation of the ratio in 14 urinary samples collected over a time span of 7 weeks by one of the EDS VIA patients, T.O., was small (5.64 ± 0.31, range 5.21–6.22) [442]. The ratio in the female patient with EDS VIB (○, [88]), however, was 0.15 and indirectly confirmed the normal activity of lysyl hydroxylase [379]. The symbols (*) indicate two of the three patients studied with alkaptonuria.[9]

Note: the urinary pyridinoline ratio is independent of age and distinguishes both groups without overlap (for further comments, see Footnote 9).

Sometimes, confounding factors or unrelated associated diseases [398] detract from its recognition.

(1) The recommended, simple, reliable, and noninvasive laboratory test is the measurement by HPLC of total urinary lysyl pyridinoline ("deoxypyridinoline") and hydroxylysyl pyridinoline ("pyridinoline") cross-links after hydrolysis, a test that has a very high degree of sensitivity and specificity [389,427,435,441]. The ratio of the two compounds is in an abnormally high, narrow range, 5.97 ± 0.99 (mean ± 1SD; range 4.2–8.1) in 17 proven cases, as opposed to the low, narrow value of 0.20 ± 0.10 in controls, and is age-independent [442] as it is in controls [425] (Fig. 26). Obligate heterozygous parents have normal values [427]. Furthermore, this high ratio is not observed in other inherited or acquired collagen disorders, and the normal ratio is not changed by ascorbate or penicillamine and is only minimally influenced by renal dysfunction [441][9].

(2) The determination of the hydroxylysine content of dermis freed from the epidermis using an amino acid analyzer following hydrolysis [88,445] is also easy and quick, although more invasive. The results may be expressed as lysyl residues/1,000 amino acid residues (normal mean 3.85 ±

[9]The urinary total lysyl pyridinoline (LP) to hydroxylysyl pyridinoline (HP) ratio is unchanged in alkaptonuria (see Fig. 26) and under ascorbate and penicillamine treatment; however, it is abnormally low in Bruck syndrome (MIM 259450, [439]).

Purified lysyl hydroxylase has been reported to be inhibited by homogentisic acid in a linear, noncompetitive, and reversible way, with a K_i of 120–180 μM, and biosynthesis of hydroxylysine-derived intermolecular collagen cross-links in organ cultures of embryonic chick calvaria was inhibited by homogentisic acid in a dose-dependent manner [443]. The authors speculated that these results may explain the predilection of alkaptonuric complications for hydroxylysine-rich tissues such as articular cartilage, heart valve, vascular wall, and bone. To address the question of potential inhibition of hydroxylation of helical and nonhelical lysyl residues by homogentisic acid and related metabolites, we measured urinary total lysyl pyridinoline and hydroxylysyl pyridinoline in three alkaptonuric adult individuals, the oldest one symptomatic (M.Y.) and the two younger ones asymptomatic (A.A., G.A.-L.). Pyridinolines were normal in total amounts excreted and relative proportions (i.e., 0.16–0.18) [444]. These results imply that under *in vivo* conditions the claimed inhibition of lysyl hydroxylase by homogentisic acid or its derivative benzoquinoneacetate seems not to be operative, at least not in the organs that are the main source of urinary pyridinolines.

An 11-year-old girl (P.J.) with Wilson disease had an unchanged urinary LP to HP ratio of 0.22 before and after 16 months of treatment with penicillamine 3 × 300 mg daily; the ratio was 0.16 after a 12-month period in which penicillamine was replaced by oral zinc (100 mg/kg body weight) and 0.15 after a 12-month period of combined treatment with the same doses of zinc and penicillamine.

For details regarding the lack of influence of ascorbate on the urinary LP to HP ratio, see "Treatment" in EDS VI.

0.42, range 3.0–4.24, $n = 8$), as the ratio of hydroxylysine to hydroxyproline (normal mean 0.042 ± 0.006, range 0.033–0.047, $n = 8$), or as the ratio of hydroxylysine to lysine (normal mean 0.15 ± 0.02, range 0.11–0.18, $n = 8$) (values calculated from Steinmann et al. [88]).

(3) The determination of enzyme activity in cell extracts is done on a research basis only. The assay is not very sensitive and requires fresh, biosynthetically [^{14}C]- or [^{3}H]-lysine-labeled unhydroxylated collagen (protocollagen) prepared from embryonic chick calvariae or tendon cells as a natural substrate [418]. Because enzyme activity depends on cell density [367], the specific activity of the substrate, and cofactor composition, appropriate control cells have to be included in each assay. The results may be expressed as the amount of hydroxy[^{14}C]lysine formed, as the amount of tritium released, determined as [$^{3}H_2O$], or as the extent of decarboxylation of 2-oxo[1-^{14}C] glutarate, per number of cells or μg of protein in the cell extract or, preferably, as the ratio of lysyl hydroxylase activity to prolyl hydroxylase activity, the latter serving as a reference enzyme [418,432].

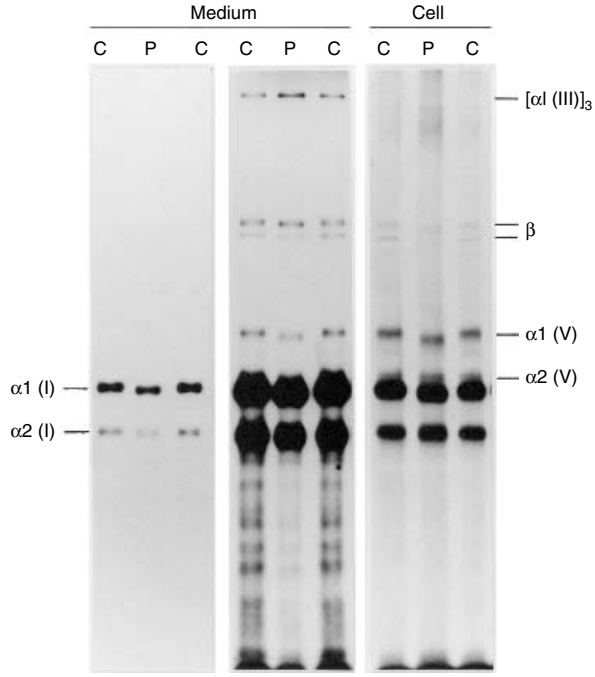


Figure 27. Lysyl residues in collagens from patients with kyphoscoliotic type of EDS (EDS VI) are underhydroxylated. Pepsin-treated radiolabeled procollagens from medium and cells synthesized by fibroblasts from case 23 with EDS VIA (P) and two controls (C) were electrophoresed on a 5% SDS-polyacrylamide gel, processed for fluorography, and exposed to x-ray films for 3 hours (**left panel**) or 7 days (**middle and right panels**). The α1(I), α2(I), and α1(V) chains and the β11 and β12 components from the patient's cells migrate slightly faster than those from the controls; the faster electrophoretic mobility is not apparent in the large collagen III molecules consisting of three disulfide-linked α1(III) chains but is obvious after reduction to free α1(III) chains (not shown). This is an indirect demonstration that collagens are underhydroxylated. Similar results were obtained after electrophoresis of collagen extracted from skin and visualized by silver staining; direct measurement of amino acids in dermis from the patient showed a marked deficiency in hydroxylysine (not shown) and proved the indirect conclusions derived from these observations. (Reprinted from Jarisch et al. [398] with permission.)

A synthetic tridecapeptide containing [^{3}H]-labeled lysine and corresponding closely to residues 98–110 of the collagen α1(I) chain has also been used as a substrate to determine residual activity in case 11 [390].

(4) It is difficult to demonstrate a decrease in the extent of hydroxylation of lysine residues in collagen produced by cultured fibroblasts from patients with EDS VI because the level of collagen hydroxylation in cultured cells is normally higher than it is *in vivo* (see above). However, a somewhat faster electrophoretic mobility of α chains on SDS-polyacrylamide gel electrophoresis is noticed due to relative underhydroxylation and underglycosylation (Fig. 27, [398]).

(5) Mutation analysis of the *PLOD1* gene will become easier and will be especially useful for prenatal diagnosis and research.

The diagnosis of EDS VIB is based so far on the typical clinical findings, together with a normal hydroxylysine content in dermis, normal lysyl hydroxylase activity, and a normal ratio of urinary pyridinolines as shown in the female of the sibs described in [88] (Steinmann et al., unpublished results) (Fig. 26).

Differential Diagnosis

EDS VI is a rare differential diagnosis in the neonate with severe muscular hypotonia; neuromuscular disorders will have to be considered first. Later, the brittle cornea syndrome [445], fragilitas oculi with joint hypermobility (MIM 229200, under which condition are included several patients also designated by McKusick [37] as having EDS VIB), and other causes of corneal rupture [410] have to be considered. Cameron et al. [446,447] reported EDS VI-like patients who presented with corneal thinning, keratoglobus, blue sclerae, corneal rupture, joint hypermobility, similarly affected sibs, and often consanguineous parents. Investigations by Steinmann et al. (unpublished results) revealed that several of the patients in these latter two reports were identical and that their normal urinary ratios of pyridinolines excluded EDS VIA. Whether this condition should be labeled as EDS VIB or instead brittle cornea syndrome, because of the fragility of the cornea as opposed to the sclera, remains open. Pemberton et al. [111] reported on six of the seven members of a three-generation family who were afflicted with retinal detachment; four of these six had features of EDS and one died of a dissecting aortic aneurysm. Biglan et al. [448] reported five patients from two families with keratoglobus, blue sclerae, hyperextensibility of the small joints, sensorineural hearing alterations, and mottling of the teeth; corneal perforations developed in seven of their ten eyes after minimal trauma.

Special Management and Genetic Counseling

Anticipatory management of individuals with EDS VI includes the following organ systems.

The musculoskeletal system. Referral to, and regular follow-up by, an orthopedic surgeon for management of kyphoscoliosis. Severe kyphoscoliosis may be resistant to bracing and require surgical intervention. Such patients may be at high risk for neurologic and vascular complications consequent upon surgery for scoliosis, necessitating careful perioperative evaluation and management [449,450]; halogravity traction may be indicated before surgery or between stages of surgery in patients with severe curves, but it must be instituted carefully to avoid brachial plexus injury and other

neurologic deficits [87]. Physical therapy should be instituted for older children, adolescents, and adults to strengthen large muscle groups, particularly at the shoulder girdle, and to prevent recurrent shoulder dislocation. Routine examination for inguinal hernia and surgical referral should be carried out as necessary. The management of other complications is similar to that in EDS I, II, and III (see above).

The eye. Routine ophthalmological examination for management of myopia and early detection of retinal detachment and glaucoma. Surgical repair of a ruptured eye globe may be difficult because of the friability of the scleral tissue (e.g., case 4). Primary vitrectomy permits the successful treatment of retinal detachment if a buckling procedure cannot be performed because of scleral atrophy. However, serious complications may occur and therefore other surgical procedures should be considered, such as pneumatic retinopexy, a temporary balloon, or a dura patch with episcleral pocket [451].

The cardiovascular system. Measurement of aortic root size by echocardiogram at the time of diagnosis or by the age of five years; it may be advisable to repeat an echocardiogram at five-year intervals even if the initial examination is normal. Patients with mitral valve prolapse or valvular insufficiency should follow guidelines for antimicrobial prophylaxis. Vigilant observation and aggressive control of blood pressure is necessary to reduce the risk of arterial rupture; vascular surgery is fraught with danger.

Others. The use of pharmacological doses of vitamin C, a cofactor of lysyl hydroxylase, has been investigated and found to result in an increased urinary excretion of hydroxylysine. Over a two-year period, one patient's wound healing and muscle strength improved and the corneal diameter increased; joint laxity, skin fragility, and the hydroxylysine content of the skin, however, did not change [381]. These results are at variance with those obtained with two pairs of sibs, cases 22 and 23, cases 26 and 27, and case 33 (legend to Fig. 22), who received vitamin C 2–4 g/day in two daily doses for two weeks. The ratio of urinary pyridinolines was indistinguishable in the six urinary specimens obtained from each during each of the periods of preloading, treatment, and washout (Steinmann et al., unpublished results)[9].

Genetic counseling and prenatal diagnosis. The parents of an affected child are obligate heterozygotes and the risk of recurrence of another affected child is 25%.

Krane et al. [33] observed that lysyl hydroxylase is readily detectable in normal cultured amniotic fluid cells, raising the possibility of prenatal diagnosis. Dembure et al. [391] found that lysyl hydroxylase activity in amniotic fluid cells, whether amniocytes (AF) or fibroblasts (F), corresponded to that in dermal fibroblasts, whereas prolyl hydroxylase activity varied markedly depending on whether F or AF cells were examined and in each case was only a fraction of that in dermal fibroblasts. This apparently differential expression of prolyl hydroxylase activity in amniotic fluid cells and dermal fibroblasts is not understood. Therefore, in a pregnancy at risk, lysyl hydroxylase activity in amniotic fluid cells was determined without reference to prolyl hydroxylase. Normal activity was found, and a healthy baby was born [391]. To our knowledge, chorionic villus biopsies, as opposed to cultured chorionic villus cells, have not been investigated as an enzyme source but have been used as a source of DNA [397].

ARTHROCHALASIS TYPE OF EDS — EDS TYPES VIIA AND VIIB (ARTHROCHALASIS MULTIPLEX CONGENITA) (MIM 130060)

Diagnostic Criteria

The arthrochalasic type of EDS is inherited as an autosomal dominant trait and is caused by mutations leading to deficient processing of the amino-terminal end of proα1(I) (EDS type VIIA) or proα2(I) (EDS type VIIB) chains of collagen I because of partial loss or complete skipping of exon 6. It is characterized as follows [39]:

- Major diagnostic criteria
  - Severe generalized joint hypermobility, with recurrent subluxations
  - Congenital bilateral hip dislocation
- Minor diagnostic criteria
  - Skin hyperextensibility
  - Tissue fragility, including atrophic scars
  - Easy bruising
  - Muscular hypotonia
  - Kyphoscoliosis
  - Radiologically mild osteopenia, occasionally fractures

Historical Introduction

Hass and Hass, in 1958 [452], suggested that there is a distinct entity of loose-jointedness, which they called arthrochalasis multiplex congenita (congenital flaccidity of the joints), which "represents, to some extent, the antithesis of arthrogryposis multiplex congenita", and which may occur with or without skin changes. This disorder was classified as EDS VII [2]. In 1973, three patients with EDS VII were reported who accumulated procollagen in their skin and tendon, and it was thought therefore that their disorder resembled dermatosparaxis in cattle (see below) [453,454]. Because it was felt that the accumulation of procollagen was most likely to be due to a defect in the conversion of procollagen to collagen, the activity of the converting proteinase was determined in the culture medium of fibroblasts from these patients and found apparently to be reduced to between 10% and 40% of normal [453,454]. However, the marked clinical and ultrastructural differences between the disorder in man and that in cattle, later reported also in sheep, led Steinmann et al. [35,36] to reinvestigate one of these patients. Analysis by SDS-polyacrylamide gel electrophoresis of collagen extracted from skin or produced by fibroblasts from the youngest of the three reported patients [453] (case 5 in Table 2) disclosed, in addition to α1(I) chains, the presence of pNα2(I)-like chains, in a 1:1 ratio with normal α2(I) chains, and an absence of pNα1(I), pCα1(I), and pCα2(I) chains. The pNα2(I) chain was resistant to pepsin, α-chymotrypsin, and N-procollagen proteinase, and, further, procollagen N-proteinase activity in cell extracts was normal. Rather than there being an autosomal recessively inherited N-proteinase deficiency as in dermatosparaxis, it was concluded that there was an extensive structural abnormality in that portion of the proα2(I) chain that is normally cleaved by N-proteinase (and other proteinases), and that the patient was a sporadic heterozygote arising from a new mutation because both parents, who were phenotypically normal, lacked the mutant pNα2(I) chain in extracts of both skin [36] and cultured fibroblasts (B. Steinmann, unpublished observations). The authors speculated further that, for sterical reasons,

TABLE 2. Arthrochalasic Type of Ehlers-Danlos Syndrome (EDS Types VIIA and VIIB)

Case	Patient initials	Sex	Congenital bilateral hip dislocation	Scoliosis	Additional findings	Family history*	Gene	Mutation	References
1.	(A)	f	+			+	*COL1A2*	Intron 5 −2A>G	457
2.	(B)	m	+	+		−	*COL1A2*	Intron 5 −1G>A	457
3.	R.B.	f	+	+	Fractures	+	*COL1A2*	Intron 5 −1G>C	458,459
4.	N.D.	f	+		Wormian bones	+	*COL1A2*	Intron 5 −1G>C	460
5.	S.N.	f	+		Fractures	−	*COL1A2*	Exon 6 −1G>A	36,305,453,454,461–464
6.	(E.)	f	+			?	*COL1A2*	Exon 6 −1G>A	457
7.	M.F.	m	+	+	Wormian bones; occipital horn	−	*COL1A2*	Intron 6 +1G>A	465
8.	T.T.	f	+	+		−	*COL1A2*	Intron 6 +1G>A	466,467
9.	P.S.	m	+	+		+	*COL1A2*	Intron 6 +1G>A	468,469
10.	K.H.	f	+			−	*COL1A2*	Intron 6 +1G>A	470,471
11.	(- -)	m	+			−	*COL1A2*	Intron 6 +1G>A	472
12.	S.M.	f	+		Fractures; Wormian bones	±	*COL1A2*	Intron 6 +1G>A	473,474
13.	(C)	f	+			−	*COL1A2*	Intron 6 +1G>T	457
14.	(D)	m	+		Fractures	−	*COL1A2*	Intron 6 +1G>T	457
15.	K.K.	m	+			±	*COL1A2*	Intron 6 +1G>T	475
16.	L.G.	m	+		Wormian bones; large head and wide patent fontanelles	−	*COL1A2*	Intron 6 +2T>C	465
17.	W.A.	f	+			−	*COL1A2*	Intron 6 +2T>C	461,476,477, Figure 2 in 456;478–480
18.	L.W.Y.	m	+			−	*COL1A2*	Intron 6 +2T>C	481
19.	(F)	f	+	+	Fractures	−	*COL1A2*	Exon 6 deletion	457

(*Continued Overleaf*)

TABLE 2. (*Continued*)

Case	Patient initials	Sex	Congenital bilateral hip dislocation	Scoliosis	Additional findings	Family history*	Gene	Mutation	References
20.	K.D.	f	+	−	Fractures	−	*COL1A1*	Genomic deletion of exons 5+6	475
21.	?	?	+			−	*COL1A1*	Intron 5 −2A>G	482
22.	(G)	f	+		EDS VII C-like features	−	*COL1A1*	Intron 5 −1G>A	457
23.	S.E.	m	+	+	Fractures, Wormian bones, dentinogenesis imperfecta	−	*COL1A1*	Intron 5 −1G>A	475
24.	?	?	+			−	*COL1A1*	Intron 5 −1G>C	482
25.	T.H.	m	+	?	Fractures, osteopenia, dentinogenesis imperfecta, Wormian bones?, rectal prolapse	−	*COL1A1*	Intron 5 −1G>T	483
26.	N.W.	f	+	+	Fractures	−	*COL1A1*	Exon 6 −1G>A	484
27.	R.M.	f	+	+	Fractures	−	*COL1A1*	Exon 6 −1G>A	455,485–487
28.	?	?	+			−	*COL1A1*	Exon 6 −1G>C	482
29.	E. D.	f	+			?	nc		Fig. 6-15 in 2;36,453,461
30.	S.E.	f	+			−	nc		Fig. 6-28 in 2;36,453,454,461,464
31.	A.P.	m	+				nc	normal *COL1A1* and *COL1A2* genes	460
32.	Ma San	f	?				nc		36,461,464
33.	Mel Neg	m	?				nc		36,461
34.	Es Ho	f	?				nc		36,461

*The symbols designate positive (+), negative (−), or questionable (?) family history; the symbol (±) refers to possible parental mosaicism. nc denotes normal structure of collagen produced by fibroblast cultures as judged by SDS-polyacrylamide gel electrophoresis.

molecules containing pNα2(I) chains would interfere with fibrillogenesis and cross-linking, thus leading to abnormal collagen fibrils and increased solubility of collagen. After the description of a similar patient, in whom the condition was due to a mutant pNα1(I) rather than pNα2(I) chain [455], the classification of EDS VII was subdivided into types VIIA and VIIB, respectively, depending on whether the α1(I) or α2(I) chain was affected [38]. The classification EDS VIIC (MIM 225410) is reserved for the procollagen N-proteinase deficiency analogous to dermatosparaxis in animals. Details of the patients discussed below are summarized, with case numbers and initials, in Table 2.

Clinical Findings

The hallmark of EDS VII is the involvement of ligaments and joint capsules. The disorder is characterized by severe multiple joint hypermobility, recurrent subluxations and luxations of both small and large joints, and ligamentous tears. All patients are ascertainable in the newborn period, although the correct diagnosis is still frequently only made years later. Congenital bilateral hip dislocation is the rule, and muscular hypotonia is prominent (Figs. 28–30); both factors predispose to the high incidence of breech presentation (cases 2, 7, 15, 22, 29; see Table 2) and delayed gross motor development. Short stature, if present, is due to severe thoracolumbar scoliosis and hip dislocation (cases 27, 29). In striking contrast to dermatosparaxis, the skin is only moderately affected; it is usually thin, velvety, and moderately extensible, and is occasionally affected by poor healing of wounds, with atrophic and hemosiderotic scars, especially in the adult. The facies appears to be normal, despite descriptions of a chubby appearance, epicanthal folds, hypertelorism, a depressed nasal bridge, and micrognathia (Figs. 28–30). Judging by six affected members from a three-generation family, intrafamilial variability is slight (case 1) [457].

The newly recognized occurrence of recurrent fractures, Wormian bones, and abnormally wide fontanelles (Table 2) indicates that the EDS VII phenotype includes bone changes and fragility similar to those reported in mild osteogenesis imperfecta [465]. Wormian bones were noted initially in one family (case 12), seducing the observers into calling into existence still another form of EDS [473], but have since also been observed in cases 4, 7, 16, and 23 (Table 2). X-rays of one of our patients disclosed bone changes such as discrete occipital horns, exostoses, a thickened calvarium, and Wormian bones [465] (Fig. 30; case 7).

Defect and Pathogenesis

The molecular defect in all 28 cases elucidated to date is remarkably homogeneous and results in the loss of exon 6, or part of it, of the mature mRNA coding for one of the α1(I) chains or the α2(I) chain (Table 2). The deleted peptide is the junctional segment (N-telopeptide) linking the N-propeptide to the major triple-helical domain. Loss of this short segment, 24 and 18 amino acid residues encoded by exon 6 in *COL1A1* and *COL1A2*, respectively, results in union of these latter domains and a lack of the N-proteinase cleavage site (Pro–Gln and Ala–Gln at positions 4–5), the critical cross-linking lysyl residue (at positions 13 and 9, respectively), and the cleavage sites for proteinases such as pepsin and α-chymotrypsin. The apparent paradox of a deletion yielding a larger than normal protein is thus resolved.

Studies by Wirtz et al. [476] showed that in EDS VIIB (case 17), the α1(I) N-propeptide, although cleaved from the α1(I) chain by the N-proteinase or (less likely) by nonspecific proteases, is retained by noncovalent association with the mutant pNα2(I) chain in native mutant collagen molecules, both *in vivo* and *in vitro* (Fig. 31); the α1(I) N-propeptide was readily demonstrable by western blotting of skin extracts, and rotary shadowing of pepsin-treated collagen produced in culture disclosed kinked molecules, which were longer than their normal counterparts (Fig. 31E). These data suggest that the retention of a partially cleaved but essentially intact N-propeptide in mutant collagen may play a critical role in the pathogenesis of this disease.

As a consequence of the persistence of the N-propeptide and/or the missing critical cross-linking lysyl residue, the solubility of collagen is increased three to four fold [36,453,470,477] (cases 5, 10, 17) and the ratio and pattern of β-components is abnormal [36,455,470] (cases 5, 10, 27). Only one-quarter of the normal content of histidinohydroxymerodesmosine, the major residue derived from skin collagen upon borohydride reduction, is found, but there is a similar to normal content of the mature cross-links, hydroxylysyl and lysyl pyridinoline, in fascia and bone [470]. Extracts of bone contain significantly more pNα2(I) than normal α2(I) chains. This solubility difference between bone and the soft tissues probably results from basic differences in the chemistry and molecular sites of their collagen cross-links [470].

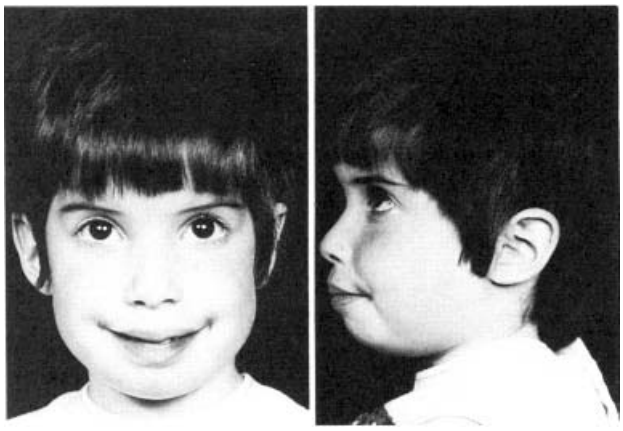


Figure 28. Arthrochalasic type of EDS (EDS VIIB). S.N. (28 Dec 1968), case 5 in Table 2, is the first patient in whom a structural mutation in proα2(I) chains was demonstrated [35,36]. At the age of 4 years, she had a ''dish-like'' face and mild retrognathia, which, however, cannot be considered typical of the syndrome because it is absent in cases 17 and 7 (Figs. 29, 30) and is not apparent in the patient herself at a later age.

She had a neuromuscular workup because of breech presentation and hypotonia at age 2 months, did not sit until age 1 year, stood with support at age 2 years, and when she began walking was noticed to have a waddling gait, which led to the diagnosis of bilateral hip luxation, necessitating several surgical interventions. The ligamentum teres of the right hip was found to be torn at surgery. She also had generalized, pronounced joint laxity.

Follow-up at age 22 years revealed an adult height of 157.5 cm (her target height was 158.5 cm); joint laxity and muscle strength had improved after puberty, whereas her skin had remained soft, velvety, and fragile and tended to form keloids. She was studying to become an elementary teacher (C. Nash, personal communication, 1991). Follow-up at age 30 years disclosed that she had had three luxations of the patella and two fractures of the femur after minor trauma in the preceding 8 years (S. Nash, personal communication, 1999).

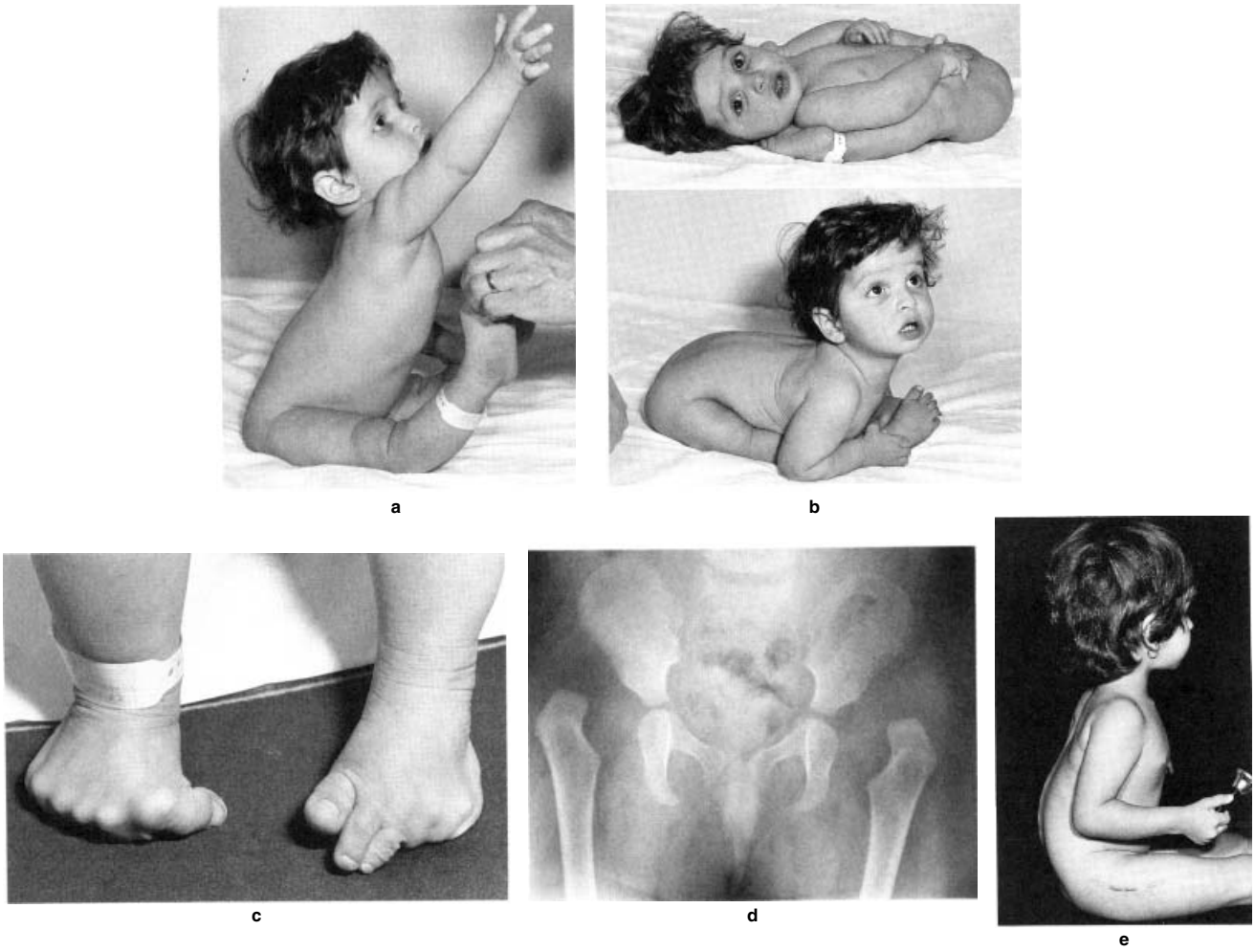


Figure 29. Arthrochalasic type of EDS (EDS VIIB). W.A. (17 Feb 1982), case 17 in Table 2, a 12-month-old girl, born to healthy, unrelated parents from Libya. Her younger brother is healthy, and cultured amniotic fluid cells obtained during his delivery produced normal collagen I. (**a**) Subluxations of both knees, moderate thoraco-lumbar kyphosis. (**b**) Congenital hip luxations. (**c**) Marked hyperlaxity of the small joints. (**d**) Radiograph showing bilateral hip dislocation with lateralization and elevation of both femurs. (**e**) Thoracolumbar kyphosis and keloid formation at age 2.7 years, one-and-a-half years after successful operative correction of the hip dislocation. (a–c are reprinted from Steinmann and Gitzelmann [456] with permission.)

Figure 30. Arthrochalasic type of EDS (EDS VIIB). This 34-year-old subject with EDS type VIIB (M.F., case 7 in Table 2) was originally referred to us by Dr. Katarina Raslova (Bratislava, Slovakia). The patient was born from a breech presentation with dislocations of both hips and the left knee; although the hips were surgically reduced at the age of 2 years, he could not walk and had to crawl around on all fours until surgical correction of the left knee at age nine. Thereafter, he was able to walk without aid for a short period of time but soon had to use crutches because of recurrent dislocation of the right ankle, upon which surgical arthrodesis was finally performed. His skin has always been somewhat fragile, but wound healing is not delayed. His only internal complication has been a symptomatic bladder diverticulum, which was resected at age 17. In spite of these severe handicaps, he has an above-average intelligence and has led an independent life, doing much traveling; he drives an adapted car. He has had one unaffected child by a first wife and another healthy child by his second wife after negative prenatal diagnosis [260].

The photographs show the sagging skin around the eyes and mouth (**a**, **b**; the xanthoma in the left upper eyelid is probably due to coincident hyperlipoproteinemia); marked ulnar deviation of fingers II to V (which cannot be fully extended) and subluxation of the proximal interphalangeal joint of the right thumb (**c**); malposition and deformation of toes (**d**); arthrodesis has been performed on the right ankle joint. Note the normal stature (172 cm) and body proportions and the thin, hyperpigmented scars on both shins and knees (**e**).

X-rays show a peculiar combination of thickening of the calvarium with a pumice stone-like appearance, Wormian bones, calcification of the falx cerebri, and calcification of a tendon insertion in the occipital region (''occipital horns''; see arrow) (**f**, **g**); exaggerated lumbar lordosis with moderate osteoporosis of vertebral bodies (**h**); arthrotic degeneration of the knee joints with marked osteophyte formation at insertion sites on the patella (**arrows**) (**i**); reduction of articular cartilages and subluxation of several joints in both hands and feet (**j**, **k**). Note also the short metacarpals III–V, a small osteophyte (probably also in a tendon insertion) at the distal extremity of the ulna, and a previous fracture of the left fourth metatarsal.

Such radiological changes have not been observed in EDS VII so far, apart from Wormian bones (Tables 2 and 3), and may point to the natural history of the disorder as observed in this 34-year-old patient. ''Occipital horns'' and calcification of tendon insertions have been considered characteristic of EDS IX, now the occipital horn syndrome (see below and Chapter 14, this volume).

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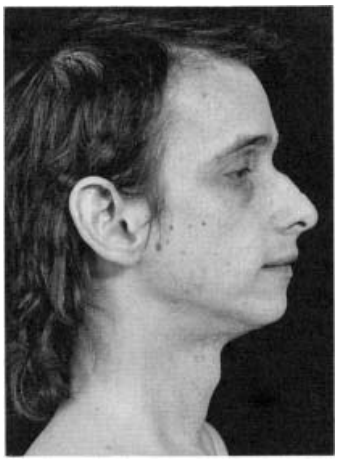

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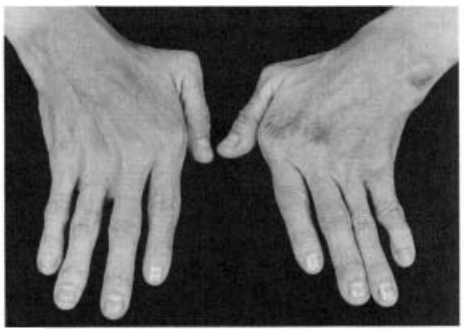

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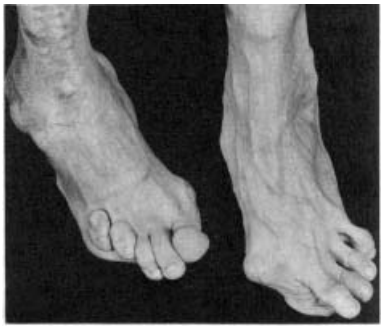

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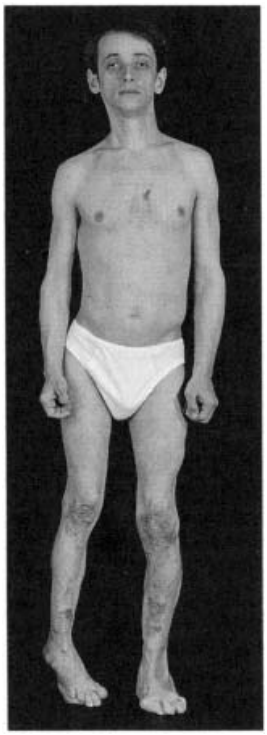

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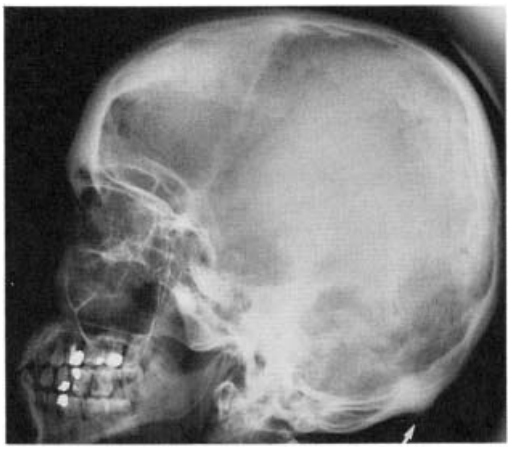

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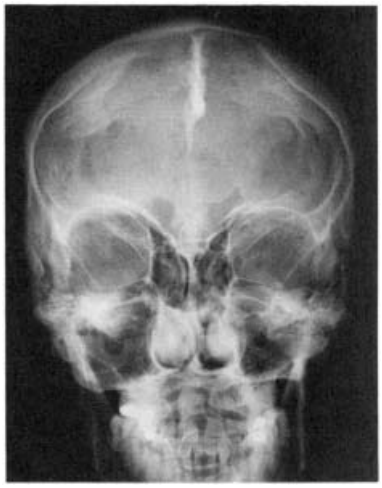

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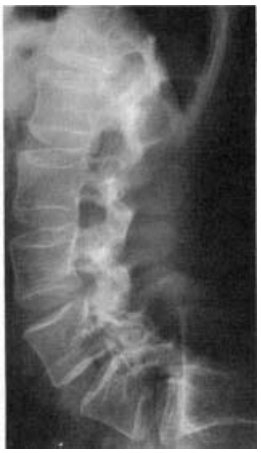

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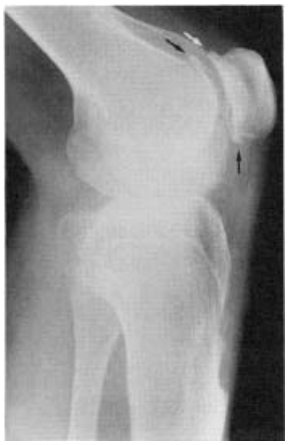

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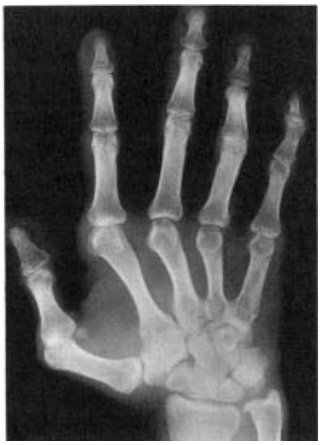

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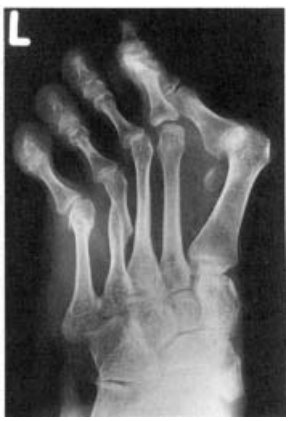

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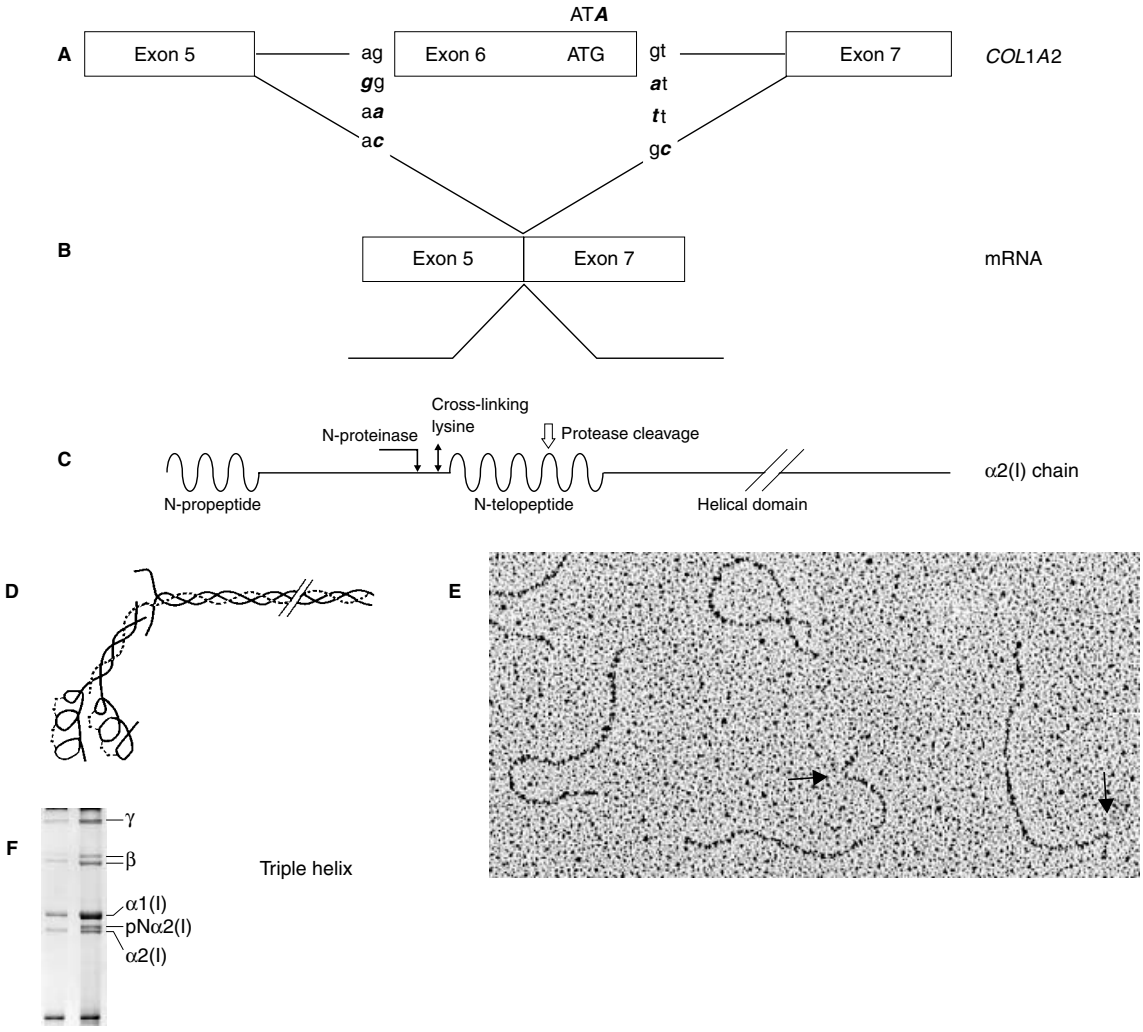

Figure 31. Schematic representation of the pathogenesis of arthrochalasic type of EDS (EDS VIIB). (**A**) Exon-intron structure of *COL1A2* with acceptor (ag) and donor (gt) splice sites adjacent to exon 6. In bold italics are mutations as listed in Table 2. (**B**) Skipping of exon 6 or deletion of exon 6 leading to fusion of exons 5 and 7 in the α2(I) mRNA. (**C**) The deletion of exon 6 removes the N-telopeptide, which contains the N-proteinase cleavage site, a lysine cross-link site, and protease cleavage sites. (**D**) Structural model of an abnormal collagen I molecule containing a mutant pNα2(I) chain, as indicated by a dotted line. Both cleaved N-terminal peptides of the α1(I) chains, as indicated by solid lines, are retained through triple-helical binding to the mutant N-terminal propeptide of the α2(I) chain and form a kink. (**E**) Rotary shadowing electron microscopy of pepsin-treated collagen produced by fibroblasts. Approximately one-half of the molecules present a kink (**arrows**) at their N-terminal ends and are somewhat longer than normal (312 vs. 297 nm). (**F**) SDS-polyacrylamide gel electrophoresis of collagen extracted by neutral salt from skin of patient S.N. [36] (**right lane**) and a control (**left lane**). The mutant pNα2(I) chain migrates more slowly than the normal α2(I) chain, and both chains are in a one-to-one ratio; pN collagen I, is more soluble than fully processed collagen I, as shown by the increased amount of its derived α, β, and γ chains. (Model in (D) adapted from experimental rotary shadowing data and reprinted from Wirtz et al. [476] with permission. (E) reprinted from Wirtz et al. [476] with permission.)

Two very interesting patients with EDS VIIB (cases 3 and 4) have been observed [458,460]. In both, a G to C change at the splice acceptor site of intron 5 activates a cryptic splice site in exon 6, removing 5 amino acid residues, including the N-proteinase cleavage site, but preserving the lysyl residue cross-link site. The fact that both mother and son, and father and daughter, respectively, were affected with typical EDS VII indicates that the persistence of the N-terminal propeptide is more deleterious than the absence of the lysyl residue or, alternatively, that in the presence of the propeptide, the lysyl residue is either not susceptible to lysyl oxidase activity or that following lysyl oxidase activity the lysyl aldehyde is unable to participate in cross-link formation for sterical reasons. However, studies of collagen solubility and cross-links have not yet been performed to answer this question.

A further instructive patient from a family with nine affected members in three generations was reported by Sippola et al. [488]. He presented features of both EDS VII and osteogenesis imperfecta (generalized osteoporosis,

platyspondyly, Wormian bones, blue sclerae, but no fractures) similarly to the patient shown in Figure 35, and carried a 19 bp deletion that caused skipping of exon 11 of *COL1A2* [489], thus deleting one of the lysyl residues involved in intermolecular cross-link formation. The presence of the shortened proα2(I) chain in procollagen synthesized by the proband's fibroblasts both lowered the thermal stability of the molecules and prevented or delayed their processing by procollagen N-proteinase [490]. The decrease in thermal stability was apparently not sufficient to produce exclusive degradation of the protein. Instead, the extreme laxity of joint ligaments in the proband was probably explained by the effect of the incompletely processed N-propeptide on fibril assembly [488]. Thus, the deletion disturbed both the helical domain and the cleavage site of the molecule, thereby resulting in the dual phenotype. The variable phenotypic expression in the proband's family was remarkable and may be explained by the possibility that the 19 bp deletion did not produce exon skipping *in vivo* with the same efficiency in everyone who inherited the defect [489], although *in vitro* the abnormal splicing was not changed by varying either the temperature of the fibroblast cultures or the salt concentration. In contrast, temperature-dependent alternative splicing has been demonstrated *in vitro* in case 5 [305] and subsequently in case 26 [484], but its significance *in vivo* remains unknown. A further case has been reported who had features of both osteogenesis imperfecta and Ehlers-Danlos syndrome [491].

We do not sufficiently understand why the abnormal collagen in humans produces major manifestations in joints and ligaments, whereas the skin, unlike that in dermatosparactic animals, is less affected; it may be that fibril formation is particularly disturbed by the pN-peptide where fibrils are tightly packed, as in tendons.

Genotype-Phenotype Correlation

Interfamilial and intrafamilial variability seems to be slight. In EDS VIIB, the heterotrimeric collagen I molecules consist of one population of normal collagen I and one population of abnormal collagen I containing the mutant α2(I) chain. In contrast, in EDS VIIA, three-quarters of collagen I molecules would be expected to be abnormal because they contain one or two mutant α1(I) chains, and thus the phenotype would be expected to be more severe than that of EDS VIIB. However, among the reported cases of EDS VIIA, three involve substitutions for the last nucleotide of exon 6 (cases 26–28, Table 2). This results in alternative splicing so that in part of the product the exon sequence from mRNA is deleted, whereas in the remainder normal splicing is permitted and the protein product contains an isoleucine rather than methionine at position 3 of the first Gly–X–Y triplet of the triple helix. The normally spliced product containing an amino acid substitution diminishes the dysfunctional effect of the three-quarters of abnormal molecules; this results in a less severe clinical phenotype that is indistinguishable from that of EDS VIIB. In contrast, in case 22, there is complete skipping of exon 6 from mutant proα1(I) chains; this leads to three-quarters of the collagen I molecules being abnormal and dysfunctional and explains the more severe phenotype, which almost resembles EDS VIIC, in which none of the collagen I molecules are processed [465].

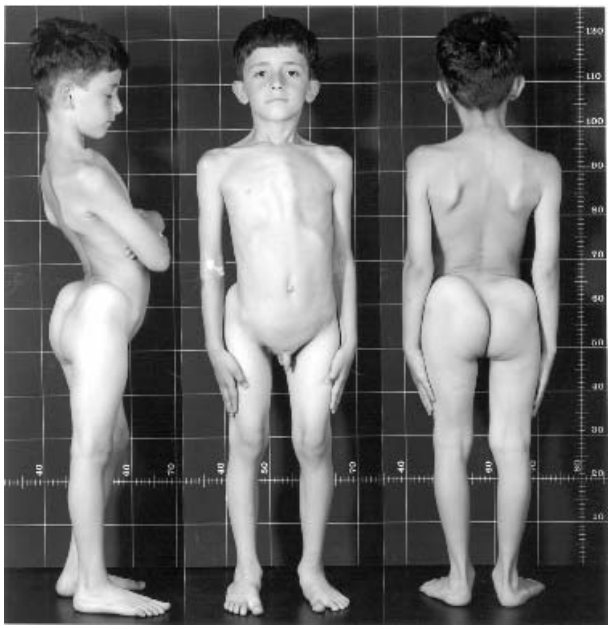

Figure 32. Arthrochalasic type of EDS (EDS VIID). A.P. (30 Sep 1993; case 31), at age 7 years, presents with bilateral hip dislocation, hyperlordosis, flat feet, pigeon-breast-like deformity, and scapulae alatae. His skin is moderately hyperelastic and soft, does not present abnormal scars, and has no tendency toward bruising. He has generalized joint laxity, muscular hypotonia, and a waddling gait due to congenital hip dislocation. The eyes and internal organs are normal, and height and weight are on the 50th centiles.

He is the fifth child of healthy, unrelated parents from Kosovo; the four older siblings are not affected. His birth and neonatal adaptation were normal, but it was noted that something was ''wrong'' with his hips; gross motor development was delayed.

Collagen fibrils in dermis were ultrastructurally normal in diameter and contour. The collagens produced by his fibroblasts were normal in amount and structure. These findings are similar to those in case 29 (see Fig. 33d) and unlike those observed in EDS VIIA, VIIB, and VIIC. He is therefore temporarily grouped as EDS VIID.

Hass and Hass [452] state that ''arthrochalasis multiplex congenita is a distinct clinical entity characterized by a multiple congenital flaccidity of the joints. It may be associated with laxity or hyperelasticity of the skin defined as Ehlers-Danlos syndrome, or it may exhibit hypermobility of the joints without involvement of the skin.''

Locus heterogeneity is exemplified by several patients (cases 29–34, Fig. 32) who are clinically similar to EDS VIIA/B but whose fibroblasts produce normal procollagen that is converted normally to collagen by pepsin or α-chymotrypsin [36]. Using the assay of Prockop and Tuderman [492], a somewhat lower N-proteinase activity has been determined, which reflects methodological problems rather than biological aberrations [36,461]. The normal structure of collagen I extracted from the skin of cases 29 and 31 and produced by fibroblasts, the absence of pNα1(I) and pNα2(I) chains in skin extracts, normal procollagen N-proteinase activity, and a normal electron-microscopic appearance of dermal collagen fibrils (Fig. 33d) clearly distinguish these patients from those with EDS VIIA and VIIB. It remains uncertain whether patients such as these are examples of EDS VII of unknown cause, temporarily called

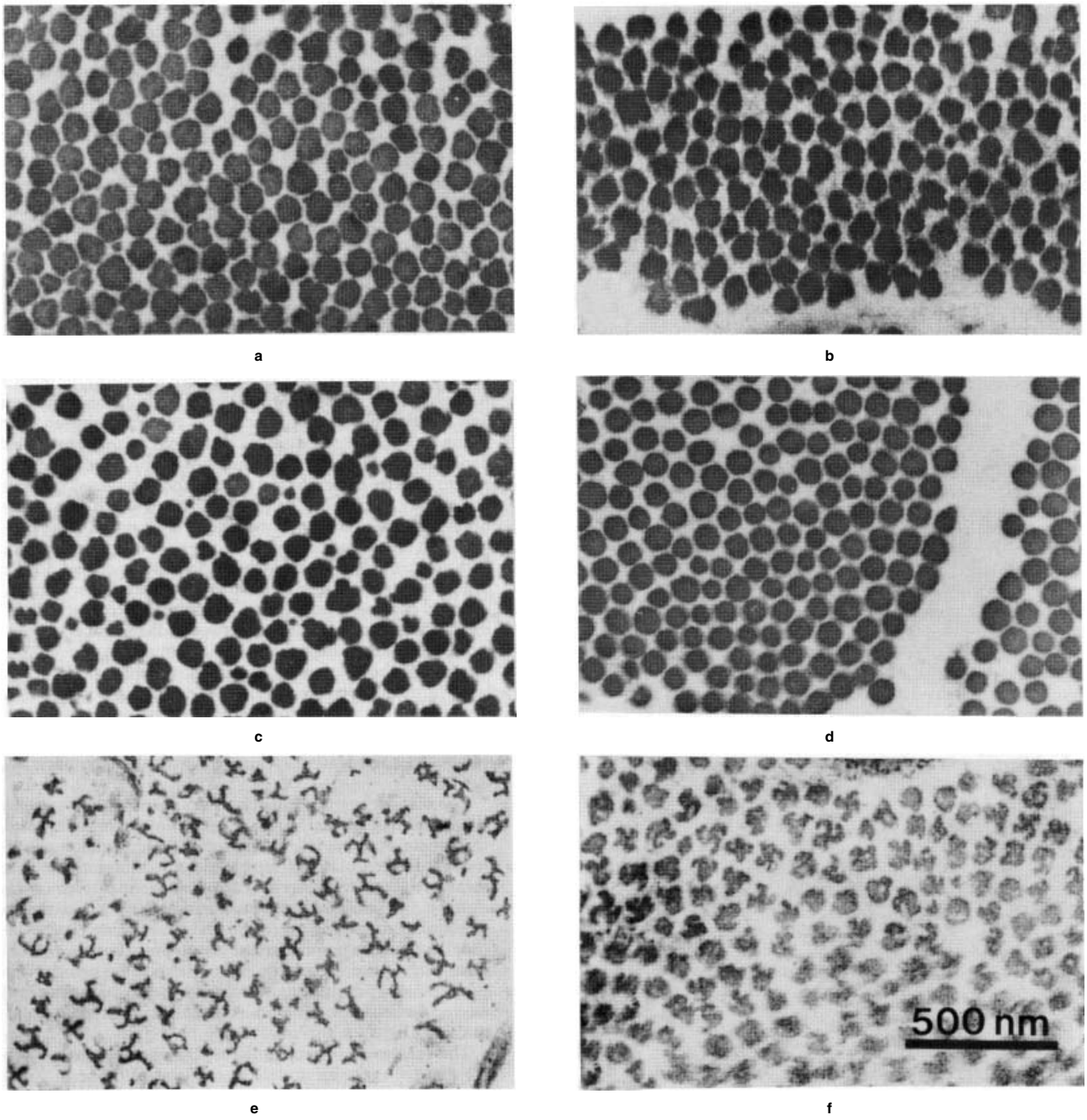

Figure 33. Ultrastructural appearance of collagen fibrils in the dermis of patients with arthrochalasic type of EDS (EDS VIIB and VIID) and in dermis and tendon of a dermatosparactic sheep. Electron-microscopic pictures of collagen fibrils in cross section from the dermis of patients with EDS VII (**a**–**d**), and the dermis (**e**) and tendon (**f**) of a dermatosparactic sheep. (**a**) Case 5, at age 10 years; (**b**) case 17, at age 1 year; (**c**) case 7; (**d**) case 29, at age 38 years (EDS VIID). Staining was with phosphotungstic acid and uranyl acetate, and the magnification is identical in all photographs. In the case of the patients, pictures were taken 0.5 mm below the epidermal-dermal junction, and the diameters of 1,000 fibrils were measured and are given below as mean ±SD.

In the three patients with defined EDS VIIB (**a**–**c**), the collagen fibrils are irregular in contour but have normal diameters with slightly increased variability: 89.0 ± 11.7 nm in (a), 86.5 ± 16.7 nm in (b); 79.5 ± 14.2 in (c). In contrast, both shape and diameter of the fibrils in the patient with EDS VIID (d) are normal and indistinguishable from controls (90.3 ± 12.4 nm).

The skin of the newborn dermatosparactic lamb was moist and thicker than normal, with a jelly-like consistency, while histologically the collagen consisted of loosely woven thin fibrils. On electron microscopy, the cross sections showed a multibranching appearance of the fibers, resembling ''hieroglyphics'' (**e**); longitudinal sections showed that the fibers had a twisted, ribbon-like appearance. In tendon, cross sections showed a more rounded appearance of the fibers with thicker and less-developed branches, particularly in areas where the fibers were packed more tightly together (**f**). (Figures a–d are unpublished pictures from Ursula Lüthi and A. Vogel, and Figs. e and f are from Fjølstad and Helle [493] with permission.)

EDS VIID, or whether they represent the extreme end of severity of EDS III. It may be of note that cases 29–31 have smooth, hyperelastic skin but without skin fragility.

Morphology

The skin collagen fibrils have a smaller and more variable diameter than normal. They are irregular in outline and also appear to be more loosely and randomly organized into fibrils resembling a loosely wound rope (Figs. 33a–c) [470,485]. A similar fibril outline is also evident in sections of fascia and decalcified bone [470]. All these changes, however, are much more subtle than those observed in dermatosparactic animals (see below) (Fig. 33e) and in cases 22 and 25, in whom three-quarters of collagen I molecules are abnormal.

Genetics

EDS VIIA and VIIB are inherited as autosomal dominant traits, and some of the sporadic cases have been shown to be heterozygous for a new mutation (Table 2). Mosaicism in the mother of case 12 is likely the cause of her milder phenotype as opposed to her affected children. The apparently higher incidence of EDS VIIB is puzzling but may have to do with differences in the structures of intron 6 in *COL1A1* and *COL1A2*.

The pedigree of case 30 (Fig. 6.15 in [2]) suggested autosomal recessive inheritance because of consanguinity of the parents and the presence of two apparently affected sisters. Re-evaluation, however, revealed that both sisters have normal joints, skin, and height and complain only about "troubles with the knees." It is therefore more likely that case 30 is a sporadic case also arising from a new mutation (B. Steinmann, own observation, May 1978).

Diagnosis and Differential Diagnosis

The diagnosis is first made on clinical grounds, supported by protein data pertaining to collagen extracted from skin or produced by fibroblasts (Fig. 31F), and proven by the demonstration of complete or partial loss of exon 6 in cDNA and of the mutation in genomic DNA. The existence of individuals with a clinical picture of arthrochalasis multiplex congenita but normal collagen I structure, normal procollagen N-proteinase activity (cases 29–34), and normal electron microscopic findings in skin (case 29, Fig. 33d) indicates further heterogeneity; these patients who do not fall into any one of the three type VII variants A, B, or C may temporarily be grouped as EDS VIID.

Congenital hip dislocation is also found in the Larsen syndrome (MIM 150250, MIM 245600), as an isolated disorder (MIM 142700), and in an as yet less well-defined disorder (i.e., dislocation of the hip, congenital, with hyperextensibility of fingers and facial dysmorphism) (MIM 601450).

The risk to healthy parents of having another affected child is small and due to the possibility of parental mosaicism. The mother of the four patients reported by Viljoen et al. [473] (case 12) was the least severely affected individual within the family and may represent a symptomatic mosaicism of the mutation in *COL1A2*, although the fibroblasts examined do not disclose any mosaicism; other of her cells, however, have not been investigated. Alternatively, her milder phenotype may simply be the expression of intrafamilial variability (G. Wallis, personal communication, 1991). The risk of an affected person transmitting the disorder is 50%, but prenatal diagnosis should be possible by either DNA or protein studies on a chorionic villus biopsy if the defect in the index case has been characterized. Biochemical analysis of a chorionic villus biopsy in a pregnancy at risk of the spouse of case 7 was normal and a healthy girl was born as predicted [260].

Management

The orthopedic outcome is often unsatisfactory because premature degenerative arthritis of the hips and other joints occurs.

The principal orthopedic problem in EDS VII patients is bilateral congenital dislocation of the hips. To gain an insight into the management of this problem, Giunta et al. [465] reviewed the treatments and their outcomes in 16 patients from 12 families. Several of the patients listed in Table 2 were not included because insufficient information was available from their published reports. Stable reductions were infrequently achieved following closed reduction with orthoses or hip spicas. Anterolateral open reductions with capsular plication, even in infancy, were also inadequate because most of the patients continued to have subluxated or dislocated hips. The poor outcome of the latter procedure is likely to be due to early stretching of the capsulorrhaphy sutures. In contrast, the addition of an iliac osteotomy of the Pemberton or Salter type with or without femoral osteotomy achieved some good results (cases 14, 17, 27).

From published results [494] and the findings of Giunta et al. [465], it appears that open reduction with capsulorrhaphy and iliac osteotomy, with the addition of femoral osteotomy in some cases, is required to achieve and maintain stable reduction. These requirements are similar to those shown to be necessary to achieve and maintain stable reduction in other laxity conditions such as Down and Larsen syndromes. As with the latter conditions, careful planning of osteotomy is required to ensure that adequate acetabular coverage of the femoral head is achieved in the functional positions of the limb [465].

Generalized joint hypermobility is worst in infancy, when marked muscular hypotonia accompanies the severe ligamentous laxity. Motor development is consequently slow, and aids such as knee-ankle-foot and ankle-foot orthoses are often required to stabilize the lower limb joints for standing and walking. As muscle tone improves, the knee-ankle-foot orthoses may be reduced to ankle-foot orthoses. Surgical procedures to stabilize the knees and the patellofemoral joints have been used occasionally. Orthotic stabilization is likely to be more reliable. Ankle or subtalar fusions have been undertaken in a few cases but, as shown in case 7, the arthrodesed joints need to be in an optimal position for this to be successful [465].

Recurrent and/or persistent dislocations, as well as hypermobility of upper limb joints, are also frequently disabling. Operative procedures appear rarely to have been undertaken in the upper limbs, and one would predict that capsulorrhaphy and osteotomy would not stabilize these joints [465]. Arthrodesis may be useful in stabilizing some small joints, such as the metacarpophalangeal joints of the thumbs, although fusion rates cannot be predicted.

Postural thoracolumbar kyphosis due to hypotonia and ligamentous laxity is a feature of infants (cases 16 and 17, Fig. 29). The spinal posture improves as the children gain in muscle power. However, structural scoliosis has been reported in eight cases (cases 2, 3, 7, 8, 9, 19, 26, 27). Spinal fusion was undertaken in cases 8 and 9, although few details are available concerning curve patterns and surgery. Spondylolisthesis of L5-S1 was noted in case 19.

The findings in the 20 molecularly proven cases of EDS VIIA and VIIB reviewed [465] show how difficult the

management is and how important early diagnosis may be. The homogeneous nature of the molecular defects allows laboratories with the appropriate expertise rapidly to establish the diagnosis, after which the clinical problems, in particular those relating to the dislocated hips, can be predicted. Adequate physical and occupational therapy and orthotic management can be given to assist with standing, walking, and activities of daily living. Appropriate surgical treatment of the dislocated hips should also diminish the frequency of hip redislocation, recurrent dislocation, avascular necrosis, and premature osteoarthritis. However, more experience correlating detailed orthopedic management and long-term outcome is needed before sound recommendations can be made [465].

Comment

In the initial study [453], it was found, and later confirmed [461], that affected fibroblasts produce more collagen in culture than normal cells. The authors speculated that the procollagen extension peptides might act as feedback inhibitors of collagen synthesis. Evidence in support of this hypothesis has been presented [495–497]. This is another example of findings in a disease leading to further general insight into biological control mechanisms.

DERMATOSPARACTIC TYPE OF EDS — EDS TYPE VIIC (MIM 225410)

Diagnostic Criteria

The dermatosparactic type of EDS is inherited as an autosomal recessive trait caused by deficiency of procollagen N-terminal proteinase as a result of homozygosity or compound heterozygosity of mutant alleles coding for the enzyme (in contrast to the arthrochalasic type, which is due to mutations involving the substrate sites of procollagen I chains) and characterized as follows [39]:

Major diagnostic criteria
- Severe skin fragility
- Sagging, redundant skin

Minor diagnostic criteria
- Soft, doughy skin texture
- Easy bruising
- Premature rupture of fetal membranes
- Large herniae (umbilical, inguinal)

Historical Introduction

Dermatosparaxis in man (see Table 3) has only recently been recognized and shown to be due to an enzyme deficiency, whereas in animals it was the first defect in collagen metabolism to be defined [507] (see "Animal Models and Lathyrism" below). An earlier suggestion of dermatosparaxis in man [453] was disproved by Steinmann et al. [36], who demonstrated a structural abnormality in the patient's proα2(I) chain, the substrate, rather than a deficiency of the enzyme, N-proteinase (see EDS VIIB above).

Clinical Findings

Clinical findings are summarized in Table 3. All six patients reported to date were born prematurely after rupture of the membranes, one of them (case 1) by Cesarean section because of breech presentation; he presented bilateral inguinal tears [498,499]. In all, the skin was soft, with a doughy feel, redundant, lax, and fragile. The skin was noted to bruise easily and avulsed from the underlying soft tissue after minor trauma; it was easily approximated, and healed well in a short time, with minimal scarring.

All patients had umbilical herniae and some dysmorphic features, consisting of micrognathia, puffy eyelids with excessive periorbital skin, sagging skin, and wide open fontanelles and osteopenia (Fig. 34). One had moderate joint laxity and had not yet walked at the age of 18 months.

Joint laxity seems to become a major clinical feature with increasing age, as illustrated by case 4, who had only a moderate degree of joint laxity as assessed over the first 3 years. Chondrodysplastic changes, however, seem not to be absent (see "Pathogenesis" below).

Genetic Defect

The defect is due to an autosomal recessively inherited deficiency of N-proteinase, as has been demonstrated by the direct measurement of enzyme activity and by indirect evidence such as pN-collagen extracted from skin or produced in fibroblast cultures, which could be cleaved by the addition of N-proteinase activity [502] or normal cell extracts [498], or, less specifically, other proteases [498,502].

Procollagen N-proteinase (ADAM-TS2, a disintegrin and metalloproteinase with thrombospondin repeats, EC 3.4.24.14, MIM 604539) is a large, neutral zinc metalloproteinase, which cleaves the amino-propeptides in the processing of pN-collagens I and II to collagens I and II, respectively. It has been isolated by affinity chromatography on immobilized collagen XIV [508]. The interaction between the enzyme and collagen XIV is highly specific, which suggests that collagen XIV might be a physiological ligand, the role of which might be to immobilize the enzyme in the close vicinity of collagen I fibers, thereby allowing the processing and subsequent polymerization of newly synthesized molecules under strict spatial control [508]. Partial amino acid sequences allowed the cDNA cloning of the enzyme. The N-proteinase consists of 1,205 amino acid residues with two sequences in the N-terminal domain that are substrates for furin-like enzymes; this suggests processing of the proenzyme to its mature form as is generally the case for metalloproteinases [509]. The enzyme is expressed in all tissues rich in collagen I [509]. The human gene is located in chromosomal region 5q35 [510].

Mutation analysis of the six cases is shown in Table 3 [500]. Five of them are homozygous for the identical mutation, although their ethnic backgrounds differ: three are Ashkenazi Jewish, one is Hispanic/Mexican, and one is from an American family of undetermined background.

Pathogenesis

Skin is more severely affected than other collagen-rich tissues (tendons, blood vessels, bones, and cartilage), and this is correlated with the extent of morphological alteration of collagen fibrils. This suggests that another enzyme, which has tissue-specific expression, can remove the amino-terminal propeptides of procollagens I and II (the latter especially in cartilage), although at a lower rate than the N-terminal proteinase.

The fragility of the skin is directly related to the concentration of precursors among the collagen polymers. The persistence of the N-terminal propeptide is known to prevent the use for intramolecular and intermolecular cross-linking of the lysyl residue located in the N-telopeptide. As a consequence, the skin collagen content in one case studied was lower than normal, and the extractability of collagen under nondenaturing conditions increased [502]; this indicates defective formation of stable intermolecular

TABLE 3. Dermatosparactic Type of Ehlers-Danlos Syndrome (EDS Type VII C)

Case no.	1	2	3	4	5	6
Reference	498–500	498,500,501	500,502–504a	500,505	500,506	500
Age at description (years)	2	2	2 and 9	15	0.5	0.7
Gestation (weeks)	28	35	29.5	32	30	31
Premature rupture of membranes	+	+	+	+	+	−
Fontanelles widely patent	+	+	+	+	+	?
Edema of eyelids	+	+	+	+	+	
Blue sclerae	+	+	+	+	+	+
Postnatal skin fragility (observed at month)	+(7)	+(0)	+(12)	+(9)	+(3)	
Easy bruisability	+	+	+	+	+	
Cutis laxa	+	+	+	+	+	+
Umbilical hernia	+	+	+	+	+	+
Joint laxity	+	+	?	+	−	
Osteopenia	−	+	+	+	−	
Wormian bones	?	+	+	?	(at birth)	
Micrognathia	+	+	+	+	+	+
Short limbs and fingers	+	+	+	+	+	
Short stature	+	+	+	+	+	+
Myopia	+	?	?	+	−	
EM ribbon-like fibrils	+	+	+	+	+	+
Defective conversion of procollagen I	+	+	+	na	+	+
pN-collagen extracted from skin	+	+	+	?	?	
Sex	f	m	f	f	m	m
Consanguinity	?	−	?	−	?	?
Genetic defect [500]	Q225X/Q225X	Q225X/Q225X	W795X/W795X	Q225X/Q225X	Q225X/Q225X	Q225X/Q225X

na, not analyzed; Q, glutamine; W, tryptophan; X, stop.

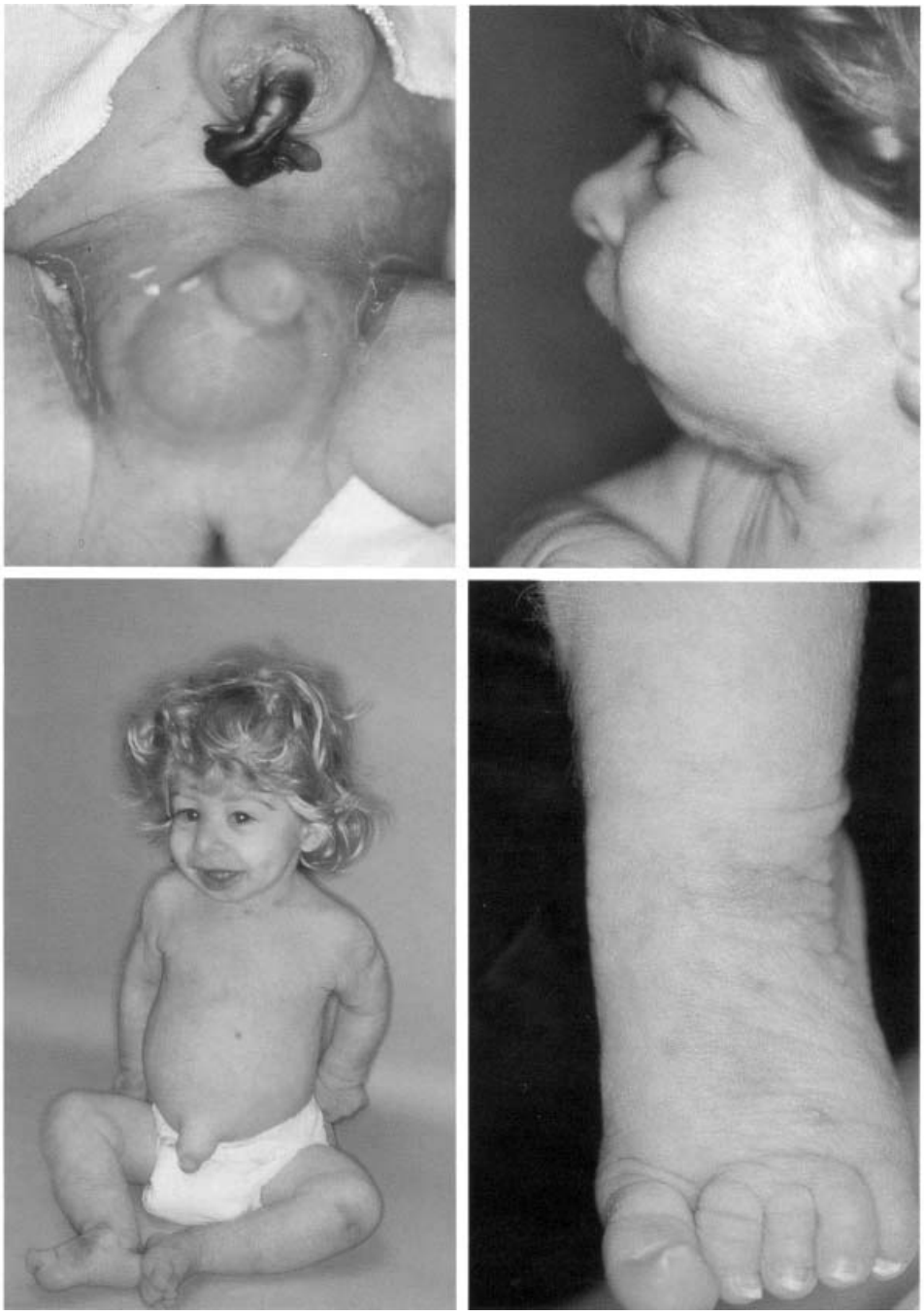

Figure 34. Dermatosparactic type of EDS (EDS VIIC). Boy with Ehlers-Danlos syndrome type VIIC. **Top left**, groin showing bilateral fissures at birth; **top right**, profile of face at 18 months of age (note the vellus hair and micrognathia); **bottom left**, frontal view at 18 months of age showing the symmetrically shortened extremities, prominently visible vasculature, multiple ecchymoses, excess skin folds, blue sclerae, and umbilical hernia; **bottom right**, closeup of left foot showing hyperconvex nails. (Reprinted from Petty et al. [501] with permission.)

cross-links, as observed in dermatosparactic animals [511]. The pattern of collagen polypeptides extracted from the skin of cases 1–3 (Table 3) comprised a large proportion of pNα1(I) and pNα2(I) chains in a 2:1 ratio, with a small proportion of completely processed α(I) chains [498,502]. The presence of apparently correctly processed collagen I is due to either residual enzyme activity or the presence of a poorly efficient alternative proteolytic pathway. Extracted pN-collagen I treated with purified N-proteinase or pepsin can be converted to collagen consisting of normal α(I) chains [502]. Extracts of normal cells cleaved procollagen I synthesized by cells from patients, demonstrating that the enzyme, not the substrate, was defective [498]; in contrast, cultured fibroblasts from three patients studied

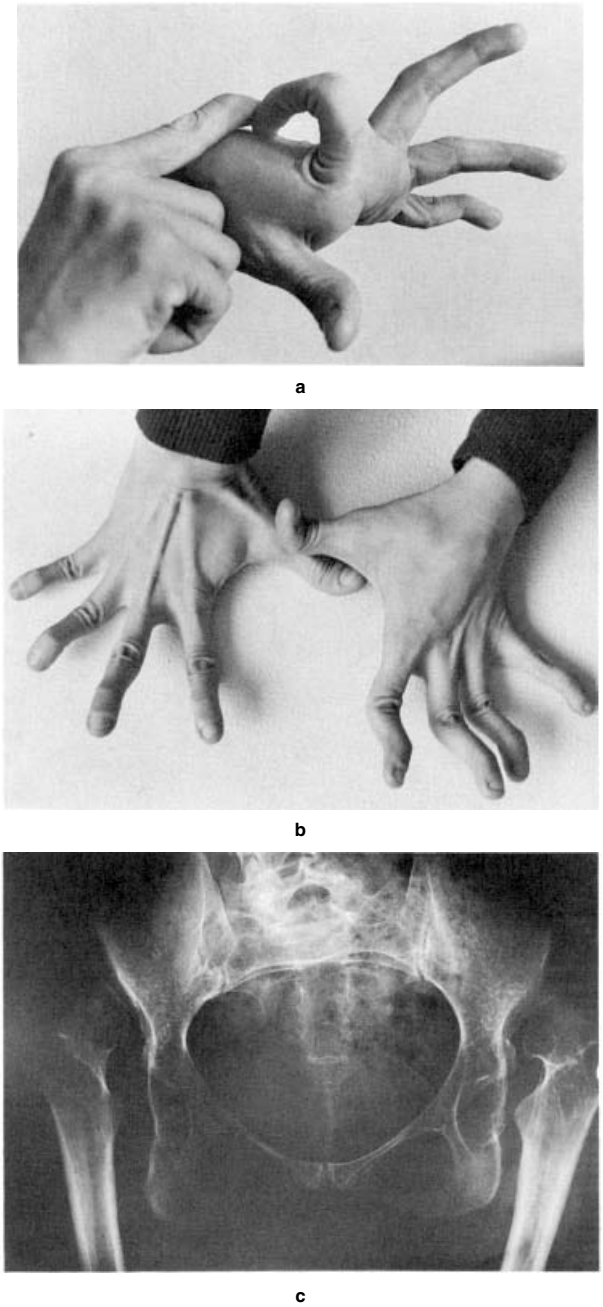

Figure 35. Marked joint laxity and osteoporosis. This female patient, in her forties, has marked joint laxity (**a**, **b**) and severe scoliosis requiring Harrington rodding. X-rays showed untreated bilateral congenital hip dislocation (**c**) and diffuse osteoporosis. She has had some fractures, suggesting that she is affected by an overlap form between EDS and osteogenesis imperfecta. She is not married and has refused a skin biopsy (own observation). Two families with similar findings have been described [488,489,491].

cleaved the N-terminal propeptides from procollagen I poorly [498,502], and collagen synthesis in cells from case 3 was increased [502], probably because of a lack of feedback inhibition by the released N-terminal propeptides, as shown in studies on cells derived from animals [495–497,512].

The accumulation of pN-collagen in tissues, mainly the skin, leads to disturbed fibrillogenesis. Instead of forming bundles of polymers running in parallel, the procollagen fibrils are poorly oriented and the space between them is much enlarged with numerous cells, among them mast cells [503], and may contain an increased amount of hyaluronate and thus water, as in dermatosparactic animals [513]. Transmission electron microscopy of the dermis reveals ribbon-like collagen fibrils and hieroglyphic profiles (Figs. 33e and 33f) characteristic of those observed in the skin of dermatosparactic animals [498,502,505,506,514]; (for a scanning electron micrograph of dermal collagen, see [498]). Collagen fibrils of the sheets around vessels, nerves, and adnexa are similarly affected, whereas the dermo-epidermal junction basement membrane and anchoring fibrils appear normal, indicating that collagens IV and VII are not involved [498], thereby indicating substrate specificity of the N-proteinase.

Watson et al. [474] have proposed a model of fibril formation in EDS VIIC in which the intact N-propeptides are located at the surface of the hieroglyphic fibrils. Partial cleavage of the *in vitro*-synthesized abnormal collagen by N-proteinase allows the N-propeptide to be incorporated within the body of the fibrils with conversion of the hieroglyphic outlines to the ragged outlines characteristic of EDS VIIA and VIIB.

Skin fragility seems to decrease as a function of age, as exemplified by case 4, a tendency also observed in animals. This may be explained by the increasing density of collagen fibers in skin during development and the slower collagen turnover as observed at a more advanced age in animals [515].

Diagnosis and Differential Diagnosis

The diagnosis is suspected clinically. The ultrastructural findings of skin collagen fibrils are typical but may be almost indistinguishable from those in certain patients with EDS VIIA (see case 22 in Table 2). It must be emphasized that light microscopy does not allow detection of these changes. This is well-demonstrated by case 4 (Table 3), where routine paraffin sections of samples of skin and tendon taken on several occasions showed no remarkable features, whereas later, samples reprocessed for electron microscopy from paraffin blocks showed hieroglyphic collagen fibrils in dermal samples and, to a lesser extent, in tendon [506]. Biochemical confirmation is based on the electrophoretic demonstration of pNα1(I) and pNα2(I) chains of collagen I extracted from dermis in the presence of protease inhibitors or detected in fibroblast cultures in the presence and/or absence of added dextran sulfate [487]. Determination of N-proteinase activity is performed on a research basis only. However, direct mutation analysis has become feasible (Table 3) [500] and will allow prenatal diagnosis.

Comment

Dermatosparaxis represents a good example of the unity of biomedical research. The study of dermatosparaxis was initiated by clinical veterinarians, taken up by biochemists and cell biologists with the help of physical chemists, and continued by clinicians and molecular biologists to determine the cause of the disease in man. This collaboration has also largely contributed to the better understanding of the physiologic processes involved in collagen fibrillogenesis and the regulation of collagen biosynthesis.

OTHER TYPES OF EHLERS-DANLOS SYNDROME

The number of distinct types of EDS that have already been identified indicates great heterogeneity, but clearly that heterogeneity is not exhausted by the most widely used classification and presents a pasture for splitters — although the most recent classification reverses that trend. There is no place for the passionate defense of a purely clinical classification, which can only provide the framework for a further investigation of clinical problems. The question is whether a particular set of findings represents merely a chance association or whether they can be understood either as having a cause-and-effect relationship or as sharing a common pathogenesis.

Ehlers-Danlos Syndrome Type V — X-Linked EDS (MIM 305200)

The existence of EDS V (Table 1) as a separate entity is more than questionable [39]. Its phenotype is not well-defined because it is based on only eight members of two British families demonstrating apparent X-linked inheritance [136] and one additional case traced during a follow-up of these two families [129]. All nine patients had marked dermal extensibility but only mild to moderate tissue fragility and mild scarring and bruising. Spheroids and molluscoid pseudotumors were also found. The skin was of a soft and doughy consistency. Articular laxity was mild and most evident in the digits, yet orthopedic complications (effusions, recurrent sprains, arthritis) were frequent. It may have been noteworthy, at least to the authors, that all affected persons in both families had red hair [129]. In one 53-year-old affected man, a skeletal survey excluded occipital horns and other skeletal abnormalities, and serum copper and ceruloplasmin in affected males and obligate carrier females were normal [129]. The disorder is thus clearly different from the occipital horn syndrome (EDS IX) and is a nuisance rather than a handicap to the affected persons [129].

Di Ferrante et al. [516] reported a 9-year-old boy and two maternal cousins as having EDS V. All had congenital heart disease, floppy mitral valve syndrome, which in one of them progressed to severe insufficiency and death, herniae, short stature, stretchable skin, moderate joint hypermobility, musculo-skeletal weakness with dorsal kyphosis, genu valgum, and flat feet. An atrial septal defect with left-to-right shunt, and floppy mitral and tricuspid valves with significant regurgitation, were present. The nosology of this disorder remains uncertain, but in our view it more likely belongs to an X-linked cardiovascular disease type (MIM 314400; [517]).

Other patients with EDS V have been mentioned [518], but no clinical or genetic data were given, and probably more sporadic cases have also been reported uncritically and erroneously as EDS type V [519–521].

The distinction of EDS V from autosomal dominant EDS is based solely on its apparently X-linked recessive mode of inheritance in two pedigrees. However, pedigree 2 [129,136] could be explained, as originally critically discussed [136], by autosomal dominant inheritance with incomplete penetrance in the supposed carrier female (II/5 in [129,136]). This view is fostered by the follow-up revealing that her affected son (III/1) has two children with minor stigmata of the syndrome: the 21-year-old daughter has generalized joint laxity and normal skin, the 19-year-old son has soft, extensible skin but no scars or joint laxity. In pedigree 1, the putative heterozygote mother (III/2 in [129,136]), when re-examined at the age of 60 years, had recently undergone surgery for degenerative arthropathy of the knee, which had been ascribed to articular instability. This problem, together with slow healing at the operation site, could represent minor syndromic manifestation in a female heterozygote or, again, incomplete penetrance. Close linkage to Xg blood groups and color blindness was excluded in the two families [136], and cytogenetic investigation, including high-resolution banding, yielded normal results [129]. X-linked inheritance therefore remains questionable, and thus the existence of this disorder as a distinct entity remains to be proven.

The biochemical defect was thought to be a deficiency of lysyl oxidase (protein-lysine 6-oxidase, EC 1.4.3.13; MIM 153455), the gene for which was later shown to be located on chromosome 5q23.3-q21.2 [522], a finding that is at variance with claimed X-linked inheritance of the disorder. The observation of low levels of lysyl oxidase in skin and other tissues from mice with an X-linked connective tissue disorder [523,524] was the stimulus for Di Ferrante et al., who reported a deficiency of lysyl oxidase in one patient [516]. However, methodological flaws make their measurements unreliable; lysyl oxidase, which is almost entirely secreted in culture, was estimated in fibroblast cell layer homogenate; furthermore, the activity in culture medium was measured after lyophilization and "redissolution" of the enzyme, which, however, is poorly soluble in buffers lacking urea; in the absence of any clinical or biochemical follow-up report, there remains a conspicuous silence about the nature of the disease in their patient, who also had two maternal cousins similarly affected. Siegel et al. [525], using appropriate techniques, could not confirm this result in four of the original patients reported [136]. They found greater than normal activity of lysyl oxidase and no diminution in the amount of immunologically cross-reacting material in extracts of skin from patient J.W. (subject III/2 in family 2 [136]), normal proportions of reducible cross-links in skin from all four patients studied (III/1 and III/2 of family 2 and IV/1 and IV/4 of family 1 [136]), and normal amounts of thermally stable cross-links as judged by the normal proportion of high-molecular-weight chains on SDS-polyacrylamide gel electrophoresis. Siegel et al. [525] also pointed out that an absence of cross-links would lead to continuous extension of the fibers as they slipped past each other under tension until rupture occurred and that in this situation there would be no restoring force, in contrast to what is found in EDS. Ultrastructural studies showed changes that were not qualitatively different from those in the skin of patients with EDS I or II [183]; other changes in undefined patients have been reported [518,519]. Note that Fig. 7 in [526] refers erroneously to a patient with EDS V instead of EDS IX, the occipital horn syndrome (K. Holbrook, personal confession, 1990).

Because there are no distinct clinical characteristics and no biochemical marker for EDS V, diagnosis depends solely on the mode of inheritance. In every sporadic male EDS patient, the family history should be carefully evaluated, with special emphasis on the males of the maternal tree (such as maternal uncles and maternal cousins). EDS V is readily differentiated from the X-linked EDS IX, the occipital horn syndrome, by the absence of cutis laxa and exostoses such as occipital horns and by the presence of normal amounts of serum ceruloplasmin and copper and from an X-linked congenital heart disease (MIM 314400) [517] by other criteria.

Beighton and Curtis [129] state that "the disorder is undoubtedly very uncommon; nevertheless, it has gained the asterisk of syndromic respectability in the catalogue of Mendelian inheritance in man." We feel that this is unjustified and would question the existence of this disorder as a separate entity despite the asterisk attributed to this entry in "Mendelian Inheritance in Man" [37].

Ehlers-Danlos Syndrome Type VIII — Periodontotic Type of EDS (MIM 130080)

The existence of EDS VIII (Table 1) as a separate entity is questionable [39]. On the last page of the chapter on the Ehlers-Danlos syndrome in "Heritable Disorders of Connective Tissue," McKusick [2] writes: "A condition unique in my experience, and apparently in the literature as well, is demonstrated by the wife of a colleague of mine and several of her relatives. Lesions on the shins suggest those of EDS, and slow-healing breaks in the skin at that site have occurred after blunt trauma. The skin is not generally fragile, and no unusual bruisability or stretchability of the skin has been noted. The joints are not hyperextensible. A second feature, apparently syndromally related to the lesions on the shins, is absorptive periodontosis, with early loss of the teeth. The dental and skin changes are present also in the proband's father, in several sibs of her father, and in a cousin. The lesions on the shins suggested necrobiosis diabeticorum on histologic study.... The small scars on the knees are somewhat like those of EDS, and the skin of the lateral aspect of the soles is wrinkled in the manner demonstrated by EDS-patients."

The description of this "unique condition," together with a second family, prompted Stewart et al. [527] to classify this new variant as EDS VIII. More cases have since been reported [528–533; D. Hollister, personal communication, 1990; K. Hinkel, personal communication, 1990] or have just been mentioned without clinical description [518]. In all, joint laxity, especially of the fingers, is mild to moderate, hyperextensibility is mild or absent, fragility of the skin is mild to severe, the scars are "cigarette-paper"-like, the palms and soles are excessively wrinkled [528], and there is no evidence of visceral involvement [529]. A Marfanoid habitus with arachnodactyly and slim limbs and fingers was noticed in two families [527,528]. Minor trauma produced ecchymoses that resolved normally except on the shins, where the pretibial skin healed with distinct hyperpigmented atrophic scars. Hoffman et al. [534] described a sporadic patient with EDS VIII in whom autoimmunity to collagen I appeared to be responsible for unusual features such as intractable vasculitis and resorptive arthritis and osteolysis.

Dental disease begins with numerous caries of the deciduous teeth, which are shed prematurely or normally; the permanent dentition erupts at the usual time. The onset of gingival inflammation in the second decade, and the progression of periodontal disease, leads to a generalized alveolar bone loss around all teeth, which results in the premature loss of all permanent teeth during the third decade. The teeth are morphologically normal [530]. A propensity for the rapid and extensive formation of calculus, particularly around the gingival portions of the posterior teeth, was noted [527].

In one sporadic case, histology of the gingiva exhibited the presence of peculiar, previously unidentified, cell-poor homogeneous masses underneath the epithelium with staining properties resembling those of fibrin. Whether these gingival fibrinoid deposits are a typical feature of EDS VIII will remain uncertain until similar studies in other cases have been performed [531]. Ultrastructural studies have shown a mixed population of large- and small-diameter fibrils with altered packing into fibers and fiber bundles in the reticular dermis [192,532].

EDS VIII seems to be a rare, dominantly inherited disorder of unknown cause. In three patients from a family with eight affected individuals over six generations (K. Hinkel, personal communication, 1990), collagen studies were normal (M. Raghunath, personal communication, 1991). In the large Dutch kindred reported by Hollister [530], linkage to *COL3A1* has been excluded [535]. Ultrastructural examination of skin from two patients did not show any abnormalities in the cells or matrix, and the collagen bundle architecture appeared normal [530].

Absorptive periodontosis occurs also in EDS IV. Therefore, collagen studies should be performed to exclude the latter condition with its dismal prognosis. The father of the initial propositus reported by Stewart et al. [527] died at the age of 39 years of an apparently spontaneous rupture of the duodenum with subsequent overwhelming peritonitis [530]. However, studies of fibroblasts from the propositus performed later showed normal collagens I and III (D. Cohn, personal communication, 1991), indicating that he may not have suffered from EDS IV. On the other hand, an initial diagnosis of EDS IV based on preliminary biochemical data in a separate case had to be revised following more careful studies [533].

The gene for an autosomal dominant form of juvenile periodontitis (MIM 170650) is located on chromosome 4 and linked to the trait for dentinogenesis imperfecta type III [536]; a recessive form may also exist (MIM 260950). Early loss of teeth occurs also in the Papillon-Lefèvre syndrome with keratosis palmoplantaris (MIM 245000), hypophosphatasia (MIM 146300 and MIM 241500) (see Chapter 18, this volume), and Werner syndrome (MIM 277700). Because periodontal ligaments are rich in collagen XII [537], the gene *COL12A1* could be a candidate for the disorder.

Occipital Horn Syndrome (Formerly Ehlers-Danlos Syndrome Type IX) (MIM 304150)

The phenotypic manifestations of the occipital horn syndrome (OHS) were first described by Lazoff et al. [538] in an 11-year-old boy and his two maternal uncles. The child presented with diarrhea, recurrent urinary tract infections, bladder diverticula, inguinal herniae, and peculiar "occipital horns." All three had educational problems but were not mentally retarded. One year later, a new form of X-linked cutis laxa with bladder diverticula and skeletal anomalies was reported [539]. The phenotype was associated with a deficiency of lysyl oxidase that was later inferred to result from disordered copper metabolism [540]. The condition was renamed Ehlers-Danlos syndrome type IX [126], but because of the skin laxity it was later withdrawn from the EDS nosology [38] and the accepted term is now the occipital horn syndrome. The proper term indeed should be mild Menkes disease with occipital horns (see Chapter 14, this volume, and Table 1).

Menkes disease (MD) is an X-linked inborn error of copper metabolism affecting several body systems, including the connective tissues. The clinical manifestations of MD can be quite variable, and both severe and mild forms exist. In the milder forms, the neurological symptoms are less severe, whereas connective tissue findings remain prominent. This group is likely underdiagnosed. The

classical, severe form of MD comprises 90–95% of all cases, and its clinical manifestations include progressive neurological degeneration, seizures, growth failure, arterial aneurysms, and skeletal defects, as well as characteristic hair changes (pili torti) and hypopigmentation, and death results in early childhood. The occipital horn syndrome is the mildest form of MD. Remarkable changes are inguinal herniae, bladder diverticula, skin laxity, and skeletal abnormalities, including a short trunk, deformed elbows, and genu valgum. A diagnostically important feature is the occurrence of bony protuberances on the occiput, the so-called occipital horns. Chronic diarrhea and orthostatic hypotension are other characteristic features.

MD and the occipital horn syndrome are allelic, X-linked, and due to mutations in the Menkes gene, *ATP7A*, which codes for a P-type ATPase, an enzyme located in the *trans*-Golgi network, where it transports copper into the lumen for incorporation into secreted and vesicular copper-requiring enzymes (see Chapter 14, this volume).

Ehlers-Danlos Syndrome Type X (MIM 225310) — Fibronectin-Deficient EDS

The existence of EDS X (Table 1) as a separate entity is questionable [39]. The problem of defining a new type of EDS is well-illustrated by the single family with EDS X to which McKusick has assigned a separate entry in the catalog, although without the "quality label" of the asterisk [37]. Arneson et al. [541] described four of six sibs born to apparently unaffected, unrelated parents with a mild form of EDS and dysfunction of plasma and cellular fibronectin, which was confirmed independently [542]. The proband, a 28-year-old female physician, had joint hyperextensibility, most prominently of the hands and feet and to a lesser extent of the elbows and shoulders, thin but not velvety skin with "fish-mouth" scars that was easily bruisable, petechiae, a positive Gorlin sign, mitral valve prolapse, an aortic root diameter at the upper limit of normal, and, surprisingly, striae distensae. She had once had a knee dislocation that reduced spontaneously (D. Hammerschmidt, personal communication, 1990). Electron-microscopic studies showed a lack of cohesion between collagen fibers within bundles in skin, where there was an unusually loose organization of the deep reticular dermis. Cauliflower collagen fibrils and large fibril diameters were also observed (Fig. 7a in [192]; Fig. 10 in [543]). The proband has had two uncomplicated pregnancies (D. Hammerschmidt, personal communication, 1990).

The platelet aggregation defect was correctable *in vitro* by the addition of normal human fibronectin; on the other hand, the patient's plasma failed to support the aggregation of gel-filtered platelets from controls in response to collagen. Because the measured level of immunoreactive fibronectin in plasma was normal and immunohistochemical studies have shown fibronectin to be present in the platelet α-granules, the authors assumed a minor structural or post-translational abnormality of the fibronectin molecule. In a preliminary experiment, the fibronectin was found to have an abnormal isoelectric behavior (D. Hammerschmidt, personal communication, 1990).

The inherited connective tissue disorder segregated with the platelet aggregation defect, and, since fibronectin is an important adhesive glycoprotein in connective tissues, the authors proposed that both the platelet malfunction and the mild EDS in this kindred might be explained by a defective fibronectin. This relationship, it seemed to them, was further supported by the observation that during the first pregnancy the ability of the proband's plasma to support collagen-induced aggregation of normal platelets markedly improved [544], which may have contributed to the surprising fact that hemostasis was normal, while the somewhat dilated aorta remained constant through both pregnancies and even episiotomy was needed, and healed normally (D. Hammerschmidt, personal communication, 1990). Plasma obtained six months postpartum behaved as did pregravid and first-trimester plasma samples [544].

The proband has two children, a girl and a boy. However, the girl had had approximately 75 luxations of the head of the radius by the time she was 8 years old but had no more in the subsequent two years. Her brother, as well as their two maternal cousins, has also had a luxation of the radius at least once. Studies of platelet aggregation or skin morphology have not yet been performed in them (D. Hammerschmidt, personal communication, 1991). Since no further cases have been observed and no biochemical or genetic studies have been performed, the primary defect and its mode of inheritance remain unclear. The linkage of two separate disturbances and a common underlying cause are both possible, and autosomal recessive or autosomal dominant inheritance, transmitted by one parental carrier of a gonadal mosaicism, are also both possible. If one considers the proband's daughter and son, as well as their maternal cousins, to be affected, dominant inheritance seems more likely.

Regarding the proposed association between the platelet aggregation disturbance and this mild form of EDS, it should be noted that altered production, assembly, and distribution of fibronectin has been observed in cultured fibroblasts from patients with several different types of EDS [545–547]. It is further appropriate to mention that in eight members of a three-generation family, half-normal levels of plasma fibronectin, which has a normal electrophoretic mobility, do not result in abnormalities of platelet aggregation or clinical signs of a connective tissue disorder [548].

Furthermore, there are numerous reports of EDS associated with more or less well-characterized platelet or coagulation dysfunctions [549–557]; most probably, these findings are simply coincidental, but they may have added to the bleeding tendency of the underlying EDS, thus prompting clinical investigation. In summary, the causal association between EDS, dysfibronectinemia, and platelet dysfunction remains to be established and thus we question the existence of EDS X as a separate entity in this single family.

Familial Joint Hypermobility Syndrome (Formerly EDS Type XI) (MIM 147900)

Familial joint laxity with recurrent luxations of the shoulder and patella, often with congenital hip dislocation, has been reported as an autosomal dominant condition [2,59,62,558–560]. Because of the lack of skin involvement, it has been recommended that this entity, formerly EDS XI (Table 1), be classified as a separate entity, familial articular hypermobility syndrome [37–39].

Progeroid Form of Ehlers-Danlos Syndrome (MIM 130070)

Hernandez et al. [561–563] described five sporadic male patients with mental retardation, short stature, a progeroid appearance with wrinkled facies, curly and fine hair, scanty eyebrows and eyelashes, and periodontitis in addition to typical signs of the EDS. Increased paternal age suggested to

them *de novo* mutations. A similar case has been reported by DeLozier-Blanchet et al. [564].

Kresse et al. [565] have described a five-year-old sporadic patient, born to nonconsanguineous parents, who represented a progeroid variant with signs of the EDS (hyperelastic skin and atrophic scars), developmental delay, dwarfism, craniofacial disproportion, osteopenia of all bones and dysplasia of some, and defective wound healing. Later, at age 8.4 years, it was noted that his growth rate had been normal during the last years and that he had made steady intellectual progress [566]. His fibroblasts were defective in the biosynthesis of the ubiquitous proteoglycan small dermatan sulfate proteoglycan (DS-PG II), or decorin, which consists of a core protein of M_r 36,319, with a single glycosaminoglycan chain on the serine residue at position 4, and either two or three asparagine-bound oligosaccharides (see Chapter 4, this volume). The fibroblasts synthesized normal amounts of core protein but converted it into mature proteoglycan only in reduced amounts; the remainder was secreted in a glycosaminoglycan-free form, the tyrosine residues of which were normally sulfated [567]. This abnormality is explained by the marked deficiency (~5% residual activity) of galactosyltransferase I (xylosylprotein 1,4-β-galactosyltransferase, XGALT1; EC 2.4.1.133) as measured in cell homogenates using artificial substrates [568]. This enzyme normally catalyzes the second glycosyl transfer reaction in the assembly of the xylose/serine-linked proteoglycans. The mutant enzyme was thermolabile and had K_m values different from normal for several substrates. Because the parents have intermediate enzyme levels, the disorder seems to be inherited as an autosomal recessive trait. The disorder has been shown by two groups to be due to compound heterozygosity for two mutant alleles, Ala186Asp and Leu206Pro, of β4Gal-T7 (*XGalT-1*), and enzymatic measurements in expression systems of both cDNAs showed 10–50% and 0% activity, respectively [569,570]. Why the activity of galactosyltransferase II (galactosylxylosylprotein 3-β-galactosyltransferase, EC 2.4.1.134) is reduced to ~20%, and whether the large dermatan and heparan sulfate proteoglycans are also affected, remains to be determined [568]. Quantitative immunogold electron microscopy of a skin biopsy revealed that the collagen fibrils of the patient were decorated with much less decorin than those from an age-matched control [566]. It was suggested that the glycosaminoglycan-free core protein present in the patient's tissues is more rapidly metabolized than intact proteoglycan. Unfortunately, no information is available about the thickness and morphology of the patient's skin. Further studies on this and future cases will certainly provide important insights into physiological and pathophysiological roles of decorin.

Reduced transcription and expression of decorin mRNA has been observed in conditions that have in common muscle hypotonia, little subcutaneous fat, joint and skin laxity, easy bruisability, and poor mineralization of bone, as observed in progeroid and progeria patients, and the neonatal Marfan syndrome (MIM 1154700) [604], as well as in the Wiedemann-Rautenstrauch syndrome (MIM 264090). However, in this last condition, such a defect was present only during infancy and hence should be considered as a secondary phenomenon, which leads to a fault in the regulation of decorin gene transcription [571].

Another example of a disturbance in proteoglycan synthesis is given by Fushimi et al. [572], who describe a sporadic case of isolated adrenocorticotrophic hormone deficiency with features characteristic of the EDS (fragility, hyperextensibility and easy bruisability of the thin skin with easily visible veins, molluscoid pseudotumors, but an absence of hypermobility of any joints). Immunohistochemical analysis using anti-dermatan sulfate-proteoglycan antibodies and studies of the biosynthesis of glycosaminoglycans in cultured fibroblasts suggested a defect either in the synthesis of a proteoglycan core protein or in the synthesis of dermatan sulfate side chains in the patient's skin. These results suggested that the dermal findings were due to a lack of dermatan sulfate proteoglycans. The reason for the thinness of the skin, and hence deficiency of collagen, in spite of the normal amounts and proportions of collagen produced by the fibroblasts, is unknown, but the findings suggest that dermatan sulfate proteoglycans play an important role in the formation, function, and maintenance of the structural integrity of the skin. The absence of joint laxity may be explained by the fact that the core proteins of dermatan sulfate proteoglycans in tendon and skin are genetically different [573], but dermatan sulfate in tendon could not be analyzed in this case. This report also illustrates that in the absence of joint laxity the nosology of the EDS itself becomes loose.

Unspecified Types of the EDS and Chance Associations

There are many enthusiastic claims reporting "new forms" of the EDS, such as that of Viljoen et al. [473] in members of a family later shown to have EDS VII [474] (see above), among others [104,109 (MIM 225320), 574,575 (MIM 130090)] waiting to be confirmed clinically and/or characterized biochemically or to be withdrawn because of a recognized chance association with EDS, such as occurred in cases of, for example, neurofibromatosis [576], α2-macroglobulin deficiency [577], α1-antitrypsin deficiency [578], epidermolysis bullosa [579], cystic fibrosis [398], and bilateral focal polymicrogyria [580]. Other authors have been caught in semantic traps of different syndromes [581].

ANIMAL MODELS AND LATHYRISM

The advantages of studying animals with heritable disorders of connective tissue are obvious: (1) they provide adequate quantities of material from different tissues for morphological, biochemical, and biomechanical studies and the establishment of cell cultures; (2) they allow the comparison of paired affected and unaffected littermates at all stages of embryonic, fetal, and postnatal development, with the assessment of early consequences of lethal mutations, as well as during aging within a reasonably short time period; (3) they allow experimental mating for prospective genetic studies to produce heterozygous, homozygous, and compound heterozygous animals; (4) they allow the comparison of similar diseases in different species. Although the eponyms of human diseases are not strictly applicable to the analogous diseases in animals, the causes and effects of these diseases appear to be directly comparable. For an overview of animals with dermatosparaxis or EDS-like conditions, see Table 4.

Dermatosparaxis

Dermatosparaxis in cattle was the first "true" collagen disease to be elucidated. Its characterization greatly stimulated research on the conversion of procollagen, led to the isolation, characterization, and sequence determination of the N-terminal propeptide extensions of proα1(I) and proα2(I) chains, and enabled the study of the regulation

TABLE 4. Natural Animal Models of EDS

Disorder and Species	Inheritance[1]	Defect	Phenotype	References (Selected)
Dermatosparaxis				
Cattle from Belgium	AR	Procollagen N-proteinase deficiency	Severe	464,500,507,511,513, 582–593
from Texas (Hereford)	AR (?)		Severe/moderate	594
Sheep from Norway (Dala)	AR	Defective conversion of pN-collagen		464,493,512,595–599,
from Australia	AR	Procollagen N-proteinase deficiency (25%)	Mild	487,600–605
from Finland	AR		Severe	606
from South Africa (white Dorper)	AR		Moderate	607
Cat	?		Mild	608–610
Dog	AR		Severe	611;G.D. Hegreberg, personal communication, 1991
Ehlers-Danlos syndrome-like conditions				
Dog	AD			612–618
	?			442,619–621
Mink	AD			613–615,622
Cat	?			623
	AD			616,618,624
Horse	ND			625
Cattle	AD?			626,627
Rabbit	?			628–631
Mottled mouse				
Blotchy mouse (Mo^{blo})	XR	Disturbed copper handling ⟶ deficient lysyl oxidase activity	Mild	523,524,632–636

[1] Inheritance: AR, autosomal recessive; AD, autosomal dominant; XL, X-linked recessive; ?, sporadic case; ND, autosomal recessive or dominant inheritance possible but more likely paternal mosaicism of an AD trait.

of fibrillogenesis and cross-link formation by the precursor peptide and the function of the cleaved peptide in the control of procollagen synthesis. Although it had been speculated for some time that high-molecular-weight precursors of collagen might exist [637,638], the actual demonstration of such molecules in tissue cultures of human fibroblasts [639], and in both rat calvariae [640] and chick embryo tendon cells [641], by three independent laboratories occurred at about the same time that dermatosparactic cattle were recognized to have an abnormal high-molecular-weight collagen precursor in skin. This immediately made it possible to prepare and purify the precursor chains in sufficient amounts and to gain the above-mentioned insights at a much higher speed.

The hallmark of dermatosparaxis (“torn skin”) is the excessive fragility of the skin with only mild or absent involvement of tendons and ligaments, which distinguishes this disorder from EDS VIIA and VIIB in man. In affected calves from Belgium, lambs from Norway, sheep from Australia, and cats, the conversion of pN-collagen to collagen seems to be disturbed, either because of a deficiency of procollagen N-proteinase activity [507,600,608] or possibly because of an absence of the collagen-binding protein annexin V (anchorin CII), which could function as a “presenter” of procollagen to the enzyme [595]. The amount of pN-collagen in skin roughly correlates with the extent of the enzyme deficiency, the clinical severity, and morphological abnormalities; for example, skin from the severely affected lambs contained almost exclusively pN-collagen [582], whereas skin from the less severely affected Australian sheep contained only approximately one-third of the collagen as pN-collagen [600]. The relatively low amount of pN-collagen in these latter sheep is due to a less severe reduction (~25% of normal) in procollagen N-proteinase activity as measured in skin extracts [600]. Within one animal, variable degrees of involvement of different organs are reflected in differences in their content of pN-collagen (70% and 15% in skin and tendon, respectively; [596]) and ultrastructure (Figs. 33e and 33f).

As a consequence of the persistence of the N-propeptide, collagen fibril formation is disturbed. This is reflected by the ultrastructural changes observed in all dermatosparactic animals. In cross-section, the fibrils have a spider-like or hieroglyphic-like appearance or a markedly serrated contour; longitudinally, the fibrils resemble ribbons twisting in both directions [493,583,594,601,609,611]. Abnormal cross-link formation [511], increased solubility of collagen, and, ultimately, decreased tensile strength are the consequences of disturbed fibril formation. *In vitro* experiments show that with increasing pN-collagen content, cross sections of the formed fibrils are progressively distorted from circular to

lobulated to thin and branched structures [642,643], which indicates that there is a dose-dependent relationship. Watson et al. [474] have proposed a model of fibril formation in EDS VIIC in which the intact N-propeptides are located at the surface of the hieroglyphic fibrils. Partial cleavage of *in vitro*-synthesized abnormal collagen by N-proteinase allows the N-propeptide to be incorporated within the body of the fibrils, with conversion of the hieroglyphic outlines to the ragged outlines characteristic of EDS VIIA and VIIB.

The variability of different organ involvement has not been adequately studied. It may be influenced by differences in local residual procollagen N-proteinase activity, collagen turnover, stringency in collagen packing, or a combination thereof. The fact that with aging there is an improvement in various properties of the skin may point to an influence of collagen turnover; Piérard and Lapière [515] observed several differences between two calves 3 and 6 months old. The sheet-like arrangement of the polymers was replaced by a bundle-like organization, the ratio of collagen to procollagen increased, and the biomechanical properties of the dermatosparactic skin also tended to become more normal.

In one strain of dermatosparactic calves in which the disease was first observed [584], the mutation consists of a 17 bp deletion that changes the reading frame of the message [500], and would, if the mRNA were stable, result in the synthesis of a truncated protein. Because the mutation would prevent the synthesis of active procollagen N-proteinase, the fact that different tissues contain variable amounts of processed procollagen I suggests that another enzyme, tissue-specifically expressed, can remove the N-terminal peptide of procollagen I, although at a lower rate than the N-proteinase. Two proteins that have a high sequence homology with the N-proteinase and contain similar domains, including properdin repeats, have been described (for references, see [500]). Their physiological roles have not yet been determined. However, the regulation of their expression and their tissue-specificity suggest various functions for members of this new subfamily, which are candidates for an alternative pathway of processing procollagens I and II [500].

Ehlers-Danlos Syndrome-like Conditions

All animals with "generalized cutaneous asthenia," "conditions resembling the EDS," or "fragility and hyperextensibility of the skin" seem to inherit these as autosomal dominant traits. Most reports focus on the description of the skin, others (e.g., [619]) mention also profound joint laxity, subluxations, and osteoarthritis; radiographic and microradiographic studies have revealed subclinical involvement of bone [617]. In all animals, the defect remains unknown. Ultrastructurally, abnormalities in the packing of collagen into fibrils and fibers have been noted [617,620,624,625]. Differences in the solubility and cross-linking of collagen have occasionally been reported [612,625], but these may also be accounted for by an increased rate of collagen turnover and thus be secondary to some other primary change.

In pregnancies resulting from matings between two heterozygous cats [624], the litter size was reduced and only 12 of 34 offspring were affected; furthermore, uterotomy at gestational days 20 and 44 (normal duration is 63 days) revealed that approximately one-quarter of the placental sacs were abnormally small and contained no embryo. It was concluded therefore that the homozygous state of the "defect in collagen fibrillogenesis" would be lethal during early embryonic development [618], perhaps shortly after implantation or during early gastrulation (R. Minor, personal communication, 1990). The apparently homozygous EDS cases in man [29,134] seem therefore to be basically different from those in this animal model.

Kobayashi et al. [644] reported a 4-month-old Holstein calf with soft and hyperextensible skin, skin fragility, and a history of delayed wound healing, but without joint hypermobility. Electron microscopy showed rarefaction of collagen fibers and an increased amount of dermal ground substance. Tajima et al. [645] showed that there was a deficiency in dermatan sulfate proteoglycans in skin caused by a presumably new heterozygous mutation, a G to A transition at nucleotide position 254, which resulted in a Ser-to-Asn substitution at amino acid position 85 of the bovine proteoglycan core protein. This substitution occurred in the highly conserved Ser–Gly dipeptide repeat sequences suspected to be the O-glycosylation site of dermatan sulfate side chains. The sire and grandsire did not carry the mutation, and the dam and granddam were not available for analysis but did not present these skin manifestations. It therefore appears that the calf was affected due to a newly occurring heterozygous mutation of the gene coding for the proteoglycan core protein.

Transgenic Animals

The use of gene targeting as a means of assessing the biological function of a protein is a powerful tool, although there are certainly many instances in which overlapping functions or gene compensation can obscure the function of the missing or defective protein under study, often making comparison between affected individuals and experimental animals difficult. Some examples are given here which illustrate that certain transgenic animals were seminal in the characterization of the human counterpart, while others should prompt research into the elucidation of hitherto unknown basic defects of diseases in man.

Targeted Mutation of the Col5a2 Gene in Mice — A Model of the Classical Type of EDS

This animal model was seminal in the elucidation of the classical type of EDS in man. Homozygous mice (pN/pN) with an in-frame exon 6 deletion in *Col5a2* survive poorly, possibly because of complications from spinal deformities, and exhibit skin and eye abnormalities caused by disorganized collagen I fibrils [150]. The deletion is outside the major helix of the collagen V molecule and does not alter the reading frame of the *Col5a2* transcript but prevents normal processing of the proα2(V) chains. It results in a heterotrimeric $[\alpha1(V)]_2\alpha2(V)$ that is assembled, secreted, and integrated normally into heterotypic collagen fibrils. The abnormal collagen V is structurally defective due to an alteration in the conformation of the N-globular domain, a region thought to play a key role in the interaction between collagen V and collagens I and III [149].

Heterozygous animals (pN/+) were phenotypically normal and fertile. Likewise, the homozygous mutant mice (pN/pN) appeared normal at birth. Soon after, however, many of them were cannibalized and the remaining ones began to show a progressive hunching of the back that affected mobility and respiration. By weaning age (~3 weeks), the survival rate was 5% and their weight was ~50% of normal. Radiology revealed varying degrees of spinal lordosis and kyphosis in all. The normal morphology of the vertebrae suggested that spinal deformity was probably secondary to loss of tensile strength of the ligaments. On the other hand, intravital

double-tetracycline labeling demonstrated that bone in the mutant mice grew at a slower than normal rate.

Skin was much more fragile and stretchable upon physical examination and biomechanical testing. The amount of collagen in the dermis of mutant mice was markedly reduced, whereas the hypodermis was 4–6-fold thicker and contained a large number of hair follicles not normally localized in this layer. Ultrastructurally, the collagen fibrils were more disorganized, less tightly packed, and more heterogeneous in size. Collagen fibrils in the cornea were disorganized and thicker: mean fibril diameters were estimated to be 25 nm in controls, 35 nm in heterozygous mutants, and 50 nm in homozygous mutants. Consistent with the ultrastructural findings, the stroma but not the epithelium of the cornea appeared collapsed and was thinner than that of controls.

The lack of an apparent phenotype in the heterozygous mice is consistent with the stoichiometry of collagen V, which predicts that only half of the trimers will have an abnormal conformation. This could also explain why size differences in fibrils were noted only in the cornea, a tissue that contains fibrils of equal size and with the largest proportion of collagen V. Alternatively, heterozygous animals were not examined with more stringent analyses and were under greater physiological stress, so clinical signs may have been missed.

In summary, the deletion of the α2(V) N-telopeptide leads to a change in the conformation of the collagen V heterotrimer and the elimination of the hinge-like region. This conformational change abrogates the collagen V regulation of the growth of collagen fibrils and their subsequent packing into fibers, affecting the spatial arrangement of the fibers into tissue-specific macroaggregates [150].

The tight skin mouse (TSK/+), a naturally occurring mutant, develops a generalized connective tissue abnormality that is transmitted as an autosomal dominant trait and is caused by a large duplication in the fibrillin1 gene (see Marfan syndrome, Chapter 12, this volume). The phenotype is characterized by marked hyperplasia of loose connective tissues, thickening of the skin, pulmonary emphysema, and cardiac hypertrophy. Although both pN/pN and TSK mice exhibit cutaneous thickening, there are striking differences between the two mutant strains. However, crossing TSK/+ mice with pN/pN mice resulted in an F1 progeny without cutaneous hyperplasia [646]. This interesting example of genetic complementation may serve as a model to explain lack of penetrance in double heterozygous individuals.

Tenascin-X Knockout Mouse — A Model of the Classical Type of EDS

Mao et al. [647] have produced a *Tnx* knockout mouse. The skin of TN-X-deficient mice becomes clearly hyperextensible at about 2 months of age, and hyperelasticity is progressive with advancing age. The best phenotypic indication, beginning at about 3 months of age, is that when such mice are held by the skin of the neck, they are still able to twist around to bite the investigator due to the hyperelasticity of their skin. The joints show no obvious hypermobility.

Biomechanical studies demonstrate reduced tensile strength (60% of that of age and sex-matched unaffected littermates) and hyperextensibility of skin. The dermis is less dense and thinner in TN-X-deficient animals than in normal littermates. Ultrastructurally, dermal collagen fibrils have a normal contour and a normal mean diameter, which is, however, more variable. The most striking finding is that the density of the collagen fibrils is reduced by 40% and that the alignment of fibrils with respect to one another appears to be less perfect. These data suggest that TN-X has a role in regulating collagen fibril architecture and that its lack can cause the skin manifestations of EDS with minimal alteration of fibrillogenesis.

Decorin Knockout Mice — A Model of EDS?

To study the function of decorin in collagen fibrillogenesis, Danielson et al. [179] created mice with a targeted disruption of the decorin gene. The decorin null mice presented no gross anatomical anomalies, were normal in size, fertile, without radiologically overt bone abnormalities, without obvious behavioral deficiencies, and with normal routine chemistry.

The skin was hyperelastic, thin, and fragile, with detachment of the tail skin, not unlike in dermatosparaxis (heterozygotes were indistinguishable from control animals). Histology showed a thin dermis, a loose connective tissue in the hypodermal layer, and a sharp detachment of the skin between the deeper dermis and the fascia, with clean, sharp edges along the dissection. Electron microscopy revealed less orderly packed collagen fibrils with a much larger diameter and irregular coarse outlines in cross section. Electron microscopy of longitudinally sectioned collagen fibrils from tendon showed that the typical 67 nm periodicity of collagen I was maintained; however, numerous d bands of the D-periodicity were not occupied by orthogonally arranged proteoglycan granules as in controls. The absence of overt abnormalities in the cornea of null mice, a tissue in which decorin is normally expressed, may be explained by the larger requirement for keratan sulfate in this transparent structure and by the presence of at least five members of the small leucine-rich proteoglycan family, which could control more tightly the fibril diameter necessary for transparency.

Dermatosparaxis and decorin knockout mice. A contributing factor to the skin fragility in dermatosparaxis was thought to be the steric exclusion of a factor from the gap zone of collagen fibrils by the N-terminal propeptide, which forms a hairpin and thus blocks this region [179]. In dermatosparactic skin, several proteoglycan filaments are not closely bound to collagen fibrils but rather float freely in the interfibrillar spaces [593]. Thus, dermatosparactic animals, through the presumed exclusion of access to decorin, and decorin null animals, through a lack of decorin, were assumed to share a common pathogenetic mechanism (i.e., the obstruction/deficiency of a key regulatory molecule in collagen fibrillogenesis). Further support for this concept was provided by the lowered dermatan sulfate to hydroxyproline ratio from 4.7 in normal skin to 3.6 in dermatosparactic skin [513]. However, later experiments showed that in dermatosparactic calf fibrils the equilibrium between fibrillar and soluble decorin was shifted in favor of enhanced fibril-associated decorin [648]. Thus, the results showed that N-propeptides can distort the morphology of fibrils, that they do not inhibit the binding of gap-associated macromolecules (such as decorin), and that normal mechanical properties of skin are strongly dependent on the close association of near-cylindrical fibrils, thereby enabling maximal fibril-fibril interaction [648].

Lumican-Deficient Mice

Mice homozygous for a null mutation in lumican display skin laxity and fragility with easy bruising resembling certain types of EDS [180]. In addition, mutant mice develop bilateral corneal opacification. Skin has a markedly increased compliance and reduced tensile strength independent of any differences in skin thickness. Its fibroblasts and collagen fibers are poorly aligned. There is a significant proportion of abnormally thick collagen fibrils in skin and cornea.

Thus, lumican, a prototypic leucine-rich proteoglycan (LRP) with keratan sulfate side chains that colocalizes with fibrillar collagens, has a crucial role in the regulation of collagen assembly into fibrils in various organs, especially the cornea [180]; other LRP members, such as biglycan, fibromodulin, epiphycan, osteoglycin, and keratocan, may have similar functions.

Thrombospondin-2-Deficient Mice — a Model of Ehlers-Danlos Syndrome

Thrombospondin-2 (TSP-2) is a member of a family of five secreted, modular glycoproteins whose functions in the extracellular matrix are diverse and poorly understood. TSP-2 is predominantly expressed in dermis, tendon, ligament, perichondrium, and pericardium; it is also present in smooth muscle cells and endothelial cells. In an attempt to determine the function of TSP-2, Kyriakides et al. [181] disrupted the *Thbs2* gene by homologous recombination in embryonic stem cells and generated TSP-2-null mice by blastocyst injection and appropriate breeding of mutant animals.

Thbs2 − /− mice are normal in size and overt appearance and reproduce normally. Juvenile mice often have subtle bends in their tails that become less distinct as they mature, but the tail becomes more flexible, enabling the investigator to tie its end into a knot, a manipulation that is not possible in normal mice. The phenotype of adult mice is characterized by increased laxity and fragility of skin, tendons, and ligaments, an increase in total bone density and cortical thickness, an increased density of medium and small blood vessels, and a bleeding diathesis.

These abnormalities in structure and function are supported by anatomical analysis and mechanical testing. The weave of dermal collagen fibers in the knockout mouse is disorganized, and collagen fibrils in skin and tendon are abnormally large and irregularly contoured when examined by electron microscopy. Tensile strength measurements of skin from mutant mice support indications of increased fragility, first suggested by the tendency of skin to tear. The skin ruptures at lower loads and has increased ductility; these findings explain its increased stretchability.

A possible clue to the pathogenesis of these findings lies in the decreased attachment of skin fibroblasts from these animals to various substrata and their increased sensitivity to trypsinization. Although the binding of TSP-2 to collagens has not been studied, defective cell adhesion to a suitable substratum through lack of TSP-2 may be the primary cause. This would explain abnormal collagen fibrillogenesis, osteopetrosis-like features through abnormal osteoclast adhesion to bone or failure to form a bone-resorbing ruffled border, and the bleeding diathesis through defective adhesion of platelet aggregates to the injured subendothelium. TSP-1 has been shown to bind to von Willebrand factor (vWF), and it is possible that TSP-2 is also required, either directly or indirectly, for the binding of vWF to one or more collagens in the subendothelium. The increase in blood vessel density in mutant mice is a strong argument for the function of TSP-2 as an inhibitor of angiogenesis *in vivo*. It is of interest that findings intermediate between those in wild-type and mutant animals are observed in skin from heterozygous *Thbs2*+/− mice; this suggests haploinsufficiency for the function of TPS-2, at least in skin [181].

Fibromodulin-Null Mice — A Model of Abnormal Fibrillogenesis as in the Ehlers-Danlos Syndrome

Homozygous mice lacking a functional fibromodulin gene do not show any gross anatomical defects, grow to normal size, are fertile, and have a normal life span. In fibromodulin-null animals, virtually all collagen fiber bundles are disorganized and have an abnormal morphology; also, 10–20% of the bundles in heterozygous mice are similar to those in homozygous null mice. Electron microscopy shows that the fibrils are thinner, with irregular and rough outlines. They have a fourfold increase in the content of lumican despite a decrease in lumican mRNA; this suggests that lumican and fibromodulin, both of which belong to a family of extracellular matrix glycoproteins/proteoglycans sharing a leucine-rich repeat (LRR), have the same binding site on collagen fibrils [649].

Procollagen N-Proteinase Knockout Mice — A Model for Dermatosparaxis

Transgenic mice that are homozygous for inactive alleles coding for procollagen N-proteinase (ADAM-TS2) have fragile skin, as expected; however, surprisingly, adult homozygous males are sterile and have a marked decrease in sperm count in semen and fluid expressed from the epididymis and in mature sperm in cross sections of the testes [650]. At the moment, it is unclear whether male sterility is confined to these transgenic mice or whether the enzyme is necessary for the modification of some other and still unknown substrate because three of the six reported cases of human dermatosparaxis (Table 3) have not reached the age of reproduction and because dermatosparactic sheep and calves did not reach puberty. Male individuals with EDS VIIB, however, were fertile and produced one affected and two unaffected children (father of case 4 and case 7, respectively).

Targeted Mutations of the Col3a1 Gene in Mice — A Model of the Vascular Type of EDS

To define the role of collagen III in fibrillogenesis, Liu et al. [334] generated *Col3a1*−/− mutant mice by gene targeting. Most homozygous mutants died in the perinatal period. The precise cause of neonatal lethality was not clear because the dead pups were cannibalized before they could be examined, and light microscopic histologic analysis of live newborn homozygous mutants did not detect any gross abnormality.

The phenotype of surviving homozygous mutant mice resembled the clinical manifestations of patients with EDS IV, whereas heterozygous mice appeared phenotypically normal. Mutant mice (−/−) displayed an average survival rate of 5% at weaning age (~3 weeks), with most deaths occurring within the first 48 hours after birth. Adult homozygous mutant mice appeared normal except that they were about 15% smaller than their wild-type littermates of the same sex. Their average life span, however, was about 6 months, or one-fifth of normal.

Autopsy showed that blood vessel rupture was the major cause of death of these mice. The sites of ruptured arteries with or without aneurysm were random. In addition, mutant mice showed frequent intestinal enlargement and occasional intestinal rupture. Two-thirds of −/− mice displayed spontaneous skin lesions. Light microscopy of skin, intestine, and internal organs, including liver and lung, did not detect any overt abnormalities.

Electron microscopy showed that collagen fibrils normally located between smooth muscle cells, or between smooth muscle cells and elastic fibers, were absent or severely reduced in the media of the aorta as well as in the intestinal submucosa and serosa. Most strikingly, in the adventitia of the aorta, skin, lung, and liver, where collagen I fibrils predominate,

the number of fibrils was reduced to approximately one-third and their mean diameter was approximately twofold and highly variable.

These studies show that collagen III is not only an essential component in tissues rich in collagen III but that it also plays a critical role in collagen I fibrillogenesis and thus in maintaining the functional integrity of the organs [334]. Also, heterozygous mice with a *Col3a1* null allele are phenotypically normal under the conditions examined and, as in putative EDS IV patients with a nonfunctional allele, a milder or late-onset visceral involvement may yet be found under stress conditions (see EDS IV above).

Targeted knock-in mutation in *Col3a1* in mice with defective cross-linking of collagen III. Toman et al. [651] generated transgenic mice by microinjection of a mutated mouse *Col3a1* harboring a Lys>Met substitution at the amino acid 939 cross-linking site within the triple-helical region of the α1(III) chain. Pregnant female mice expressing the transgene at levels of >30% in relation to the normal endogenous procollagen III showed greatly diminished ability to deliver pups at time of labor. Analysis of the uterine smooth muscle function of pseudopregnant transgenic mice showed an abnormal contraction pattern upon stimulation with oxytocin, and electron-microscopic analysis of the uterus indicated a marked disorganization of the collagen bundles disrupting the normal architecture of the myometrium. The authors concluded that the mutation that prevents cross-linking of the collagen III alters the normal functioning of smooth muscle in the uterus.

Mottled Mouse Mutants

Of the series of mouse mutants involving the X-linked locus mottled (Mo), so named because of its effect on coat color, the brindled (Mo^{br}) and blotchy (Mo^{blo}) variants have been studied particularly. The nature of the basic defect is a disturbance in copper handling as a result of mutations in *Atp7a*, the murine homolog of *ATP7A*, which codes for ATP7A, a member of the copper ATPase family, and is known to be affected in Menkes disease and the occipital horn syndrome. Consequently, intracellular copper is unavailable for enzymes requiring copper [632,633,652]. For a more detailed consideration, see Chapter 14 of this volume.

Hemizygous brindled mutant males normally die at 10–14 days of age with severe neurological deterioration without showing any signs of connective tissue disorder [523,653]. Although lysyl oxidase in extracts of skin is secondarily reduced to about 60% of normal [524,654], there is perhaps insufficient time for effects on connective tissue to manifest themselves before the neurological involvement takes its toll. The brindled mutants are considered to be a model of Menkes disease in the human [633], in severe cases of which connective tissue abnormalities may be prominent [655].

The blotchy male mutant, on the other hand, shows a variety of connective tissue features, including emphysema [656], and tends to die from aortic aneurysm at about 150 days of age [523,657]. The connective tissue defects appear to be secondary to deficient cross-linking of collagen [658] and elastin, consequent on inadequate activity of the copper-dependent lysyl oxidase which functions to initiate the process and is present in the tissues at only about one-quarter of normal levels [524,654,659]. The blotchy mutant has been suggested as a model of EDS IX [633], now reclassified as the occipital horn syndrome (see Chapter 14, this volume).

Experimental Animals for the Study of Tissue Fragility

Lathyrism

The condition in which deleterious effects in connective tissues are caused by chronic ingestion of the sweet pea *Lathyrus odoratus* is called lathyrism and is due to the inhibition of lysyl oxidase by β-aminopropionitrile (β-APN), with consequent inhibition of cross-link formation in both collagen and elastin. β-APN, the toxic agent, is released *in vivo* by an amidase or protease from the precursor, β-(γ-glutamyl)aminopropionitrile, present in the legumes, and is a potent and irreversible inhibitor of lysyl oxidase, with I_{50} values of 3–5 μM (for a review, see Kagan [660]).

Lathyrism has been reviewed by Levene and Gross [661] and Barrow et al. [662]. Geiger et al. [663], by feeding growing rats with sweet pea, were the first to describe dramatic malformations of mesenchymal tissues, termed osteolathyrism as opposed to neurolathyrism [664], which included kyphoscoliosis, exostosis of the long bones, marked periosteal new bone formation, weakening of the tendinous and ligamentous attachments and of the epiphyseal plates, skin, cartilage, and healing wounds, dislocation of joints, loss of teeth, and herniae. Later, dissecting, diffuse, or saccular aneurysms of the aorta were reported (angiolathyrism) [665], and teratogenic effects such as cleft palate [666] and ectopia cordis and gastroschisis [667] were observed in 1968 and 1971, respectively. In 1954/1955, β-APN was found to be the toxic factor in *Lathyrus odoratus* causing osteolathyrism [668]. Using chick embryos, Levene and Gross [661] showed that both tissue fragility and the amount of extractable collagen were dose- and time-dependent and speculated that normal intermolecular cross-linking was affected by the lathyrogenic agent. Martin and co-workers reported that the interference by β-APN in collagen cross-linking, and in the biosynthesis of desmosine and isodesmosine, was through the specific inhibition of lysyl oxidase, the enzyme used in the first step of cross-linking of collagen and elastin [669]. Lathyrogenic compounds were shown to fall into four major groups, in order of diminishing potency: nitriles > ureides > hydrazides > hydrazines [670].

β-APN has proved to be a useful agent to administer to growing animals, or to add to organ and cell cultures, to block collagen cross-linking and thus allow the extraction in large amounts of newly formed collagen which is amenable to characterization. It is also required in the preparation of biosynthetically labeled substrate for the assay of lysyl oxidase [418]. The successful use of β-APN in the prevention of esophageal strictures after lye injury and in overcoming the restrictive effects of peritendinous adhesions in animal experiments [671] led to human clinical trials. Results with β-APN in the treatment of patients with ureteral strictures and tendon adhesions [672] indicate that the physical properties of scar tissue may be amenable to biochemical control. However, the therapeutic value of β-APN as an antifibrotic agent is, unfortunately, outweighed by its damaging systemic side effects [673].

Copper Deficiency

Because copper is a cofactor of lysyl oxidase, its dietarily induced deficiency has effects that strongly resemble those of lathyrism. Common to both conditions are the following: (1) the disease is readily inducible only in the young; (2) vascular rupture is the cause of mortality; (3) aortic tensile strength is reduced, and there is a decrease in aortic elastin; (4) elastic fiber alterations are the cardinal histopathologic

and ultrastructural vascular lesions; (5) bone deformities occur in both conditions; (6) increased solubility of elastin and collagen are due to deficient cross-linking. Lysyl oxidase activity is not detectable, or is grossly reduced, in extracts of tissues from copper-deficient animals, a fact that originally suggested copper as the naturally occurring cofactor for the enzyme. Indeed, copper-deficiency states enabled the early isolation and characterization of tropoelastin [674].

D-Penicillamine

D-penicillamine (β, β-dimethylcysteine), according to the conventional view, inhibits collagen cross-linking by interacting with the lysine-derived aldehydes, rendering them unavailable for cross-link formation, although there is evidence that its principal effect might be to block the synthesis of polyfunctional cross-links from bifunctional Schiff base precursors [675]. D-Penicillamine acts preferentially on collagen cross-linking in soft tissues, while the hydroxylysine-derived aldehydes present in bone are much less affected. At high concentrations, it also chelates copper and reduces the activity of lysyl oxidase [675] (see also Footnote 9).

The overall effectiveness of the drug in reducing the structural stability of collagen *in vivo* is well-documented in animal experiments. It has also been used to reverse collagen accumulation in patients with hepatic fibrosis and progressive systemic sclerosis, but side effects, reviewed by Steinmann et al. [676], have prevented its use in such high concentrations as in animal experiments. Side effects of penicillamine in the treatment of cystinuria and Wilson disease have been reported and include symptoms reminiscent of EDS [676] and pseudoxanthoma elasticum [677] as well as ultrastructural changes in dermal collagen and elastin [678].

In conclusion, studies using lathyrogens have been instrumental in demonstrating that the ability of collagen and elastin fibers to function is primarily dependent on a system of covalent cross-links between the polypeptide chains of the respective proteins.

CONCLUDING REMARKS

Mutations that give rise to the several different phenotypes of EDS affect collagen structure (some cases of EDS I and II, EDS VIIA and VIIB), expression (a few cases of EDS I and III), maturation (EDS VI, VIIC, occipital horn syndrome), and fibrillogenesis (EDS I/II, VII), noncollagenous proteins, such as proteoglycans (progeroid form of EDS) and tenascin-X, and potentially many more proteins of the extracellular matrix in the vast majority of cases. The EDS phenotypes reflect both disturbances in the intermolecular cross-linking of collagen (EDS VI, VII, and occipital horn syndrome) and the lack of an appropriate scaffold (EDS IV, progeroid form of EDS) on which to build the major components of the skin. The precise phenotypic findings therefore depend on the nature of a mutation and the molecule in which it occurs. Despite such a wide genetic heterogeneity, the organism has only a limited repertoire for functional and morphological changes.

RECENT DEVELOPMENTS

EDS Types II and III

Clinical Features

Absence of the inferior labial and lingual frenula has been reported in 12 patients, four with EDS II and eight with EDS III, with a mean age of 29.7 years (range 15–45 years), and suggested to be a highly specific and sensitive marker for these disorders [679]. It will remain uncertain, however, whether this is a congenital anomaly or rather an acquired trait due to tissue fragility, until prospective, longitudinal observations have been performed and the natural history is known. It is further unknown whether this clinical finding also occurs in other types of EDS.

Vascular Type of EDS (EDS Type IV)

Genetics and Pathogenesis

Because EDS IV has hitherto been associated exclusively with dominant negative *COL3A1* mutations, it has been unclear whether any *COL3A1* haploinsufficiency mutation would cause an EDS IV-like phenotype differing in severity or symptomatic range from usual, or indeed any phenotype, and therefore have escaped analysis. This uncertainty was reinforced by the finding that heterozygous *Col3a1* knockout mice are phenotypically normal [334], although late onset signs would have been missed if they had occurred after the 18 months follow-up period.

Schwarze et al. [680] have now presented data on four patients with EDS IV with *COL3A1* haploinsufficiency mutations. Three of them had frameshift mutations that led to premature termination codons in exons 27 (1832delAA), 6 (413delC), and 9 (555delT), respectively, and to allele product instability. In the fourth patient, a point mutation introduced a premature stop codon in the most 3′ exon (4294C>T; Arg1432Ter), resulting in the synthesis of truncated proα1(III) chains that were not incorporated into procollagen III molecules. All four index patients had vascular aneurysms or ruptures, and the phenotype of family members presumed to be affected was reported to be within the range of "classical EDS IV" due to structurally abnormal collagen III. Surprisingly, biochemical evidence of reduced synthesis of procollagen III was limited to dermal fibroblasts from a single patient, procollagen III synthesis in cell strains from each of the other three patients being normal. The authors concluded that a 50% reduction in the amount of structurally normal procollagen III is as deleterious to the vascular system as the biosynthesis of collagen III, seven-eighths of which is structurally abnormal, that occurs as a consequence of the more than 200 other reported mutations. However, their conclusions have some limitations in that the probands had been ascertained because of "typical" EDS IV signs and symptoms and on average they, as well as their relatives presumed to be affected, were older than the reported median age of survival for EDS IV [256]. The probands may thus simply represent the more severe range of a milder phenotype characterized by the attainment of a greater age before symptomatic arterial events occur and by longer survival.

The report by Schwarze et al. [680] gives rise to the following considerations in relation to the diagnosis of EDS IV: (1) The cornerstone of the diagnosis should remain suggestive clinical findings in the proband and similarly affected family members in conjunction with detailed medical histories; by this approach alone, however, sporadic cases are less likely to be detected. (2) In fibroblast strains with *COL3A1* haploinsufficiency, the relative proportions of collagens I and III secreted into the medium and retained by the cells do not constitute a reliable diagnostic indicator because they depend on such parameters of culture conditions as cell density [218,342]. It must also be stressed that the ratio of $[\alpha1(III)]_3$ molecules to combined α1(I) and α2(I) chains after limited proteolysis with pepsin provides a more accurate measure of synthesis of the two collagen types than may be achieved by determining the relative proportions of reduced

proα1(III) and proα1(I) chains, as done in the study under discussion [680]. Furthermore, subtle structural alterations in type III collagen are more readily detectable in $[\alpha1(III)]_3$ molecules than in proα1(III) chains. (3) Mutation analysis in individuals in whom the diagnosis of EDS IV has been made on clinical grounds has been successful in those whose cultured cells have synthesized a cohort of structurally abnormal procollagen III molecules. Now that a clinical phenotype due to haploinsufficiency has been recognized, direct genomic analysis is likely to be a more efficient way of identifying cell strains from individuals so afflicted. (4) Because the role of *COL3A1* mutations in other conditions such as isolated cerebral aneurysm [272] and abdominal aortic aneurysm [271] has been explored only at the cDNA level, non-expressed alleles or unstable mRNA products might not have been detected in a minority of individuals homozygous (or hemizygous) at known polymorphic sites; retesting of such individuals at the genomic level might therefore be warranted.

Kyphoscoliotic Type of EDS (EDS Type VI)

Clinical Features

Pregnancy in EDS VI. There remain no published reports on pregnancies of individuals with EDS VI. Two affected women are known to have delivered normally after 36 weeks without untoward consequences (R. Wenstrup, personal communication, 2001). Another affected woman with a moderately severe phenotype (case 55, see legend to Fig. 22) had an early abortion and later delivered a boy at term (B. Steinmann, personal observation).

Differential Diagnosis

Infants with Ullrich disease (MIM 254090; see Chapter 26, Part VI, this volume) may present with severe muscular hypotonia, marked joint laxity, Marfanoid proportions, and autosomal recessive inheritance, and thus may expand the differential diagnosis of EDS VI. Children with the autosomal recessively inherited Nevo syndrome (MIM 601451) have tall stature at birth, muscular hypotonia, joint laxity, kyphoscoliosis, a Marfanoid habitus, wrist drop, long spindle-shaped fingers, osteopenia, and dural ectasia, and may be confounded with EDS VI (B. Steinmann, personal observation).

Therapeutic Approaches

With the aim of local gene therapy, Rauma et al. [681] cloned human lysyl hydroxylase 1 cDNA into a recombinant adenoviral vector (Ad5RSV-LH). Transfection of human EDS VI fibroblasts from case 3 (see legend to Fig. 22) with the vector increased lysyl hydroxylase activity in these cells in a dose-dependent manner from residual values of 20% of normal to levels greater than in control cells. The adenoviral vector also successfully transfected rat fibroblasts *in vitro*, and intradermal injections of the vector in rats produced human lysyl hydroxylase 1 mRNA and elevated lysyl hydroxylase 1 activity *in vivo*. This study suggests the feasibility of gene replacement therapy to modify skin wound healing in EDS VI patients.

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APPENDIX: PATIENT SUPPORT GROUPS

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Fax: 323-651-3038
E-mail: LooseJoint@aol.com
Web site: http://www.ednf.org
Contact person: Mrs. Linda Neumann-Potash

ELECTRONIC-DATABASE INFORMATION

Online Mendelian Inheritance in Man (OMIM)
http://www.ncbi.nlm.nih.gov/omim

London Dysmorphology Data Base
http://genetics.ich.ucl.ac.uk/lddb/lddb.html

The Human Gene Mutation Database Cardiff (HGMD)
http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html

Mutations/Polymorphisms in *COL1A1*
http://www.le.ac.uk/genetics/collagen/col1a1.html

Mutations/Polymorphisms in *COL1A2*
http://www.le.ac.uk/genetics/collagen/col1a2.html

Mutations/Polymorphisms in *COL3A1*
http://www.le.ac.uk/genetics/collagen/col3a1.html

Mutations/Polymorphisms in *COL5A1*
http://www.uwcm.ac.uk/uwcm/mg/search/131457.html

Mutations/Polymorphisms in *COL5A2*
http://www.uwcm.ac.uk/uwcm/mg/search/119064.html

Enzyme Nomenclature Database
http://www.expasy.ch/enzyme/

Online Resource
http://www.familyvillage.wisc.edu/lib_e-ds.htm

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Chapter 10

Cutis Laxa and Premature Aging Syndromes

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SUMMARY

- **Cutis laxa is characterized by deep wrinkling or sagging of skin, predominantly due to defects in the elastic fiber system. Elastin may be reduced, absent, fragmented, or dissociated from the microfibrillar components of the elastic fiber. Congenital forms of the disease occur with autosomal dominant and autosomal recessive inheritance patterns, whereas acquired cutis laxa usually has a later onset that is associated with collateral inflammatory aspects.**
- **Progerias are accelerated aging syndromes involving many organ systems, in which effects on connective tissues are most likely secondary to global, metabolic defects in processes such as DNA repair. In many forms of progeria, such as Hutchinson-Gilford progeria and Werner syndrome, the *in vitro* life span of cultured fibroblasts is reduced.**
- **Autosomal dominant cutis laxa and supravalvular aortic stenosis are linked to the elastin locus. Both diseases involve skin and other elastic tissues, but there are selective effects on the designated tissue compartments. Supravalvular aortic stenosis is due to functional or actual hemizygosity at the elastin locus, a deficiency that affects the elastin-rich aortic root most profoundly. Other elastic tissues, such as skin, are less affected. Williams syndrome is due to a large-scale chromosomal deletion that usually encompasses the elastin locus; thus, most affected individuals exhibit supravalvular aortic stenosis. In autosomal dominant cutis laxa, point mutations in the 3′-coding region of the elastin transcript can affect synthesis and perhaps assembly of mutant protein. Elastic tissues throughout the body are affected to a variable extent.**
- **Autosomal recessive cutis laxa is usually more severe, with major effects on the pulmonary elastic network that lead to death in infancy. Although elastic fibers are malformed, no specific genetic defect has been identified. Findings in Costello syndrome suggest a linkage between altered metabolism of dermatan and chondroitin sulfate, the 67 kDa elastin/laminin-binding protein, and elastic fiber assembly.**
- **Cutis laxa is a trait that is associated with the phenotype of many other syndromes, including Wiedemann-Rautenstrauch syndrome, Hallerman-Streiff syndrome, Cantu syndrome, Meretoja syndrome, and others. Bone and skin are affected, but there does not appear to be a selective effect on connective tissues. Intrauterine growth retardation may occur.**
- **The copper-transport defects now known as occipital horn syndrome and Menkes disease are allelic and due to mutations in *ATP7A*. Cutis laxa arises as a result of defects in copper transport that have significant effects on the cross-linking of collagen and elastin by lysyl oxidase.**
- **Acquired cutis laxa usually arises from postinflammatory elastolysis, and it is manifest in Sweet's syndrome, Marshall syndrome, and mid-dermal elastolysis.**
- **Hutchinson-Gilford progeria is the most striking form of progeria, with early childhood signs of reduced stature, cutaneous atrophy, alopecia, and micrognathia. Fatal complications of atherosclerosis and respiratory failure occur in the second and third decades of life. Biochemical evidence of altered elastin and hyaluronan metabolism are associated with the disease but not known to be causative.**
- **Werner syndrome manifests in adolescence as a lack of the typical growth spurt, lack of subcutaneous fat, skin wrinkling with scleroderma-like changes, hyperkeratosis, and premature arteriosclerosis. Malignancies develop in the third and fourth decades. Although connective tissue metabolism is altered in several ways, the primary defect is in RecQ DNA helicase at the WRN locus. Cockayne syndrome is another late-onset form of progeria that is associated with mutations in two genes that lead to defective DNA excision repair.**

INTRODUCTION

The clearest and most inevitable signs of aging are the changes in the appearance of the skin and the shape of the craniofacial skeleton. These are due to alterations in the structural components of the dermis, bone, and cartilage, and there are several striking disease states or syndromes in which the skin, fat, and bone changes that signify advanced age appear

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prematurely. More importantly, the altered arrangement and abundance of skin and bone macromolecules are often signs of internal connective tissue changes that can impede organ function and increase morbidity. As will be discussed, the etiologies of these syndromes can be quite diverse, although two themes predominate: alterations to elastic tissue and defective DNA repair mechanisms. In the latter case, it should be clear that effects on connective tissue will be secondary to actual genetic mechanisms.

DEFINITIONS AND NOMENCLATURE

Cutis Laxa and Slack Skin Syndromes

Cutis laxa (see Table 1) is characterized by loose and inelastic skin and can be inherited [1,2] or acquired [3–5]. It was first described in the early 19th century by Graf [6]. Inherited forms of cutis laxa are characterized by generalized cutaneous laxity that is present at birth or develops soon afterward. Other organs, such as the lungs, aorta,

TABLE 1. Cutis Laxa and Slack Skin Syndromes

Disease	Inheritance	MIM	Skin Phenotype	Other Clinical Manifestations
Cutis laxa/slack skin				
Cutis laxa	AD	123700	loose, sagging skin lacking elastic recoil	pulmonary emphysema
CL type I	AR	219100	loose skin; deficiency of elastic fibers in the skin	Pulmonary emphysema; diaphragmatic hernia; diverticula of the gastrointestinal and urinary tracts
CL type II	AR	219200	markedly wrinkled and lax skin	mental retardation
CL type III	AR	219150	lax skin; defective elastic fibers	cloudiness of the cornea; mental retardation
CL X-linked (OHS)	AR	304150	defective collagen and elastin; reduced lysyl oxidase activity; poor wound healing	occipital osseous projection; ligament laxity; poor wound healing; abnormality of copper metabolism
Menkes disease	AR	309400	skin laxity and hyperextensibility	skeletal abnormality; vascular complication; mental retardation
SCARF	X-linked	312830	lax skin	joint hyperextensibility; mental retardation; facial abnormality
Deficiency of lysyl oxidase	AR	153455	skin with fewer elastic fibers	
SVAS	AD	185500	premature aging of the skin	Narrowing of aortas; heart failure
Williams syndrome		194050	premature aging of the skin	congenital heart disease; mental retardation
Patterson pseudoleprechaunism		169170	cutis gyrata in hands and feet	hyperadrenocorticism; diabetes mellitus; bladder diverticula
Wrinkly skin syndrome		278250	wrinkled skin of hands and feet	skeletal musculature poorly developed
Costello syndrome		218040	areas of redundant skin; elastic fiber degeneration	growth and mental retardation
Elastosis				
Actinic (solar) elastosis			aged skin; accumulation of elastotic material	fibrillin accumulation
Buschke-Ollendorf syndrome	AD	166700	dermal papules	osteopoikilosis
Elastoderma			skin laxity with accumulation of dermal elastin	
Elastosis perforans serpiginosa	AD?	130100	transepidermal elimination of abnormal elastic fibers	
Pseudoxanthoma elasticum	AR (AD)	264800 177850	skin laxity; calcified elastic fibers	cardiovascular complications; aneurysm; retinopathy

TABLE 1. (*Continued*)

Disease	Inheritance	MIM	Skin Phenotype	Other Clinical Manifestations
Cantu syndrome	AD or X-linked	114620	wrinkled palms and soles	joint hyperextensibility; cardiac anomalies; mental retardation
Aging Syndromes				
Progeria	AD;AR	176670	cutaneous atrophy; loss of facial and scalp hair	arthritis; respiratory complications; atherosclerosis with angina
Wiedemann-Rautenstrauch syndrome	AR	264090	progeroid appearance	intrauterine growth retardation; natal teeth
Werner syndrome	AR	277700	wrinkled skin; hyperkeratosis; atrophy of subcutaneous fat	short stature; arteriosclerosis; frequent malignancies
Cockayne syndrome		216400	lack of subcutaneous fat; senile appearance	dwarfism; retinal atrophy; deafness; mental retardation
Xeroderma pigmentosum		194400		
Mandibulo-acral dysplasia		248370	progeroid features	alopecia; postnatal growth retardation
Gerodermia osteodysplastica	AR	231070	lax skin	joint hyperextensibility; osteoporosis
Hallerman-Streiff syndrome		234100	skin atrophy; progeroid features	cataract; microphthalmia; dysmorphology of airways
Acrogeria		201200	wrinkled skin of feet and hands	joint hypermobility; mental retardation
Metageria			generalized form of 201200; loss of subcutaneous fat; thinning of skin	
Down syndrome		190685	prematurely aged skin	degenerative vascular disease; increased tissue lipofuscin; precocious aging
PAS, Okamoto type		601811		microcephaly; osteosarcoma; osteoporosis
PAS, Penttinen type		601812	hard confluent skin lesions	prematurely aged appearance; acrosteolysis; brachydactyly
Lenz-Majewski hyperostotic dwarfism		151050	skin hypoplasia; loose, wrinkled, and atrophic skin	dental enamel dysplasia; joint laxity
ROMBO syndrome		180730	lack of elastin or elastin in clumps in affected areas	
Acquired Forms				
Cutis laxa acquisita			fragmented, disorganized, and scarce elastic fibers	emphysema; aortic aneurysm; bowel diverticula
Postinflammatory elastolysis			atrophy, wrinkling, and disfigurement of the skin	
Mid-dermal elastolysis			absence of elastic fibers in mid-dermis; wrinkling	
Sweet syndrome			destruction of dermal elastin	
Anetoderma	a few congenital	250450 133690	localized sac-like skin lesions with reduced and fragmented elastic fibers	

gastrointestinal tract, and genitourinary systems are generally involved. Inheritance patterns include autosomal dominant, autosomal recessive, and X-linked recessive. In the inherited forms, the defect seems to be in the synthesis and/or assembly of elastic fibers [7–10], whereas the acquired forms result from the destruction of normal elastic fibers and are often associated with preceding inflammatory reactions, such as urticaria, eczema, or erythema, or as a reaction to penicillin, penicillamine, or other drugs. Histologically, all types of cutis laxa have decreased numbers or an absence of elastic fibers in the dermis [11–13].

Cutis laxa can often be confusing to the clinician because of its heterogeneity and varying etiology. For the purposes of this discussion, cutis laxa is defined as a condition in which the skin, in whole or in part, is inelastic and remarkably wrinkled or sagging. This is in striking contrast to the hyperextensible, hyperelastic skin seen in patients with classical Ehlers-Danlos syndrome (EDS) (see Chapter 9, this volume). Other elastic tissues are affected to a varying extent. Many terms have been used to describe this syndrome in the past, such as dermatochalasis, chalazodermia, Schlaffhaut, and cutis pendula [2]. In contrast to the Ehlers-Danlos syndrome, joint mobility in autosomal forms is usually reduced rather than increased, and the skin does not show increased fragility or a propensity to heal poorly with scarring.

Autosomal Dominant Cutis Laxa

Congenital cutis laxa with no evidence of consanguinity and milder, cutaneous manifestations is often considered to arise from (new) dominant mutations. A clear inheritance pattern has been documented in a number of cases [1,14]. Elastic fibers are either sparse or disorganized. The skin is loose, wrinkled, and lacking in elastic recoil. At least three cases with these criteria have been shown to be due to mutations in the elastin gene [15,16].

Autosomal Recessive Cutis Laxa

The autosomal recessive form of the syndrome displays a more severe phenotype than the dominant form and is often accompanied by aortic aneurysm and pulmonary emphysema. Classification is further subdivided into type I (MIM 219100), fatal in the first year of life [17]; type II (MIM 219200), associated with bone dystrophy, joint laxity, and developmental delay [18]; and type III (De Barsy syndrome; MIM 219150), which presents very severe symptoms, with ocular involvement and mental retardation [19].

In addition to the more typical forms of the syndrome, Rogers and Danks [20] reported a form of cutis laxa with intermediate severity, probably autosomal recessive, which was associated with mental and growth retardation. A unique form of autosomal recessive cutis laxa with cutaneous and systemic involvement presents with severely fragmented elastic fibers in early childhood [21]. This form of the disorder is thought to involve the overexpression of a neutral proteinase with elastolytic activity [22].

Two case reports describe another form of cutis laxa with recessive inheritance presenting with skin and joint laxity at birth and Wormian bones. Lysyl oxidase (MIM 153455) activity is reportedly reduced, but ceruloplasmin and serum copper levels are reportedly normal, and the authors propose a mechanism distinct from the X-linked form of the syndrome that involves copper transport (see below) [23].

X-Linked Recessive Cutis Laxa (MIM 304150)

In the X-linked, recessively inherited form of the syndrome, the cutis laxa phenotype is accompanied by effects on collagenous tissues (ligamentous laxity) and poor wound healing. The underlying mechanism is a copper-transport deficiency leading to reduced activity of lysyl oxidase, an enzyme that is essential to cross-linking of both collagen and elastin. This disorder is discussed more extensively under its new designation, the occipital horn syndrome, in Chapter 14 of this volume.

Acquired Cutis Laxa

Postdevelopmental loss of skin elasticity characterizes acquired cutis laxa, but internal organs, including the respiratory and cardiovascular systems, may become involved. The condition is rare in childhood, and elastic tissue destruction can be generalized, with a craniocaudal progression, or it may be more focal, as in the case of Marshall syndrome (focal elastolysis following Sweet syndrome) [24]. This syndrome should not be confused with the heritable disorder also named Marshall syndrome (MIM 154780). Patients with acquired generalized elastolysis reportedly have a local increase in granulocyte elastase [25].

Progeria

Progeria and accelerated aging syndromes (see Table 1) are a collection of syndromes that manifest several features of accelerated aging, including cutaneous changes such as thin, wrinkled, or sagging skin, premature atherosclerosis, early onset of tumor growth, and bone dystrophy [26,27]. Early-onset forms of progeria include geroderm(i)a osteodysplastica (Bamatter-Franceschetti syndrome), Hallermann-Streiff syndrome, and Wiedemann-Rautenstrauch syndrome, or neonatal progeroid syndrome. Among the forms that show postnatal onset are Cockayne syndrome, Hutchinson-Gilford progeria syndrome, Werner syndrome, and mandibuloacral dysplasia [28]. Skin fibroblasts from progeria patients show a limited *in vitro* life span [29], and faulty DNA repair mechanisms are implicated in several instances [30,31]. Although skin atrophy is common to several subtypes, skin recoil is not altered in progeroid syndromes. Many of the progeroid syndromes show postnatal skeletal and developmental abnormalities that involve connective tissue, but no single matrix component has been implicated. The distinct forms of cutis laxa are excluded from the progeria category (autosomal recessive cutis laxa (MIM 219100), De Barsy syndrome (MIM 219150)) because elastic tissue is selectively affected.

MATRIX COMPONENTS INVOLVED IN CUTIS LAXA AND PREMATURE AGING

Elastic Fibers

Elastin and elastic fibers are reviewed extensively in Chapter 3 of this volume. The elastic properties of nearly all connective tissues are provided by the unique vertebrate structure, the elastic fiber. This is a composite structure that is abundant in the extracellular matrix of dermis, large and medium blood vessels, the lung interstitium and pleura, the vocal folds, the intervertebral ligaments, and elastic cartilage. Elastic fibers are a minor component of many other tissues, including Bruch's membrane of the eye, the cornea [32–34], and the periosteum [35]. The principal component of the elastic fiber is elastin, comprising 70–90% of the peptide material, and the remainder is a mixture of microfibrillar proteins organized into the 15 nm microfibril. The predominant molecule in this latter structure is fibrillin.

Elastin (MIM 130160)

Genetic organization. The single *ELN* (elastin gene locus) resides at 7q11.23 as a highly dilute, multiexon structure spanning more than 40 kb. Individual exons

encode hydrophobic or cross-link domains. Alternative splicing commonly excludes several exons. A 6 kb promoter region has been identified [36,37], and there is a large conserved (1.6 kb) 3′ untranslated region [38] that may regulate mRNA stability [39]. In addition, mRNA stability and TGF-β response elements have been identified within exon 30 of rat elastin mRNA [16].

Biosynthesis. The only intracellular, post-translational modification is the occasional hydroxylation of proline when this amino acid occurs in the appropriate position to act as a substrate for prolyl hydroxylase. Nevertheless, ascorbic acid, a cofactor of prolyl hydroxylase, can antagonize elastin synthesis, probably through an oxidative stress mechanism [40].

Tropoelastin appears to become associated with a molecular chaperone, s-galactosidase (an alternatively spliced, inactive form of β-galactosidase), in the secretory pathway. This lectin may play a dual role as both a means of preventing spontaneous self-aggregation of the hydrophobic monomer before secretion and as part of a cell-surface recognition mechanism [41–43]. Altered proteoglycan catabolism in Hurler syndrome appears to affect elastogenesis via interactions with the 67 kDa elastin/laminin-binding protein [44]. The most crucial modification of elastin is the oxidative deamination of lysyl residues by the copper-dependent lysyl oxidase in the extracellular space to catalyze the condensation of the interchain, tetrafunctional cross-links desmosine and isodesmosine. Several new lysyl oxidase genes have been identified [45–47].

Degradation. The bulk of elastin is accumulated during late fetal life and rapid growth, and turnover of the molecule is exceptionally low. Under normal circumstances, the turnover of elastin can be measured in months to years. Thus, degradation of elastic fibers, particularly in the skin, can lead to irreversible loss of these structures and loss of skin elasticity. Elastin accumulation can rapidly be reactivated in other tissues, however. In blood vessels, neosynthesis is readily brought on by injury or hypertension [48,49], and lung fibrosis can also sharply up-regulate elastin gene expression and accumulation [50].

Breakdown of (cutaneous) elastic tissue is characteristic of intrinsic and accelerated aging. A number of proteinases can degrade elastin (see also Chapter 7, this volume), although the specific term elastase is reserved for neutrophil elastase, a serine proteinase with broad substrate specificity, and a member of the matrix metalloproteinase family, MMP-12 [51]. This zinc metalloenzyme is predominantly produced by macrophages [52]. Both of the gelatinases, MMP-2 and MMP-9, are also relatively efficient elastases that can be secreted by fibroblasts and other mesenchymal cells [53]. Matrilysin (MMP-7) can also degrade elastin. Smooth muscle cells appear to express a unique elastase related to adipsin [54]. As discussed below, many of the acquired forms of cutis laxa appear to arise from either localized or generalized elastin destruction.

Given the high potency of neutrophil elastase, multiple defense mechanisms are normally in place to limit proteolytic action. The most specific and effective inhibitor is α1-antiproteinase (see also Chapter 17, this volume). However, α_2-macroglobulin can certainly block elastase access to macromolecular substrates [55], and secretory leukocyte proteinase inhibitor has significant anti-elastase activity [56].

Microfibrillar Proteins

Microfibrils form the core of the elastic fiber, eventually becoming obscured to microscopic detection as elastin is deposited around them during development. In addition to fibrillin-1 and fibrillin-2, microfibrils contain the microfibril-associated glycoproteins MAGP-1 and MAGP-2, fibulin-1 and fibulin-2, emilin, and LTBP-2 (see also Chapter 3, this volume). In certain sites, such as the dermal-epidermal junction and the trabecular meshwork of the eye, diminishing proportions of elastin transform the tinctorial properties of the elastic structures, leading to the designations elaunin and oxytalan fibers. These microfibrillar components can be affected to a limited extent in acquired forms of cutis laxa. There is a loss of microfibrils perpendicular to the epidermis in cutis laxa acquisita, although fibrillin (MIM 134797) persists in the middle and deep dermis [57], but fibrillin has not been linked genetically to cutis laxa [58]. The fibrillins are closely related to latent TGF-β binding proteins (LTBPs), and the elastic fiber may serve as a reservoir for this potent cytokine [59].

Proteoglycans and Hyaluronan

Proteoglycans and hyaluronan (see Chapter 4, this volume) are crucial to maintaining the hydration state of many connective tissues, and proteoglycan destruction is frequently associated with the age-related degeneration of the joint. In the skin, turgor is regulated, at least in part, by proteoglycan content. These large, negatively charged, hydrophilic macromolecules are not associated with normal elastic fibers, but they can be detected in several pathological conditions affecting elastic fibers. From fetal life onward, the content of the glycosaminoglycan, hyaluronan, diminishes in skin. In addition to changing the mechanical properties of skin, hyaluronan is reported to affect the quality of wound healing. A lower hyaluronan content and a higher hyaluronidase content are associated with a granulation and scarring response, while the converse is characteristic of a fetal regenerative response. Age-related losses are noted in the articular cartilage proteoglycan, aggrecan. The principal proteoglycan found in association with aggrecan is the small proteoglycan, decorin, comprising a 28 kDa polypeptide core and a single dermatan sulfate side chain. Interestingly, an abnormality in small proteodermatan sulfate biosynthesis was reported by Quentin et al. [60,61] in a patient presenting with features of Ehlers-Danlos syndrome and progeria (MIM 130070) (see Chapter 9, this volume). There were multiple abnormalities of connective tissue, including short stature, loose but elastic skin, osteopenia of all bones, delayed wound healing, defective deciduous teeth, and hypermobile joints. The primary defect appeared to be a deficiency of galactosyltransferase I that normally catalyzes the second transfer reaction to xylosyl acceptors on decorin. Two mutations have been identified [62]. As a result, decorin was secreted as an unmodified core protein.

Collagen

Many of the age-related changes in connective tissue are due to altered collagen function; (for a detailed review of collagen biochemistry and molecular biology, see Chapter 2, this volume). Osteoarthritis, osteoporosis, and cutaneous atrophy are hallmarks of aging. They are thought to be due, at least in part, to imbalances between the synthesis and degradation of collagen and other connective tissue components. Senile purpura is consistent with a weakened collagenous structure in the microvasculature.

Biosynthesis. The quantity and quality of collagen production are affected by age. One of the most notable developmental changes is the switch from type III to type I collagen

during perinatal maturation of the skin, but this decline continues for some time. Type III collagen is thought to regulate the form and extensibility of the collagen fiber [63]; thus, reduced elasticity of connective tissues can arise from a lack of type III molecules in both the human disease Ehlers-Danlos syndrome type IV and the *Col3A1* null mouse (see also Chapter 9, this volume). Blood vessels and other extensible structures are most severely affected by overt deficiencies in type III collagen. Fiber diameter is altered in skin and blood vessels. The synthetic rate of collagen in skin fibroblasts shows a moderate decline with age, but this decline is not sufficient to account for phenomena such as cutaneous atrophy or susceptibility to trauma.

Degradation. Collagen destruction is a hallmark of joint and skin aging. The extent of cutaneous atrophy in human skin is correlated with elevated expression of matrix metalloproteinases, including MMP-1 and MMP-2 [64]. Many forms of skin ulceration are coupled to the aging process, and these chronic lesions call into play inflammatory cell proteinases, including neutrophil collagenase (MMP-8) and elastase, among others [65]. It is likely that a modest decline in synthetic activity, when coupled to an exaggerated expression of proteolytic enzymes, can lead to the slow, progressive loss of dermal connective tissue. Although the age-related synthetic capacity of cartilage is less well-characterized, the poor reparative potential of cartilage, once damaged, is well-known.

CUTIS LAXA AND ELASTIC TISSUE DISEASES

Cutis laxa is a relatively rare connective tissue disorder characterized by genetic heterogeneity and clinical variability [12,14–16,66]. The common trend is loose skin with reduced elasticity and a prematurely aged appearance.

Clinical Classification

Cutis laxa (slack skin syndrome) is a collection of diseases that are typified by loose and/or wrinkled skin that imparts a prematurely aged appearance [1]. Various classification schemes have been applied, including distinction based on apparent pattern of inheritance: autosomal recessive, autosomal dominant, X-linked, sporadic, and acquired. More recently, one group of investigators [18] suggested a segregation of the recessive forms into type I (MIM 219100; skin laxity without bone involvement) and type II (MIM 219200; skin laxity with bone involvement) and further amended the McKusick classification [67] to include type III (MIM 219150; skin laxity with mental retardation; De Barsy syndrome). Unfortunately, none of these classifications provides insight into differential mechanisms, although the autosomal dominant forms (MIM 123700) are linked to elastin gene mutations [15,16]. Among the acquired forms, the syndrome has been subdivided.

Common Features

All forms of cutis laxa exhibit clear involvement of the skin, although face, hands, feet, joints, and torso may be differentially affected. Unlike the Ehlers-Danlos syndrome, cutis laxa syndrome always involves elastic tissue, although various organs may be relatively spared.

Cutaneous aspects. The typical aspect of the cutis laxa patient is loose, sagging skin lacking elastic recoil [68]. This is in marked contrast to the hyperelasticity apparent in classical Ehlers-Danlos syndrome (see Chapter 9, this volume). These properties are nearly always attributable to loss, fragmentation, or severe disorganization of dermal elastic fibers [8,69] (Fig. 1). Some, but not all, forms of congenital cutis laxa exhibit reduced elastin production by skin fibroblasts, some of which are due to missense frame shift mutations in the elastin gene [15,16]. Other congenital forms show an abnormal structural arrangement of elastin and its improper association with the microfibrillar component of the elastic fiber [8]. In one familial form of cutis laxa [70] and many forms of cutis laxa acquisita, elastin degradation and increased elastolytic activity can be seen to progress as affected areas or lesions enlarge.

Pulmonary aspects. Despite the heterogeneity of cutis laxa, many forms of the syndrome include pulmonary complications, especially emphysema [1,71–76]. This emphasizes the convergent effects of cutis laxa on the form and function of elastic fibers. Emphysema is most prevalent in the severe, autosomal recessive form of the syndrome. Milder changes, sometimes described as chronic obstructive pulmonary disease, are described in autosomal dominant [1,72,77] and acquired [3,25,78–80] forms of cutis laxa. Cutis laxa patients are often reported to have altered voice characteristics, which are particularly noticeable in acquired forms. Voice alteration (hoarseness) is consistent with deterioration of the rich bed of elastic fibers present in the vocal folds [81–83].

Vascular aspects. Cases of congenital cutis laxa with dilatation of the ascending aorta and hypoplasia of the pulmonary arteries have been reported [1,84]. Aneurysm has been noted in both recessive and acquired forms.

Other organ involvement. Other common abnormalities in cutis laxa include bladder and gut diverticula, diaphragmatic and inguinal hernias, and changes in joint mobility. With the exception of type II and type III autosomal recessive cutis laxa (De Barsy syndrome), bone involvement is not remarkable.

Autosomal Cutis Laxa

Clinical aspects of the syndrome and its heritable pattern were described by Graf as early as 1836 [6], and the predominant patterns of inheritance were described clearly by Beighton [1,2]. The morbidity of the syndrome is related to the pattern of inheritance, the recessive forms being much more severe.

Autosomal Recessive Cutis Laxa

Autosomal recessive cutis laxa was described in the earlier literature by several investigators and reviewed by Beighton [2]. In the type I form (MIM 219100), the phenotype of loose, wrinkled skin, a consequent prematurely aged appearance (Fig. 2), and cardiopulmonary complications is recognized at or shortly after birth. The consequences of internal organ involvement are often fatal in infancy [85]. Beighton noted the frequent association of the syndrome with consanguinity and/or in multiple offspring of the same parents, and characteristic kindreds were described in his 1972 publication [2]. Although elastic fibers and elastic tissues are clearly involved in this condition, the biochemical basis is as yet unknown. Examination of fibroblast strains and dermis from some cutis laxa patients revealed lower elastin expression and ultrastructural findings consistent with defects in elastic fiber assembly [8]. Other congenital cases of cutis laxa show reduced elastin mRNA levels in cultured fibroblasts [7], although lower elastin mRNA levels are not universally associated with these severe forms.

Type II cutis laxa (MIM 219200) is an additional autosomal recessive form of the syndrome that is accompanied by bone dystrophy that was originally described in an affected

Figure 1. Ultrastructural heterogeneity of elastic fiber morphology in dermal biopsies from cutis laxa patients. All micrographs are at an original magnification of 10,000. **A**: small elastic fibers are indicated by arrows. Elastin and microfibrillar components are present but in markedly reduced amounts. **B**: another patient with reduced abundance and altered morphology of elastic fibers in the reticular dermis. The arrow points to elastic fibers. **C**: elastic fibers from the reticular dermis of a patient with autosomal dominant cutis laxa with a 2132delC missense mutation. Note the marked absence of amorphous elastin (arrows) in these fibers. **D**: another cutis laxa patient with abundant elastin but high levels of intrafibrillar microfibrils (arrows). **E**: in this patient, elastic fibers are marked by an excess of microfibrillar material (arrows) and a paucity of amorphous elastin. **F**: a cutis laxa patient who exhibited separate elastin (heavy arrow) and microfibrillar aggregates (fine arrow) as well as an overall reduction in the abundance of each component. (Reprinted from [8] with permission.)

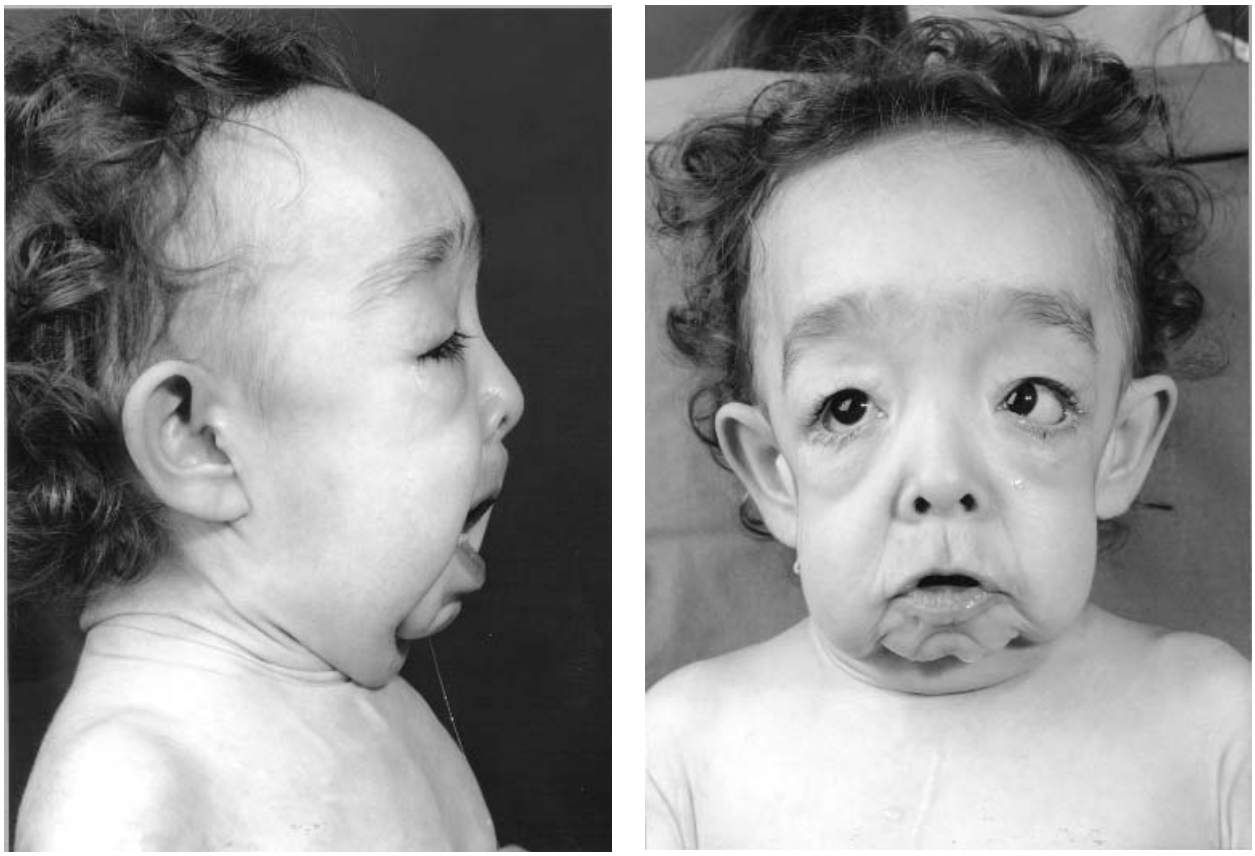


Figure 2. S.A. (d.o.b. 4 Oct 96), a 1.5-year-old girl with severe cutis laxa. She was the first and only child of consanguineous Turkish parents. Birth was normal after an uncomplicated pregnancy at term and neonatal adaptation was good; the child at birth had weight 3410 g, length 49 cm, and head circumference 34.5 cm. Both parents are unaffected, although the mother carried a Gly610Arg polymorphism in one *ELN* allele.

S.A. had numerous hospitalizations and the following cumulative diagnoses and complications of severe cutis laxa: upside-down stomach, which was successfully operated upon with ensuing gastroesophageal reflux; pseudodiverticulosis of the colon; diverticulae of the bladder with recurrent urinary tract infections; mitral valve prolapse and insufficiency of the mitral and aortic valves; recurrent respiratory infections; respiratory failure, oxygen-dependent pneumopathy, and pulmonary hypertension; bilateral inguinal herniae; anal prolapse; progressive short stature. (Courtesy of Dr. B. Steinmann.)

female [86]. Japanese investigators described a male patient and reviewed the literature [87]. Unlike type I cutis laxa, hip dislocation is accompanied by growth retardation. Hatamochi et al. [88–90] reported elevated levels of MMP-1, MMP-3, and MMP-9 expression in autosomal recessive cutis laxa fibroblasts and suggest that the pathology of this disease may be due to excess proteinase activity, possibly due to elevated levels of an AP-1-binding transcription factor.

De Barsy syndrome, the type III form of autosomal recessive cutis laxa (MIM 219150), is a progeroid syndrome first described by De Barsy et al. [91] in an infant girl who had cutis laxa with defective development of elastic fibers in the skin. The phenotype is not restricted to elastic tissue, however. The patient presented with cloudiness of the cornea and mental retardation (Fig. 3). Karnes et al. [19] extended these observations by describing a case with aged appearance, dwarfism, mental retardation, right esotropia and myopia, lax skin, lax joints, and elastic fiber deficiency. These authors studied elastin gene expression in cultured skin fibroblasts and observed reduced elastin mRNA steady-state levels compared with age-matched control skin fibroblasts. Stanton et al. [92] presented two cases characterized by severe cutis laxa, growth and mental retardation, a characteristic facies, and joint hypermobility. Jung et al. [93] reported that congenital cutis laxa type III also involves collagen fibers. They described a 6-year-old girl presenting aberrations of collagenous and elastic tissues, increased collagen type I mRNA expression associated with increased protein synthesis, and increased collagenase gene expression in cultured fibroblasts.

Autosomal Dominant Cutis Laxa (MIM 123700)

Dominantly inherited forms of cutis laxa (MIM 123700) were definitively documented in two kindreds by Beighton [2], although earlier citations support this categorization. Loose skin hanging in folds is obvious in infancy (Fig. 4), and elastic fibers are reduced in number. Beighton suggested that the dominant pattern was consistent with a mutation in the structural protein elastin [1]. Molecular and cellular investigations by Tassabehji et al. [15] and Zhang et al. [16] have confirmed dominant inheritance in affected kindreds, although a lack of elastase inhibitors has been suggested as an alternative explanation [94]. Corbett et al. [77] reported two cases (mother and daughter) with a benign

Figure 3. de Barsy syndrome. The 22-month old patient shows laxity of abdominal, thoracic, and facial skin. The patient could not sit up unaided. She showed characteristic facial grimacing and clenching of the hands and wrist. (Reprinted from [19] with permission.)

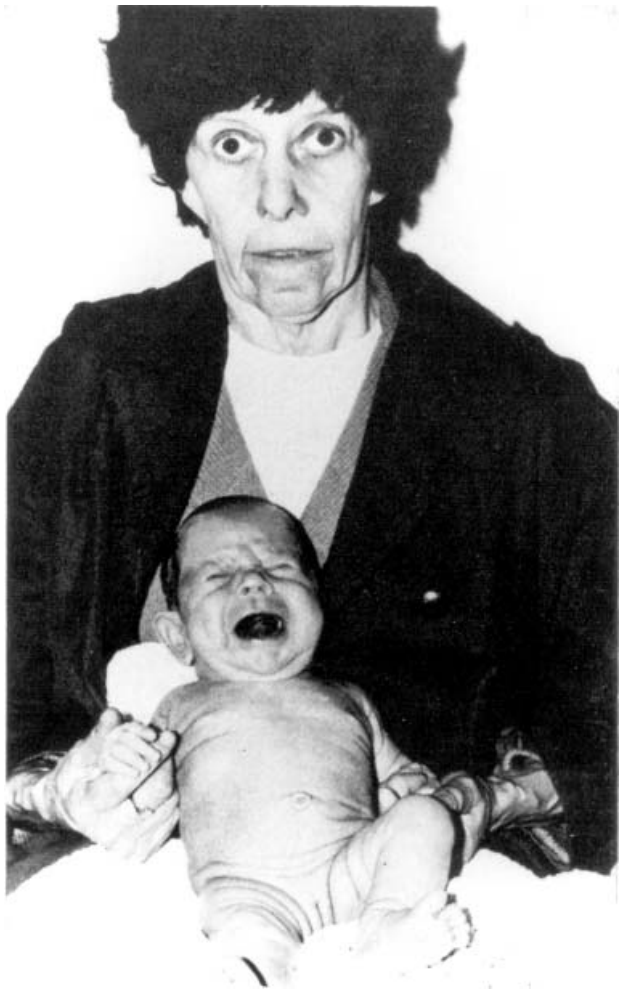


Figure 4. Examples of autosomal dominant cutis laxa. Illustration of the phenotype in two generations. The mother was 28 at the time of the photograph. Skin laxity and an aged appearance are evident in both. (Reprinted from [2] with permission.)

form of autosomal dominant cutis laxa, confined to the skin, with early onset of emphysema. This classification is probably erroneous, because both patients were heterozygous for α1-antitrypsin mutations as well as being smokers.

Biochemical Findings

Although patterns of inheritance were not clearly described in initial studies, several strains of skin fibroblasts derived from autosomal dominant cutis laxa patients demonstrated low elastin production accompanied by low or undetectable elastin mRNA levels [7,8]. Subsequently, Zhang et al. [9] demonstrated that reduced mRNA levels in cells from two kindreds with autosomal dominant cutis laxa were attributable to mRNA instability, an abnormality that could be reversed by TGF-β.

Type VI collagen. A 1985 *in vitro* study of one cutis laxa fibroblast strain implicated type VI collagen overproduction [95]. Gene expression and protein production of type VI collagen were studied in fibroblasts from patients with congenital cutis laxa and compared with normal strains. Increased quantities of collagen type VI and microfibrils were noted in the strain of skin fibroblasts derived from a 2-year-old patient [95]. In a follow-up study, both collagen type VI mRNAs and corresponding proteins were increased in four other cutis laxa cell strains, and this phenomenon may have been secondarily related to skin changes in the patients from whom the cell strains were derived [96].

Other matrix molecules. Laminin deficiency has been reported in one case of congenital cutis laxa that presented also cardiovascular abnormalities, emphysema, and a diaphragmatic hernia [97]. The same investigators cited an additional 17 cases that may encompass a new disease class.

Proteinase involvement. Increased elastase activity has been reported in cultured dermal fibroblasts from lesional skin of cutis laxa patients [98]. In one report, an Italian family had three of four siblings affected with hemolytic anemia and pulmonary emphysema, and the only sib to survive into adolescence exhibited cutis laxa together with elevated serum elastase activity [99]. A patient with Marshall syndrome combined with α1-antiproteinase (α1-antitrypsin) deficiency exhibited both cutis laxa and pulmonary emphysema [24]. Cathepsin G expression has been implicated in cutis laxa with possible involvement in two generations [100].

Genetic Basis

Both Tassabehji et al. [15] and Zhang et al. [16] have described mutations in the elastin gene in autosomal dominant cutis laxa (Fig. 5). The cellular consequences appear to be different in the three cases. In the study by Zhang et al., decreases in elastin protein production and

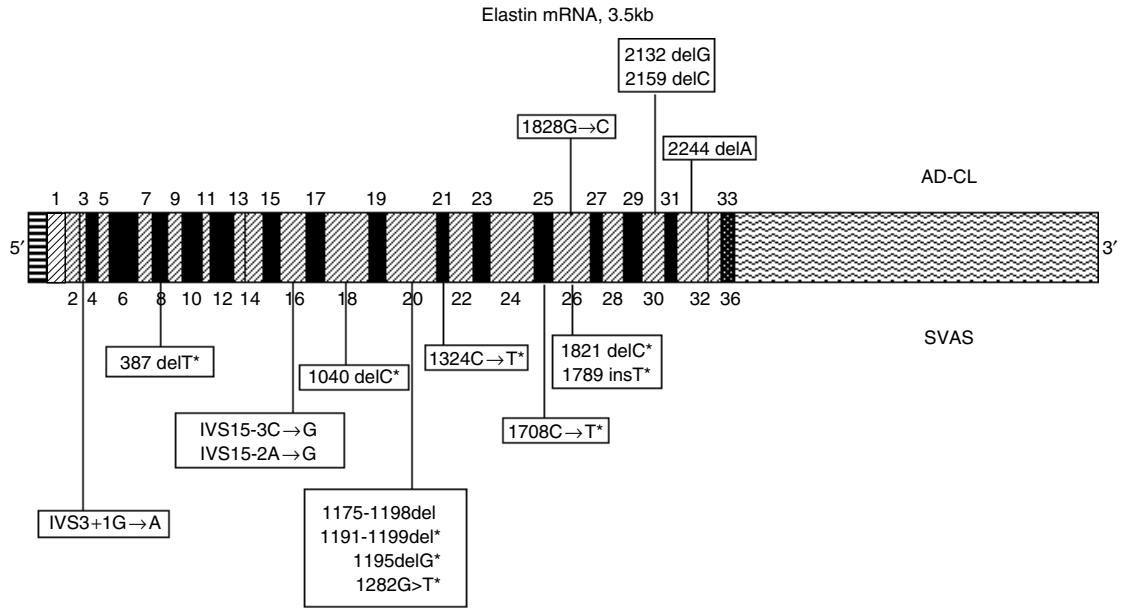


Figure 5. Mutations in the elastin gene. The entire coding sequence of 3.5 kb is depicted in relation to exon notation and domain structure as follows: 5′UTR ▤; signal peptide ▨; cross-linking domains ■; hydrophobic domains ▨; C-terminal region (exon 36) ▩; and 3′ UTR ▤. Mutations in supravalvular aortic stenosis (SVAS) are shown below the cDNA structure, while mutations in cutis laxa (AD-CL) are shown above. In one instance, the same mutation has been documented in two separate cases. A number of the SVAS mutations shown below the graph involve splice sites. In general, SVAS mutations tend to be located toward the 5′ end of the gene and generally result in premature termination codons (asterisks), while the cutis laxa mutations are confined to exons 30 [16] and 32 [15] in the 3′ region and encode missense C-termini. The nonconservative 1828G → C mutation in exon 26 (Gly610Arg) is reported to be polymorphic with a 7% allelic frequency in a Caucasian population and has been identified in one severely affected cutis laxa patient (Fig. 2). These data and a compilation of known polymorphisms have been summarized by Urban et al. [189].

unstable elastin mRNA in skin fibroblasts from two kindreds were linked to two distinct point deletions in the 5′ portion of exon 30 (2132delG; 2159delC), an exon that encodes a typical hydrophobic domain in elastin. The presence of either mutation and the observed alternative splicing of exon 32 led to a missense translation product extended by 25 amino acids; however, no significant elastin protein was produced by cultured fibroblasts from either patient in the absence of TGF-β. The structure of the mutant mRNA or protein led to instability of both normal and mutant transcripts, possibly related to a negative feedback regulation [101] or the alteration of mRNA stability sequences present in exon 30 [16]. In contrast, the mutation described by Tassabehji et al. [15] (2244delA) had no apparent effect on elastin production or mRNA stability and occurred in exon 32. This deletion resulted in a missense translation product in which the 37 C-terminal amino acids of tropoelastin were replaced with a novel sequence of 62 amino acids. Although exon 32 is frequently absent from mature elastin mRNA, transcripts from both mutant and normal alleles were present in cultured skin fibroblasts, and an abnormally large form of tropoelastin was detected in culture supernatants. Thus, in this case, mutant protein may act dominantly to alter elastin assembly. One implication of these studies is that truncations or extensions near the C-terminus of the protein may have limited consequences on elastin function in organs other than the skin. On the other hand, the findings from both of these studies suggest that phenotypic rescue by alternative splicing of mutant exons could spare other organs [15,16].

X-Linked Recessive Cutis Laxa (MIM 304150)

X-linked cutis laxa has had a complex history. It was first described by Byers et al. [102] as a cutis laxa syndrome, while a similar disease had previously been identified as a novel syndrome presenting, among other features, occipital horns [67,103]. Subsequently, Taieb et al. [104] reported that the X-linked cutis laxa described by Byers et al. was the same entity as that referred to at that time as Ehlers-Danlos syndrome type IX (see Chapter 9, this volume) by other investigators [105]. This syndrome is no longer classified as EDS type IX but now corresponds to the occipital horn syndrome, a disorder of copper transport (see Chapter 14, this volume). In this syndrome, the cutis laxa phenotype is accompanied by effects on collagenous tissues (ligamentous laxity) [106] and poor wound healing. Occipital horn syndrome and lysyl oxidase are closely interrelated by the dependency of this enzyme on tissue copper availability. The underlying disease mechanism is a copper-transport deficiency leading, among other effects, to reduced activity of lysyl oxidase, an enzyme essential to cross-linking of both collagen and elastin [106–108] (see Chapters 2 and 3, this volume). The specific genetic lesion results in the deficiency of a Cu^{2+}-ATPase located on the X chromosome (MIM 300011; *ATP7A*). Similarly, direct deficiency of the enzyme, resulting from either toxic inhibition through the ingestion of lathyrogens such as β-aminopropionitrile, or dietary copper deficiency, has an effect on the degree and extent of collagen and elastin cross-linking that can manifest, in part, as cutis laxa.

Menkes Disease (MIM 309400) and the Occipital Horn Syndrome (MIM 304150)

Menkes disease and the occipital horn syndrome (see also Chapter 14, this volume) are allelic variants of X-linked cutis laxa [109–112], being X-linked recessive disorders of copper transport with a reduction in fibroblast lysyl oxidase activity due, in part, to a pretranslational mechanism [113] as well as to dependence of the enzyme on copper for catalytic activity. Increased skin laxity is accompanied by skin hyperextensibility, joint hyperextensibility, skeletal abnormalities, neurologic degeneration, mental retardation, and vascular complications [105]. Unlike the occipital horn syndrome, Menkes disease is usually a lethal condition, although it can exist in a milder form [114–116]. There are two highly polymorphic CA repeats that should be useful for prenatal diagnosis and carrier detection in Menkes disease and the occipital horn syndrome [117]. Martins et al. [118] have reported ultrastructural findings concerning elastic fiber alterations of the skin in three patients with this syndrome. The elastic fibers of the reticular dermis presented a paucity of the central amorphous component whereas the microfibrillar component looked normal. Elastic fiber disruption and dye and antibody binding suggest an abnormal association of elastin with glycosaminoglycans [119].

SCARF Syndrome (MIM 312830)

Koppe et al. [120] described two male maternal cousins with lax skin and joint hyperextensibility, skeletal abnormalities, retardation, and facial abnormalities. The fact that the two young patients were related through their mothers may suggest X-linked recessive inheritance. The acronym SCARF stands for skeletal abnormalities, cutis laxa, craniostenosis, ambiguous genitalia, retardation, and facial abnormalities. This rare form only differs from the occipital horn syndrome in terms of the genital ambiguity and dental abnormalities.

Deficiency of Lysyl Oxidase

Lysyl oxidase, an extracellular, copper-dependent enzyme located on chromosome 5 (MIM 153455), has been reported, without supporting data, to be deficient in two cases of congenital cutis laxa with an autosomal recessive inheritance [23]. Cutaneous elastic fibers were diminished, and the symptoms declined with age. Spinal osteoporosis and joint laxity were present. Serum copper and ceruloplasmin levels were normal in the affected individuals. A family with several affected offspring showing mild cutis laxa, emphysema, and joint laxity of variable penetrance may fall into this category [121].

Cutis Laxa Acquisita

Clinical Aspects

Perhaps even more heterogeneous than the inherited forms of cutis laxa is the collection of acquired conditions that lead to late (adult) onset of elastic fiber destruction. Though genetic associations are lacking, knowledge of these forms can be useful in differential diagnosis. The disorders can be generalized and progressive or may be localized to the face or digits. Cutis laxa acquisita has been associated with multiple myeloma [5], altered antiproteinase status [100], and a wide variety of inflammatory conditions [24,122–135]. Tsuji provides a good summary of earlier cases [136].

Postinflammatory Elastolysis

Elastolysis and cutis laxa as a consequence of inflammation (Fig. 6) has been described by Lewis et al. [132]. This form of acquired cutis laxa has a childhood onset, and its characteristics are malaise, fever, and the presence of large urticarial or papular eruptions that evolve to bring about the destruction of elastic tissue, resulting in areas of atrophy, wrinkling, and severe disfigurement of the skin. Its course is relatively benign, and it is distinguishable from anetoderma with cutis laxa and other forms of elastolysis by its clinical features. It was first described in children from Africa and South America living in tropical or subtropical climates; Lewis et al. [132] reported the first case in a white child.

Other Forms of Elastolysis

Mid-dermal elastolysis. This form of cutis laxa acquisita [137] is a rare, acquired condition presenting as erythematous patches, an absence of elastic fibers in the mid-dermis, and wrinkling [123]. The condition has been compared and contrasted with other elastic tissue abnormalities such as solar elastosis, anetoderma, postinflammatory elastolysis, and heritable forms of cutis laxa [131,138]. As may be true for other acquisita forms, this condition may have an inflammatory pathogenesis [138].

Sweet syndrome with acquired cutis laxa. This rare pediatric skin disease starts with an acquired neutrophilic dermatosis (Sweet syndrome) that is followed by the destruction of dermal elastin producing acquired cutis laxa [139–142]. In one case, inflammation and destruction extended to the aorta [140]. The original reports were

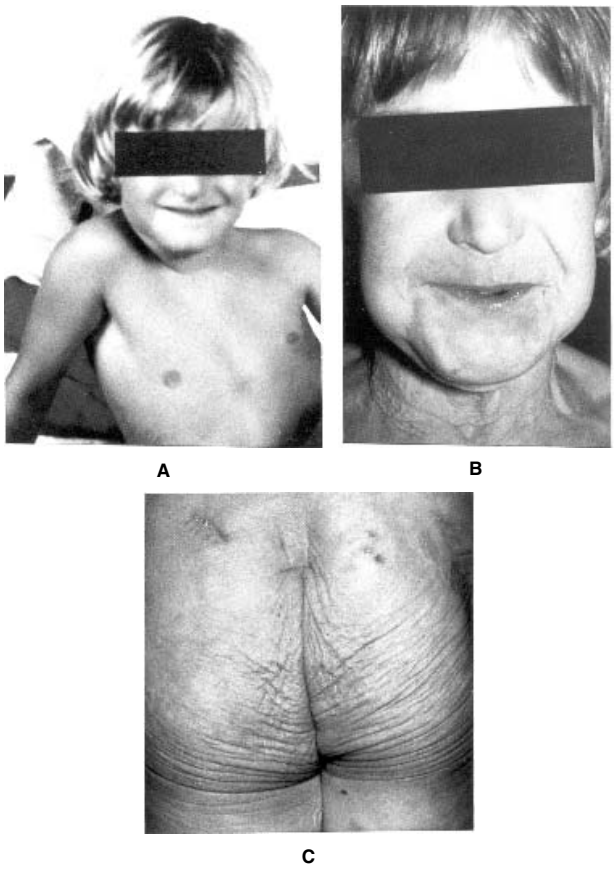


Figure 6. Postinflammatory elastolysis and cutis laxa. Face before (**A**) and after (**B**) development and resolution of urticarial lesions in a five-year-old male. Panel **C** illustrates the buttocks region. (Reprinted from [132] with permission.)

based on observations by Marshall on African children in whom lesions were possibly created by insect bites [143,144]. Hwang et al. [24] reviewed several cases of Sweet syndrome and presented a novel case with classic signs and also α1-antitrypsin deficiency. The cause of Sweet syndrome is unknown, but the α1-antitrypsin deficiency may have allowed the unregulated destruction of dermal elastin by neutrophil elastase.

Anetoderma. In this disorder, localized flaccid, sac-like skin lesions develop over several years. Within the lesions, elastin fibers are reduced in number and fragmented. This has been determined in studies using both radioimmunoassay of desmosines, elastin cross-links [145], and light and electron microscopy [146]. Both macrophages and lymphocytes with plasma cells have been localized in the affected areas [147] and, more recently, image analysis has demonstrated a reduced number of elastic fibers in tensional skin of three patients [148]. This last study also reported an increase in the activity of progelatinases A and B (MMP-2 and MMP-9) in tissues and the activation of progelatinase A in organ cultures from explants of affected skin [148]. Another case study suggested that autoimmunity could be involved [149], which is a common thread in many cutis laxa studies. Other cutaneous insults such as the application of leeches can incite similar lesions [150]. Histologic examination of areas so treated showed a deficiency of elastic fibers, probably due to the release of elastase from leukocytes.

A few cases of congenital anetoderma have been reported (MIM 250450; MIM 133690), some in association with skeletal abnormalities [151,152]. Michaelsson [153] described the Rombo syndrome (MIM 180730), a familial disorder with vermiculate atrophoderma, and other cutaneous abnormalities. This dominant, four-generation disorder showed histological features during the early stages that included irregularly distributed and atrophic hair follicles, milia, dilated dermal vessels, and lack of elastin or the presence of elastin in clumps. Related localized elastolysis and cutis laxa also occur in annular atrophic lichen planus [154].

Localized loss of elastic fibers can also occur in atrophoderma, which presents as circular, depressed lesions in the dermis [155]. Elastin production was reduced and elastase levels increased in cultured skin fibroblasts from affected sites in one patient with a diagnosis of atrophoderma that was based on similar perivascular infiltrates of mononuclear cells [156]. The more recent case report describes similar lesions in a 61-year-old black female, and the authors suggest the term atrophoderma elastolytica discreta for this entity [155].

Pathogenesis

Role of the neutrophil. Because the neutrophil represents the richest source of elastolytic enzymes, including elastase and cathepsin G, involvement of this cell type might be expected. Several instances of neutrophil involvement in acquired cutis laxa may be found in the literature [139–141,157,158]. In a number of cases, generalized elastolysis associated with IgA deposits and infiltrates of eosinophils and neutrophils has been described [125,128,138]. Marshall syndrome shares this pathogenetic mechanism. A genetic component was excluded in one study of identical twins [159].

Role of the lymphocyte. Elastic tissue breakdown and disappearance often begins with an inflammatory episode. This may be accompanied by an obvious source of irritation, but this is not always the case [138]. The immune system shows multiple connections with acquired forms of cutis laxa, but autoimmunity is rarely implicated. A number of cases of multiple myeloma show association with cutis laxa acquisita as a paraneoplastic syndrome [3,5,160–164], but the accumulation of IgG on elastic fibers has only been noted in one such instance [3]. IgG associated with elastic fibers was noted in a case of plasma cell dyscrasia [126], and Tsuji suggested a possible autoimmune component [136]. Acquired cutis laxa has been reported in association with cutaneous T-cell [124] and B-cell lymphoma [127].

Blood vessels. Vascular involvement has been noted in acute neutrophilic dermatosis (Sweet syndrome) [165]. Vascular damage is clear in pseudoxanthoma elasticum (PXE; see Chapter 11, this volume), although the typical abnormality of elastic fibers in this instance is calcification rather than degradation [166]. Pseudoxanthoma elasticum can exhibit signs of cutis laxa [167], and multiple alterations to the elastic fiber have been noted [168–170]. Congenital vascular abnormalities may be present [84,171].

Lungs. Pulmonary involvement is occasionally found in association with cutaneous manifestations of cutis laxa acquisita [5,100,136]. Although not truly representing an acquisita form of the syndrome, one family with generalized elastolysis and elastic fiber fragmentation exhibited clear and early signs of pulmonary emphysema [22,99]. Pulmonary artery stenosis may also be present in acquired cutis laxa [28,84,104,172].

Supravalvular Aortic Stenosis (SVAS; MIM 185500) and Williams syndrome (MIM 194050)

SVAS is an autosomal dominant condition that presents as narrowing of the ascending aorta and other arteries, which can lead to elevated intracardiac pressure, myocardial hypertrophy, heart failure, and death. It can be inherited as an isolated trait [15,173–177] or as part of a second disease, Williams syndrome, which is a developmental disorder involving the vasculature, the central nervous system, and connective tissue. SVAS is due to functional hemizygosity at the elastin locus, whereas Williams syndrome deletions encompass a much larger segment of chromosome 7 [178]. The SVAS patient described by Tassabehji et al. [15] presented at birth with a heart murmur. At the age of 3 years, echocardiography suggested SVAS on the basis of narrowing of the ascending aorta and poststenotic dilatation. A brother had died suddenly in the first year of life and at autopsy had been noted to have had spontaneously repaired SVAS, repaired central pulmonary artery stenosis, and marked ventricular hypertrophy. The aortic valve and proximal aorta were markedly dysplastic, with extreme thickening beyond the valve. The proband's mother had presented to cardiologists in childhood with a murmur, and a clinical diagnosis of aortic stenosis had been made.

Some of the main features of Williams syndrome are congenital heart disease, premature aging of the skin, and poor visual-motor integration [178–185]. Williams syndrome patients have a remarkable spectrum of cognitive alterations, including mental retardation that is accompanied by unusual musical and linguistic skills and an outgoing personality [186]. There are also connective tissue changes, including vascular involvement, hypertension, and premature aging of the skin, but other significant features such as mental retardation and an unusual set of behaviors are consistent with the deletion of a larger block of genetic information.

Moderate changes in skin texture that may relate to elastic fiber architecture have been reported [187].

Molecular Genetics

Since 1993, a number of investigators have defined SVAS as a group of mutations at the elastin locus (7q11.23) [173–175,179,181,188,189]. In the study reported by Ewart et al. [174], the disease phenotype was linked to gross DNA rearrangements (35 and 85 kb deletions and a translocation in chromosome 7) in three SVAS families. However, large-scale rearrangements of the elastin gene have not occurred in the majority of autosomal dominant SVAS cases examined to date. A number of point mutations that support functional hemizygosity have been defined in SVAS (Fig. 5). Li et al. [177] showed that *ELN* point mutations cosegregated with SVAS in four familial cases and were correlated with SVAS in three sporadic cases. Two of the mutations were nonsense mutations causing premature termination in exons 21 (1324C → T) and 25 (1708C → T), respectively, one was a single base pair deletion (1821delC), and four were splice site mutations in introns. In one sporadic case, an *ELN* deletion arose *de novo* and caused a frame shift, resulting in a premature stop codon in exon 28. In one patient with SVAS, Tassabehji et al. [188] identified the insertion of a T in codon 606 of exon 26 of the *ELN* gene (1814insT), which produced a frame shift predicted to cause premature termination 10 codons downstream. Thus, mutations in SVAS that do not ablate large portions of the *ELN* locus are likely to create functional hemizygosity through the generation of translation products that undergo premature termination and, at least in some cases, nonsense-mediated decay. Six new SVAS mutations and 11 new single-nucleotide polymorphisms (SNP) have been identified [189]. Five SNPs were intronic, and five of six expressed SNPs were from a single individual. Interestingly, three of the SVAS kindreds had mutations in exon 20. Compound heterozygosity was suggested in one proband, with one apparently normal parent carrying a frame shift mutation (387delT) and the other a short, in-frame deletion in exon 20 (1175-1198del). The one broadly expressed polymorphism, 1828G → C in exon 26, had a prevalence of 7% and predicts a Gly610Arg amino acid change that could have functional consequences. In contrast to pure SVAS, Williams syndrome involves the deletion of a large (100 kB) region at the 7q11.2 locus that nearly always encompasses the *ELN* locus and that includes the deletion of at least five other structural genes [178,180,182,190].

Messenger RNA levels and levels of tropoelastin expression have not been determined in most cases of SVAS. A C to G transversion at the acceptor splice site of exon 16 was shown to create a truncated elastin transcript that was expressed at very low levels [191]. Urban and colleagues demonstrated that one mutation, 1195delG, leads to haploinsufficiency due to nonsense-mediated decay that is reversible by cycloheximide treatment of cultured fibroblasts. These cells also produced reduced amounts of tropoelastin [189]. *ELN* (elastin gene) haploinsufficiency appears to affect the ascending aorta selectively, most likely due to the high concentration of elastin in this tissue as a result of the selective expression of the elastin gene during perinatal development and the high mechanical load placed on the tissue by cardiac output. Other elastic tissues are affected to a lesser extent, if at all. Although penetrance of the mutation is complete, there is very wide variability in the extent and severity of mutational consequences, confirming the important influence of other genes and environmental influences on the expression of this defect.

Relationship to Other Elastin Gene Defects

Molecular genetic findings have now shown that autosomal dominant cutis laxa is allelic with SVAS at the elastin locus. Although there are cutaneous manifestations in SVAS, the predominant pathology is severe, progressive disorganization of the arterial wall. In contrast, the autosomal dominant cutis laxa mutations identified to date are all predicted to encode peptides that are extended at their C-termini and whose C-termini contain missense sequences. The only confirmed cutis laxa mutations occur in the 5′ region of the transcript (exons 30 and 32) [15,16].

Other Congenital Forms or Syndromes with Features of Cutis Laxa

Patterson Pseudoleprechaunism Syndrome (MIM 169170)

Patterson and Watkins [192] described a 10-month-old boy with marked cutis gyrata (deeply furrowed skin) of hands and feet and a generalized skeletal disorder. He developed hyperadrenocorticism, diabetes mellitus, and bladder diverticula and died at age 7.5 years from gram-negative sepsis. Enlargement of the zona fasciculata of the adrenal glands was found at autopsy [193].

Wrinkly Skin Syndrome (MIM 278250)

Wrinkly skin syndrome is characterized by wrinkled skin on the hands and feet, with an increased number of wrinkles on the palms and soles, poorly developed skeletal musculature, and a prominent venous pattern over the anterior thorax [194]. The syndrome may occur together with mental retardation and microcephaly, in one such case of which there was a decrease in the number and length of elastic fibers in a skin biopsy from a wrinkled area [195]. Likewise, a mother and two sons with wrinkly skin syndrome presented with wrinkling of the abdominal skin as well as that of the dorsum of the hands and feet, decreased elastic recoil of the skin, an increased number of palmar and plantar creases, musculoskeletal anomalies, microcephaly, mental retardation, and an aged appearance. Fragmented elastic fibers were evident in the dermis [196]. These last authors reported an interstitial deletion involving band q32 of chromosome 2 and also noted that two collagen genes, *COL3A1* (MIM 120180) and *COL5A2* (MIM 120190), located in the same general region of 2q, may be candidate genes for wrinkly skin syndrome. They compared the phenotype of their patient with those of 20 previously reported individuals with the same deletion and emphasized the overlapping features of del 2q and wrinkly skin syndrome. The co-occurrence of mental retardation and elastic fiber deficiency is shared with DeBarsy syndrome and Costello syndrome, perhaps suggesting a common mechanism.

Other Syndromes

Other congenital anomalies that exhibit cutis laxa features include ablepharon-macrostomia syndrome (MIM 200110) and the related, possibly allelic condition, Barber-Say syndrome (MIM 209885) [197,198]. There is marked wrinkling and laxity of the skin and a prematurely aged appearance in both syndromes, and elastic fiber abnormalities have been noted in several cases [199,200]. However, there were no ultrastructural abnormalities of either elastic fibers or microfibrils at the electron-microscopic level in one case in which an 18q deletion was present [201]. Furthermore, hyperelastosis was reported in the index case of the Barber-Say syndrome [197]. Because of distinctive

clinical manifestations that are shared by the ablepharon-macrostomia and Barber-Say syndromes, it has been suggested that they may represent defects in the same gene [198].

Another syndrome, the arterial tortuosity syndrome (MIM 208050), presents with arterial tortuosity and alteration of the vascular elastic fibers accompanied by skin hyperelasticity, which may be confused with cutis laxa. It has been suggested to be an autosomal recessive connective tissue disorder [202]. The latter authors reported histologic findings at autopsy of one affected child showing arterial changes involving disruption of elastic fibers of the media and fragmentation of the internal elastic lamina. Other reports suggest an association with Ehlers-Danlos syndrome, based on skin and joint hyperextensibility [203,204].

Costello Syndrome (MIM 218040)

This condition was first described in 1977 [205]. Children with this syndrome present with short stature; redundant skin of the neck, palms, soles, and fingers; curly hair; papillomata around the mouth and nares; and mental retardation [205,206]. Further review of several cases [207] and a ten-year follow-up [208] of previous cases [209,210] led to the conclusion that Costello syndrome should be included in the differential diagnosis of cutis laxa in association with postnatal growth retardation and developmental delay. A further case described clinical findings that included loose skin of the neck, hands, and feet; deep palmar and plantar creases; a typical coarse face with thick lips and macroglossia; relative macrocephaly; mental retardation; short stature; arrhythmia; a large size for gestational age; and poor feeding [211]. The infant died of rhabdomyolysis at the age of 6 months. The major pathologic findings were fine, disrupted, and loosely constructed elastic fibers in the skin, tongue, pharynx, larynx, and upper esophagus, but fibers were normal in the bronchi, alveoli, aorta, and coronary arteries. The degeneration of elastic fibers was confirmed in the skin of another, previously described patient from Japan [212]. Autopsy also showed degeneration of the atrial conduction system, calcification and ballooning of skeletal muscle fibers with infiltration of macrophages, and myoglobin deposits in the collecting ducts of the kidney, consistent with rhabdomyolysis. Clinical findings in 14 cases showed increased elastic fiber fragmentation. Lack of elastic fiber attachment was observed in one patient [213]; however, normal elastin fibers have been reported by other authors [207,214–216].

Although the cause of Costello syndrome remains unknown, possible autosomal recessive inheritance [217–219] and an autosomal dominant, *de novo* mutation to explain the sporadic occurrence of the syndrome [220] have been suggested. An underlying metabolic defect, possibly a lysosomal storage disorder, has been proposed [217].

Defects in elastic fiber assembly may be coupled to mental retardation and other abnormalities in Costello syndrome through an excess level of galactosyl sugars, derived from chondroitin sulfate proteoglycans such as biglycan and CD44, that interfere with the action of the 67 kDa elastin/laminin-binding protein [221]. This study showed that six strains of Costello syndrome skin fibroblasts secreted normal levels of tropoelastin but that they failed to incorporate the precursor into insoluble elastin or immunoreactive elastic fibers. The elastin-binding protein appeared to be shed from the cell surface at an abnormally high rate, and intracellular pools of biglycan and CD44 appeared elevated. The cellular phenotype was reversed by addition of chondroitinase ABC. This concept has been reviewed [222].

Trisomy 7p

This disorder can include cutis laxa as part of the spectrum of multiple congenital anomalies [223]. Redundant skin at the neck and joints as well as cardiovascular abnormalities have been reported. Because the elastin locus is on the 7q arm of chromosome 7, elastin gene dosage would be unaffected.

Recurrent ctb(7)(q31.3)

This syndrome consists of one case that presented as neonatal cutis laxa with a Marfanoid phenotype. The child died of severe cardiovascular problems at 22 weeks. There was a chromosomal break (ctb) at 7q31.3 that led to an examination of laminin status. Fibroblast strains could not be established. The investigators proposed either an absence of, or an abnormality in, the B1 chain of laminin as a consequence of the chromosomal anomaly [97].

Meretoja Type Amyloidosis (MIM 105120)

Neurologic signs in this disorder (also known as amyloidosis V or Finnish type amyloidosis) derive from an inheritable systemic amyloidosis (amyloid deposition in the perineurium and endoneurium) that affects facial muscle tone and causes a bloodhound-like facies that could be confused with cutis laxa. In several Japanese cases, the phenotype is due to a mutation in gelsolin that results in extracellular deposition of the protein as amyloid material [164,224,225]. Cutis laxa with typical dermal elastolysis can also be associated with amyloid deposits [162,164,226–228].

Cantu Syndrome (MIM 114620)

Cantu et al. [229] reported four unrelated girls with an apparently identical syndrome consisting of mild mental retardation, short stature, macrocranium, a prominent forehead, hypertelorism, exophthalmos, cardiac anomalies, cutis laxa, wrinkled palms and soles, joint hyperextensibility, wide ribs, and small vertebral bodies. Each was the product of nonconsanguineous parents and appeared to be sporadic cases. In each case, the age of the father was advanced. The authors suggested the possible presence of a *de novo* autosomal or X-linked dominant mutation. Further reports suggested an autosomal recessive pattern based on affected sibs [230–232], but the most recent analysis has once again favored the concept of new, dominant mutations [233].

Congenital Hemolytic Anemia with Emphysema and Cutis Laxa (MIM 235360)

This syndrome presented in three of four sibs from a consanguineous mating as severe congenital hemolytic anemia and early onset of pulmonary emphysema. One of the probands demonstrated at age 15 severe pulmonary emphysema and cutis laxa [99]. These cases can be considered a unique form of cutis laxa.

Elastoses

Excessive elastin accumulation (elastosis) can arise from both genetic and environmental causes. The examples discussed below do not include common pathological states that involve elastin, such as hypertension and other fibroproliferative disorders.

Actinic (Solar) Elastosis

The sun is a major cause of dermal alteration in photoaging. Chronic exposure of the skin to UV leads to the accumulation of so-called elastotic material in the mid- and deep dermis. This material is formed by the

generation of large and irregular clumps of elastin together with other poorly defined extracellular matrix components by an unknown mechanism. Elastin expression is increased, and fibrillin expression and accumulation are also elevated [234]. Elastic fiber accumulation appears both to displace and mask collagen accumulation at affected sites [235], leading to altered appearance and biomechanical properties. In transgenic mice harboring an elastin promoter linked to β-galactosidase, UV activation of the promoter correlates with elastosis [236–238]. In contrast to the situation in photoaged skin, elastic fiber content tends to diminish with chronological age in nonsun-exposed areas [239].

Buschke-Ollendorff Syndrome (Osteopoikilosis; MIM 166700)

Buschke-Ollendorff syndrome is an autosomal dominant disorder characterized by uneven osseous formation in bone (osteopoikilosis) and yellowish, fibrous skin papules containing elastic fibers (dermatofibrosis lenticularis disseminata). This disease is not characterized by a prematurely aged appearance. Collagen and elastin fibers appear thickened and distorted by electron microscopy, and *in vitro* production of these proteins by skin fibroblasts is elevated [240]. Osteopoikilosis may occur independently of skin lesions. Although bone involvement may suggest a nonelastin etiology, elastic fibers are a component of the periosteum, and their abundance is elevated during fracture repair [35].

Elastoderma

Elastoderma is a very rare acquired syndrome, involving skin laxity and excessive accumulation of dermal elastin without calcification, in contrast to the calcification seen in pseudoxanthoma elasticum. Only two cases have been described. The first, a 29-year-old female, showed the development of loose and pendulous skin over the trunk and right arm with a superficial resemblance to acquired cutis laxa [241,242]. Although excessive elastin accumulation was noted, the skin lacked normal recoil. Elastic fibers were reportedly pleiomorphic. The etiology is unknown. The second case was a 27-year-old man with a 2-year history of prematurely aged skin localized to the anterior and posterior of his neck, accompanied by a marked accumulation of thin, intertwined elastic fibers, particularly in the papillary dermis [243].

Elastosis Perforans Serpiginosa (MIM 130100)

Elastosis perforans serpiginosa is a rare skin disease characterized by transepidermal elimination of abnormal elastic fibers [244]. Elastic material can be organized as large fibers or as infiltrating material with an inhomogeneous organization. It is a rare disease, found principally in young individuals, and is characterized by keratotic papules clustered in an arcuate arrangement. Histologically, the lesions show a hyperkeratotic and acanthotic epidermis, communicating channels extending from the dermis to the surface, and increased dermal elastin. It is occasionally associated with Marfan syndrome, pseudoxanthoma elasticum, Ehlers-Danlos syndrome type IV, osteogenesis imperfecta, and Down syndrome. The pathogenesis of the condition remains unclear. One family with isolated elastosis perforans serpiginosa has been reported in which inheritance was autosomal dominant with variable expression [245].

Pseudoxanthoma Elasticum (PXE; MIM 264800,177850)

The prime characteristic of pseudoxanthoma elasticum (see also Chapter 11, this volume) is the accumulation of calcified, fragmented elastic fibers in flexural skin. Skin laxity may be present. Effects extend to other elastic tissues, including Bruch's membrane of the eye (forming angioid streaks), and cardiovascular complications due, at least in part, to calcification and cracking of vascular elastic laminae [168,169,246]. Patients are at risk of blindness and gastrointestinal hemorrhage. A consensus conference reviewed the features and classification of PXE [247]. Lax or redundant skin is part of a distinctive constellation of signs that strongly implicate elastic tissue as a primary target. Elevated fibroblast elastase activity has been reported [98]. Elastin has been excluded as a candidate locus [246]. Linkage of both autosomal recessive and dominant PXE to a locus at 16p13.1 has been shown [248–252], and PXE-causing mutations have been identified at the locus of the ABC transporter gene (*ABCC6*), the product of which is associated with multidrug resistance [253,254].

PREMATURE AGING SYNDROMES

Premature aging disorders can be divided into several groups, depending on the time of onset, which can be prenatal, neonatal, postnatal, or postadolescent. Many of these syndromes exhibit connective tissue changes common to innate aging, including wrinkling and atrophy of skin, loss of subcutaneous fat, bone fragility, advanced atherosclerosis, and skeletal deformities. In most cases, the biochemical and genetic bases of the pathology are unknown. Many of the premature aging syndromes show evidence of possible convergent mechanisms in the sense that signs such as micrognathia, alopecia, and a beaked nose are common among many of the progeroid forms.

A hallmark of many of the aging syndromes is atrophy and wrinkling of the skin. The skin may have a parchment-like texture, and there is often an absence of subcutaneous fat. Unlike cutis laxa, loose, sagging skin is a rarer observation. The collagen content of the skin is reduced as determined by histological evaluation. Muscle development can be poor or atrophic, and many of the syndromes show micrognathia and/or facial hypoplasia. Growth may be mildly to severely retarded. Atherosclerosis is accelerated and advanced. Most severely affected individuals die of complications of coronary artery disease.

Hutchinson-Gilford Progeria (MIM 176670)

This form of progeria was independently described by Hutchinson in 1886 [255] and Gilford in 1904 [256,257]. It is the rare but classical archetype of premature aging syndromes (Fig. 7). Although patients may not exhibit growth retardation at birth, this problem is evident within the first few months of life, and growth rapidly falls below the 10th percentile, often ceasing after the fifth year. Patients have striking features of segmental aging, including cutaneous atrophy, micrognathia, a beaked nose, a large cranium, corneal clouding, loss of facial and scalp hair in the first years of life, crowding of teeth, and a narrow chest. Mental retardation is absent. Arthritis and respiratory complications arise in the first decade, and atherosclerosis with angina is prevalent. Life span ranges from 7 to 27 years, with coronary artery disease being the most common cause of death [258]. Both autosomal dominant [259,260] and autosomal recessive inheritance patterns [261–263] have been suggested. Germline mosaicism has been suggested in a few familial cases, one of two brothers [264], another of nine offspring of two sisters [265], and a consanguineous Libyan family [266]. Maciel [262] reported a Brazilian family with

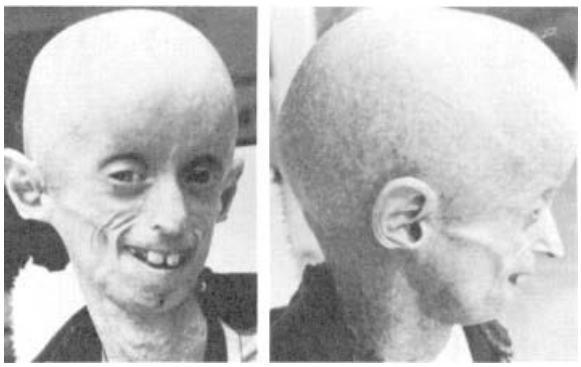


Figure 7. Characteristic facial features of a patient with Hutchinson-Gilford progeria. This 10-year-old female shows classic craniofacial disproportion, loss of hair and subcutaneous adipose tissue, absent earlobes, and irregular skin pigmentation. She died three years later of congestive heart failure. (Reprinted from [260] with permission.)

clear autosomal recessive inheritance, but McKusick [67] questions the authenticity of the diagnosis (MIM 176670). Brown et al. [267] described identical twins with progeria who died of heart failure a month apart at age 8 years. There was evidence for a possible deficit in growth hormone and a suggestion that a gene for progeria could be located on chromosome 1 [267]. On the other hand, a common trait of high elastin production as observed in fibroblasts from one mother and her progeroid child could indicate an autosomal recessive inheritance [263].

Biochemical Findings

Progeroid fibroblasts in culture have a very limited life span [268]. Histologically, Colige et al. [269] have reported the presence of abundant and abnormal elastic fibers as well as thickening of basement membrane in the skin of one patient. In fibroblasts from some patients, elastin mRNA expression and elastin synthesis are elevated [269,270]. Unlike normal skin fibroblasts, the addition of TGF-β does not affect these parameters, suggesting that elastin production is already maximally stimulated [263] (Fig. 8). Likewise, collagen type IV production was high in cultured fibroblasts from one patient as well as steady-state levels of elastin and type IV mRNA [269]. While fibroblasts from affected individuals responded to the addition of EGF by a decrease in the level of mRNA for fibrillar collagens and elastin, collagen type IV remained highly expressed [271].

In addition to abnormalities in matrix proteins, reduced antioxidant protection by vitamin E was suggested by Ayres and Mihan as a cause of the disorder [272], while Goldstein and Moerman showed increased thermolability of fibroblast and erythrocyte enzymes [273,274]. A recessive inheritance pattern was suggested on the basis of intermediate thermolability values in both parents. Brown and

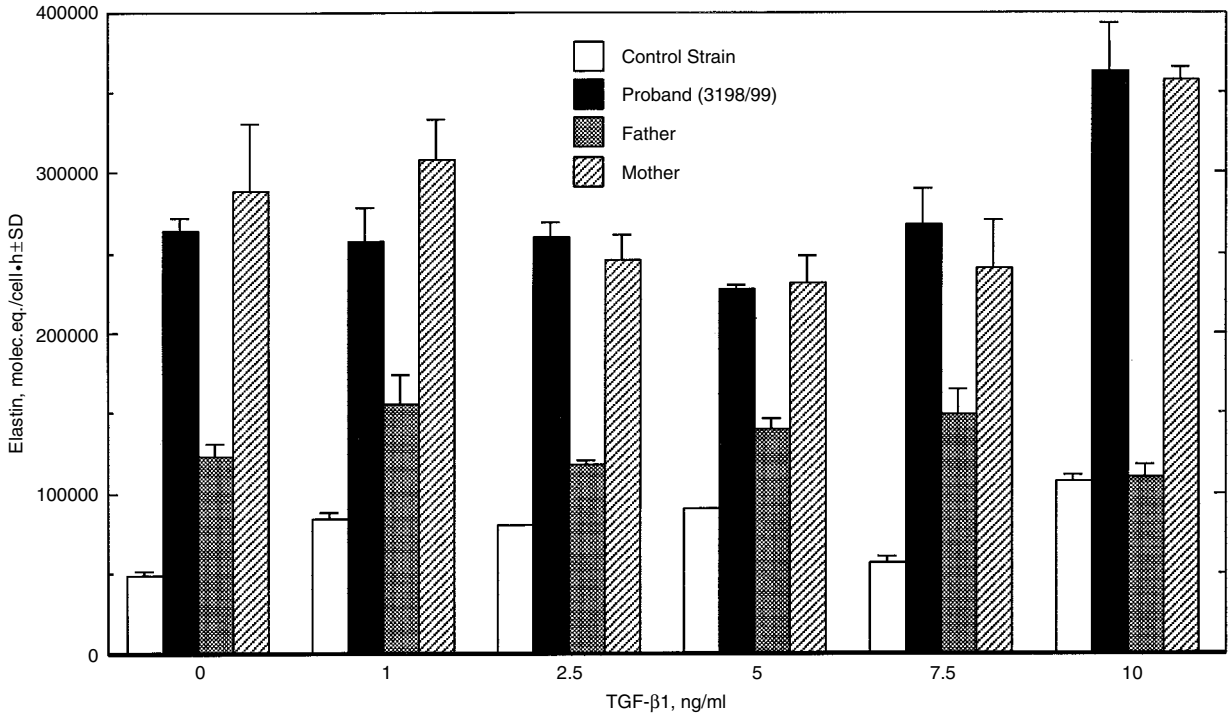


Figure 8. Elastin production in progeria fibroblasts. Evidence for dominant inheritance of high production and lack of a TGF-β response. Skin fibroblasts were cultivated to confluence in 5% fetal calf serum, and elastin production was analyzed by competitive ELISA over the next 48 hours. Production was expressed on a per cell basis in a control strain as well as in fibroblasts from a progeroid patient and the two parents. Basal elastin production is illustrated at the 0 ng/ml dose of TGF-β1. While elastin production in control cells was doubled by the highest dose of TGF-β1, production appeared to be constitutively elevated, at least under standard cell-culture conditions, in the proband and mother. The father's production level was intermediate. Ordinate: elastin production expressed in molecular equivalents per cell •h $\times 10^{-5}$, ±SD. (Reprinted from [263] with permission.)

Darlington [275] reported contrary evidence using glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) as markers. Fibroblasts from patients with Hutchinson-Gilford progeria and several other progerias show greatly reduced *in vitro* life spans that could be consistent with defects in an error correction mechanism. However, the DNA helicase mutations reported in Werner syndrome [276] have not been identified in progeria [277].

Brown reported reduced IGF-1 but normal growth hormone levels and very high basal metabolic rates in several progeria patients [259]. Interestingly, growth hormone therapy reduced metabolic rates. Brown and his colleagues have also identified excess urinary excretion of hyaluronan as a common feature of Hutchinson-Gilford progeria and Werner syndrome, suggesting that excess hyaluronan synthesis is associated in each case with a lack of vasculogenesis [278,279]. Hyaluronan is certainly a key factor in the hydration of many connective tissues, including cartilage and skin. Another study reported increased secretion of fibronectin and collagen by cultured skin fibroblasts from a patient with Hutchinson-Gilford progeria [280]. An unusual aspect of connective tissue metabolism in progeria is the paradoxical overexpression of elastin [263,270] and collagen IV [281] by progeroid skin fibroblasts. The familial segregation of elastin overexpression may suggest a recessive aspect to the syndrome [263] (Fig. 8). Colige et al. [271] have also reported a reduced mitotic responsiveness of progeroid cells to EGF, and Giro and Davidson [263] noted a diminished response of elastin synthesis to TGF-β, although elastin production may have already been maximal. Quentin-Hoffmann et al. [61] reported that several patients with progeroid-like symptoms presented abnormalities in the biosynthesis of chondroitin/dermatan sulfate proteoglycans. It has also been shown that in infantile progeroid patients there is deficient expression of decorin [282]. Another study has suggested that polymorphisms in signal peptide sequences may be linked to premature aging [283].

Age-related atrophy of skin and other connective tissues is consistent with excessive matrix catabolism. Studies by Millis et al. [64,284] have illustrated the overexpression of several matrix-degrading enzymes by aging fibroblasts. Our own studies with the aged rat reflect this as an overexpression of MMP-2 (72 kDa gelatinase) in skin. Indeed, the screening of an expression array with mRNA from normal and Hutchinson-Gilford progeria fibroblasts provides unequivocal evidence for overexpression of several MMPs in these strains (Davidson, Giro, and He, unpublished findings). As part of a gene-chip-based screen of alterations common to progeria and intrinsic aging, it has been suggested that aging involves the accumulation of errors in the mitotic machinery of cells. These studies also detected altered expression of a number of matrix molecules and MMPs [285].

Wiedemann-Rautenstrauch Syndrome (Pseudohydrocephalic Progeroid Syndrome; MIM 264090)

This neonatal progeroid syndrome is a rare, autosomal recessive disorder first reported by Rautenstrauch and Snigula in 1977 [286] and Wiedemann in 1979 [287]. Affected children have intrauterine growth retardation, a progeroid appearance at birth and during infancy, and natal teeth. Death occurs in infancy or early childhood [28,288,289]. Hou and Wang described a Chinese girl without intrauterine growth retardation [290]. Arboleda et al. [291] found disturbance of bone maturation as well as of lipid and hormone metabolism. Key findings include prenatal growth deficiency, a lack of subcutaneous fat except in the flanks, buttocks, and anogenital area, sparse scalp hair, beaking of the nose, a persistent anterior fontanelle, macrocephaly, and congenital heart defects. Sudanophilic leukodystrophy is a common laboratory finding, consistent with a generalized abnormality of lipid metabolism. One case report describes another patient and compares the findings among six individuals [292], and a further report describes five additional cases [293].

Werner Syndrome (MIM 277700)

Werner syndrome is a rare, autosomal recessive progeria with substantial connective tissue involvement [294]. An important difference in comparison with other progerias is the later onset of symptoms. These begin during the second decade with a lack of the typical adolescent growth spurt. Consequently, Werner syndrome patients have short stature, slender limbs, and a stocky trunk. In common with other progeroid syndromes, the nose is beaked and there is atrophy of subcutaneous fat. There are scleroderma-like skin changes, especially over the extremities, bilateral cataracts, subcutaneous calcification, hyperkeratosis, hoarseness, a high-pitched voice, and premature arteriosclerosis. These features progress over several decades. Wrinkled skin, a wizened appearance, and premature graying of the hair are other significant features (Fig. 9). Affected patients frequently develop malignancies in the third and fourth decades [295]. Death usually occurs in the fourth or fifth decade from malignancy or cardiovascular disease.

Connective Tissue Abnormalities

Classical signs of Werner syndrome include diffuse osteoporosis and calcification of the soft tissues and blood vessels as well as calluses or deep ulceration of the skin over the lower extremities [295]. A few specific biochemical abnormalities have been noted: elevated urinary excretion of hyaluronan [296,297]; a diminished response of collagenase expression to stimulation by PDGF or basic FGF; increased basal expression of collagenase by fibroblasts [298]; increased fibroblast expression of collagen I [299]; and decreased expression of collagen VI [300]. Several matrix genes were found to be overexpressed in a Werner syndrome cDNA library that was used in a candidate gene approach; these were *COL1A1* (collagen α1(I) chain), *COL1A2* (collagen α2(I) chain), *FN1* (fibrillin 1), *THBS1* (thrombospondin 1), *ON/SPARC* (osteonectin/SPARC), *HSPA1-1* (heat shock protein 70 1A), and *IGF-BP3* (insulin-like growth factor binding protein-3) [301]. Interestingly, many of the products of these genes are induced by TGF-β. Unlike Hutchinson-Gilford progeria fibroblasts, elastin production in Werner syndrome fibroblasts is normal; it is slightly increased by TGF-β and slightly decreased by bFGF (M.G. Giro and J.M. Davidson, unpublished observations) (Fig. 10). Increased collagenase expression is consistent with the behavior of normal fibroblasts near the limits of their replicative life spans [64]. None of these defects can be directly linked to the known molecular defect in Werner syndrome, decreased activity of a RecQ DNA helicase [276,302].

Molecular Genetics

Although there had previously been a number of genetic hypotheses concerning Werner syndrome [303], positional cloning firmly and finally established the basis

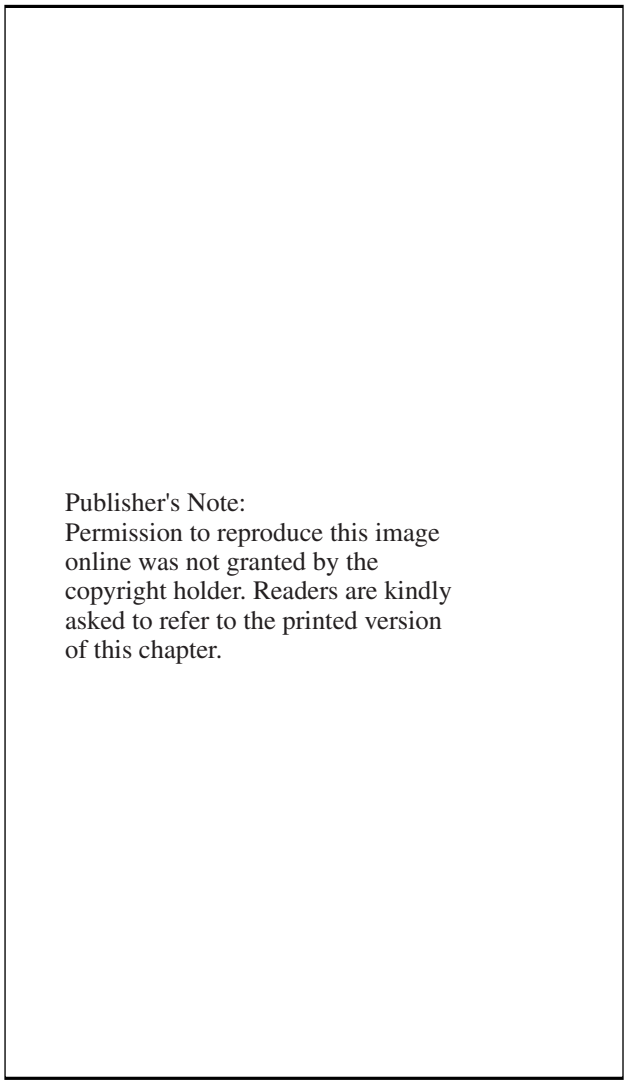


Figure 9. Clinical features of the Werner syndrome. **A**: This 54-year-old man demonstrates a characteristic bird-like facies with a beaked nose and premature graying of hair. **B**: The patient demonstrates scleroderma-like features of the feet, with acrosclerosis and recalcitrant ulcers. (Reprinted from [377] with permission.)

of the disease in 1996 [276,302,304–306] and a large number of publications have ensued that describe mutations within the Werner (*WRN*) gene locus [307]. Most of the reported mutations predict premature truncation in the C-terminal region of the *WRN* gene product, a region that contains helicase activity [276,308,309] as well as a nuclear translocation signal [310,311]. In the case of one mutation, the mRNA product is unstable [312]. Defects in RecQ helicase activity could account for chromosomal instability [313,314] and high mutation rates [315].

Cockayne Syndrome (MIM 216400)

In 1936, Cockayne described two siblings with dwarfism, retinal atrophy, deafness, and mental retardation. A precociously senile appearance due to lack of subcutaneous fat and sunken eyes gives the syndrome progeroid aspects; sensitivity to sunlight was also described [316]. No abnormality was observed in fibroblast collagen synthesis from one patient [317]. In our own studies of elastin synthesis in three strains of cultured skin fibroblasts, we found that elastin production was reduced in two cases and could be highly stimulated by the addition of 10 ng/ml of TGF-β, while 100 ng/ml of bFGF reversed this effect (Fig. 10). Two major subtypes have been described within the syndrome — type I (type A) and type II (type B) [318] — both of which exhibit defective repair of UV-mediated DNA damage [319]. Cockayne syndrome type I is due to mutations in the *CKN1/CSA* gene [320], the product of which contains so-called WD40 domains, and has been implicated as a transcription-repair coupling factor [321] in studies with mutant yeast homologs; however, the gene appears to function in the context of other RNA polymerase II transcription factors [322]. Cockayne syndrome type II is due to mutations in the *ERCC6* (*CKNB/CSB*) gene, the product of which is a DNA-dependent ATPase [323] that appears to function, at least in part, as an RNA polymerase II transcription factor [324,325]. The consequence of the CSA and CSB mutations, as in xeroderma pigmentosum (MIM 194400), is defective nucleotide excision repair.

Other Progeroid Syndromes

Mandibuloacral Dysplasia (MIM 248370)

The progeroid features in this syndrome appear 1–2 years after birth and include hypoplasia of the jaw and clavicles, late closure of cranial fontanelles and sutures, a thin, beaked nose, prominent eyes, alopecia, short stature, and postnatal growth retardation [326,327]. Changes in the fingertips, including shortened terminal phalanges and atrophic skin, may be indicative of a diffuse connective tissue involvement. This very rare disease occurs mostly in families of Italian origin [328]. A lethal, neonatal form of the disorder has been described [329].

Gerodermia Osteodysplastica (MIM 231070)

This condition was first described by Bamatter in 1950, and the author called affected individuals "Walt Disney dwarfs" [330]. Hunter et al. [331] reported that this autosomal recessive condition exhibited lax but not hyperelastic skin, most markedly over the extremities, joint hyperextensibility, osteoporosis, which may be associated with fractures, vertebral collapse, and Wormian bones. The cutaneous features are illustrated in two siblings and another proband (Fig. 11). Three key findings are severe osteoporosis, joint hyperextensibility, and cutis laxa. Eich et al. [332] described two sibs with gerodermia osteodysplastica who, in addition to the characteristic manifestation of the disease, presented a metaphyseal peg indenting the epiphyses of the long bones, particularly at the knees. The peg was only visible from the age of 4 to 5 years to epiphyseal closure, and the authors proposed that it could be a new bone marker specific to gerodermia osteodysplastica. Al Torki et al. [333] described the disease in a Bedouin family in which two sisters had the disorder and the older sister had a prematurely aged face with loose and wrinkled skin, joint laxity, and osteoporosis involving the thoracolumbar vertebrae.

Acrogeria (MIM 201200)

Gottron [334] first reported two cases of acrogeria in whom hands and feet had appeared aged since infancy because of thin skin. The same disease has also been called metageria when more generalized [335] and acrometageria

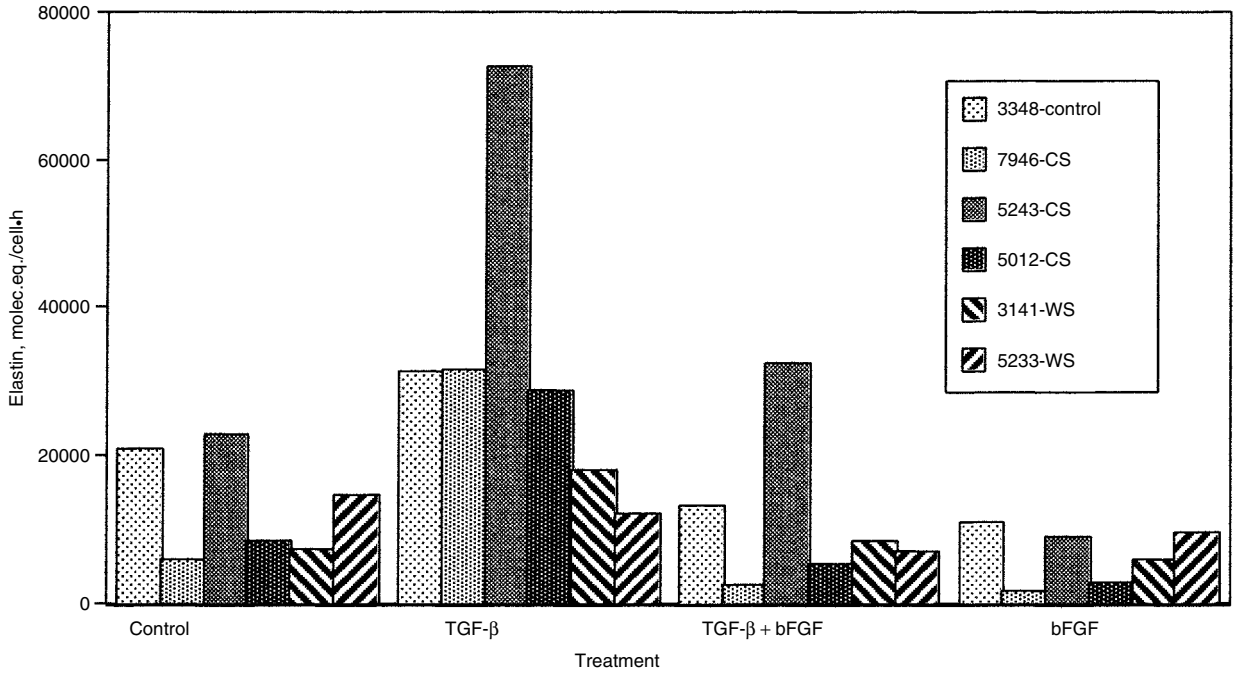


Figure 10. Elastin production in Cockayne syndrome (CS) and Werner syndrome (WS) fibroblasts and their cytokine responses. Normal (National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (NIGMS) strain GM3348), Cockayne syndrome (National Institute on Aging Cell Repository (NIA) AG7946, AG5243, AG5012), and Werner syndrome fibroblasts (NIA AG3141, AG5233) were treated with serum alone, TGF-β1(10 ng/ml), TGF-β1 (10 ng/ml) plus basic FGF (100 ng/ml), or basic FGF alone (100 ng/ml). Culture supernatants were analyzed for elastin production as described in Figure 8. Werner syndrome strains were only slightly affected by the growth factors. Cockayne syndrome fibroblasts increased elastin production nearly fourfold in response to TGF-β1. This effect was attenuated by bFGF, and bFGF alone inhibited elastin production in Cockayne syndrome fibroblasts.

by Greally et al. [336]. In addition to wrinkled skin of feet and hands, patients with this disorder have joint hypermobility and mental retardation.

Metageria, the more generalized form of acrogeria, is characterized by loss of subcutaneous fat, thinning of the dermis, multiple telangiectasias, and mottled hyperpigmentation. In cultured fibroblasts from the skin of a patient and his sister, Hunzelman et al. [337] found some decrease in the production of type I collagen, which was reversed when TGF-β was added to the culture medium. De Groot et al. [338] described an affected mother and son with conspicuous vessels over the trunk and elastosis perforans serpiginosa, which was also a feature of the female described by Gottron as well as two other patients, but this rare inherited form lacks the characteristic scarring or arterial and intestinal rupture characteristic of Ehlers-Danlos syndrome type IV. No defects in type III collagen have been noted, but the reader should refer to the chapter on EDS (Chapter 9, this volume) for a discussion of possible phenotypic overlap.

Hallermann-Streiff Syndrome (MIM 234100)

This is a sporadic syndrome with major craniofacial and ocular abnormalities, including dyscephaly, cataracts, microphthalmia, and dental abnormalities. Stature is short but proportionate. Skin atrophy is reported in 60–70% of cases [339,340]. Mandibular hypoplasia and a beaked nose are features shared with many other progeroid syndromes [341]. Major complications relate to airway and respiratory mechanics, probably related to dysmorphology of these structures.

Down Syndrome (MIM 190685)

Down syndrome has features of a "segmental" progeroid syndrome, meaning that tissues and organs are differentially affected. Patients present with degenerative vascular disease, premature hair loss and graying, increased tissue lipofuscin, and markedly reduced life expectancy. Neuropathological changes include precocious aging and dementia [342]. These features are apparently the result of overexpression of genes located on chromosome 21. Down syndrome fibroblasts have a reduced *in vitro* life span [343].

Premature Aging Syndrome, Okamoto Type (MIM 601811)

Severe growth and developmental abnormalities were noted in the 15-year-old daughter of unrelated parents. Microcephaly, osteosarcoma, and osteoporosis were significant connective tissue findings in this condition [344].

Premature Aging Syndrome, Penttinen Type (MIM 601812)

A 10-year-old Caucasian male presented a prematurely aged appearance with pronounced acroosteolysis and brachydactyly [345]. Distinctive cutaneous findings included hard, confluent skin lesions that clinically and histologically resembled those of juvenile hyaline fibromatosis (MIM 228600).

Lenz-Majewski Hyperostotic Dwarfism (MIM 151050)

Patients with this disorder exhibit a progeroid appearance that includes dental enamel dysplasia, skin hypoplasia, and joint laxity. Gorlin and Whitley [346] reported a patient with strikingly loose and wrinkled atrophic skin of the

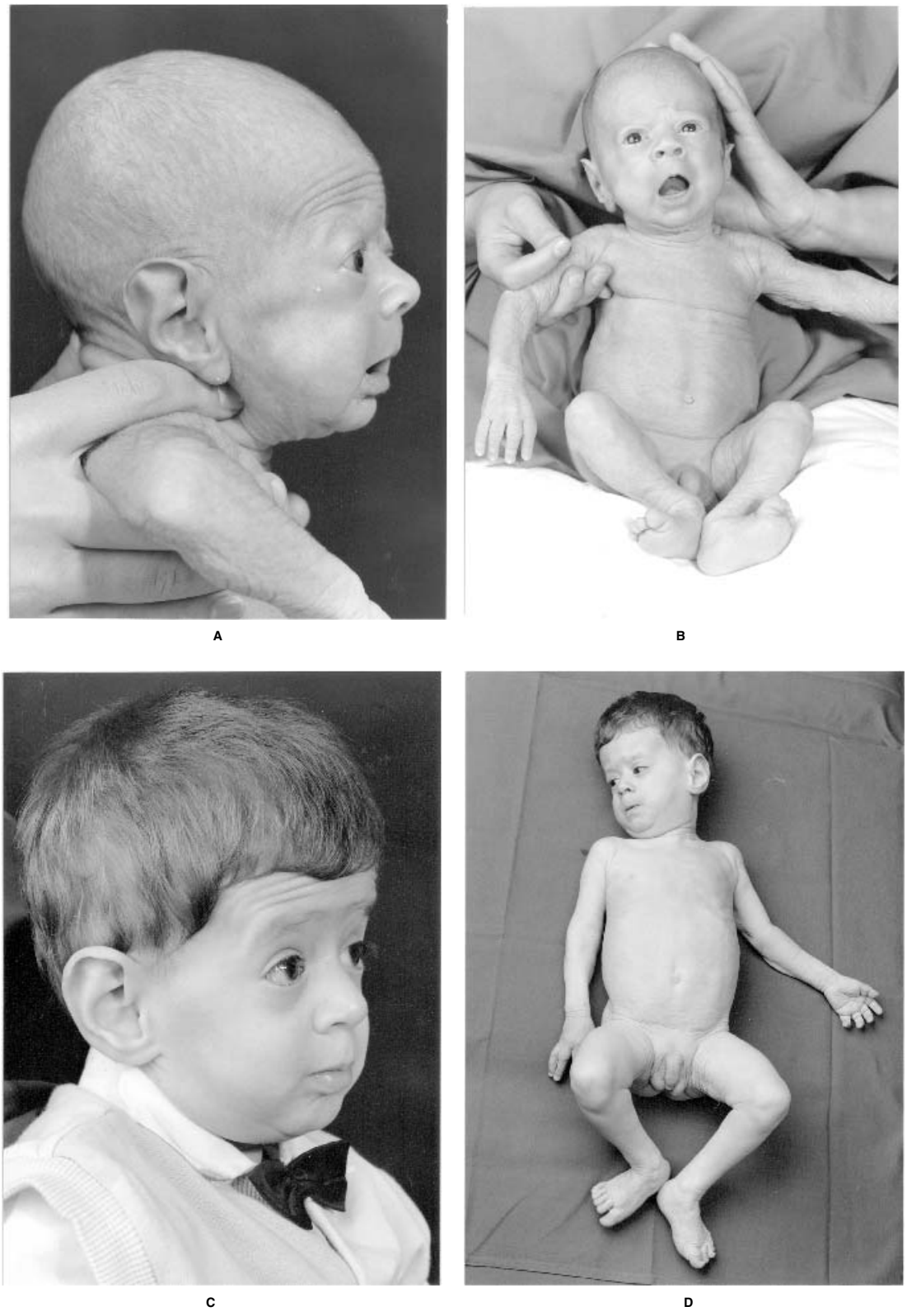
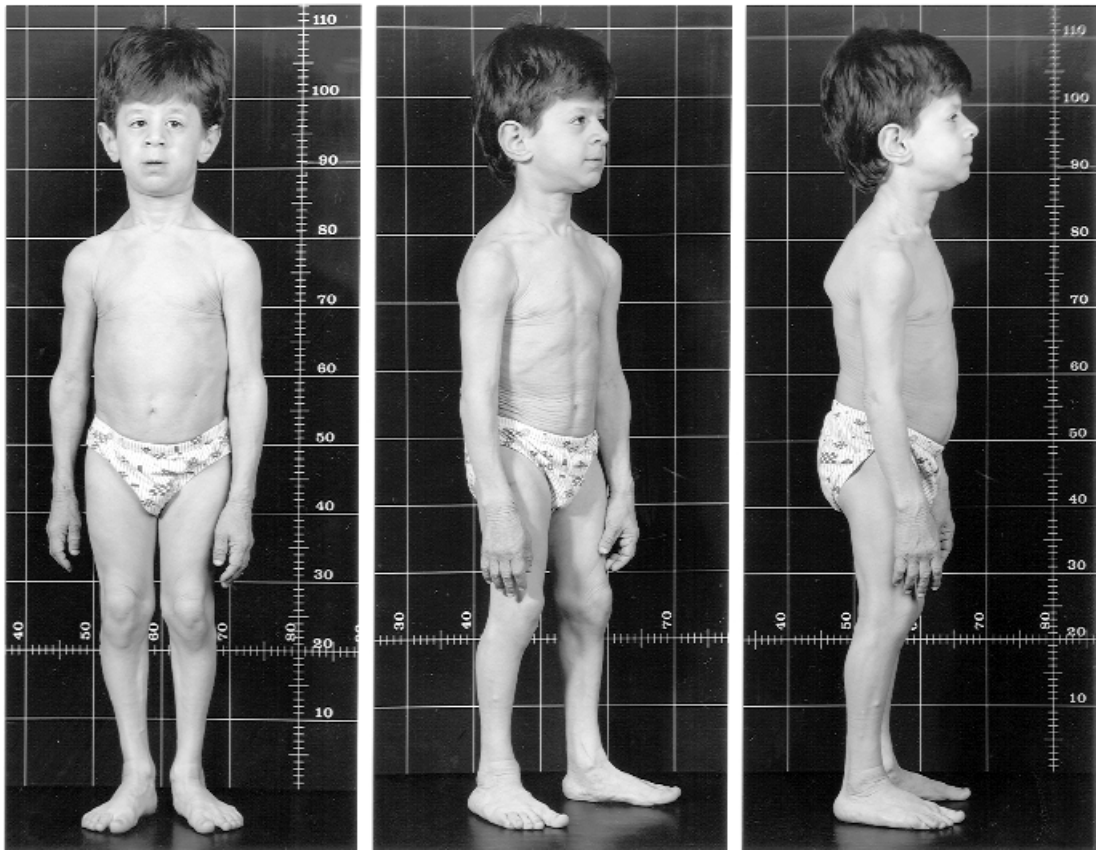
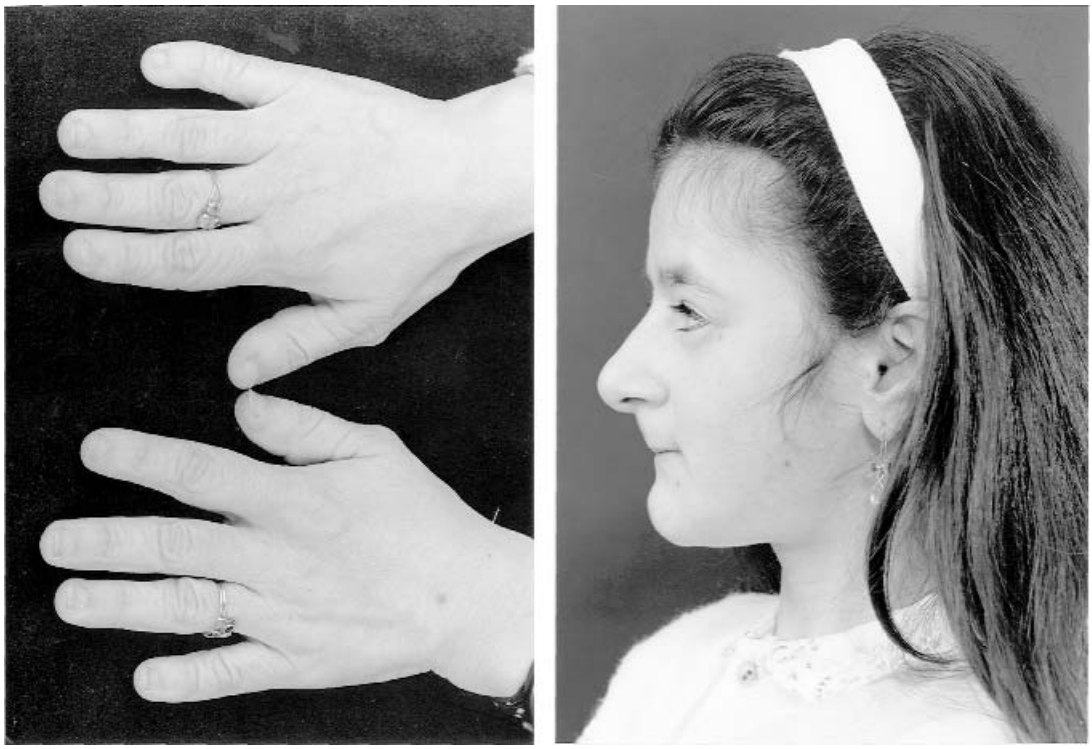


Figure 11. Gerodermia hereditaria osteodysplastica. V.R. (d.o.b. 20 May 90) at 3 weeks of age (**A** and **B**), at 2 years (**C** and **D**), and at 6 years (**E**), and his older sister M.R. (d.o.b. 14 Nov 79) at 12 years (**F** and **G**). Extensive wrinkling and an aged facies are especially obvious at younger ages. Congenital hip dislocations, multiple spontaneous fractures, marked osteopenia, Wormian bones, and short stature are common to both, findings that were initially interpreted as osteogenesis imperfecta in the sister. The parents are healthy and unrelated but originate from the same region in Italy. (All illustrations courtesy of Dr. B. Steinmann).



E



F G

Figure 11. (*Continued*)

hands with short digits and partial syndactyly. Scalp veins were prominent through thin, wrinkled, and atrophic skin. There was bilateral narrowing of the nasal choanae and nasolacrimal duct obstruction. Skeletal maturation was retarded, with progressive sclerosis of the skull, facial bones, and vertebrae, broad clavicles and ribs, and short or absent middle phalanges. Chrzanowska et al. [347] described the 10-year-old son of young, healthy, unrelated parents, who had a progeroid appearance, bilateral symmetrical shortening of the fingers and metacarpals, short toes, and loose, thin skin with marked hyperlaxity.

MURINE GENETIC MODELS

Experimental models can provide substantial insight into the etiology and pathogenesis of cutis laxa and premature aging. Transgenic and recombinant mouse models have proven to be extremely informative in understanding certain aspects of connective tissue biology. This volume contains numerous examples representing the remarkable rate of advancement offered by genetic manipulation of whole animals. Although the microfibrillar proteins are intimately related to elastogenesis, other chapters discuss fibrillin and other elastin-associated proteins in detail. This discussion will be more restricted.

Elastin

Mammalian elastin promoter sequences have been defined by combining sequence and functional studies of the upstream regions of the elastin gene of man [36,348–350], cow [351–353], and rat [354,355]. Uitto et al. have developed a transgenic mouse that expresses the human elastin promoter (6 kB) linked to the *CAT* gene. This has proven to be a useful model for evaluating molecules that regulate elastin accumulation in photodamaged skin, such as glucocorticoids, TGF-β, and psoralens [236,356–359]. Elastin null mice have been produced by homologous recombination [360]. It was hypothesized that heterozygotes would present a phenotype resembling that of SVAS. Interestingly, however, mice hemizygous for elastin show a compensatory increase in the number of elastic lamellae despite a reduction in aortic elastin mRNA and protein [361]. The murine model does not completely parallel the response in the human aorta to SVAS mutations. Essentially the same genetic lesion manifests in man as severe disorganization of the vessel wall as well as there being effects on other elastic tissues. Mice that are homozygous for the elastin gene deletion exhibit a lethal, obstructive arterial disease, the development of which is not dependent on hemodynamics, thrombosis, or interactions with circulating cells [360]. These latter findings underscore a critical role for elastin in the homeostasis of the arterial wall.

Premature Aging

A number of animal species have been used to examine the relationship between intrinsic aging and connective tissue, but only recently have new strains been identified that exhibit phenotypes akin to progeroid syndromes.

Klotho: A Prematurely Aging Mouse Strain

A new murine gene locus has been defined by a targeted deletion that leads to many signs of premature aging, including atherosclerosis, emphysema, hypogonadism, skin atrophy, and calcification of soft tissues [362]. Mice homozygous for the mutation die within 3–4 months. The mouse *Klotho* gene encodes a novel, single-pass transmembrane protein with homology to β-glycosidases [363], which is predominantly expressed in kidney and amplified by renal stress [364]. There also appear to be effects on bone metabolism in the Kl^{-}/Kl^{-} mutant mouse that resemble osteopenia [365]. The human gene has been localized to chromosome 13q12 and, as does the mouse gene [366], expresses two transcripts as a result of alternative splicing [367]. It is not known how the status of *Klotho* gene expression relates to normal or accelerated aging in man.

Senescence-Accelerated Mouse

This term covers a widely studied group of inbred mouse strains that has undergone extensive selection for reduced life span [368]. Strains with a shortened life span exhibit many manifestations of accelerated aging, including atherosclerosis [369], reduced life span of dermal fibroblasts [370,371], and bone loss [372–376].

RECENT DEVELOPMENTS

Cutis Laxa

A two- to threefold increase in elastolytic activity in lesional skin fibroblasts from a 21-year-old female with acquired cutis laxa has been reported [378]. No significant mutations could be detected in the coding region of the *ELN* gene in five further cases of apparent autosomal recessive cutis laxa, nor was elastin production by dermal fibroblasts found to be markedly altered *in vitro*. Although polymorphisms in exons and introns could be detected, none can definitively be linked to disease (X. Zhao and J.M. Davidson, unpublished observations).

A further patient with the wrinkly skin syndrome has been described, in whom there was a considerable loss of elastin and fragmentation of microfibrils associated with wrinkly features and poor elasticity over the abdomen and the dorsum of the hands and feet [379]. Loss of mid-dermal elastic fibers has also been reported in two cases of lupus erythematosus [380]. In addition to forms of cutis laxa acquisita being associated with cutaneous lymphoma, T-cell lymphoma, and myeloma, it has been suggested that anetoderma can arise in cutaneous B-cell lymphoma [381]. One of the acquired forms, granulomatous slack skin disease, has been reported to have been treated successfully with systemic, recombinant interferon-γ [382]. Although decreased elastin expression in Costello syndrome fibroblasts has been reported [383], the *ELN* locus has been excluded in a separate study [384].

Supravalvular Aortic Stenosis

The analysis of new cases of SVAS has reinforced the concept that mutations toward the N-terminus of elastin lead to functional haploinsufficiency, while C-terminal missense or truncation mutations can lead to the cutis laxa phenotype [385]. Metcalfe et al. have reported 35 mutations associated with SVAS [386], and many more are likely to exist (Z. Urban, personal communication). The predominant mechanism for haploinsufficiency appears to be nonsense-mediated decay. A small fraction of the SVAS mutations result in the production of mutant tropoelastin molecules, the coacervation properties of which are altered *in vitro* [387]. Exon 26 is reported to contain crucial sites for coacervation [388,389]. A novel 1281delC mutation with variable penetrance is the fifth example of an SVAS mutation in exon 20 [390]. Skin elastic fibers have been suggested to be a useful diagnostic feature for Williams syndrome, and, by analogy, SVAS [391]. The clinical relevance of mouse models of elastin haploinsufficiency has been reviewed [392].

Premature Aging Syndromes

Hutchinson-Gilford Progeria

There are a number of further, descriptive studies of Hutchinson-Gilford progeria [393–397]. An additional reported pathologic feature of the condition is an expansion of the mesangial matrix, leading to glomerulosclerosis [398]. Of interest, are the scleroderma-like features of several patients [394,395]. Unlike the situation in some cases of Hutchinson-Gilford progeria, telomere shortening was not a feature in a newly identified case of Wiedemann-Rautenstrauch syndrome [399]. On the other hand, two studies in gene profiling have led to the suggestion that there are genes altered in common in progeroid and elderly skin fibroblasts, but not in *in vitro* aged skin cells [400]. Furthermore, the expression of MMPs 1, 2, and 3, is commonly elevated in aged and progeroid fibroblast cultures (L. He and J.M. Davidson, unpublished findings). A more detailed profile by Ly et al. [401], suggests that genes associated with the mitotic machinery may be selectively prone to error, leading to chromosomal abnormalities.

Werner Syndrome

It has been suggested that defective helicase domain-containing proteins represent a common mechanism leading to the aging phenotype, not only in Werner and Cockayne syndromes, but also in other conditions such as xeroderma pigmentosum, trichothiodystrophy, Bloom syndrome, X-linked α-thalassemia/mental retardation syndrome, and Juberg-Marsidi syndrome [402–404]. In Werner syndrome, the enzyme RecQ is distinguished from other helicases by a physically separable and functionally distinct 3′-5′ exonuclease activity [405,406]. Although there are examples of WRN mutants lacking either 3′-5′ exonuclease or helicase activity, the preponderant genotype is thought to be consistent with a functionally null phenotype, since the WRN protein is generally absent in affected individuals [404,407]. Possibly, genetic instability arises in *WRN* mutants through an interaction of WRN protein with DNA polymerase-δ, an enzyme involved in DNA replication and repair [408]. WRN protein has also been shown to interact with the catalytic subunit of a DNA-dependent protein kinase, and its activity to be regulated by phosphorylation-dependent complex formation [409]. Glycophorin A mutation rates show an age-dependent increase in the development of variant sequences, and there is also evidence for genetic instability in mutant WRN heterozygotes [410]. In a murine model of Werner syndrome expressing a helicase from which the C-terminal region had been deleted, dermal fibroblasts derived from homozygous null mutants showed a very mild phenotype *in vitro*, and fibroblasts derived from a double null mutant lacking also p53 exhibited an increased mortality rate [411]. The WRN protein seems to interact with telomerase, although their actions can be separated. The introduction of telomerase activity into Werner and other progeroid fibroblast strains can certainly extend the replicative lifespan of these cells [412], but genomic instability persists [413]. Perhaps the best explanation for tissue-specific effects of *WRN* mutations derives from studies of T lymphocytes which, unlike fibroblasts, are able to upregulate telomerase activity independently of the presence or absence of functional WRN protein, and thereby maintain some resistance to the lifespan shortening effects of the genetic defect [414]. However, lifespan extension is not sufficient to overcome important aspects of WRN function; lymphoblasts from Werner syndrome patients are indeed long-lived, but there is still a marked elevation of genetic instability. Such studies do emphasize the differential effects of the *WRN* mutation on different cell populations [415], and they may help to explain some of the selective effects on connective tissues.

PATIENT SUPPORT GROUPS

Williams Syndrome Association
WSA National Office
1312 N. Campbell, Suite 34
Royal Oak, MI 48607-1555
Tel: (248)541-3630
Fax: (248)541-3631
Email: WSAoffice@aol.com
Web site: http://www.williams-syndrome.org

Williams Syndrome Foundation
University of California
Irvine, CA 92697-2310
Tel: (949)824-7259
Email: hmlenhof@uci.edu
Web site: http://www.wsf.org (this site provides links to many other national associations)

Williams Syndrome Foundation (UK)
161 High Street
Tonbridge
Kent TN9 1BX
England
Web site: http://www.williams-syndrome.org.uk (this site provides links to many other national associations)

Sunshine Foundation (Progeria)
Sunshine Foundation
Sunshine Foundation Dream Village
5400 C.R. 547 North
P.O. Box 255
Loughman, FL 33858
Tel: (800)457-1776
Email: SUNFOUND@aol.com
Web site: http://www.sunshinefoundation.org

International Progeria Registry
Human Genetics
Institute for Research
1050 Forest Hill Road
Staten Island, NY 10314
Tel: (718)494-5333
Email: wtbibr@aol.com

Research Trust for Metabolic Diseases in Children
46 Park Road
Chandlers Ford
Hampshire S053 2ES
England
Tel: +44-23 802 53863
Fax: +44-23 802 53863

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Chapter 11

Pseudoxanthoma Elasticum

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SUMMARY

- **Pseudoxanthoma elasticum (PXE) is a heritable systemic disorder that affects elastic tissue and is characterized by systemic manifestations, typically first in skin and then in the eyes and cardiovascular system. There is consensus about the diagnostic criteria defining the disease.**
- **The disorder manifests primarily as an autosomal recessive disease, and is caused by mutations in the *ABCC6* gene encoding the transmembrane transporter protein ABCC6, situated at chromosome 16p13.1. The heterozygous carrier may express a forme fruste. Although familial clinical PXE in three consecutive generations with apparent disease transmission from diseased parent to child has been reported, genetic haplotype analysis in 81 families did not identify affected sib pairs as heterozygotes at the locus. Although this finding does not absolutely exclude the existence of a dominant PXE trait, it clearly indicates its rarity, if it exists at all.**
- **The prevalence of PXE is approximately 1 in 75,000, but this could be an underestimate due to some affected individuals having only mild manifestations and unawareness among physicians of the disease. There is an unexplained 2:1 predominance in females.**
- **The diagnostic and histopathological hallmark of PXE is degenerative calcification with subsequent disintegration and destruction of the elastic tissue of the body. Elastic tissue is involved in the mid- and deep dermis of the skin, in Bruch's membrane of the eye, in the endocardium of the heart, and in the lamina elastica interna and externa of arteries. The pulmonary elastic tissue is spared. An increased content of glycosaminoglycans in affected elastic tissue has been demonstrated.**
- **The clinical spectrum of the disease ranges from mild manifestations to severe, occasionally lethal cardiovascular complications. The skin typically shows yellow-orange papules and plaques symmetrically in flexural sites, with gradual extension, and subsequent loss of elasticity with redundant folding of the skin. While the skin lesions are cosmetic in nature, the eye manifestations begin with angioid streaks and progress to macular degenerative retinopathy complicated by retinal bleeding and central visual field blindness. Cardiovascular disease encompasses a wide spectrum from mental fatigue syndrome to early cardiovascular death due to myocardial infarction or, very rarely, gastrointestinal hemorrhage. Typically, it manifests with hypertension, intermittent claudication, mitral valve prolapse, and angina pectoris.**
- **The cardiovascular phenotype may manifest as a dominant trait and show striking histomorphological similarities to idiopathic arterial calcification of infancy (IACI). Although IACI is most likely an independent heritable disease, its potential clinical overlap with PXE, its comparatively earlier manifestation, either intrauterine or typically within 6 months of birth, and its lethal course give some justification for hypothesizing IACI as the homozygous, potentially lethal manifestation of the dominant cardiovascular PXE trait.**
- **Although there is no treatment for the basic defect, a prophylactic lifestyle, prophylactic medical monitoring, and genetic counseling are highly recommended to prevent complications from arising.**

INTRODUCTION

Definition

Pseudoxanthoma elasticum (PXE) is a hereditary, systemic disorder of connective tissue. Its transmission is primarily autosomal recessive (MIM 264800). A possible dominant inheritance pattern (MIM 177850) has been described. The elastic fibers of the skin, eyes, and cardiovascular system degenerate and slowly become calcified, subsequently losing their structural integrity and elastic function. This causes a clinical spectrum of disease involving these three organ systems. The disease shows considerable clinical heterogeneity, independent of the mode of inheritance. There is a 2 to 1 female predominance. A gene locus for both the recessive and dominant forms of the disease has been mapped to chromosome 16p13.1, suggesting allelic heterogeneity, by which different molecular mutations of the same gene give rise to recessive and dominant variants of the same disease [1–3]. Mutations in the gene *ABCC6* encoding the transmembrane transporter protein ABCC6 have been shown to cause PXE (see Recent Developments).

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History

First Description

PXE is probably as old as mankind, although there is no documentation of the disease before the nineteenth century. The first description of the clinical signs and symptoms of PXE appeared in the medical literature in 1884 as a case report by Balzer [4]. However, he did not recognize the involvement of the elastic tissue and its aberrant calcification in the pathogenesis of the clinical features he described and attributed them to xanthomas instead. Some of the histological features of the disease had already been described before Balzer's clinical description by Rigal in 1881 [5], but Rigal also attributed the skin lesions to xanthomas.

Recognition as an Independent Disease Entity

The case presentation by Balzer in 1884 [4] and a clinically similar one by Chauffard in 1889 [6] were reviewed by Darier and others from 1891 [7] to 1896 [8,9] with regard to their clinical and histological similarities and compared with the known clinical and histological characteristics of xanthomas. Darier recognized these cases as representing a unique and independent disease entity of the elastic tissue with clinical and histological features distinct from xanthomas and, perhaps in an overzealous effort to distinguish the two different disease entities, coined the term "pseudoxanthome élastique." This term remains the most widely accepted name for the disorder. The fundamental histological feature of PXE — namely, calcification of the elastic fiber — was first described by von Tannenhain in 1901 [10] and further verified by Finnerud and Nomland [11], who used microincineration methods to establish the fact that the minerals in the elastic fibers were calcium salts. Multinuclear giant cells in close proximity to vessels in the periphery of typical histological skin lesions were first recognized by Balzer [4] and confirmed by Bodin in 1900 [12]. De Schweinitz, at the time in all likelihood unaware of PXE as a new disease entity, had first described in 1897 the familial occurrence of angioid streaks in family members most likely suffering from PXE [13]. It was then Werther, in 1902, who was probably the first to observe familial aggregation of PXE cases and who suggested the cause of the disease to be congenital, pioneering the idea of a genetic cause underlying PXE [14].

Recognition as a Systemic Disorder of Connective Tissue

The nosologic position of PXE continued to unfold in the later nineteenth and early twentieth centuries as angioid streaks were first described by Doyne [15] in 1889, named by Knapp [16] in 1892, and recognized by Kofler [17] in 1917 as originating from crazing of Bruch's membrane. Thirty-three years after Darier's initial recognition of PXE, a Swedish ophthalmologist, Grönblad [18], and her colleague Strandberg [19], a dermatologist, in 1929 made the first unequivocal connection between the skin and eye manifestations of PXE. The recognition of PXE as a general disorder of the elastic tissue was imminent and resulted in a new eponym, the Grönblad-Strandberg syndrome. Giesen [20] confirmed the involvement of the mucosal membrane, which had first been described by Balzer [4], in a typical case of PXE in 1936.

The cardiovascular aspects of PXE were studied by Carlborg et al. [21–23], Scheie [24], and Touraine [25,26] from the 1940s on. They were the first to comprehensively describe the cardiovascular manifestations of PXE and therefore to complete the full canonical triad of skin, eye, and cardiovascular aspects of PXE, which has remained essentially unchanged ever since.

Many reports of individuals and large cohort groups and literature reviews were published between 1950 and 1990 and served to outline in greater detail variations in the natural course of PXE and identify environmental risk factors that can alter the natural course. Major clinical reviews have been conducted by Eddy and Farber [27], Goodman et al. [28,29], McKusick [30], and Neldner [31].

Eponymous Designations

Typically for the descriptive medical science of the late nineteenth and early twentieth centuries in Europe, new discoveries were named after their discoverers. Arbitrary examples of eponymous PXE designations are: Grönblad-Strandberg syndrome, Darier-Grönblad-Strandberg syndrome, and Grönblad-Strandberg-Touraine syndrome. The most common of these is the Grönblad-Strandberg syndrome. Today, these eponyms are still used in the European medical literature. In view of the more common international use of the term pseudoxanthoma elasticum to describe not only the skin involvement but also the inherited systemic connective tissue disorder, with additional retinal and cardiovascular manifestations, and its convenient abbreviation, PXE, it is recommended that the use of eponyms be discontinued.

EPIDEMIOLOGY

The prevalence of PXE in the general population is estimated to be approximately 1 in 75,000. It is believed that many cases may go undiagnosed for the lifetime of an affected individual so that the exact prevalence may be more common. Because genetic linkage analysis is now available and the gene will be identified in the near future (see Recent Developments), more examples of minimal or forme fruste expression will be detected, which can represent disease expression in the heterozygote. Therefore, the true incidence will become known with greater accuracy.

There seems to be no geographic or racial predilection for PXE. All studies of large cohort groups of individuals with PXE or literature summaries of individual case reports have shown an approximate 2 to 1 female-to-male ratio [30,31].

The mean age of clinical onset is 13 years. There are rare reports of the onset of skin lesions at age 2 or 3 years and even more rare reports of their being present at birth [28], although none of the latter were biopsied for proof. Delayed onset in the fifth or sixth decade of life has been reported. Such cases may simply have gone undetected for many years. In very mild cases, skin lesions are most likely present but so minimal as to be overlooked, even by physicians, unless there is a specific request to look for minimal PXE and to biopsy any suspicious areas. Such cases represent the far end of the clinical spectrum of PXE.

CLINICAL ASPECTS

Skin

The most apparent clinical feature of PXE is the skin involvement, which in most cases is the first clinical manifestation and the one on which the diagnosis is based. If systemic involvement does antedate the skin lesions, it is seldom recognized and can at best be considered a presumptive diagnosis, which needs further confirmation. Whereas previously the diagnosis of PXE in such cases could only be confirmed after the appearance of pathognomonic

skin signs, the discovery of the gene locus now allows diagnosis through linkage analysis in families with proven clinical disease in at least one other family member for both recessive and dominant forms of the disorder.

Clinical Signs and Symptoms

The primary cutaneous expression of PXE is small, 2–5 mm, yellow-orange papules of rhomboidal or irregular shape, bounded by normal skin, which coalesce into larger plaques. These yellow-orange papular lesions with a rough, cobblestone-like, Moroccan leather or chicken skin texture are highly characteristic. They usually first come into view on the lateral neck and gradually appear, over several years, in the more distal flexural sites. They develop symmetrically and are initially confined to flexural areas (lateral neck, axillae, antecubital fossae, groins, and popliteal spaces) of the body, although they may gradually encompass larger areas (Figs. 1–4). In a clinically heterogeneous fashion, the skin lesions in some patients may extend well beyond the flexural sites. Anogenital and oral mucous membranes are affected in some, but not all, patients. The oral mucosal lesions are usually confined to the inner aspect of the lower lip. There are also rare cases with normal skin appearance on visual inspection but with minimal diagnostic histological changes in skin biopsies taken from predilection sites [32–35]. This finding can be interpreted both as representing preclinical disease and as minimal disease expression in a heterozygous individual. About 2–3% of individuals with PXE will develop small circles of 2–3 mm diameter, encrusted papules called elastosis perforans serpiginosa (EPS) or perforating PXE. EPS is not unique to PXE, although in other disorders with EPS, the extruded elastic fibers will not be calcified. The long-term cutaneous changes are diffuse thickening, loss of elasticity, and loss of the early surface signs, resulting in sagging and/or redundant folding of the skin, particularly in the axillae. This may appear 20–40 years after the initial onset of the disease and may interfere with clothing. In some cases with facial involvement, this phenomenon may produce a "hound-dog" look. Solar elastosis can give a similar appearance but usually occurs at a later average age and is confined to the face and lateral neck. Except

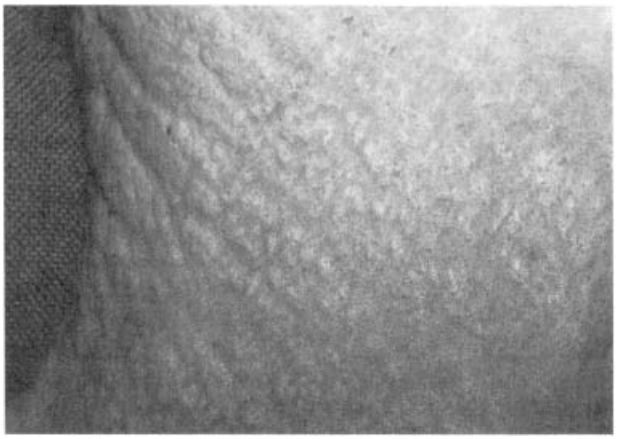

Figure 1. Close-up photograph of typical and pathognomonic early skin lesions on the lateral neck of a 21-year-old female with PXE. This is the most common site of origin of PXE skin involvement and always presents in a symmetrical distribution.

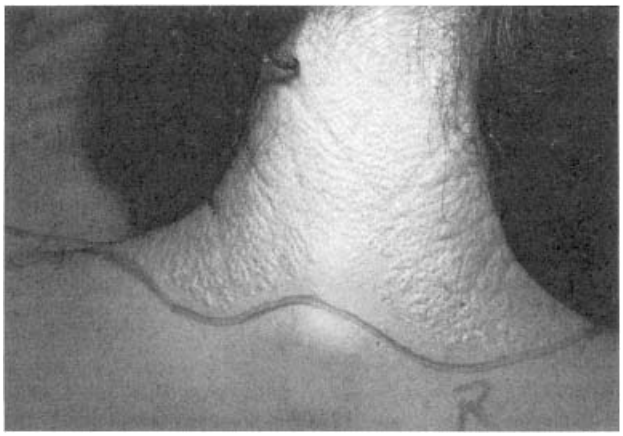

Figure 2. Posterior neck lesions of a more advanced case in a 30-year-old female.

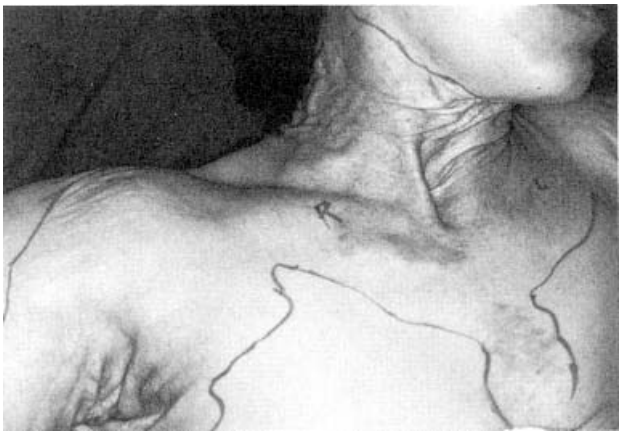

Figure 3. Advanced PXE in a 45-year-old female with extensive neck lesions that have joined with the axillary lesions. Thickened, folded, affected skin, most pronounced in the axillae, is a sign of PXE of long duration.

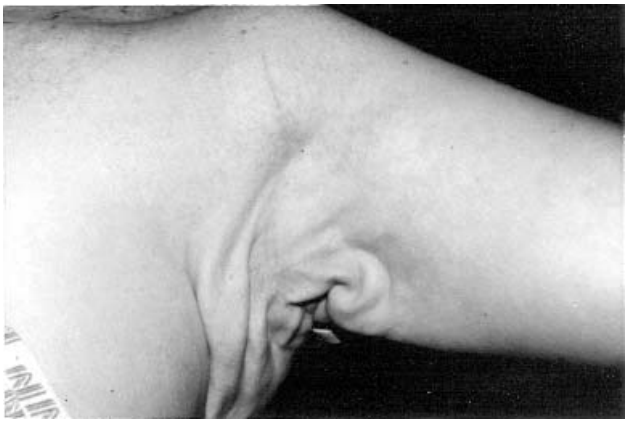

Figure 4. Axillary involvement producing deep folds when the arm is lowered. The surface features of PXE skin are often lost in such areas.

for mild itching in some individuals, the skin lesions are asymptomatic.

Eyes

The PXE retinopathy, clinically demonstrated by funduscopic examination, follows a somewhat predictable course but also one with great clinical heterogeneity.

Clinical Signs and Symptoms

The retinopathy of PXE consists of the following findings:

- Mottled *peau d'orange* hyperpigmentation
- Angioid streaks
- Atypical (Bruch's membrane) drusen
- Subretinal membranes (nets) with neovascularization
- Retinal hemorrhages with macular scarring
- Central vision loss with "legal blindness" (20/200 visual acuity OU [both eyes])
- Preservation of peripheral vision

The mottled *peau d'orange* hyperpigmentation (Fig. 5) is probably the earliest retinal finding, but it is seldom recognized until there is a medical indication for a retinal examination later in life. Its most common time of origin is unknown; it is, however, present in most cases when the earliest angioid streaks are found. Its specificity to PXE is unknown. The mottled hyperpigmentation is best seen at the periphery of the retina on funduscopic examination. At times, the pigment may form clumps of dark-staining material scattered throughout the retina. The reason for, and significance of, this clumping is also unknown.

Bruch's membrane is the retinal layer that contains elastic fibers and is therefore a potential site for calcification. It usually becomes sufficiently calcified about two to five years after the skin lesions first appear (at an average age of 13 years) and subsequently develops cracks or splits, called angioid streaks (Fig. 6). Angioid streaks are totally asymptomatic, and their presence often remains undetected until later in life (past age 40 years), when retinal hemorrhages begin to occur, unless a retinal examination has been performed earlier. They usually appear as irregular, often jagged, pigmented lines radiating out from the disk, like spokes on a wheel, but may also encircle the disk as peripapular streaks. At times, they develop white borders,

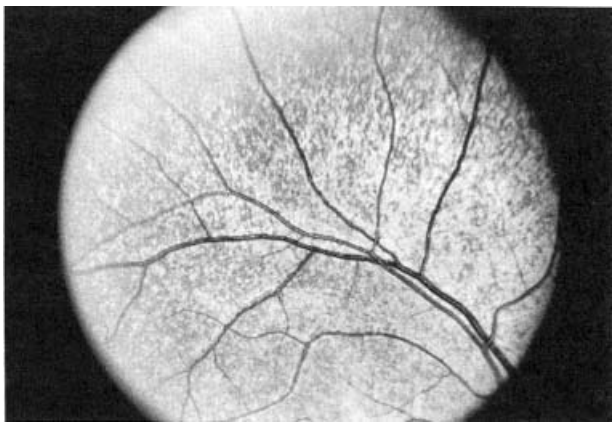

Figure 5. Mottled *peau d'orange* hyperpigmentation of the lateral margin of the posterior pole of the retina. Such pigmentation is nearly always more pronounced peripherally.

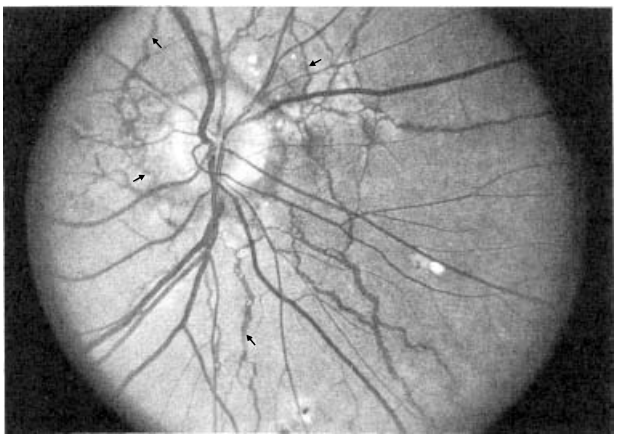

Figure 6. Multiple angioid streaks (small arrows), some radiating outward from the disk and others in a peripapillary distribution around the optic disk. Note the whitish "rock-like" drusen.

suggesting scar formation around the streaks, although the exact etiology of these white borders is unknown. Some streaks will develop paired pigmented spots on either side, which have been called "owl's eyes" and are again of unknown etiology and significance. There are at least 17 other conditions in which angioid streaks have been observed (see Table 1), so they cannot be considered pathognomonic for PXE, but, sooner or later, essentially 100% of individuals with PXE will develop streaks. The most notable examples of disorders other than PXE in which angioid streaks occur include Marfan syndrome, Ehlers-Danlos syndrome, β-thalassemia, Paget disease of bone, and sickle cell anemia [36,37].

The drusen bodies appear as small, shiny, white, "rock-like" deposits scattered throughout the retina, with no particular pattern or distribution. They are seen in about half of all patients, and their numbers will vary from one or two spots to large "showers" of drusen. Their etiology is unknown.

With time, small vascular loops grow up through the cracks in Bruch's membrane (angioid streaks) from the underlying choroid vessels by the process of neovascularization. As the vessels enlarge, particularly if the eye is traumatized,

TABLE 1. Disorders Other than PXE in Which Angioid Streaks Have Been Observed

Paget disease (Osteitis deformans)
Marfan syndrome (MIM 154700)
Ehlers-Danlos syndrome (see Chapter 9, this volume)
β-thalassemia
Sickle cell hemoglobinopathies
Hereditary spherocytosis (MIM 182900)
Idiopathic thrombocytopenic purpura (MIM 188030)
Tumoral calcinosis (MIM 211900)
Lead poisoning
Cowden syndrome (MIM 158350)
Pituitary tumors
Acromegaly
Familial polyposis (MIM 175100)
Nevus of Ota (oculodermal melanocytosis)

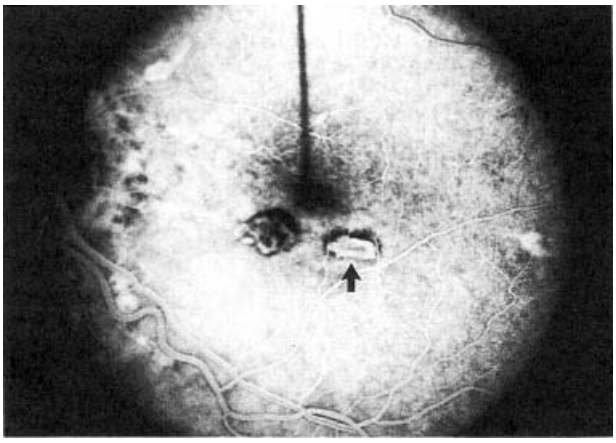

Figure 7. Example of a fluorescein angiogram. A typical subretinal net (membrane) is shown (arrow).

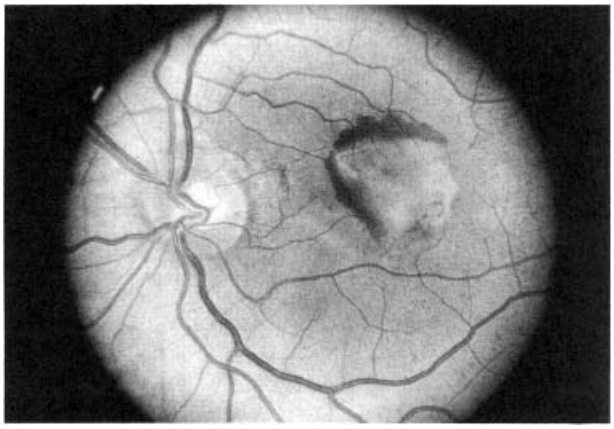

Figure 9. A healed retinal hemorrhage (4 months after the event) with the resulting white gliotic scar in the macula resulting in loss of central vision.

they may begin to leak clear serous fluid and form a so-called subretinal membrane or net (Fig. 7). At this juncture, the patient will notice wavy lines at the subretinal net site on an Amsler grid examination. Once this stage is reached, a frank retinal hemorrhage is imminent and usually occurs within days or a few weeks. Retinal hemorrhages (Fig. 8) most commonly seek out the macula and leave a white opaque scar (Fig. 9) as they heal over a period of several weeks. The end result is a loss of central vision, much the same as in the wet form of age-related macular degeneration (AMD), but through a different mechanism.

When a retinal hemorrhage occurs in one eye in an individual past age 40 years, a second hemorrhage in the same or opposite eye will commonly occur within one to five years, although longer intervals have occurred [31]. By the sixth decade of life, nearly 100% of affected individuals will have had a retinal hemorrhage of some degree. Retinal hemorrhages may occur before age 40 years, but, if so, they usually follow blunt trauma to the eye. It is important to emphasize to patients that, even if multiple retinal hemorrhages occur, peripheral vision will be preserved to the extent that they will be able to get around on their own, although they will be unable to drive a car or read standard-sized print. This will usually reach the point of 20/200 visual acuity, which is the definition of beginning legal blindness.

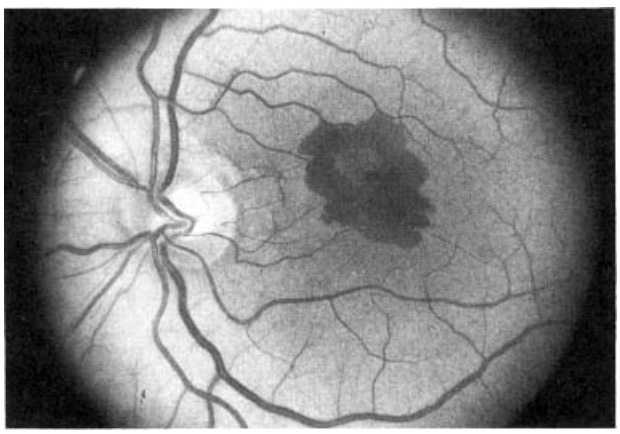

Figure 8. A fresh retinal hemorrhage, several days old, in the macular area, which is the most common site for retinal hemorrhages in PXE.

Cardiovascular System

General Considerations

The cardiovascular manifestations of PXE result from characteristic degenerative changes that occur in the elastic tissue of the endocardium and all arteries, although they are predominantly seen in arteries of the muscular type. Secondary aberrant calcification of the arterial internal elastic lamina and myoelastic tissue (media and external elastic lamina), with their subsequent fragmentation and proliferative destruction and continued calcification (Fig. 10), therefore primarily affects the peripheral (femoral, external iliac, renal, splenic, and coronary) arteries [21,24,28,31,38]. This may be accompanied by various degrees of intimal thickening due to a patchy proliferation pattern of the fibroelastic components [38].

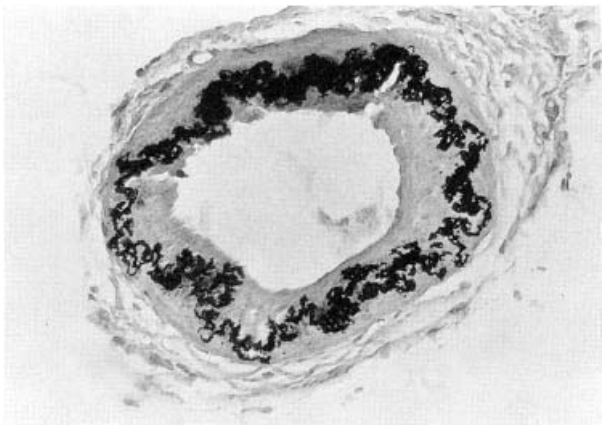

Figure 10. Medium-sized artery from a deep breast biopsy showing calcification of the elastic media. Elastic tissue stain (Verhoeff-van Gieson) shows dark irregularly shaped, fragmented calcified elastic fibers. ×225.

Comparison with Similar Cardiovascular Phenotypes

The medial calcification in Mönckeberg-type arteriosclerosis resembles that seen in PXE [21,38]. This form of focal calcific arteriosclerosis in the elderly is primarily characterized by widespread medial calcification particularly in the arteries of the lower limbs [39,40], but it has also been described in the arteries of the upper extremities, the splenic and renal arteries [41], and the mesenteric and coronary arteries [42]. Although medial calcification can be extreme, leading to circumferentially arranged rings of calcium within the arterial wall, Mönckeberg sclerosis does not usually cause cardiovascular complications unless an independently occurring process of intimal sclerosis with luminal narrowing accompanies it. This is usually not documented before the seventh decade of life. Intimal proliferation and calcification is, by definition [39], not an integral part of Mönckeberg calcinosis. The lamina elastica interna remains intact.

In PXE, cardiovascular complications can occur as early as within the first decade of life [23,43–45] but, in general, do not do so earlier than the end of the third or the beginning of the fourth decade [21,31,46]. Death at an early age due to cardiovascular complications in PXE has rarely been reported [47], or PXE may not have been recognized as the underlying disorder causing early cardiac death [45]. In any case of unexplainable early cardiac death with histopathological features of generalized calcification of the arterial myoelastic tissue, PXE has to be considered as one of the differential diagnoses.

The idiopathic type of generalized arterial calcification in infancy (IACI) (MIM 208000) is a third, clinically dramatic variant of arterial myoelastic degenerative tissue calcification, which can affect all arteries [48–50]. It is rare, of unknown etiology, and morphologically characterized by diffuse, degenerative calcification of the arterial internal elastic lamina. This is usually associated with fragmentation of the internal elastic lamina and a locally prominent, occasionally obliterative, patchy fibroelastic intimal proliferation pattern. The clinical course of IACI may lead to intrauterine death [51–53] or premature delivery around 32 weeks of gestation [54]. In live births, it is usually lethal in 85% of cases within the first 6 months (range 1 day to 27 months) [55,56], in most cases as a result of coronary artery occlusion due to excessive fibroelastic hyperplasia of the intima [48,55–57]. Prolonged survival, in one case up to adulthood, has been reported [58–61]. Based on its familial aggregation [54,62–74], it has been considered to be an independent congenital disease entity, most likely with an autosomal recessive inheritance pattern [60,74]. A comparison of different clinical features of cardiovascular manifestations in IACI, PXE, and Mönckeberg sclerosis of the elderly is given in Table 2.

It is important to emphasize that PXE and IACI differ with regard to their individual time courses of the typical clinical onset of cardiovascular complications, whereas the histopathological features of both disease entities show striking similarities [57,75]. Moreover, morphological changes similar to those described in PXE skin and endocardium at the ultrastructural level [76–78] were recognized within the internal elastic lamina of the coronary artery of an infant who died from IACI [75]. In fact, there is one reported case of a baby with IACI born to a mother with PXE [79]. To the extent that IACI most likely represents an independent disease entity in which the morphological similarities to PXE as regards structural changes within the elastic tissue are caused by a different etiological factor and the familial association of PXE and IACI in one case [79] is just a coincidental occurrence, these findings, however, still raise the intriguing question of whether IACI could represent at least in part, due to the overlap of its clinical spectrum with PXE, the extreme of the earliest fatal cardiovascular manifestations of PXE. Considering the recessive mode of inheritance of IACI, parents, as heterozygous carriers, would most likely not have clinical signs and symptoms of PXE if IACI were the earliest manifestation of any PXE abnormality. Because their children with IACI die of cardiovascular complications before the development of classical signs and symptoms of PXE, the diagnosis of PXE in these families cannot be established. Even if parents of babies with IACI did have signs or symptoms of PXE, these would most likely remain unnoticed because PXE is not generally considered as a potential differential diagnosis in relation to IACI.

Clinical Signs and Symptoms

Vascular morphological changes in PXE produce symptoms of intermittent claudication, coronary artery disease, hypertension, stroke, gastrointestinal hemorrhage, and abdominal angina. Aberrant calcification of the degenerated elastic tissue of the heart may result in diffuse endocardial

TABLE 2. Comparison of Clinical Characteristics of Cardiovascular Disease in PXE with Histopathologically Similar Vascular Phenotypes of Degenerative Aberrant Calcification Involving Primarily the Myoelastic Tissue of Arteries

Disease	Typical site of arterial manifestation	Typical age range of disease onset	Prognosis of clinical course of disease
Idiopathic arterial calcification of infancy (IACI) MIM 208000	Predominantly lamina elastica interna	0–2 yr,	Typical course: lethal
		Delayed onset up to 22 yr of age	Delayed onset: coronary artery disease (CAD), hypertension
PXE MIM 264800 MIM 177850	Predominantly lamina elastica interna and media but also lamina elastica externa	35–50 yr	Typical course: peripheral vascular disease and/or CAD
		Progressive course: 0–20 yr	Progressive course: lethal due to CAD (in the majority of reported lethal cases) or, rarely, other vascular complications
Mönckeberg's arteriosclerosis	Predominantly media	60–80 yr	Typically benign

fibroelastosis of the atria, ventricles, and atrioventricular valves [38,80,81] with clinical manifestations of mitral valve prolapse [82] or other forms of valvular heart disease [83] or restrictive cardiomyopathy [80,84].

Intermittent claudication of the lower extremities is the most common cardiovascular symptom of PXE, occurring in 30% of patients [31], usually from 30 years of age and older [31,85] and ultimately in nearly all patients to some degree, although it may occasionally be observed as early as 9 years of age [43]. The peripheral artery calcification is usually patchy such that the ischemia produced progresses slowly enough to allow for the development of collateral circulation in the extremities [85–87]. Therefore, the serious consequences of gangrene and total loss of an extremity are rare in PXE and usually coincide with other risk factors of vascular disease [85]. Severe stenosis and occlusion of the ulnar, radial celiac, splenic, renal interlobar, and femoral arteries have been observed angiographically and/or Doppler ultrasonographically [21,28,34,43,85,88]. Symptoms of intermittent claudication of the upper extremities are rare, but early fatigability has been reported. The rarity of intermittent claudication in the upper extremities may result from the development of an obviously sufficient arterial collateral blood supply to the radial and ulnar vascular bed originating from the interosseous artery and is related to the lesser muscular mass of the upper extremity [30].

Abdominal angina results from celiac artery stenosis [88] and is the second most frequent abdominal complaint in PXE [30]. Hypertension, an additional risk factor for vascular disease and hemorrhage, was observed in 8–22% [27,31] of patients studied. Hypertension has also been observed as the presenting manifestation of PXE in childhood [88–90].

Involvement of the major vessels and the heart does occur but is rare. More severe cardiovascular complications can occur. Myocardial infarction has been reported only rarely, but examples of very young patients 13 years [44], 18 years [47], and 19 years old (unpublished data) who succumbed to myocardial infarction have occurred. There is also an uncertain number of cases of early cardiac death due to PXE in whom the diagnosis of PXE as the causative underlying disorder had not been established at the time of death [45]. The incidence of strokes is rare [31]. A more common, potentially serious complication is gastric hemorrhage, with hematemesis as the presenting sign. This complication has been reported variously in from 8% to 19% [31,91] of patients. Gastric hemorrhage is slightly more common during pregnancy and in younger patients (under age 29 years). Death due to gastrointestinal hemorrhage in PXE has been reported [91].

Pathophysiology

Comparable to the loss of elasticity in skin, the gradual loss of arterial elasticity secondary to the gradual destruction of the vascular myoelastic tissues with subsequent calcification is the most obvious and logical pathophysiologic mechanism for the cardiovascular manifestations in PXE. This mechanism has a continuous, potentially "vicious cycle" effect on the arterial vascular system and the heart with unphysiologically increasing vessel stiffness (vascular impedance) and subsequent hemodynamic complications. Although increasing arterial impedance is, to some extent, a physiological process occurring with increasing age, there has been no doubt since the original and groundbreaking hemodynamic studies of Carlborg in 1944 [21] that the physiological process of age-related increased vascular stiffening is accelerated in PXE beyond the physiological pace.

Carlborg demonstrated in two morphologically distinctly structured arterial segments, elastic conduit vessels and arteries of the muscular type, different hemodynamic effects which were specific for the morphological type of arterial segment. Elastic conduit vessels showed a significant premature increase in arterial stiffening in PXE in comparison with normal controls, as documented by an increased pulse wave velocity and an increased pulse pressure amplitude. Increased vascular stiffness may be associated with hypertension. The functional impairment of the elastic properties of the conduit vessels is related to the histomorphological findings of destruction of the arterial myoelastic tissue (Fig. 10). The impaired compliance of the arterial conduit in storing and transforming the intermittently ejected left ventricular stroke volume into a continuous flow toward the periphery increases the left ventricular workload, resulting in left ventricular hypertrophy. Increased left ventricular muscular mass and wall tension may functionally enhance the morphological changes within the coronary artery system toward an early manifestation of coronary insufficiency in PXE, with angina pectoris or myocardial infarction, among others, as possible clinical manifestations. Elevated pulse pressure has been suggested to be involved in the development and progression of large vessel atherosclerosis [92] and small vessel disease [93–96]. In fact, pulse pressure, although not an ideal measure of conduit vessel function, has been shown to be an important determinant of cardiovascular and, in particular, coronary events [97–103].

Carlborg's functional findings in the peripheral arteries of the muscular type were different from those observed in the elastic conduit vessels. As the functional result of the morphological changes in muscular arteries that cause diminished arterial elasticity in PXE, he found symmetrically in the radial artery (arteria radialis) and the anterior tibial artery (arteria tibialis anterior) characteristically changed sphygmograms and a symmetrically abnormally low pulse wave velocity in comparison with healthy subjects.

The sphygmograms showed a prolonged crest time, a very low height and amplitude of the pulse curve, and a prolonged and convex shape of its descending limb. These findings were in accordance with his clinical findings of a weakened peripheral pulse with a low and slowly increasing amplitude in patients with PXE. In arteries of the muscular type, the amount of elastic tissue is considerably less than in the elastic conduit vessels. The elastic properties of the muscular arteries are therefore noticeably influenced by the natural viscoelasticity of the arterial smooth muscle layer. The effect is pronounced in PXE due to the destruction of the elastic laminae and the medial calcification. The PXE sphygmograms of peripheral arteries therefore suggest a marked arterial smooth muscle hysteresis effect in these vessels. This effect is characterized by a reduced elastic modulus of the vascular smooth muscle and its decreased distensibility within the arterial wall at a given pressure over a given time interval. The normal pulse duration is too short to fully distend the arterial smooth muscle layer, resulting in diminished arterial wall oscillations. The pathophysiological consequences are the same as have been described for the changes within the elastic conduit vessels — that is, loss of energy during the continuous propagation and transformation of the pulse wave into continuous blood flow and nutrient supply along the arterial bed to the end organs,

with the subsequent, potentially "vicious cycle" adaptations of the cardiovascular system.

In contrast to the peripheral muscular arteries of the extremities, comparable morphological changes in the submucosal gastric arteries result in increased vascular fragility, with subsequent gastrointestinal hemorrhage. Other etiological factors, such as increased gastric acidity with mucosal erosions and subsequent damage to the submucosal arteries, may also be involved. The retinal arteries in PXE also show increased fragility, leading to retinal hemorrhage.

Pregnancy

Pregnancy in PXE has been studied, but further long-term assessment in a larger number of patients is warranted. Viljoen et al. [104] found a slightly higher first-trimester abortion rate compared with the general population and a slightly greater incidence of hypertension. In general, pregnancy was well-tolerated and uncomplicated — an observation confirmed by Neldner and Yoles [31,105]. There are two reports of normal infants from mothers with PXE being "small for age", but a direct relationship with PXE is unverified [106]. A long-term assessment of the effect of pregnancy on the natural course of PXE did show a positive correlation between pregnancy and the long-term overall severity of the disease, which also correlated with the number of pregnancies [31]. If this result is confirmed in a larger number of patients, it would be prudent for women with PXE to limit their number of full-term pregnancies. The unusually high quantities of calcium ingested by many pregnant women (up to 2–3 g daily) may be a contributing factor and should be limited to 1200 mg daily, the RDA (recommended daily allowance) for pregnancy. Calcification of the placenta is known to occur in PXE but also occurs in other conditions. Its frequency and potential significance are unknown.

DIAGNOSIS

Clinical Diagnosis

The initial diagnosis of PXE is based, in most cases, on the presence of the highly characteristic yellowish, cobblestone-like papular skin lesions in bilaterally symmetrical flexural sites (neck, antecubital spaces, axillae, groins, popliteal spaces) (Figs. 1–4). The lateral neck is usually the first site to be involved, and the trained eye can make the diagnosis instantly. A small 3 mm punch biopsy from an affected site is adequate to give positive histological confirmation of calcified elastic fibers in the mid- and deep dermis. (See "Diagnostic Tests" below and also Figs. 11, 12).

The retina is the second place to look for signs of PXE by funduscopic examination. It must, however, be stressed that the characteristic angioid streaks (Fig. 6) may not be present in the early stages of the disorder; therefore, early skin lesions may be present while the retina appears normal. Angioid streaks most commonly first appear a few years after the onset of the skin lesions.

The initial clinical assessment of cardiovascular involvement in PXE includes taking a thorough history for symptoms of intermittent claudication, angina pectoris, gastrointestinal bleeding, hypertension, and exercise-related fatigue of the upper and lower extremities and making a comparative evaluation of the pulse status at different peripheral locations. With the manual measurement of the blood pressure in both upper and lower extremities, the arterial pulse pressure should also be recorded separately for each of the extremities.

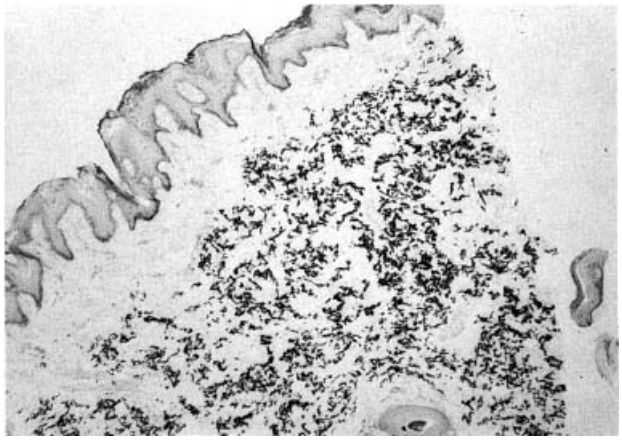

Figure 11. PXE-affected skin. Von Kossa stain. The black staining fibers in the reticular dermis are elastic fibers filled with calcium salts. ×20.

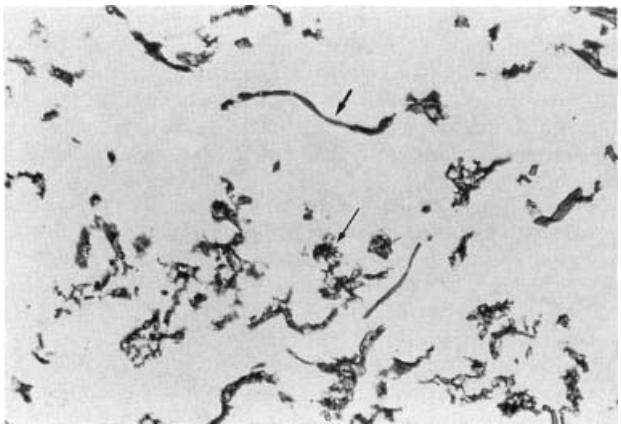

Figure 12. Von Kossa stain. Higher magnification (×250) of sample shown in Figure 11. At this magnification, elastic fibers with early calcification (shorter arrow) and also severely mineralized and ruptured (long arrow) elastic fibers are visible.

It is important to realize that at the time of the first diagnosis of PXE not all three sites of disease expression will necessarily show clinical manifestations of the disease. It is, however, certain that with increasing disease duration all sites will manifest clinical signs and symptoms of the disease but with considerable clinical heterogeneity. Regularly obtained cardiovascular parameters will allow a prospective evaluation of the development of cardiovascular disease in PXE patients.

Prenatal Diagnosis

The identification of the gene locus on chromosome 16 [1,2] permits the prenatal prospective diagnosis of the mutation through linkage analysis in families with a proven case of PXE in at least one other child.

Diagnostic Classification

Historical aspects. Pope's individual and historically justified attempt to describe a wide spectrum of clinical manifestations of PXE, with their subsequent classification into

different diagnostic and potentially etiological and prognostic subclasses [107–109], became obsolete with increasing knowledge about the disease over time. Pope's classification was replaced [110] because his clinical observations had not been made in earlier investigations [27,28] and could not be confirmed in later studies [31]. An additional systematic shortcoming of this classification had been the inevitability of needing to reclassify patients during their individual natural disease course.

Current consensus. At the consensus conference in 1992 [110], it was concluded that all patients seem eventually to merge into a single classic phenotype of the disease, which involves the skin, the eyes, and the cardiovascular system, although considerable clinical heterogeneity of individual expression of the disease at various points in time can be observed. This conclusion, based on clinical observations, implied the concept of pleiotropy of PXE to explain the many different clinical facets of the disease. To guide, facilitate, and unify the diagnostic process for PXE, three major diagnostic criteria (characteristic skin involvement, characteristic histopathological features of lesional skin, characteristic ocular disease) and two minor diagnostic criteria (characteristic histopathological features of nonlesional skin, family history of PXE in first-degree relatives) were defined (Table 3) [110]. This categorization, although not perfect, has proven to be a clinically useful diagnostic aid in evaluating the disease status of different family members because it has become clear that category I defines not only the full-featured clinical syndrome of PXE but also characterizes at the molecular level the genetic homozygote or compound heterozygote for *PXE* on chromosome 16. Category II with its subcategories (a–d) defines at the molecular level the heterozygous carrier state, which may be clinically associated with angioid streaks and/or the minor diagnostic criteria. (For more details, see "Genetics" below.)

Future consensus. Although the diagnostic criteria and their categorization in combination with molecular haplotype data are helpful, although incomplete, in diagnosing PXE and counseling families with PXE, this classification will be replaced with a molecular classification. Now that the PXE gene has been identified, different mutations may be associated with different phenotypic patterns of the disease.

Diagnostic Tests

Light microscopy. The presence of fragmented and calcified elastic fibers in the mid- and lower dermis of affected skin is essentially pathognomonic for PXE (Figs. 11, 12). The only exceptions are the rare acquired forms of localized acquired cutaneous PXE (LAC-PXE), for which the history and clinical presentation are vastly different from classical hereditary PXE. (For further details, see "Histopathology," below.)

Electron microscopy. Although electron microscopy is not routinely used for the diagnosis of PXE, the ultrastructure of calcified elastic fibers is also highly characteristic and pathognomonic for the disorder and may therefore be used if confirmation of the diagnosis is needed. Ross et al. [111] demonstrated that calcified elastic fibers can be detected in skin biopsies of clinically unaffected relatives, such as parents and siblings of patients in families with a recessive mode of inheritance. This morphological finding thus characterized those relatives as heterozygous carriers of *PXE*. (For further details, see "Histopathology" below.)

Ultrasonography. The following tests are recommended as an integral part of a standard clinical workup for each patient with PXE.

1. Doppler ultrasound. The ankle/brachial index (or A/B ratio) compares the arterial occlusion pressures of the upper and lower extremities on each side of the body. The occlusion pressure for the brachial arteries is measured with a sphygmomanometer and that for the dorsalis pedis and posterior tibial arteries in both ankles is measured by Doppler ultrasound. This is a fast, inexpensive, and noninvasive means of determining the competency of the vasculature and blood supply of the lower extremities for screening purposes. The A/B ratio usually correlates quite well with the presence or absence of intermittent claudication and is therefore useful for initial baseline data and follow-up studies.
2. 2-D color-coded ultrasound. This technique is excellently suited to screening for peripheral vascular disease in general and has been used for the diagnosis of vascular disease in PXE [34]. Its high resolution allows the evaluation of arteries, particularly with regard to

TABLE 3. Consensus Diagnostic Categorization of PXE Enhanced by Haplotype Analysis Data for the Chromosome 16 Locus in 81 Families

Category I (three major criteria)	Category IIa (one major criterion and two minor criteria)	Category IIb (one major and one minor criterion)	Category IIc (one major and one minor criterion)	Category IId (two minor criteria)
characteristic flexural skin lesions				
ocular disease in adults	angioid streaks	angioid streaks	angioid streaks	
elastic fiber calcification, lesional skin	elastic fiber calcification, nonlesional skin	elastic fiber calcification, nonlesional skin		elastic fiber calcification, nonlesional skin
	family history of PXE in first-degree relatives		family history of PXE in first-degree relatives	family history of PXE in first-degree relatives

Family members in category I invariably carry two disease alleles. In contrast to those in category I, family members in all subclasses of category II invariably carry one disease allele and one normal allele, as defined by the combined genotypic and phenotypic observation in each family. Genetically, this characterizes family members in this diagnostic category as heterozygous carriers of a disease allele.

morphological changes of the arterial wall structure (thickness and degree of calcification). The color-coded Doppler signal allows a functional assessment of local vessel patency and stenotic or obliterative processes along the vascular bed. In skilled hands, this technique is highly accurate and the first choice for a prospective diagnostic assessment of vascular disease in virtually any location, although peripheral arteries are particularly easy to access.

3. Abdominal ultrasound. With this diagnostic screening tool, Garel et al. [112] demonstrated a pattern of increased echogenicity at the renal corticomedullary junction in six children with renovascular hypertension. Two of these children had PXE. The hyperechogenicity pattern was considered to be due to a diffuse calcification process involving the elastic tissue of the small arteries (interlobar and/or arcuate arteries of the kidney). These patterns of kidney hyperechogenicity have been confirmed in other PXE patients [88,113–115] and also shown to occur in pancreas [88,114] and spleen [114] in these patients. Although this pattern is not pathognomonic for PXE [112,115], it is highly suggestive of vascular changes due to PXE in members of a family with inherited PXE.
4. Echocardiography. This method is indispensable in cardiology and, in PXE, is particularly useful and necessary in evaluating and monitoring cardiac function foremost with regard to morphological changes of the endocardium and potentially subsequently developing valvular heart disease (valve thickening, stenosis, and insufficiency, such as mitral valve prolapse). Diagnosis of these changes makes an antibiotic endocarditis prophylaxis necessary shortly before dental or surgical treatment. Excessive thickening of the endocardium may result in a restrictive cardiomyopathy, which can be diagnosed by echocardiography. Reduced ventricular contractility and local wall mobility, suggestive of myocardial ischemia due to coronary insufficiency, can also be visualized.

Prognostic Laboratory Tests

The prognosis for the progression of cardiovascular disease in PXE is not controlled solely by the defective PXE gene but is additionally influenced by general polygenic risk factors for cardiovascular disease. Therefore, the following tests are recommended for the detection of additional risk factors for vascular disease in PXE.

Lipid profiling. A complete lipid profile (cholesterol, triglycerides, HDL-C, and LDL-C) is most important and should be obtained initially and then followed from time to time over the lifetime of the patient. Abnormal lipid values portend more severe cardiovascular adverse effects.

Fasting glucose test. Any evidence of familial diabetes mellitus, the most common endocrine disease with vascular complications similar to those seen in PXE, should be thoroughly evaluated. Although there is no causal relationship with PXE, the independent, considerable additional risk of vascular disease as a long-term complication of an unrecognized fasting hyperglycemia and the high frequency of diabetes mellitus in western societies make every effort necessary to diagnose abnormal glucose metabolism in PXE patients. This will prevent additional vascular complications through the early recognition of an additional common vascular risk factor and its subsequent therapeutic control.

Specialized Procedures

Endoscopy. Esophageal endoscopy is not indicated unless there are signs of acute gastrointestinal bleeding. Gastrointestinal tract bleeding can occur anywhere in the gastrointestinal tract; however, the stomach is almost always the site of the bleeding. Even with active bleeding, the endoscopic findings are usually nonspecific, showing only a diffuse oozing of blood, without specific focal bleeding points. In some patients, a submucosal, yellowish cobblestone-like pattern can be observed [116,117].

Angiography. Invasive arteriography is indicated for the planning of invasive treatment of severe vascular disease that cannot be managed by conservative procedures.

Magnetic resonance imaging (MRI), computed axial tomography (CAT). These tests are not routinely indicated but have been used on occasion to document aberrant intracranial calcification in PXE patients with symptoms most likely ascribed to a neurological category.

Differential Diagnosis

The differential diagnosis of classical hereditary PXE is usually not difficult. Characteristic skin lesions in the typical flexural sites will, in all likelihood, represent hereditary PXE, which can be confirmed histologically by a skin biopsy (Figs. 11, 12). There are, however, a number of disorders in which angioid streaks have been reported (see Table 1) such that their presence cannot be considered pathognomonic for PXE, although in most instances they are highly suggestive. There are also a number of conditions in which there are skin lesions that superficially resemble PXE and must therefore be considered in the differential diagnosis (see Table 4).

The occasional association of PXE with other hereditary disorders of connective tissue is well-documented, particularly Marfan syndrome, Ehlers-Danlos syndrome, and Paget disease of bone. Less common associations have been reported with tumoral calcinosis and congenital hyperphosphatasia.

Conditions with Histological but no Clinical Resemblance to Hereditary PXE

Localized acquired cutaneous PXE (LAC-PXE). LAC-PXE [118], also called "periumbilical perforating PXE" [119,120], is the best-known example of such a condition. We prefer the former term to emphasize the nonhereditary nature

TABLE 4. Conditions that May Be Associated with or Resemble PXE

Marfan syndrome
Ehlers-Danlos syndrome
Idiopathic hyperphosphatasia
Hyperphosphatemia
Tumoral calcinosis
Vitamin D toxicity
Renal disease with elevated Ca × P product
Penicillamine therapy
Milk-alkali syndrome
β-thalassemia
Localized acquired cutaneous PXE
Saltpeter contact
White papulosis
Papillary dermal elastolysis
Buschke-Ollendorf syndrome

of the disorders included in LAC-PXE. Furthermore, the skin lesions in LAC-PXE are not always strictly periumbilical and do not always show examples of perforating lesions. LAC-PXE has been reported most commonly in black, multiparous, hypertensive, obese females. Type II diabetes has also been described in some. Most such patients do not have angioid streaks; however, one reported patient [120] did do so, which raised the question of a possible hereditary component, although the streaks could also have been acquired. The patient was a 77-year-old, multiparous, obese female with hypertension and severe renal disease with an elevated Ca × P product. After 6 months of hemodialysis and with a normalized Ca × P product, her skin lesions decreased markedly in size, indicating the reversible nature of the basic, *acquired* process. In an example of typical *hereditary* PXE, a reduction in diameter of the lateral neck lesions was seen after several months of a low (400 mg per day) calcium diet [76], again indicating the possible reversible nature of the skin lesions in their early stages and suggesting calcification of elastic fibers in PXE to be a secondary effect.

Saltpeter contact. Contact with saltpeter in farmers with forearm skin exposure from carrying fertilizer bags represents another entity of acquired, highly localized cutaneous patches with a histologically PXE-like appearance [121]. The skin contact areas usually remain localized to the forearms without any of the stigmata of hereditary PXE. Such localized lesions have been reported to last up to 50 years [122].

Conditions with Clinical but no Histological Resemblance to Hereditary PXE

White papulosis of the neck, acquired elastolysis of the papillary dermis. There are several connective tissue disorders that have been described with a PXE-like appearance. These include "white papulosis of the neck" and "acquired elastolysis of the papillary dermis," which is also called "PXE-like papillary dermal elastolysis" [123,124]. Both conditions present flesh-colored papules that commonly occur on the neck but also in other nonflexural sites. They display none of the other signs of hereditary PXE, and the elastic fibers show varying degrees of elastolysis or collagen fibrosis but no calcification. The collagen fibers usually show fibrotic changes.

Penicillamine. Long-term penicillamine therapy can also lead to the production of skin lesions similar to those of hereditary PXE as one of its adverse side effects [125,126]. Again, however, histology shows no calcification of elastic fibers but does show a degenerative alteration of them, called a "bramble bush" deformity.

Hyperphosphatemic tumoral calcinosis (MIM 211900). This condition is, in some patients, associated with angioid streaks of the retina and may also show skin lesions clinically similar to those of PXE. The histological findings in this disease are quite distinct from those in PXE.

Familial hypoalphalipoproteinemia (MIM 107680). This disease, particularly the Detroit type of high-density lipoprotein deficiency (combined deficiency of apolipoproteins A-I and C-III), has been shown to be associated with infiltrative xanthoma of the neck and antecubital fossa [127–129]. These skin features may be misinterpreted clinically as signs of PXE, but the distinct histological appearance of the lesions, the extremely low plasma level of HDL cholesterol, and other distinct clinical manifestations (xanthelasma, arcus cornealis) allow the unequivocal differential diagnosis of hypoalphalipoproteinemia from PXE.

Buschke-Ollendorf syndrome (osteopoikilosis) (MIM 166700). This disease (see also Chapter 10, this volume) manifests skin lesions that can look strikingly similar to the skin appearance in PXE. They also show a symmetrical distribution on the forearms, but are commonly found on the trunk, buttocks and feet, where PXE does not occur. Although the skin disturbance may resemble signs of PXE, the differentiation of both diseases is easy because in osteopoikilosis there is in most cases typical bone involvement, no ocular disease, and a different histopathology.

Conditions with Both Clinical and Histological Resemblance to Hereditary PXE

β-thalassemia. In recent years, there has been an increasing number of reports of cases of β-thalassemia resembling the clinical manifestations of PXE [37,130–133]. Some patients have histological changes in skin biopsies similar to those seen in PXE but do not show angioid streaks, while others have angioid streaks and minimal or no skin involvement. Aessopos et al. [133] found that six of eight patients studied with β-thalassemia had positive skin biopsies with PXE-like lesions and that 52% of a larger group ($n = 40$) had angioid streaks. Cardiovascular manifestations have been mild or have not been detected. β-thalassemia patients commonly have iron overload with high serum ferritin levels, raising the additional interesting question of a possible role for iron in a calciphylaxis-type reaction.

GENETICS

Mendelian Inheritance

Pedigree analysis of families in which PXE occurs demonstrated that PXE is, unequivocally, a genetically predetermined disorder [28,30,31,107–109,134–136]. The disease, although highly variable in its clinical expression in different individuals, nevertheless cosegregates as a Mendelian trait. This means that a particular genotype at one locus is both necessary and sufficient for the trait to be expressed. This definition takes into account that the expression of a Mendelian trait or character most likely involves a large number of genes and environmental factors. (See also "Diagnosis-Current Consensus" above.) "Sporadic" cases, which are also commonly observed, usually represent the autosomal recessive form of the disease or could be new dominant mutations.

Autosomal Recessive Trait

The disease presents primarily and in the majority of families (greater than 90%) as an autosomal recessive trait. This requires that those who are affected according to the diagnostic criteria of category I (see Table 3) be homozygotes or compound heterozygotes at the gene locus. Large, genetically isolated, consanguineous families displaying cosegregation of the PXE trait through multiple generations have been described [137,138].

Autosomal Dominant Trait

Individuals heterozygous at the gene locus express a disease phenotype that is, in principle, indistinguishable from that seen in recessive PXE. Families manifesting PXE in three consecutive generations have occasionally been described in the literature [109,134]. Clinically detected disease expression in three consecutive generations of a family, with transmission of the trait from an affected parent to a child in at least two successive generations,

makes the existence of dominantly inherited PXE possible. Nevertheless, it must be rare (less than 10%) compared with the number of reported families with a most likely recessive inheritance of the disease. The mode of inheritance can clinically be defined unambiguously in only a small number of pedigrees, thereby leaving a certain margin of error, even in the assessment of pedigrees with the occurrence of a PXE affection status in three consecutive generations. The situation may be complicated further by issues of penetrance, late disease onset, or the occurrence of new mutations.

Penetrance

The degree of penetrance of PXE has never been formally investigated. Preliminary analyses in the families we have studied [1–3] indicate that penetrance of the recessive disease may actually be complete or close to complete by the fourth decade of life because almost all individuals that eventually develop PXE do so by their early thirties. In fact, the problem appears more to be late recognition of a manifest illness by physicians not fully aware of the disorder. A reliable estimate for the penetrance of potentially dominantly inherited PXE cannot be given due to the small number of families comprising three or more generations with clinically detectable disease in at least two of them.

Molecular Genetics

Candidate Gene Studies

The first molecular studies to investigate the genetics of PXE were undertaken in 1992 [139] and 1994 [140] and used the candidate gene approach. These studies were either inconclusive or negative. In the former investigation, which examined the possible role of lysyl oxidase in some ten families, genetic linkage to this enzyme was excluded; likewise, no evidence for linkage was demonstrated for the gene encoding fibrillin-2 (*FBN2*) in a family with six affected members, although the power was naturally insufficient to exclude linkage [139]. The latter study investigated the elastin gene as a potential cause of PXE through cosegregation analysis of a gene-inherent RFLP (restriction fragment length polymorphism) in one family with PXE [140].

Identification of the Gene Locus

Applying a two-stage strategy, our group performed, as a first step, a genome-wide screen of 73 affected sib pairs from 38 families with PXE using a commercially available set of single-sequence-length polymorphisms (SSLPs) to identify the chromosomal location of the causative gene, designated *PXE*. Sib pair genotype data analysis with nonparametric allele-sharing algorithms identified a significant excess of allele sharing for three markers on the short arm of chromosome 16 [1,2]. In a second step, we mapped *PXE* to a 3 cm interval on chromosome 16p13.1 by linkage analysis in all available members of 50 families with PXE using a highly polymorphic high-resolution panel of SSLPs and calculating linkage twice: first under the assumption of a recessive, and second under the assumption of a dominant mode of inheritance in all families [1,2].

Heterogeneity

The linkage results with multipoint lod score values of greater than 21 assuming a recessive model and of greater than 16 assuming a dominant model of inheritance suggest considerable homogeneity of the study sample. Further heterogeneity analysis confirmed this suggestion and predicted allelic heterogeneity at the chromosome 16 locus, where allelic variants of a single gene most likely account for recessive and dominant forms of PXE [1,2]. Although possible because it cannot be definitely excluded, there is no convincing evidence in our set of family data for locus heterogeneity of the disease. In fact, others have confirmed our mapping data in different collections of families [141].

High-Resolution Mapping and Genomic Structure of the 500 kb PXE Locus

Haplotype analysis of informative PXE families narrowed the locus to an interval of less than 500 kb [3]. This interval is defined by the markers *D16B9621* and *D16B764*. This region could be covered by three overlapping YAC clones, and a second-layer BAC contig was constructed.

Candidate Genes at the Locus

Two multidrug resistance protein genes, *MRP1* [142] and its homolog *MRP6* [143], represent two possible candidate genes within the *PXE* region on chromosome 16p and belong to the gene superfamily of ATP–binding cassette (ABC) polytopic integral membrane transport proteins. Members of this gene superfamily are also involved in the transport of proteins, amino acids, inorganic ions, large polysaccharides, and peptides in prokaryotes and eukaryotes [144]. Many known ABC genes cause heritable human diseases such as cystic fibrosis (MIM 219700) (*CFTR*) [145] and recessive Stargardt macular dystrophy (MIM 248200) (*ABCR*) [146]. Typically, mutations in ABC genes, which have been shown to be responsible for specific genetic disorders, cause defects in the energy-dependent transport of specific substrates. Therefore, mutations in drug resistance genes of the ABC superfamily could cause PXE through a transport defect eventually leading to the accumulation of substrates such as calcium and/or glycosaminoglycans within the extracellular matrix. Both genes are currently under investigation for PXE-specific mutations by our group due to their physical location within the *PXE* locus on chromosome 16p13.1.

An expressed sequence tag (EST) of a transcript named *pM5*, of unknown function but with domains homologous to those of metalloproteinases, was mapped close to the locus of *PXE* on chromosome 16 by radiation hybrid mapping and into the locus by genetic mapping. This gene had been identified while screening a melanoma cDNA library on the basis of homology to the metal-binding domain of human fibroblast collagenase and had the anticipated function of coding for a serine protease [147]. The physiologic function of this gene has not been determined. Its domain homology to metalloproteinases makes it a potential candidate for *PXE*.

Three similar copies of a novel gene, with different predicted splicing forms, are found in the *PXE*-relevant region [3,148]. This gene has been characterized as encoding a nuclear core complex interacting protein (NPIP). Additional paralogs of this gene are present in several duplicons [148] on chromosome 16 outside the immediate region of interest for PXE, raising the possibility of a novel gene family.

Editors' note added in proof: The genetic basis of PXE is now known to reside in the gene *ABCC6*, as described in the section "Recent Developments", below.

Molecular Analysis of Mendelian Inheritance Patterns

When Mendel introduced the qualitative terms "recessive" and "dominant" to describe inheritance, he intended to reduce the very complex and quantitative phenomenon of allelic control of phenotypic traits to the two extreme ends of a continuous spectrum. At the "dominant" end of the spectrum, one mutated allele of a gene is necessary and sufficient to result in expression of the Mendelian trait, whereas at the "recessive" end of the spectrum, two

allelic variants are needed to express the same trait. This reductionist approach was very helpful to Mendel to convey a revolutionary concept that has proven to be useful in clinical medicine for a coarse and greatly simplified categorization of different inheritance patterns for the same disease. This is important for the individual and familial risk assessment applied in genetic counseling.

Correlating the consensus diagnostic criteria [110] and their subsequent arbitrary, diagnostic categorization of the PXE trait with our familial haplotyping data, it is evident that all sib pairs fulfilling the diagnostic criteria of category I (Table 3) invariably share two alleles and are homozygotes or compound heterozygotes at the locus. These individuals show the full clinical picture of the disease and therefore carry the recessive trait. Siblings with signs of PXE according to category II diagnostic criteria share with siblings belonging to category I only one allele and are heterozygotes as defined by the combined genotypic and phenotypic observations in each family. We were unable to identify sib pairs who belonged to category I and shared only one allele at the locus. We did not detect PXE as defined by diagnostic category I in three consecutive generations of any family in our study. Therefore, most family members with signs of PXE according to diagnostic category II and with a single disease allele at the locus are heterozygous carriers of the recessive trait. At this time, there is no positive confirmation by haplotype analysis for the existence of a dominant PXE trait. Although the haplotype data in our families cannot definitely exclude the existence of a dominant PXE trait at the molecular level, they do provide further evidence, in addition to the rarity of families with unequivocal category I disease in three consecutive generations that are not consanguineous, that a dominant PXE trait must be rare, if it exists at all. The haplotype data further confirm that those heterozygous for the recessive trait may develop angioid streaks or a positive skin biopsy over time. At the time of the consensus conference, it was not clear whether the diagnostic criteria of category II defined the heterozygous carrier of the dominant or recessive disease.

Molecular Genetic Considerations in Relation to Cardiovascular Disease

It is important to note that, in contrast to other polygenic diseases associated with increased vascular impedance, accelerated arterial stiffening in PXE has a broad hereditary base; in other words, it is predominantly influenced by allelic variants of a single gene. PXE could therefore develop as a "monogenic model disease" for the analysis of the molecular pathways and the identification of genes responsible for the development of premature vascular aging and subsequent cardiovascular complications. Moreover, it has been observed that although the canonical triad of clinical PXE is predominantly a recessive trait, the vascular disease can manifest as a dominant trait [149]. This identifies heterozygotes as susceptible to early clinical manifestations of cardiovascular disease. It remains to be seen whether heterozygotes in PXE families show an increased risk of early manifestation of cardiovascular disease in general or whether such risk is associated with certain allelic variants of *PXE*.

The vascular histomorphological similarities of idiopathic arterial calcification of infancy (IACI) with the vascular and skin lesions in PXE, the association of PXE with at least one case of IACI, and the comparatively extremely early manifestation of cardiovascular death in IACI in the majority of cases raise the intriguing question of whether IACI is the homozygous lethal manifestation of a dominant cardiovascular PXE trait associated with specific allelic variants of *PXE*. Because this remains a speculative question for the time being, *PXE* will, after its identification, be a prime candidate for exclusion as the molecular cause of IACI, particularly in consideration of the obvious difficulties in undertaking a gene mapping study in this disease.

HISTOPATHOLOGY

Light Microscopy

By light microscopy, fragmented and calcified elastic fibers in the mid- and lower dermis of affected skin can be demonstrated as the essential pathognomonic finding in hereditary PXE. These findings may also be observed to a lesser extent in carriers of the disease. As discussed under "Differential Diagnosis" above, the two exceptions to this are the rare acquired forms LAC-PXE and "contact–PXE" through saltpeter, which show a similar histopathology. In well-established lesions, the diagnosis can be made with ordinary hematoxylin and eosin or elastic tissue stains; however, the von Kossa stain (Figs. 11, 12) is the "gold standard" in that it stains specifically for calcium salts and dramatically shows the calcified elastic fibers that confirm the diagnosis. Calcified and fragmented elastic fibers can also be demonstrated in the myoelastic tissue of arteries (Fig. 10). Uncalcified elastic fibers appear essentially normal with elastic tissue stains before their mineralization. The dense deposits of glycosaminoglycans (polyanions) in the dermis can usually be demonstrated with alcian blue stain. The numerical increase in elastic fibers in the dermis may or may not be detectable by light microscopy. Although of unknown significance, it is common to see many mast cells in the PXE dermis. They may be concentrated in perivascular locations or scattered throughout the dermis. As many as 12–15 mast cells per high-power field may be seen.

Electron Microscopy

The ultrastructure of calcified elastic fibers, particularly the initial observation that calcium salts are confined to the central core of the fiber, is also highly characteristic and pathognomonic for the disorder [31,150] (Fig. 13).

There is always a darker, more electron-dense band surrounding the central calcified core, which is of unknown

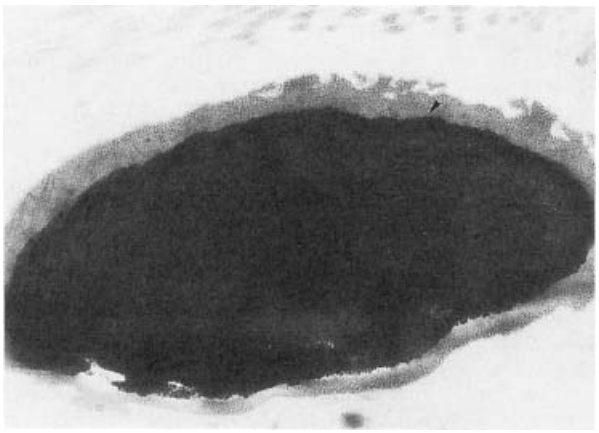

Figure 13. Electron micrograph (×30, 000) of an early mineralized elastic fiber. Note the band of normal-appearing elastic fiber and microfibrils at the periphery and the solid electron-dense, calcified core. A discrete, sharp peripheral margin to the mineralized elastin is commonly seen (arrowhead).

significance (Fig. 13). As the process evolves, the core calcification of some fibers seems to clear, leaving an electron lucent area, but with the peripheral dark band remaining. The natural course appears to be a gradual complete calcification of the fiber, with only tiny sites of normal-appearing elastic fiber at the periphery remaining. The fiber then seemingly "bursts," leaving a totally fragmented structure (Figs. 14, 15).

Collagen fibers, especially those adjacent to calcified elastic fibers, occasionally show partial or total calcification. Interference with fiber formation to produce so-called flower figures is also common [78] but not specific for PXE. Less often, masses of fine filaments (microfibrils?) and amorphous granular material are seen in the same area and described as granulofilamentous material. Such masses are of unknown origin but are thought possibly to represent an abortive effort to make new elastic fibers.

Figure 14. Electron micrograph (×12, 000) of a more advanced stage of calcification showing nearly total involvement with beginning fragmentation of the elastic fiber.

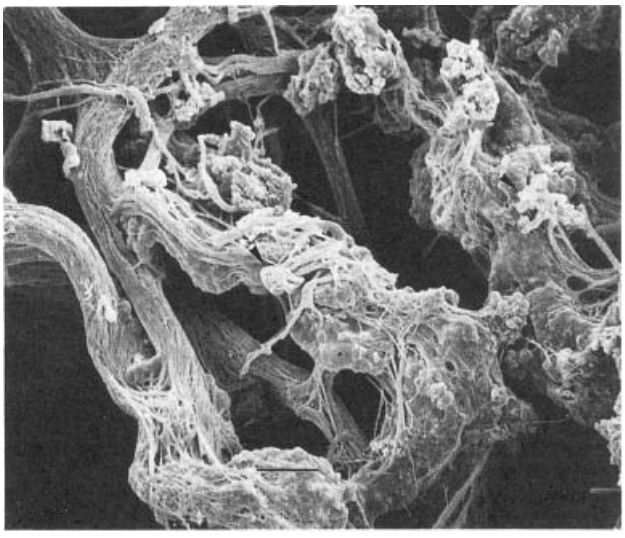

Figure 15. Scanning electron micrograph of elastic fibers showing the abrupt sites of mineralization. Bar = 1 μm. (Courtesy of Dr. Takuo Tsuji.)

BIOCHEMISTRY

There are three unequivocal biochemical abnormalities recognized in PXE:

- Elastic fiber mineralization predominantly by calcium carbonate and calcium phosphate, although other elements, especially magnesium and possibly iron, aluminum, and copper, may also be involved.
- Increased deposition of glycosaminoglycans in the affected dermis.
- Increased production of elastic fibers in affected skin.

Cutaneous Elastic Fibers and Amino Acid Composition

Only a few attempts have been made to isolate and analyze PXE elastic fibers for amino acid content, and these have yielded varying results. This is due, at least in part, to the difficulty of extracting pure elastic fibers from skin. Different extraction methods yield different concentrations of amino acids from the same dermis. Increases in the content of polar amino acids (aspartic and glutamic acids, lysine, arginine) have been observed somewhat more consistently than others [31]. The significance of this is unknown other than there being a possible theoretical role for glutamic acid as a precursor of γ-carboxyglutamic acid, which has a specific affinity for calcium and has been implicated in bone mineralization.

Glycosaminoglycans

After the seminal observation that elastic fibers were the target in PXE, the next observation at the molecular level was that there was an increase in glycosaminoglycans (GAGs), formerly known as mucopolysaccharides (MPS), throughout the affected dermis [151]. The excessive deposition of GAGs in affected skin has led to speculation that these compounds might be involved in the pathogenesis of PXE [152]. As previously noted, most observers have held the view that elastic fibers are morphologically normal before mineralization; however, one report [77] suggested that they appear more granular than normal, and that this granularity is secondary to an increased content of polyanions within the fiber, on the basis of increased staining with osmium-ruthenium red following EDTA demineralization.

Earlier studies in PXE [151] showed the GAGs to be mainly hyaluronan and chondroitin sulfate (mainly chondroitin 4-sulfate B). More recent studies by Longas et al. [153] showed relatively large amounts of dermatan sulfate in addition to hyaluronan and chondroitin 4-sulfate B in the skin and urine of PXE patients. These authors also reported an altered form of chondroitin 6-sulfate in urine. This observation was confirmed by Walker et al. [154], who identified chondroitin 6-sulfate using immunohistochemical staining. Tiozzo Costa et al. [155] found decreased amounts of sulfated glycosaminoglycans in cultured PXE skin fibroblasts compared with normal controls and increased production of a high-molecular-weight fraction of sulfated proteoglycans.

Two multidrug resistance protein genes, *MRP1* [142] and its homolog *MRP6* [143], are located within the *PXE* locus on chromosome 16p. Apart from their specific protective function of preventing cytotoxicity through transmembranous transport of cell toxic substances, they may also be nonspecifically involved in the transport of inorganic ions, large polysaccharides, and peptides in prokaryotes and eukaryotes [144]. The unspecific transport

function of these genes and their localization within the *PXE* locus are the reasons for their consideration as candidate genes for PXE (see "Candidate Genes at the Locus" under "Molecular Genetics" above).

Proteinases, Elastases, and Elastogenesis

The possibility of the elastic fibers in PXE being uniquely sensitive to enzymatic degradation by proteinases has been considered as a factor in the pathogenesis of the disorder but no conclusive link has yet been established.

Various species of elastase are produced by the pancreas, polymorphonuclear leukocytes, platelets, macrophages, fibroblasts, smooth muscle cells, synovial membranes, and bacteria. However, these and serum elastase inhibitors, such as α1-antitrypsin and α2-macroglobulin, have been sparsely studied, and no clear implication for a role in pathogenesis has yet emerged. Pasquali Ronchetti et al. [156] reported that both the calcified and uncalcified elastic fibers in PXE had a normal lytic response to porcine pancreatic elastase and that the dermal granulofilamentous material described by Danielsen [157] was sensitive to degradation by elastase and hyaluronidase. It was also partially removed by trypsin and chondroitinase but not by collagenase. The great stability and resistance of normal elastic fibers to chemical destruction and normal catabolism suggests that an abnormal elastolytic enzyme might be required to alter a fiber and specifically create a highly localized environment in or around it that would allow the mineralization process to proceed without initially destroying the fiber.

The elastic fiber precursor, tropoelastin, is known to be highly sensitive to degradation by proteinases, including leukocyte and pancreatic elastases at physiologic pH and ionic strength, and therefore might be uniquely sensitive to an abnormally active PXE proteinase. The age of an individual fiber may also be important. The more rapid destruction of elastic fibers could also be the feedback stimulus to synthesize more elastic tissue, which is known to occur in PXE. Perhaps the granulofilamentous material represents an abortive attempt by the fibroblast to synthesize new elastic tissue but one that is at least partially blocked at a post-translational stage such that tropoelastin becomes the granular portion of the granulofilamentous material rather than moving on to become mature elastic fibers.

Schwartz et al. [158] demonstrated an elastase-like proteinase that degraded a synthetic substrate, succinyl-trialaninyl-p-nitroanilide (SANA), in cultured PXE lesional fibroblast medium and was not found in normal control fibroblast cultures. The nature of the proteinase and its kinetics however, were, not studied.

Gordon et al. [159,160] studied proteoglycanase activity in the medium of cultured PXE fibroblasts. They found a cysteine proteolytic enzyme with proteoglycanase activity, which was markedly (500-fold) enhanced by the addition of zinc and, to a much lesser degree (70-fold), by the addition of $CaCl_2$. Normal control fibroblasts did not produce this enzyme. The authors did not further purify or study the enzyme.

A gene named *pM5*, with homology to metalloproteinase domains, was identified at the *PXE* locus [3,141,147,148]. Its homology to metal-binding domains of human fibroblast collagenase, a matrix degrading metalloproteinase, suggests it as a potential candidate for *PXE*.

Calcium, Phosphorus, and Vitamin D Metabolism

There is good evidence that any condition resulting in an elevated Ca × P product can present as a complication aberrant calcification in the skin, arteries, and other organs, causing large necrotic ulcers and arterial occlusion secondary to vascular calcification [161]. There are also reported examples of unusual combinations of PXE with an elevated Ca × P product, which appears to accelerate greatly the natural course of PXE. Elevated serum phosphorus is usually associated with high levels of alkaline phosphatase, which is normally involved in calcification and remodeling of bone. Chronic renal disease and hyperparathyroidism are two conditions that commonly display a high Ca × P product, usually in excess of 60. Alkaline phosphatase activity is high during childhood and adolescent growth spurts, which is also the average time of onset of PXE and its most rapid period of progression.

Some of the rare congenital and idiopathic disorders of calcium and phosphorus metabolism at least suggest a possible aggravating role of aberrant calcium and phosphorus metabolism in the pathogenesis of PXE. In rare instances, PXE has been noted in association with chronic idiopathic hyperphosphatasia, which is characterized by high levels of serum alkaline phosphatase activity and with elevated levels of serum calcium and phosphorus. One such case was followed by Mitsudo [162] from age 6 years to death at 18 years. This individual had moderately elevated levels of serum total calcium (10.7 mg/dl) and phosphorus (5.8 mg/dl) with a Ca × P product of 62. At death, his alkaline phosphatase was very high. All aspects of his PXE progressed with great speed, resulting in hypertension at age 6 years, retinal hemorrhages at age 9 years, and a fatal stroke at age 18 years.

Tumoral calcinosis (MIM 211900) is predominantly an autosomal recessive disorder of children and young adults in which large localized soft tissue calcium deposits occur, most commonly around major joints. Several cases have been reported in association with PXE in which the PXE had an early-childhood onset and progressed more rapidly than usual [163]. Most of these patients also had elevated serum phosphorus levels with a high Ca × P product.

Self-induced dietary disorders, such as the milk-alkali syndrome or hypervitaminosis D, will result in hypercalcemia. Prolonged hypercalcemia impairs renal function, which, in turn, causes hyperphosphatemia. When the Ca × P product reaches critical levels, soft tissue calcification commonly occurs but not specifically affecting elastic fibers or in a PXE distribution. Mallette and Mechanick [163] reported a 60-year-old white male with PXE, hyperphosphatemia, and elevated 1,25-dihydroxyvitamin D levels. His clinical course was complicated by severe, occlusive peripheral vascular disease, retinal hemorrhages, and hypertension. His milk intake had exceeded 4.5 liters per day for many years.

The Fibroblast

Because the fibroblast is the source of all the extracellular matrix of the skin, it is most likely the origin of any abnormal tropoelastin, GAG, or proteinase, although aberrant post-translational modification of any otherwise normal extracellular connective tissue matrix component cannot be ruled out. Much more is known of collagen synthesis and turnover, in which at least ten specific enzymes are required for synthesis and degradation, many of which require additional cofactors and cosubstrates (see also Chapter 2, this volume). With electron microscopy, PXE fibroblasts appear metabolically active with a large, rough endoplasmic reticulum filled with granular material. However, they are morphologically indistinguishable from fibroblasts in any non-PXE healing or rapidly growing site.

MANAGEMENT

There is currently no specific or causal treatment available for the disease.

Prophylactic Lifestyle

Prophylactic measures can be taken by any patient with PXE in an attempt to prevent, or at least postpone, known complications of the disease as long as possible. Most physicians, unfortunately, neglect the prophylaxis of complications of hereditary disorders. There are many recommendations, and the earlier in life they are started, the more effective they will be. The recommendations outlined below are primarily designed for the prevention of adverse effects rather than for the treatment of an existing complication.

General:

- A proper attitude toward any chronic disorder is important. A healthy balance between excessive concern on one hand and a denial or disregard of preventive measures on the other is important. Learning to cope with PXE may take many years, especially for adolescent and younger individuals.

Activities:

- A regular exercise program at a level that is well-tolerated should be developed. If nothing more, it should involve a vigorous daily walk.
- Head trauma can cause retinal hemorrhages. Sports involving potential head trauma or very heavy lifting should be avoided, especially in the adolescent years. These include such activities as boxing, soccer, football, rugby, hockey, heavy weight lifting, and so forth. Sports such as track, swimming, or bicycling are recommended.

Tobacco Use:

- The use of tobacco in any form should never be started. If it is being consumed, it should be stopped immediately.

Dietary:

- A normal weight for age and height should be maintained.
- A dietary calcium intake at or slightly below the recommended dietary allowance (RDA) of 800 mg per day should be maintained, even during the childhood/adolescent years. A well-balanced diet, rich in antioxidants, is also recommended. A good starting point is to follow the current recommendation of eating five servings of fruit and vegetables each day.

Prophylactic Medical Care and Symptomatic Treatment

- Any slight vomiting of blood or the appearance of black tarry stools can be a sign of stomach bleeding. This is a medical emergency and requires immediate attention.
- Routine eye examinations should be carried out by a retinal specialist, preferably with experience in treating PXE and a conservative attitude toward laser treatment of retinal hemorrhages. The value of laser therapy for retinal hemorrhages in PXE is controversial and believed indicated only in very selective situations. Excessive ultraviolet light exposure should be avoided by wearing good UV-filtering sunglasses when outdoors in the bright sun for prolonged periods.
- The complete lipid profile should be checked regularly (cholesterol, triglycerides, HDL-C, and LDL-C). An abnormal result in any of the four categories is cause for concern and should be vigorously treated by dietary means and, if necessary, with medication.
- Blood pressure should be checked regularly. If elevated, an initial effort should be made to control it through diet, weight loss, and exercise. Drug treatment should be added if necessary.
- Echocardiography should be carried out at regular time intervals. If endocardial thickening of the heart valves has been diagnosed, which may result in functional valve impairment causing mitral valve prolapse (MVP), antibiotics should be taken before surgical or dental procedures.
- The long-term daily use of aspirin or any other substance of the ibuprofen group of analgesics should be avoided. They are also good anticoagulants and could precipitate or aggravate a retinal or gastrointestinal hemorrhage. Occasional use is safe. In the case of cardiovascular complications that require the long-term use of anticoagulants, a specialist should be consulted.

ANIMAL MODELS

There are no known animal models of pseudoxanthoma elasticum.

RECENT DEVELOPMENTS

Beat Steinmann and Peter M. Royce

Identification of the PXE Gene

Four groups have reported independently, within a short time of each other, that mutations in the *ABCC6* gene (formerly known as *MRP6* or *eMOAT*) are responsible for causing PXE [164–167]. A historical account of the events leading to discovery of the gene has been given by Pennisi [168].

ABCC6 — The Gene Encoding a Transporter for an Unknown Substrate

The *ABCC6* gene is located on chromosome 16p13.1, spans approximately 73 kb, containing 31 exons, and codes for an mRNA approximately 6 kb in size with an open reading frame of 4.5 kb. The polypeptide ABCC6, also called MRP6 (multi-drug resistance protein 6), is a member of the ATP-binding cassette (ABC) transmembrane transporter subfamily C, and comprises 1503 amino acid residues with a calculated molecular mass of 165 kDa. It is located in the plasma membrane, has 17 membrane-spanning helices, grouped into three transmembrane domains (TMD1-3), and two intracellular nucleotide binding domains NBD1 and NBD2 (see Figure 1 in [169]), each of which is built of approximately 200 highly conserved amino acid residues containing the Walker A and B domains that bind and hydrolyze ATP, as well as a C-terminal motif that is critical for the function of the protein as a transmembrane transporter [170]. ABCC6 is highly homologous to ABCC1, with a sequence identity of 45%, and the respective genes, *ABCC6* and *ABCC1*, both containing 31 exons, are located in the same region, being orientated in opposite directions.

The 4.5 kb ABCC6 mRNA is expressed in several secretory tissues, but primarily in kidney and liver. Immunolocalization studies have identified a basolateral location of ABCC6 in both hepatocytes and renal epithelial

cells [171]. ABCC6 is a transporter for an as yet unidentified physiologic substance, although experimentally it is known to transport a non-physiologic substrate, the cyclopentapeptide Trp–Asp–Pro–Val–Leu, the endothelial receptor antagonist BQ-123, and amphipathic anion conjugates.

Mutations in the *ABCC6* Gene (MIM 603234)

Le Saux et al. [169] studied 122 unrelated patients with PXE and compiled all 43 *ABCC6* mutations known to date. Thirty-six *ABCC6* mutations were characterized in their population, among which 28 were novel variants. Twenty two alleles have been missense variants, nine have had small insertions or deletions, six have contained nonsense mutations, two have contained mutations likely to result in aberrant mRNA splicing, two have had large deletions, and one has had a contig deletion comprising both flanking genes, *ABCC1* and *MYH11*del [169].

Although most mutations appeared to be unique variants, two disease-causing alleles occurred frequently in apparently unrelated individuals. One, Arg1141Ter was found at a frequency of 18.8% and was preponderant in European patients. The other, a large deletion, *ABCC6*del exons 23-29, occurred at a frequency of 12.9% and was prevalent in patients from the United States. This latter 16.5 kb *Alu*-mediated deletion was described in four multiplex families with PXE inherited in an autosomal recessive pattern by Ringpfeil et al. [172], and in a further such family by LeSaux et al. [169]. In each of the families the proband was a compound heterozygote for a nonsense mutation and the approximately 16.5 kb deletion mutation spanning the site of the nonsense mutation in *trans*. The nonsense mutation was thereby reduced to hemizygosity in these patients. The deletion mutation was shown to extend from intron 22 to intron 29, resulting in an out-of-frame deletion of 1213 nucleotides from the corresponding mRNA, and causing the elimination of 505 amino acid residues from the ABCC6 polypeptide. The deletion breakpoints were precisely the same in all families, which were of different ethnic origins, and haplotype analysis suggested that the deletions had occurred independently. Deletion breakpoints were embedded within *Alu*Sx repeat sequences, suggesting *Alu*-mediated homologous recombination as a mechanism.

The distribution pattern of *ABCC6* mutations showed a cluster of disease-causing variants in a region of the gene encoding the C-terminal end of the protein, in contrast to the neutral variants, in which alterations appeared to be evenly distributed. Indeed, most mutations (36 of 43), including deletions, were located in the 3′ portion of the gene, and, remarkably, 24 (56%) mutations were located in exon 24 and exons 28-30, the latter of which encode the NBD2 domain. The fact that only a few mutations affect NBD1 indicates that the two nuclear binding domains are functionally nonequivalent [169]. This supposition is supported by the fact that in the case of *ABCC1*, which is highly homologous to *ABCC6*, mutations affecting NBD2 abolish its function whereas those affecting NBD1 decrease transport activity by about 70% [173]. In keeping with this, homozygous or compound heterozygous mutations in the region of *ABCC6* encoding NBD1 may not contribute significantly to the development of the PXE phenotype, because an intact NBD2 could sustain residual activity (approximately 30%) which could be comparable to the overall transport capacity (50%) of an asymptomatic individual carrying a single disease-causing allele.

Among the 122 patients studied by Le Saux et al. [169], putative disease-causing mutations were identified in approximately 64% of the 244 chromosomes studied, and 85.2% of them were found to have at least one disease-causing allele. The mutation detection rate achieved in the study indicated that a significant number of mutations escaped detection by SSCP (single-stranded conformation-dependent polymorphism) and CSGE (conformation sensitive gel electrophoresis); the results also suggest that a fraction of undetected mutant alleles could be either genomic rearrangements or mutations occurring in non-coding regions of the gene [169].

Genetics

Nonsense, missense, deletion, and insertion mutations are associated with sporadic, autosomal recessive and autosomal dominant forms of PXE. Large deletions may lead to haploinsufficiency. The molecular mechanisms associated with both autosomal recessive and autosomal dominant PXE due to missense mutations are not clear, but a number of similar phenomena have been described. For instance, glycine substitutions in *COL7A1* severely affect folding of collagen VII, causing dominant dystrophic epidermolysis bullosa (DEB), whereas similar glycine mutations which have little effect on the folding of collagen result in autosomal recessive DEB [174].

Pathogenesis

The pathogenesis of PXE remains unknown. It is possible that, because of an aberrant transport function of ABCC6 in the kidney and/or the liver, the lack of an unknown substrate(s) in the blood could lead to secondary changes in the dermis and the arterial wall that lead to calcification of elastic fibers and the development of PXE. Indeed, for these reasons, Bergen [166] commented that the molecules presumably transported by ABCC6 may be essential for extracellular matrix deposition or the turnover of connective tissue at specific sites, and Uitto et al. [175] have suggested that perhaps PXE is more a heritable metabolic disorder than a primary connective tissue disease. In this respect it would be akin to alkaptonuria. In keeping with this concept, acquired PXE-like symptoms have been associated with altered renal function; elastic fiber calcification has been shown, for example, in a patient with chronic renal failure and periumbilical PXE [120], in another with calciphylaxis and renal failure [161], and in many with β-thalassemia and sickle-thalassemia after prolonged transfusion and chelation therapy [130–133], treatments that are known to influence renal function. It is possible, therefore, that abnormal renal function in patients with the acquired disorder results in altered levels of ABCC6 which, in turn, lead secondarily to symptoms of PXE.

Alternatively, subtle changes in the expression of the ABCC6 transporter in affected tissues in patients with PXE may be instrumental in inducing an inappropriate extracellular matrix environment for the correct assembly of elastic fibers. However, by RT-PCR analysis using RNA isolated from tissues affected by PXE, Bergen et al. [166] detected expression of ABCC6 in retina, skin, and vascular tissue, but only in very small amounts, which makes the pathogenic role of this route less likely.

No significant correlation could be established between the disease-causing alleles and the clinical manifestations in the 26 patients for whom the pertinent information was available [169]. However, the small size of the cohort may have been a factor in the lack of any obvious genotype-phenotype relationship. Although the severity of the phenotype seemed to be related to the age of the patients, it appeared not to be mutation dependent because the

mutations identified in them (premature termination codons and missense mutations located in NBD2) all predict the complete loss of ABCC6 function. It is therefore reasonable to assume that PXE mutations predispose carriers to the typical PXE clinical lesions, but that the contribution of other factors — such as trauma in the case of ocular lesions, and, perhaps, diet or lifestyle — may determine the age of onset of clinical manifestations and their severity.

Further functional studies of the substrate specificity of ABCC6 and the effect of its reduced capacity are needed to elucidate its role in maintaining elastin fiber integrity and its role in the development of PXE.

FUTURE INVESTIGATIONS

The next step will be the identification of further disease-causing mutations and their correlation with the clinical spectrum of the disease. To create a *PXE* knockout mouse, the murine homolog needs to be identified. The generation of a knockout or knockin mouse will allow the experimental study of gene function in relation to cardiovascular, ocular, and dermal phenotypes of the disease. It will be intriguing to study whether non-*PXE* allelic variants of *PXE* can be associated with early manifestations of cardiovascular disease or with the occurrence of early macular degeneration in the general population. All of these investigations will eventually provide potential molecular targets for the design of forms of treatment that either prevent or alleviate potential complications of the disease in patients.

PATIENT SUPPORT GROUPS

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Chapter 12

Marfan Syndrome and Other Microfibrillar Disorders

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SUMMARY

- **Marfan syndrome (MFS) is a pleiotropic, autosomal dominant disorder of connective tissue that affects 1–3 per 10,000 individuals, regardless of ethnicity. The condition is characterized by abnormalities in the ocular, skeletal, and cardiovascular systems. The eye shows lens dislocation and early myopia. Retinal detachment, early glaucoma, and cataract are occasional findings. The skeleton demonstrates overgrowth of the bones and joint hypermobility. The cardiovascular system shows enlargement of the aortic root with a predisposition for dissection or rupture. Mitral valve prolapse is common. Aortic or mitral valve regurgitation can cause myocardial dysfunction.**
- **MFS can cause major morbidity and mortality if untreated. The average age of death, usually due to aortic dissection, for affected individuals who receive no treatment is in the fourth decade. Left ventricular failure due to mitral valve regurgitation and volume overload is the leading contributor to mortality in young children with a severe variant of MFS.**
- **Improvements in diagnostic, medical, and surgical practices have resulted in an extension of life expectancy in MFS to near normal. Close echocardiographic follow-up of aortic root size, the use of medications to decrease hemodynamic stress, and prophylactic surgical repair of the ascending aorta are mainstays of the therapeutic protocol.**
- **All cases of MFS for which a basic defect has been defined demonstrate heterozygosity for a mutation in the *FBN1* gene, which is located at 15q21.1 and encodes the extracellular matrix component fibrillin-1. This glycoprotein is the major constituent of extracellular microfibrils, which are abundant in both elastic and nonelastic tissues.**
- **The diagnosis of MFS remains based largely on clinical criteria. Molecular adjuncts to diagnosis are applicable in selected circumstances. Conditions that show clinical overlap but do not meet criteria for the diagnosis of MFS have been grouped separately. Included among these are the MASS phenotype, familial thoracic aortic aneurysm or dissection, familial ectopia lentis, and familial tall stature. Defects of *FBN1* have only been found in a minority of patients and families warranting a diagnosis of one of these conditions.**
- **Congenital contractural arachnodactyly (CCA) affects predominantly the skeleton and is caused by mutations in the *FBN2* gene, which is located at 5q23-q31 and encodes fibrillin-2.**
- **The fibrillins are structurally related proteins that are both incorporated into microfibrils but show divergent temporal and spatial patterns of expression. Current evidence suggests that perturbation of both the structural and regulatory roles of microfibrils underlies the phenotypic abnormalities in MFS and selected related disorders.**

INTRODUCTION

Marfan syndrome was one of the charter members of Victor McKusick's initial compilation of Mendelian conditions that he termed "heritable disorders of connective tissue" [1]. This initial nosology encompassed six syndromes that shared little in common other than the presumption that different defects in components of the extracellular matrix were at fault. Because this category of human disease has expanded to over 200 phenotypes, the nosology has continued to be based largely on phenotypes, despite the growing knowledge about causation at the biochemical and genetic levels.

The disorders discussed in this chapter, with MFS being the most prominent and best-understood example, share many phenotypic features and have as the cause of at least some cases of each abnormalities of one or another of the components of the extracellular microfibril, hence the term "microfibrillopathies" used by some. Fibrillin-1 and fibrillin-2 are the best-studied components of the microfibril, and a subset of the microfibrillopathies has been called "fibrillinopathies" by some.

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MARFAN SYNDROME

Historical Introduction

> I present to the Society a little girl five and one-half years old, suffering from a congenital deformation of the four limbs, for which I have not found a precedent from the authors whom it was possible for me to consult.
> A.-B. Marfan, 1896 [2]

Among the heritable disorders of connective tissue, Marfan syndrome is especially noteworthy because of its worldwide distribution and relatively high prevalence, its clinical variability, and its pleiotropic manifestations, some of which are life-threatening. Marfan syndrome is second only to osteogenesis imperfecta with regard to when the phenotype was first described, when even earlier literature references occurred, when it was correctly interpreted as a generalized disorder of mesenchyme, and in the number of published articles addressing various aspects of the condition. The most extensive critical review of the literature of the first half of the twentieth century is in McKusick's monograph [3].

Antonin-Bernard Marfan (1858–1942) was Professor of Pediatrics in Paris; little could he have suspected that his name and interest in him as a physician would persist because of a brief case presentation made to the local medical society [2]. At the Société Médicale des Hôpitaux de Paris on February 28,1896, he described a 5-year-old girl, Gabrielle P., who had disproportionately long, thin limbs and digits, the latter so remarkable that he termed them *pattes d'araignée*, or spider fingers [2]. Her musculature was sparse, but on numerous examinations, including electromyography, no myopathy was evident. She had fibrous contractures of the fingers and knees, a long, narrow skull (dolichocephaly), and tall stature. Her eyes, heart, and intellect were normal, or at least aroused no clinical suspicion. She was the last of five children of a father in his early thirties, and the family history, sketchy as it was, did not suggest similarly affected relatives. Marfan was baffled as to causation and concluded with a plea, the sense of which is frequently uttered at gatherings of clinical geneticists today: "Above all, I have intended to present a case which seemed exceptional to me. I beg my colleagues to tell me if they have seen analogous cases" [translated by E. L. Fox].

A brief follow-up report of Gabrielle at age 11, including skeletal radiographs, revealed thoracolumbar kyphoscoliosis, perhaps an anterior chest deformity, and the first signs of the tuberculosis that was to end her life a few years later [4]. By 1938, when Marfan [5] reviewed the subject of dolichostenomelia, meaning excessively long limbs (the term he preferred), more than 150 similar cases had been reported, congenital displacement of the lens (ectopia lentis) [6] and disease of the mitral valve [7,8] had been associated with the skeletal phenotype, the concept of the *forme fruste* had (unfortunately) been introduced, and Gabrielle P. had died at 16 years of age, apparently without an autopsy. Furthermore, Marfan knew that both sexes and sequential generations in families were affected, which he recognized as being consistent with a Mendelian dominant trait. The idea of genetic mutation was quite clearly stated. In terms of pathogenesis, Marfan considered several possibilities, including Weve's [9] concept of a developmental defect of mesodermal tissues and an abnormality of pituitary function. The chief detraction of the mesodermal hypothesis was the apparent involvement of the ocular lens, which is ectodermal; the pathologic involvement of the zonules in ectopia lentis and their ectodermal embryologic origin had not been described. However, neither was Marfan particularly enamored of an endocrine pathogenesis.

More than 40 years after he had described Gabrielle P., Marfan understood a great deal about "his" syndrome, although his only reference to the eponymous designation, made without evident favor or disfavor, was to cite Weve's [9] suggested designation, "dystrophia mesodermalis congenita typus Marfan." The only major breakthroughs missed by Marfan, who died in 1942, were the recognition of aortic involvement in 1943 [10], the creation of the heritable disorders of connective tissue nosology, with Marfan syndrome a charter member, by McKusick in 1955 [11], and the discovery of the nature of the basic defect in 1991 [12]. Marfan never hinted that the mass of clinical information about dolichostenomelia in his day prompted a reconsideration of the diagnosis in his first patient. Thus, one could imagine the senior pediatrician in France bristling a bit had he heard the notion, first suggested in 1971, that Gabrielle P. did not have what we understand today as being Marfan syndrome but congenital contractural arachnodactyly [13,14]. Of course, Marfan's young patient may have had subtle lens dislocation, mitral valve prolapse, and aortic root dilatation, with all of these manifestations never looked for because of her premature death from an infectious disease. In 1938, Apert [15], a student of Marfan's and a definer of syndromes himself, codified use of the eponymous designation "Marfan syndrome," and the term may well still be in common use 50 years from now.

Phenotype, Natural History, and Prognosis

Marfan syndrome (MIM 154700) is a *pleiotropic* condition, meaning that diverse and even seemingly unrelated manifestations stem from a single mutation. The range of pleiotropism has largely been a function of the industriousness of the clinician and the sensitivity of the methods of analyzing the phenotype. As a result, the number of abnormalities in MFS has steadily increased. In addition, pleiotropism is expanding because affected individuals are living longer, and both primary and secondary evidence of the underlying connective tissue abnormality is emerging in mid- and late adulthood.

Most textbook descriptions of the phenotype and diagnostic criteria focus on the three organ systems prominently involved: the skeleton, the eye, and the heart and aorta. However, the skin, fascia, lungs, skeletal muscle, and adipose tissue are also clearly involved, and occasionally one of these areas constitutes the principal clinical concern. In this section, the clinical involvement (see also Table 1) and natural history of each area are discussed.

The Skeletal System

Height and proportions. In some respects, little improvement can be made to Marfan's description of the typical skeletal changes in a child with "dolichostenomelia." The birth length is long, and the stature is tall, mostly due to disproportionately long legs (Fig. 1), although the height of vertebrae is increased to some extent. Growth curves based on cross-sectional and longitudinal observations (Fig. 2) [16] have been constructed for affected Caucasians and show that height tends to increase parallel to, and on average slightly above, the 97th percentile. Skeletal maturation is not markedly different from that of unaffected children and

TABLE 1. Diagnostic Criteria for Marfan Syndrome

Summary: For the *index case*, two systems must qualify as ''major criteria'' and a third must be ''involved.'' Alternatively, if a mutation in *FBN1* is present, then one system must qualify as a ''major criterion'' and a second must be ''involved.''
For a *near relative of an index case*, one system and the family history must each qualify as a ''major criterion'' and a second organ system must be ''involved.''

Skeletal System
A ''major criterion'' is defined by the presence of at least four of the following:
- pectus carinatum
- pectus excavatum severe enough to require surgery
- reduced upper-to-lower segment ratio *or* arm span-to-height ratio ≥ 1.05 (both in the absence of severe scoliosis)
- positive wrist *and* thumb signs
- reduced extension of the elbows ($\leq 170^{\circ}$)
- medial displacement of the medial malleolus associated with pes planus
- protrusio acetabuli of any degree (ascertained on radiographs, CT, or MRI)

''Involvement'' of the skeletal system is defined by the presence of two of the preceding features *or* the presence of one of the preceding features and two of the following: pectus excavatum not requiring surgery; joint hypermobility; high-arched palate; facial features—at least two of dolichocephaly, malar hypoplasia, enophthalmos, retrognathia, down-slanting palpebral fissures.

Ocular System
A ''major criterion'' is defined by ectopia lentis of any degree.
''Involvement'' of the ocular system is defined by the presence of at least two of the following:
flat cornea; increased axial length of the globe (>23.5 mm); hypoplastic iris or hypoplastic ciliary muscle causing decreased miosis.

Cardiovascular System
A ''major criterion'' is defined by either dissection of the ascending aorta *or* dilatation of the ascending aorta with or without aortic regurgitation and involving at least the sinuses of Valsalva.
''Involvement'' of the cardiovascular system requires the presence of at least one of the following: mitral valve prolapse with or without mitral regurgitation; dilatation of the main pulmonary artery, in the absence of valvular or peripheral pulmonic stenosis, under the age of 40 years; calcification of the mitral annulus under the age of 40 years; dilatation or dissection of the descending thoracic or abdominal aorta under the age of 50 years.

Pulmonary System
''Involvement'' is defined by either spontaneous pneumothorax or radiographic evidence of apical blebs.

Skin and Integument
''Involvement'' is defined by either striae atrophicae or a recurrent or incisional hernia.

Dura
A ''major criterion'' is the presence of lumbosacral dural ectasia.

Family/Genetic History
A ''major criterion'' is defined by one of the following: a first-degree relative who independently meets these diagnostic criteria; the presence of a mutation in *FBN1* that is likely to be pathogenic; or the presence of a haplotype around the *FBN1* locus inherited by descent and unequivocally associated with diagnosed Marfan syndrome in the family.

adolescents, in keeping with apparently normal homeostasis of the endocrine system. Height may be extreme with occasional males exceeding 2.1 m; however, most adult men are less than 1.95 m and most adult women do not reach 1.85 m.

The arm span is exaggerated. A span greater than 1.05 times the height, provided vertebral deformity has not foreshortened stature, is considered abnormal. However, if the span is measured as a straight line from fingertip to fingertip, then flexion contractures of the elbows will underestimate the true length of the long bones of the arms. The dolichostenomelia of the legs has traditionally been expressed by the upper-to-lower segment ratio (US/LS), in which LS is measured from the top of the symphysis pubis to the floor and subtracted from stature to obtain US. The ratio is abnormally low in most patients with the Marfan syndrome but can reflect more than long legs if the upper segment is reduced by vertebral column deformity. The major limitation of the US/LS is its dependency on age, sex, and race. The only standard curves were developed years ago [3] and are somewhat cumbersome to use. Regression of US on LS produces a tight relationship that is independent of these confounding factors [16]

$$\frac{\text{US (cm)} - 24}{\text{LS (cm)}} = 0.6$$

but the equation has not been tested for its ability to discriminate between normally and abnormally proportioned people.

Exaggerated length of the bones of the hands and feet results in several striking manifestations. Arachnodactyly is best left a subjective impression and is shown by the Walker-Murdoch wrist sign — the combination of thin wrist and long digits results in the overlap of fingers I and V when the wrist is grasped (Fig. 1h). Attempts to objectify the measure, based on finger length or radiographs (the metacarpal index [17] or metacarpophalangeal pattern [18]), put more weight and importance on the finding than is warranted by its specificity.

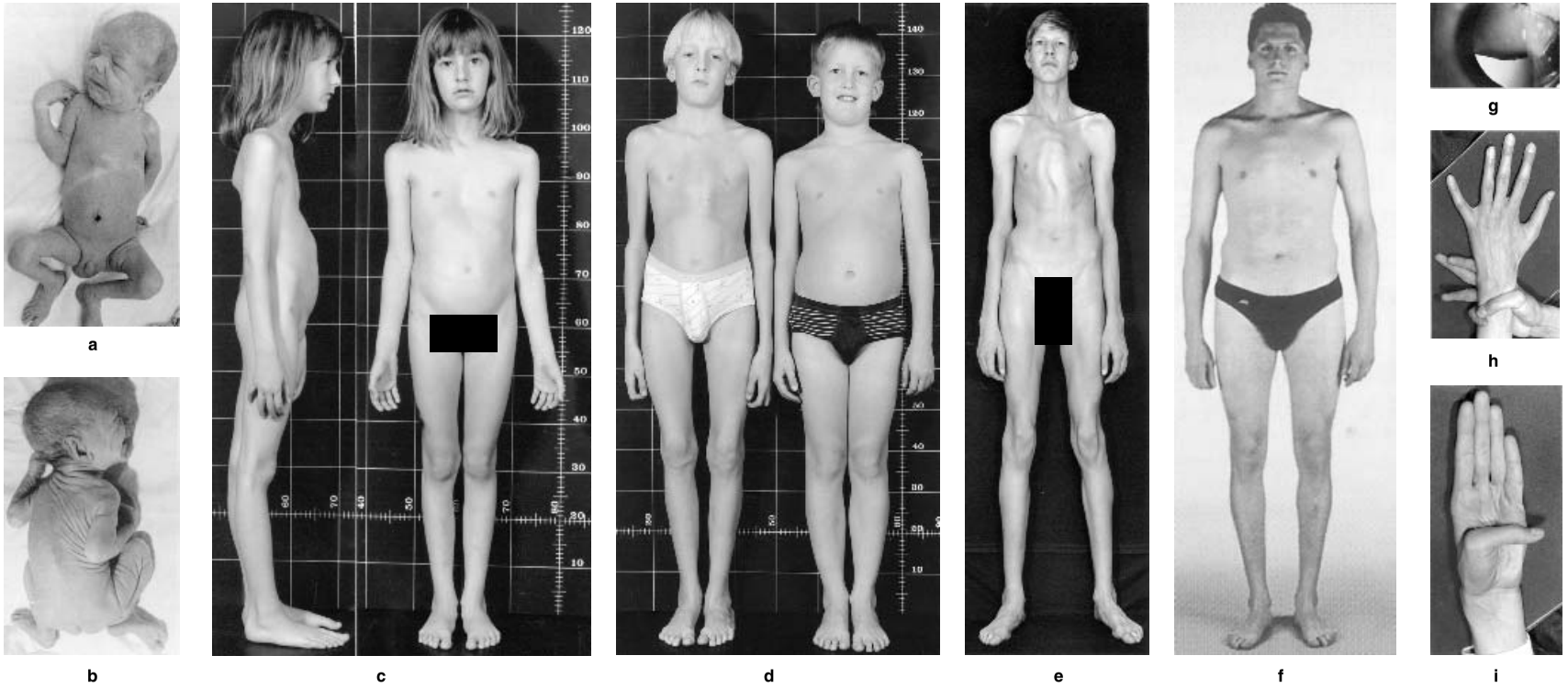


Figure 1. Marfan syndrome (MFS) and congenital contractural arachnodactyly (CCA). a, b: One-week-old patient with **neonatal lethal MFS** who died at age 10 weeks from global valvular insufficiency. Note the old-appearing expression, cutis laxa, the large and soft ears, and contractures of the elbow, hip, wrist, and knee joints. **c**: Girl with **classical MFS**, age 5.5 years, with an earnest and older-looking facial appearance, mild pectus excavatum, a flat thoracic cage, and height 4 cm above the 97th centile. **d**: 9-year-old boy with **CCA** and his healthy dizygotic twin brother. Note dolichostenomelia, asymmetric pectus carinatum, and contractures of the fingers (''arachnodactyly'') and elbows. **e**: **Classical MFS.** 15-year-old youth with nonfamilial tall stature (213 cm), an older-appearing facial expression, long limbs, especially the distal portions, asymmetric pigeon breast, sparse subcutaneous fat, and poorly developed muscles. He had several surgical repairs of diaphragmatic herniae and died at age 23 years of pericardial tamponade after dissection of the ascending aorta. **f**: 28-year-old man with no apparent external stigmata of **MFS**; his height is 192 cm, the aorta is dilated, and both lenses are luxated. **g**: **MFS.** Luxation of the lens to the temporal upper side. Note the parallel aligned zonular fibers that deform the edge of the lens. **h**: Positive Murdoch or wrist sign: the small finger and the thumb of one hand touch each other or overlap while encircling the opposite wrist. **i**: Steinberg or thumb sign: the thumb when folded across the palm extends beyond the ulnar edge of the hand. (Courtesy of Dr. B. Steinmann, Zurich.)

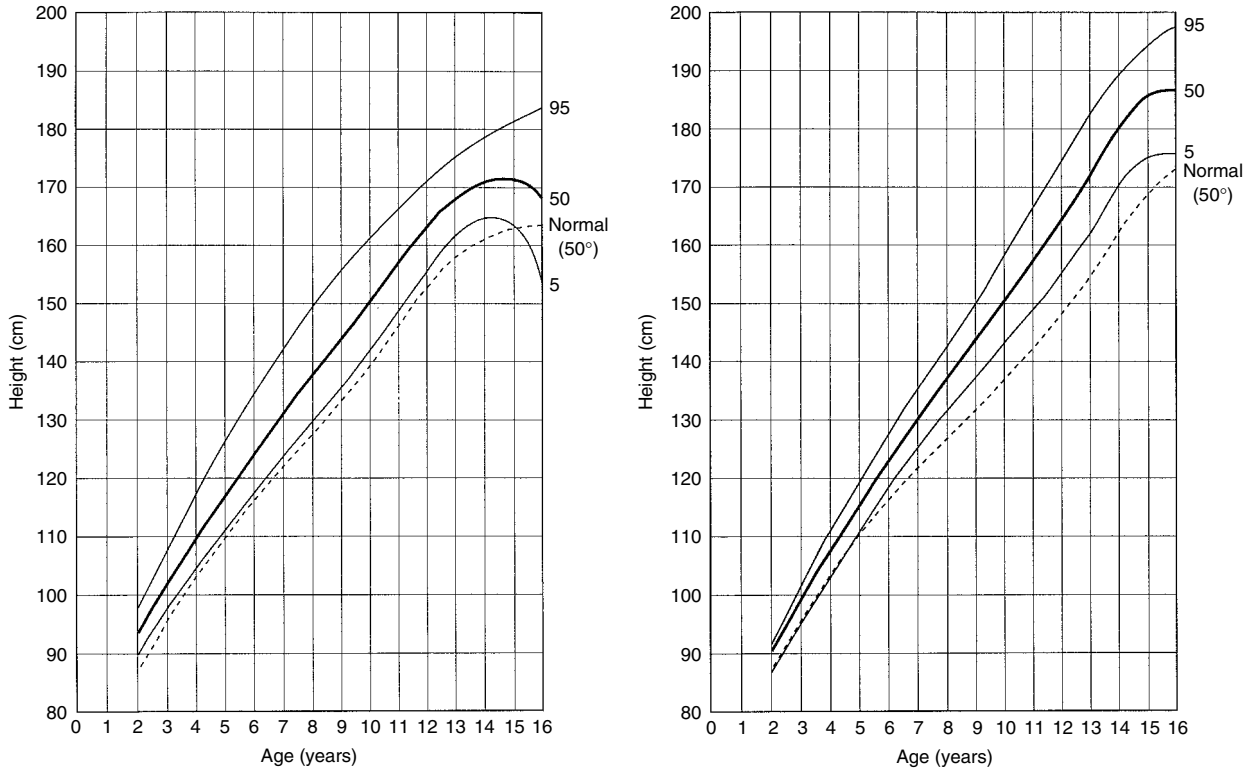


Figure 2. Growth curves for height in girls and boys with Marfan syndrome. **Left panel**: girls; **right panel**: boys.

The elongated foot often requires AA or narrower shoes; the narrow heel is particularly difficult to fit properly.

Weight. Most children and adolescents with MFS weigh less than the 50th percentile for age, which gives them a strikingly low weight/height ratio. When adipose tissue is accumulated in adulthood, it is usually central, especially around the hips. Weight is also reduced because of decreased skeletal muscle mass and occasionally, in severely affected children, by feeding difficulties or cardiac cachexia.

Craniofacies. The skull is long and narrow (dolichocephaly), the palate high and narrow, and the mandible hypognathic, retrognathic, or both. The eyes are usually deeply set (enophthalmos), in part due to a deficiency of retro- and peri-orbital fat. The brow appears prominent, whereas the malar region is hypoplastic and flat. The overall appearance can be "myopathic." McKusick [3] has described the facial expression as "lugubrious," and it is the facies, coupled with the tall stature, that explains why young children seem older than they are (Fig. 1c). The ears are normally placed, but the helix may be soft, simple, or crumpled, the latter occasionally as striking as described in contractural arachnodactyly [13,19,20]. The teeth show malocclusion on account of both crowding and overbite.

Anterior chest. The ribs, being tubular bones, tend to overgrow and in doing so push the sternum posteriorly, anteriorly, or both. The result is a funnel chest (pectus excavatum), pigeon breast (pectus carinatum), or a combined defect (Fig. 1c, e). The last is quite typical of MFS; the left costochondral junctions usually protrude, presumably because the underlying heart buttresses the ribs. The shape of the thorax may change dramatically during childhood; on several occasions, we have witnessed a deep pectus excavatum evolve over the course of a decade into a pectus carinatum. Until maturation of the skeleton, alteration in the contour of the rib cage should be anticipated.

Vertebral column. Abnormal curvature of the spine is common and may begin at any time before skeletal maturity; depending on severity, it may progress at any age [21–23]. However, estimates of prevalence, especially those emanating from orthopedic centers, must be interpreted with caution because of bias of ascertainment favoring severe cases. A reduction in the usual high thoracic kyphosis to a "straight back" or an outright reversal is most prevalent and need not be associated with scoliosis; accentuated high thoracic kyphosis is less frequent. Scoliosis typically begins in childhood and progresses most rapidly during growth spurts. Single or multiple curves affecting any level of the vertebral column may occur. A spine not deformed beyond about 10° by late adolescence is unlikely to become distorted. However, for severe curves (40° or more), completion of linear growth is no guarantee of future stability. The consequences of marked thoracic spinal deformity in MFS are little different from those of idiopathic scoliosis (i.e., restrictive pulmonary disease and cor pulmonale). Severe scoliosis of the lumbar spine may lead to chronic back pain subject to acute exacerbation.

Spondylolisthesis is relatively more common than normal in MFS, but a true prevalence is unknown [24]. Typically, the lower lumbar levels are affected. Instability of the cervical spine is also of unknown prevalence and occasionally causes clinical problems [25]; anesthesiologists should be aware of potential laxity before attempting endotracheal intubation.

A common skeletal manifestation in the spine is secondary to dural ectasia. The radiologic findings, often incidentally noted, include thinning of the laminae of L4 through S2 and widening of the neural foraminae.

Pelvis. Congenital dislocation of the hip occurs infrequently but at an increased incidence compared with the general population [26]. Protrusio acetabuli is much more frequent than in the general population, is more common in MFS than appreciated [27], can be evident in children, and is progressive but generally asymptomatic through young adulthood [28]. The findings are often incidentally noted on plain radiographs or CT scans. However, as with most of the skeletal manifestations mentioned thus far, the extended natural history of protrusio acetabuli is unknown because until recently most adults did not survive their cardiovascular problems.

Joints. Although the appendicular joints of MFS patients are commonly perceived as being lax, normal mobility and even outright contractures are frequent. During early life, little disability usually results from laxity of the hands, wrists, elbows, shoulders, hips, and knees. Occasionally, acquisition of motor skills is delayed, but this is often impossible to separate from muscular hypoplasia. Similarly, handwriting may suffer from weakness and instability of the wrist and fingers. The patella may dislocate repeatedly, causing pain, transient locking of the knee joint, and chondromalacia. In general, however, repeated dislocations are uncommon. Longitudinal laxity of the hand is responsible for the positive "thumb sign," also known as the Steinberg sign, in which the thumb, when apposed across the palm, extends well beyond the ulnar border of the hand (Fig. 1i). Laxity of the ankle and foot produces the most common problems. Although the arch of the foot may be normal, or even exaggerated (pes cavus), a "flat foot" (pes planus) is one of the most common skeletal manifestations. At its most mild, pes planus is due to stretching of the plantar fascia and other ligaments of the midfoot. At its worst, calcaneoplanovalgus exists, and the medial malleolus is displaced medially; as a result, body weight is not centered over the foot, and secondary disturbances of knee and hip function develop, especially during childhood. Later in adulthood, degenerative arthritis often develops, especially in the hips and knees [29]. Osteoporosis is more common in MFS and at earlier ages than in the unaffected population [30,31].

The Ocular System

The first description of ectopia lentis with joint laxity may well have been recorded two decades before Marfan [32]. The most comprehensive studies have been those of Cross and Jensen [33] and Maumenee [34]; both of these series consisted largely, but not exclusively, of patients referred to the Johns Hopkins Hospital for other than ophthalmic concerns.

The globe. Typically, the globe is elongated, a feature that can be defined by the axial length as determined by ultrasound [34]. Exaggerated axial length contributes to myopia, is a risk factor for retinal detachment, and is more likely to be present in association with ectopia lentis. The sclera may be thin, and thereby appear blue, but this is not a consistent finding.

The cornea. The distribution of corneal diameters is shifted upward in Marfan syndrome, with some in the megalocornea range. Corneal clouding is not a feature. Study of the radius of curvature (keratometry) has proven useful from the diagnostic, pathogenetic, and management perspectives [34]. The cornea tends to be flatter than normal; the degree of reduction in curvature is positively associated with the presence of ectopia lentis but bears little relation to the severity of astigmatism. Keratoconus is distinctly uncommon.

The iris. The iris is often homogeneous or "velvety" in appearance. Transillumination is possible in ~20% of patients. Dilation of the pupil is often difficult with standard mydriatics because of hypoplasia of the dilator pupillae muscle.

The lens. Adequate evaluation for ectopia lentis requires optimal pupillary dilatation and slit-lamp examination. Under these circumstances, and with bias of ascertainment minimized, about 60% of patients have some degree of lens displacement by age 4 years (Fig. 1g). Dislocation can be in any direction, with superior, superotemporal, and backward preferred. Ectopia lentis is often congenital and may be detected first by a parent or grandparent through iridodonesis, or fluttering of the iris as the eye attempts to accommodate. Some progression of displacement can occur, being most common during periods of rapid growth. The ocular zonules tend to be normal in appearance, both in number and organization. In a minority of cases of clear MFS, the zonules break, as is the rule in homocystinuria. This distinctive behavior of the zonules accounts for the lens tending to fall inferiorly and lodge in the anterior chamber in homocystinuria, whereas both behaviors are much rarer in MFS. Microspherophakia is uncommon. Development of cataracts is accelerated in MFS, especially in displaced lenses. Cataract is one of the pleiotropic manifestations that will become more prevalent and better understood as patients live longer.

The retina. Changes associated with myopia, such as thinning of the choroid, retinal holes, and lattice degeneration, are the most common findings. Detachment occurs in a small minority and used to be predisposed by intraocular surgery, especially lensectomy. Newer surgical techniques have greatly reduced this risk.

Visual acuity. Most patients with MFS attain corrected visual acuity of 20/40 or better in at least one eye [34]. This optimistic prognosis even pertains in the presence of ectopia lentis. The most common cause of poor vision in one or both eyes is amblyopia, nearly always secondary to inattention to conservative methods of improving acuity during infancy and early childhood. Retinal detachment usually impairs acuity to some further extent and can cause blindness.

The Cardiovascular System

Life expectancy. A preponderance of clinical attention in the person with MFS is directed at the aorta. The reason for this bias is readily evident from the important study by Murdoch and associates [35] of life expectancy and causes of death. By the early 1970s, when the retrospective review of medical records on which Murdoch based his conclusions was conducted, medical and surgical therapy had had no beneficial impact on survival. The causes and ages of death of 270 patients were ascribed, and the latter were compared with age-adjusted mortality rates for the United States population. On average, men and women survived about two-thirds as long as expected, although the life-table mortality curves began to deviate unfavorably in infancy. When the cause of death was known, the cardiovascular system was the culprit in over 90% of cases, with aortic dissection, aortic regurgitation, and congestive heart failure (presumably from chronic valvular regurgitation) the leading

conditions. A more recent assessment of outcome in Marfan syndrome suggests that life expectancy now approximates that for the general population [36]. Advances in the management of cardiovascular complications were cited as primary contributors to improved prognosis.

The heart. The first reported cardiovascular manifestation of MFS involved the mitral valve [7]. Early in the twentieth century, the thought emerged that septal defects were common, but this has not been confirmed by later, less biased experience. Cardiomyopathy, out of proportion to valvular problems, appears occasionally, and some have speculated that a primary defect in cardiac muscle occurs [37]. Whether this is a widespread but generally subclinical tendency, a manifestation of only some biochemical defects, or a chance association of idiopathic dilated cardiomyopathy, which is not rare, with MFS remains to be explored. The most consistent manifestations in the heart involve the atrioventricular valves and the conduction system [3,11,38–42].

The earliest clinically evident changes occur in the mitral valve [43–47]. The patients affected most severely are often burdened with a severe phenotype [45,46,48]; severe mitral regurgitation may lead to early congestive heart failure, failure to thrive, pulmonary hypertension, and demise in infancy. However, children with MFS less severe in the skeletal and ocular systems may still have severe mitral regurgitation develop before adulthood [47,49]. The underlying connective tissue disturbance is reflected in redundancy of the atrioventricular valve leaflets, stretching of the chordae tendineae, and dilatation of the valve annulus; in brief, these are the same features as in mitral valve prolapse in its nontrivial form [40,50]. The mitral valve is invariably more severely affected than the tricuspid valve, presumably because of its location in a higher-pressure system. Physiologically, the prolapsing mitral valve in MFS behaves as in the idiopathic variety.

The clinical findings vary with both physiologic maneuvers and pathophysiologic alterations [51]. The prevalence of mitral valve prolapse, defined by either auscultation or echocardiography, increases with age through young adulthood and, during at least that age range, is more common in women than in men. Both of these characteristics are qualitatively similar to mitral valve prolapse in the general population. However, there are several important departures in MFS. First, more than one-quarter of people with MFS have progression of the prolapse to mitral regurgitation, and worsening of the valvular leak, by adulthood [49]. Second, a much higher percentage have marked redundancy of the valve leaflets detected on echocardiography (Fig. 3) [52]. Third, calcification of the mitral annulus accompanies mitral valve prolapse in a higher proportion of patients with MFS [3,40,53]. Remaining questions include whether the risk of endocarditis is higher in all patients with MFS or just those with mitral valve prolapse, how often mitral valve prolapse is associated with dysrhythmia, and what the long-term course of mitral valve prolapse will be as patients with Marfan syndrome survive to advanced ages because of aortic surgery.

The aortic valve is often thinned and myxomatous. Although these changes are intrinsic to the fundamental connective tissue defect, they become exaggerated as the three valve cusps are stretched by the expanding sinuses of Valsalva. Aortic regurgitation is occasionally due to cusp prolapse, a fenestrated cusp, or valvular endocarditis. Acute aortic leaks are usually due to aortic dissection. However, the vast majority of aortic regurgitation is due to progressive dilatation of the upper attachments of the cusps at the sinotubular ridge [51].

Electrical disturbances are common in MFS and a not infrequent cause of sudden death. Unfortunately, few extensive or unbiased studies of ambulatory electrocardiographic recordings have been done [41]. There is a predilection for both supraventricular and ventricular dysrhythmia as primary disturbances (i.e., unassociated with valvular regurgitation, electrolyte disturbance, cardiac surgery, or the like). Occasional patients have primary conduction delay or aberrant pathways. Both atrial and ventricular dysrhythmia are often associated with mitral valve prolapse.

No electrocardiographic changes can be considered typical of MFS. Patients with mitral valve prolapse often have the inferior T wave inversions seen in that condition [54]. A personal impression is that limb lead aVL often shows a Q wave, usually interpreted even in young children as suggestive of anterior ischemia, although patients almost never have any reason to suspect inadequate myocardial perfusion. Development of anterior electrical forces across the precordium is delayed (i.e., does not appear until V4–5) in patients with pectus excavatum and leftward rotation or shift of the heart.

The aorta. Dilatation of the sinuses of Valsalva often begins *in utero*, as evidenced by neonates having enlargement of the aortic root on echocardiography [3,45,47,55]. This fact has several implications. First, dilatation initially affects the most proximal portion of the aorta. Second, in some patients the aortic wall is so intrinsically abnormal that the relatively minor hemodynamic stresses of fetal life are sufficient to promote irreversible stretching of the wall. Third, if therapy to prevent dilatation is to be effective, it must be started early, at least in some cases; this point is discussed in the section on management.

The rate of dilatation is unpredictable. In general, children with considerable dilatation are more likely to develop clinically important aortic root aneurysms sooner than other children with MFS. Echocardiography and β-adrenergic blockade therapy arrived on the scene at about the same time, so that large, unbiased longitudinal studies of untreated patients have never been performed; hence, the detailed natural history of ascending aortic involvement has been gleaned from small series and case reports [42,56–59].

A recurring problem is how to interpret the aortic root dimension of an echocardiogram (Fig. 4). At least three issues must be addressed. The first concerns which dimension to use for diagnostic purposes. The size of the ascending aorta clearly depends on age and body size; sex and blood pressure are not important determinants in children [60,61]. Devereux and colleagues [62] have examined the ascending aorta by cross-sectional echocardiography and measured the dimensions at four levels: the annulus, midsinuses of Valsalva, supraaortic ridge, and proximal ascending aorta. Measurements obtained solely by M-mode echocardiography (in widespread use until the early 1980s) may approximate the annular diameter rather than that of the sinuses of Valsalva. These days, the cross-sectional echocardiogram in the parasternal long-axis view should be used to generate an M-mode tracing or directly measure the point of maximal dilatation at the sinuses of Valsalva. Thus, the nomograms, and graphs that related aortic dimensions obtained by the earlier M-mode technique to body size [60,61], may underestimate the diameter of the sinuses and if relied on may produce a false-positive diagnosis of aortic

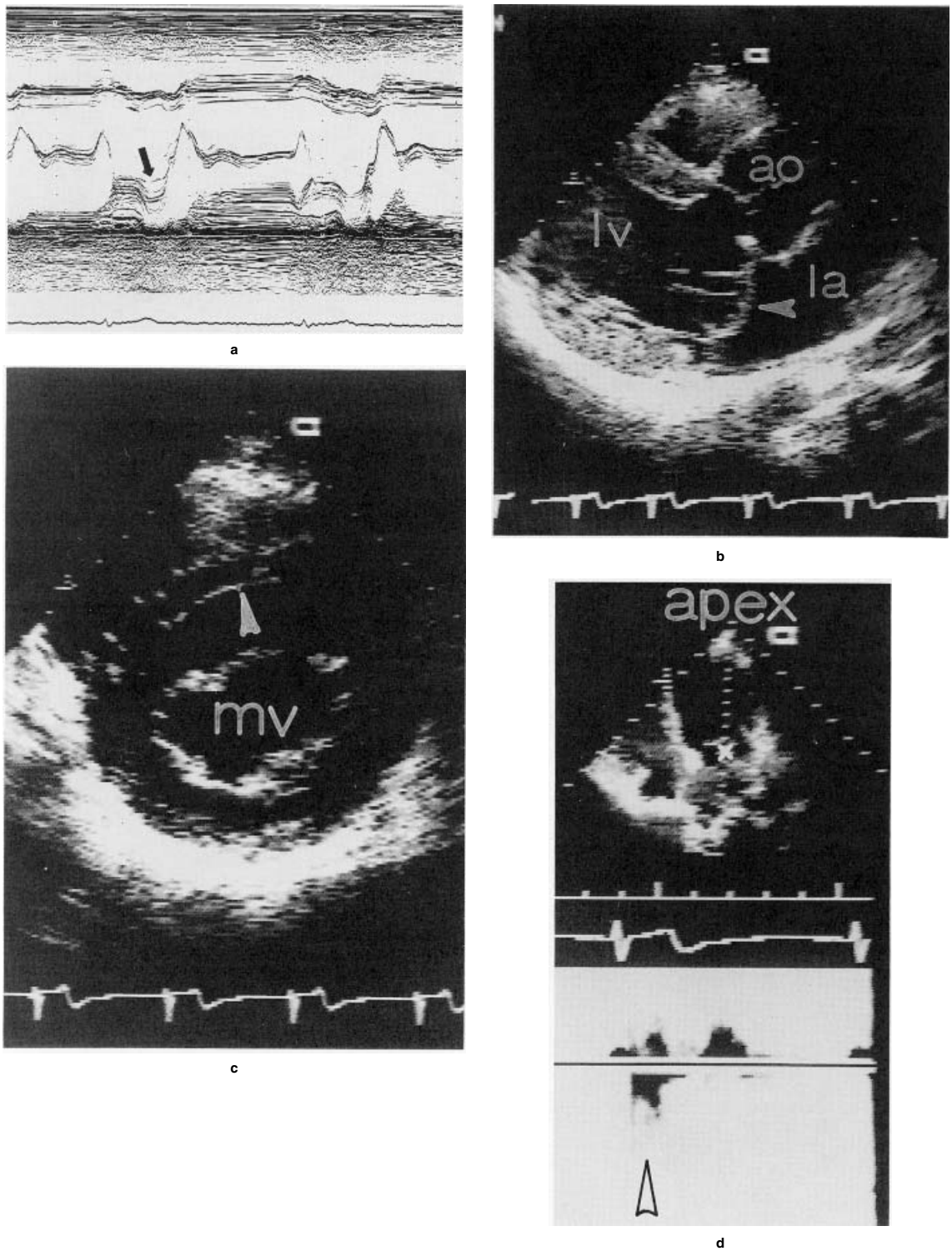
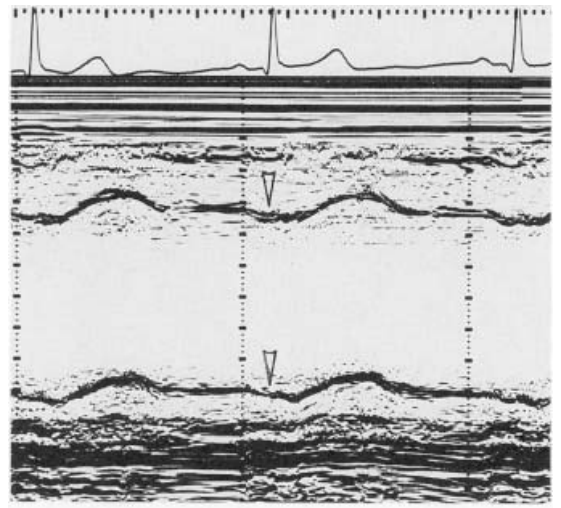


Figure 3. The mitral valve in Marfan syndrome as viewed by echocardiography. **a**: A one-dimensional (M-mode) tracing; the arrow points to the mid-systolic prolapse. **b**: A cross-sectional (two-dimensional) scan in the parasternal long-axis view; the arrowhead points to the prolapsing mitral leaflets. la, left atrium; lv, left ventricle; ao, aortic root. **c**: A cross-sectional parasternal short-axis view of the left ventricle. The letters ''mv'' are surrounded by the patulous mitral leaflets, open in diastole. The arrowhead points to the left ventricular endocardium. **d**: Pulsed-wave Doppler examination of the mitral valve. The **upper** part of the figure shows a cross-sectional echocardiogram in the apical four-chamber view; the ''x'' marks the sampling space for Doppler interrogation and is located just to the atrial side of the mitral valve. The **lower** part of the figure shows the Doppler tracing, with the arrowhead pointing to turbulent flow away from the sampling space (downward in the figure) in systole, indicative of mitral regurgitation.



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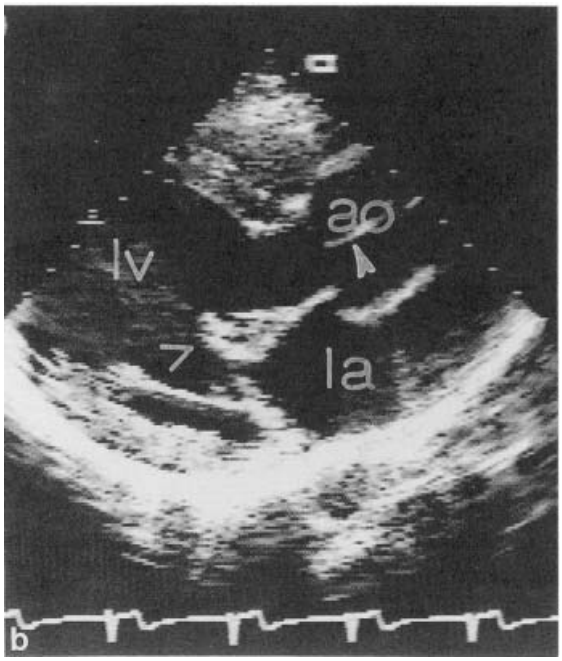

b

Figure 4. The aortic root in Marfan syndrome as viewed by echocardiography. **a**: An M-mode view in which the scale is divided into centimeters (between the large dots) and the arrowheads point to the anterior surfaces of the anterior and posterior walls of the aortic root; the diameter at end-diastole (at the Q wave of the EKG) is 58 mm, well above the upper limit of normal for this adult of 37 mm. **b**: A cross-sectional, parasternal long-axis view, labeled as in Figure 3b. The open arrowhead points to a redundant anterior mitral valve leaflet and the closed arrowhead to the aortic valve, here closed in diastole. The aortic root surrounding the aortic valve measures 41 mm (the dots along each edge of the sector are 1 cm apart).

dilatation [62]. Other attempts to define "normal" values, although employing cross-sectionally derived dimensions, are not appropriate because of a limitation to the annular measurement [63]. A useful graph for infants and children relating sinus of Valsalva diameter to body surface area is shown in Figure 5. Adults pose additional problems because body surface area is more often distorted by obesity; hence, correlation coefficients are not as high. This may account, in part, for the looser relationship between aortic size and body surface area for adults over 40 years. We have used the slightly different approach of analyzing the cross-sectionally directed M-mode echocardiographic dimension at the sinuses of Valsalva [64]. By using a regression equation [65],

$$\text{aortic root dimension (mm)} = 24.0\ (\text{BSA})^{1/3} + 0.1\ (\text{age}) - 4.3\ (+/-18\%)$$

(in which BSA, the body surface area, is in m^2 and the age is in years), the expected aortic diameter is calculated and is divided into the patient's measurement to generate an "aortic ratio." Because age and body size are incorporated, a plot of a "normal" aortic ratio against time is a horizontal line of 1.0. This approach can be used to assess dilatation (a ratio of 1.18 is the upper 95% confidence limit of 1.0) and changes with time. Other approaches have been defined [66].

The second issue concerns prognosis. In rough terms, the risk of developing aortic regurgitation is related to aortic diameter. In adults, aortic regurgitation is rarely present when the diameter is less than 40 mm and almost always present when it is above 60 mm [51]. Valve incompetence generally is caused by stretching of the upper attachments of the cusps, and thus the diameter at the supraaortic ridge should be the critical one, although previous studies have employed sinus of Valsalva dimensions [51]. Dissection of the ascending aorta has been reported at aortic dimensions under 50 mm, and some have claimed dissection only occurs in the nondilated state [40]. We and others, however, have documented numerous examples of dissection of the ascending aorta, both with and without rupture, in patients with dilatation greater than 55 mm. Surgical repair of the aorta is recommended when the diameter reaches 50–55 mm, which emphasizes our belief that most well-managed patients can survive until dilatation progresses to that stage [67].

Finally, some have suggested that the shape of the aorta is a marker of clinical prognosis and classification. Devereux and colleagues presented cross-sectional data in which patients with MFS were categorized by whether proximal aortic dilatation was limited to the sinuses of Valsalva or was more fusiform [68]. Diffuse involvement of the proximal aorta was recognized as a risk factor for aortic complications. Unless a dissection is present, the aorta beyond the root rarely dilates in children or adults through middle age [3,69]. Whether older affected adults will be more prone to abdominal aortic aneurysms than the general population is an open question [70,71].

Dissection usually begins in the ascending aorta. Most dissections progress anterogradely to involve the entire aorta, and some extend retrogradely. The latter are particularly life-threatening because of interference with coronary artery blood flow or rupture into the pericardial sac with tamponade. Although most patients experience the classic clinical symptoms associated with the acute dissection [72], some patients are unaware that they have suffered an extensive aortic disruption [73]. Intimal tears, occasionally with circumscribed areas of dissection, are not uncommon in the dilated sinus of Valsalva and are usually recognized at the time of surgery.

Any region of a dissected aorta may dilate over time. Close follow-up is mandatory, and any of the classic clinical signs

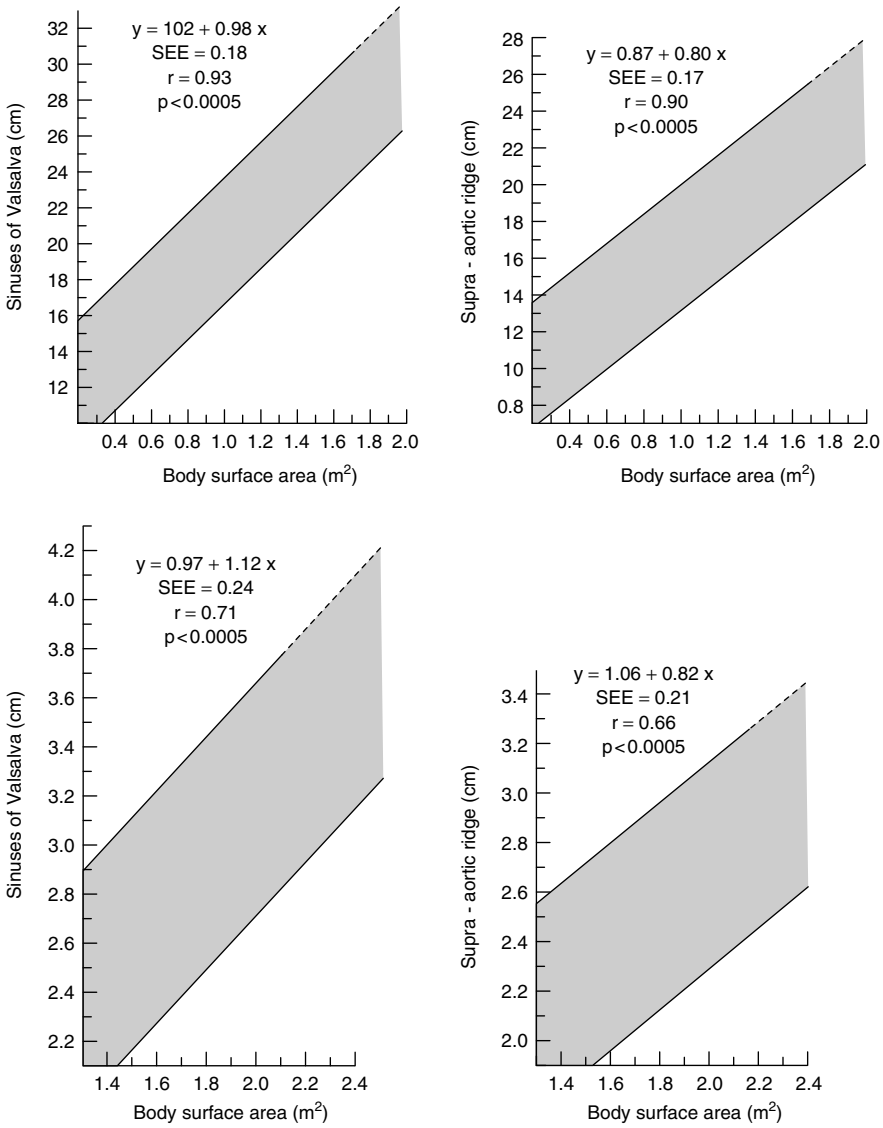

Figure 5. Assessment of the diameter of the aortic root as a function of the body surface area. The **upper** two graphs are for children and adolescents and the **lower** two for adults. The graphs on the **left** are for the aortic diameter at the level of the sinuses of Valsalva and those on the **right** are for the diameter at the supraaortic ridge (sinotubular junction). The shaded areas represent the range of values in which 95% of the population of males and females will fall. Data were from cross-sectional echocardiograms measured at end-diastole using the leading-edge technique. (Courtesy of Dr. Richard Devereux; modified from [62] with permission of the publisher.)

of progression, such as pain, limb or organ ischemia, and hematuria, should be taken quite seriously.

The Respiratory System

Given the predominance of connective tissue in the lung and airways, it is perhaps surprising that the lung is affected clinically so infrequently. The major manifestation is spontaneous pneumothorax, usually due to rupture of an apical bleb [74]. A variety of severe emphysematous changes have been reported in patients, including infants [75–82], but whether MFS predisposes to obstructive lung disease in most patients remains unclear.

Measures of static pulmonary function may be misleading if the patient's values are compared with normative values calculated on the basis of, in part, standing height [83]. Because the legs of the patient are abnormally long, lung volumes are predicted to be greater than warranted, and the patient is reported to have a reduced forced vital capacity and total lung capacity. In fact, when the predicted values are based on sitting height (a more accurate reflection of thoracic size), both are normal in most patients [83]. The exceptions are those with severe pectus excavatum, severe vertebral column deformity, or both, in whom forced vital capacity, total lung capacity, and residual volume are reduced. The pattern is that of restrictive lung disease and may become life-threatening in patients with marked thoracic deformity.

Dynamic pulmonary function has not been assessed rigorously in MFS. There is some suggestion that lung compliance is abnormal [82], but measurements are difficult and abnormalities detected must be partitioned into

intrinsic defects of lung parenchyma and secondary effects of supporting and confining structures. Severe pectus excavatum, for example, markedly alters the mechanics of deep inspiration.

The Integumentary System

In its broadest sense, this system includes not only the skin but fascial coverings of tissues and organs and even intraorgan septae. Given that the primary constituent of this system is connective tissue, it is not surprising to find pleiotropic manifestations of MFS in skin, fascia, and dura.

Skin. In general, skin elasticity is not strikingly unusual, although some patients have more distensible skin than expected, with prompt return to its original tautness; when present, this "hyperelasticity" is typical of the mild forms of the Ehlers-Danlos syndrome (see Chapter 9, this volume) and not of cutis laxa. Some patients complain of easy bruising, and several factors may be at work. First, skin of people with MFS may be thinner than that of age-matched controls, especially the deeper reticular dermis. Second, people with MFS often have decreased subcutaneous fat and hence less protective cushioning for minor contusions. Third, joint laxity, muscular hypoplasia, and poor visual acuity may contribute to a predisposition to contusions in some patients. However, no consistent abnormality of platelet function or coagulation occurs. Healing of both traumatic and surgical disruptions of fascia and skin seems grossly normal, without predisposition to wound dehiscence.

By far, the most common manifestations of MFS in the skin are striae atrophicae [84], which occur in roughly two-thirds of patients. These "stretch marks" occur in areas of flexural stress, such as the lumbar area, buttocks, shoulders, breasts, thighs, and abdomen, in approximate descending order of involvement. Striae may first appear in childhood but are usually first recognized around puberty.

In contrast to the striae common in adolescents who lack a connective tissue disorder, those in MFS persist and are not associated with being overweight. Histologically, striae atrophicae in MFS skin appear no different from stretch marks in the general population; elastic fiber fragmentation is the most striking feature [85]. The striae of pregnancy can be particularly exuberant in the woman with MFS.

Hernia. Inguinal herniae, both congenital and acquired in adolescence or later, are relatively common in MFS. Recurrence after standard surgical therapy is also common, and buttressing with prosthetic meshwork is often required for the second and subsequent repairs. From personal observations, incisional herniae, especially ventral ones, are definitely of increased prevalence and may also require buttressed repair. Herniae of other structures, such as the diaphragm, may be more common in MFS, but a coincidental occurrence is also possible [86].

The Central Nervous System

Intelligence and cortical function. Intelligence, as measured by standard tests of IQ, is unaffected [3,87]. Presence of low intelligence or frank retardation in a person with classic MFS should prompt an intensive search for a cause of the mental deficiency. In the retarded patient with some features suggestive of MFS, homocystinuria, fragile-X syndrome (MIM 309550), Shprintzen-Goldberg syndrome (MIM 182212), and chromosome aberrations should be considered.

In a pilot study of children and adolescents with MFS, Hofman and colleagues [87] found a high prevalence of neurodevelopmental problems. Attention deficit disorder, hyperactivity, and verbal-performance discrepancies are all common in the general population, and ascertainment bias or confounding factors (such as poor visual acuity, muscular incoordination, and β-adrenergic blockade) may have influenced the results of this initial study. However, some young people with MFS clearly have learning problems; whether these represent pleiotropic manifestations of the genetic mutation or epiphenomena remains to be determined.

Several instances of psychosis in people with MFS [88,89] probably represent coincidences.

Dural ectasia. Case reports of marked ectasia of the dural sac in patients with MFS have appeared over the years [90], but the widespread use of CT scanning and MRI has resulted in an appreciation of both the extent and the prevalence of this manifestation [90–94]. In about two-thirds of patients, the dura stretches, presumably under the influence of cerebrospinal fluid pressure, with three prominent effects. In the lumbosacral region, the vertebral canal becomes widened due to pressure-induced remodeling and erosion of vertebral bone (Fig. 6a). If the dura protrudes through the neural foramina, arachnoid cysts and pseudomeningoceles can develop (Fig. 6b). Finally, rare patients develop enlargement of spaces containing cerebrospinal fluid in the head, and the cisterna magnum is especially susceptible; however, no systematic survey of cephalic involvement has been made.

Dural ectasia is usually asymptomatic; the most common clinical presentations are radicular pain from nerve root compression in lumbosacral foramina and pelvic pain from meningoceles. The latter are often misdiagnosed as ovarian cysts.

Other Features

Skeletal muscle. Patients with "classic" MFS often appear "myopathic," with little skeletal muscle bulk, neonatal hypotonia, scoliosis, joint contractures, and a flat, expressionless facies [3]. Joint laxity, cardiovascular problems, or both may limit exercise in childhood and adversely affect development of muscle bulk and strength. Advice to limit exercise out of concern for aortic dilatation may further impair muscle development. Several case reports have suggested that a skeletal muscle myopathy is a pleiotropic manifestation, but adequate controlled studies have not been performed [95–97].

Adipose tissue. Adipose tissue has not been studied in MFS, which is surprising given its obvious deficiency in most children and adolescents. This distribution of adiposity is curious; most young patients lack subcutaneous fat stores throughout their bodies but as adults find that centripetal accumulation is marked, especially around the hips. On the other hand, some patients have considerable bulk, both skeletal muscle and adiposity, from early ages; given tall stature, body weight can be considerable and become an important or even limiting factor in considering cardiopulmonary bypass for cardiac surgery.

Diagnosis

Despite knowledge of the basic genetic defect, the diagnosis of MFS remains firmly rooted in the phenotype. The so-called Ghent criteria [98] are the latest iteration of diagnostic standards and are summarized in Table 1.

Diagnostic Criteria

In 1986, a committee of international consultants first suggested diagnostic criteria (the Berlin nosology) for many common heritable disorders of connective tissue, including MFS [99]. Subsequently, the Ghent criteria were offered by

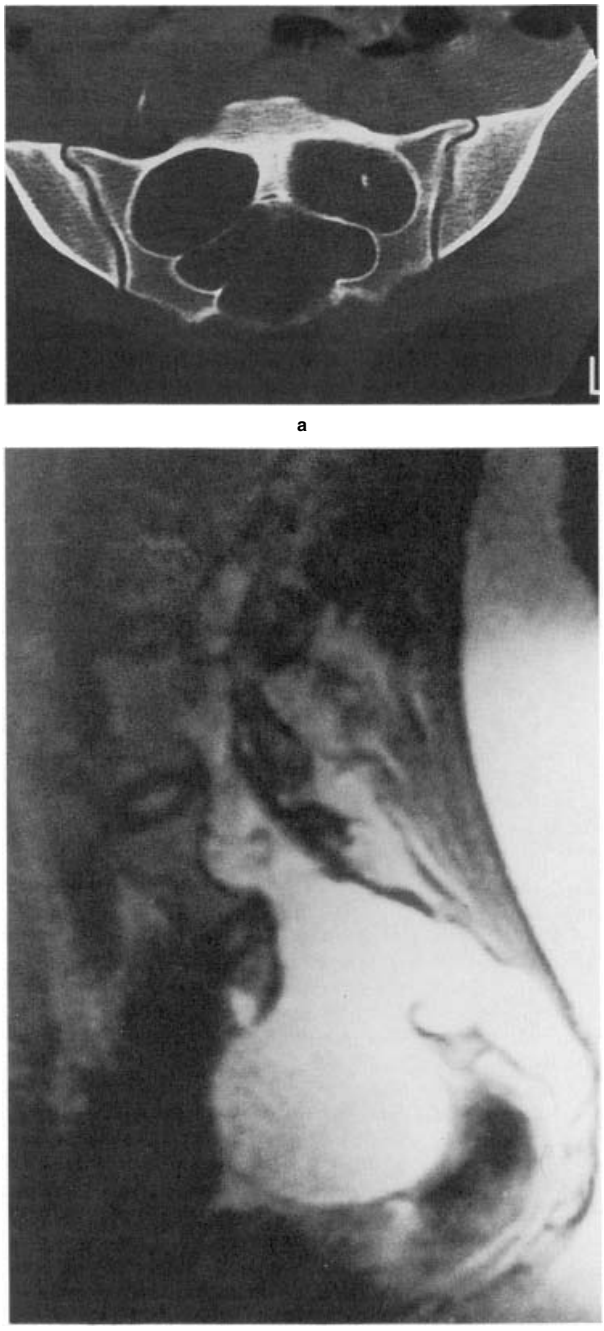

Figure 6. Dural ectasia in Marfan syndrome. a: An axial CT scan of L5, showing marked bony erosion and dilatation of the central spinal canal and the two lateral neural foramina. The scan is oriented with the dorsal surface down. **b**: A myelogram of the lumbar spine showing both dilatation of the spinal canal and a large anterior pseudomeningocele. The radiograph is oriented with the head up and the dorsal surface to the right.

De Paepe and colleagues [98] (Table 1). At one end of a phenotypic continuum resides MFS, and at the opposite end lies the general population. A variety of conditions occupies this continuum.

Diagnosis of MFS is pragmatic, based on what can be done to define the phenotype in any specific case. For example, dural ectasia is a highly specific sign but requires CT or MRI to detect, and such tests are not practical for every patient.

In addition, if a patient has no relative with any suggestion of MFS, or if the patient has been adopted and has no knowledge of relatives, then more phenotypic manifestations are required than when the family history is positive. However, one must be careful in calling the family history positive; many aneurysms, most scoliosis and tall stature, most myopia, and so forth are not due to MFS. In the best circumstance, diagnosis in a relative is based on personal examination or autopsy confirmation.

Clinical diagnosis. When the patient exemplifies the standard medical textbook description of MFS, the diagnosis is rarely in doubt. The clinical challenge comes in the less obvious cases, and here the clinician charged with determining the diagnosis must assemble a full range of objective data. In nearly all instances, the clinician will require the assistance of specialists, particularly the ophthalmologist and the cardiologist.

Thorough reviews of past medical history, current complaints, and family history are essential. The general physical examination focuses on height and body proportions, the shape of the palate and state of dental occlusion, passive joint mobility, shape of the anterior thoracic cage, shape of the vertebral column, and inspection and auscultation of the cardiovascular system.

The ophthalmologist should dilate the pupil to the maximum and inspect for any displacement, including anterior, of the lens. The visual acuity in each eye must be determined. The entire retina should be inspected for tears. Also, the globe in MFS is often elongated and the cornea is usually flattened. Lenticular cataracts often begin to appear in early adulthood. The intraocular pressures should be determined, even in children.

The cardiologist is useful for expert examination and recommending and interpreting diagnostic tests. The transthoracic echocardiogram is an essential diagnostic tool, most useful for detecting redundancy and prolapse of the atrioventricular valves and for measuring aortic diameter and ventricular size and function. Addition of Doppler techniques enhances detection of subtle valvular abnormalities.

Computed tomography (CT) and magnetic resonance imaging (MRI) are useful for defining the mediastinal relationships when severe pectus excavatum is present, the size and shape of the aortic root and entire aorta, whether a dissection is present, and whether protrusio acetabuli or dural ectasia is present.

Molecular diagnosis. Mutations in the locus that encodes fibrillin-1 at 15q21 account for all cases of classic MFS that have been studied. Other loci likely contribute to the panoply of conditions in the phenotypic continuum of MFS. However, because of the extensive intragenic heterogeneity at *FBN1* in MFS, the current technical challenges of mutation detection, and the fact that *FBN1* mutations may cause the same conditions that constitute the differential diagnosis for MFS (see Table 2), molecular diagnosis for the sporadic case has little role [98,100,101].

Two situations occur when molecular diagnosis is practical. First, if the *FBN1* mutation is known for a person with MFS, then that information can be applied to relatives and prenatal diagnosis. Second, sufficient intragenic and tightly linked polymorphisms have been described that

TABLE 2. Differential Diagnosis for Marfan Syndrome

Primarily skeletal features
Homocystinuria (MIM 236200)
Congenital contractural arachnodactyly (MIM 121050)
Klinefelter syndrome
Familial tall stature
Stickler syndrome (MIM 108300)
Primarily ocular features
Familial ectopia lentis (MIM 129600 , MIM 225900, MIM 225100, MIM 225200)
Primarily cardiovascular features
Familial mitral valve prolapse syndrome (MIM 157700)
Familial aortic aneurysm (MIM 132900)
Familial aortic dissection (MIM 132900)
Pleiotropic
MASS phenotype (MIM 157700)
Shprintzen-Goldberg syndrome (MIM 182212)

linkage analysis can be performed in families that have several available and willing relatives with MFS [102,103].

Differential Diagnosis

A primary preoccupation of the genetic nosologist is deciding whether to "lump" or "split" similar phenotypes. Evidence of genetic heterogeneity, through analysis of pedigrees, proteins, and genes, is justification for splitting similar phenotypes into distinct clinical disorders. However, not all genetic heterogeneity necessitates splitting; both allelic and locus heterogeneity of the type I procollagen genes produce clinically inseparable osteogenesis imperfecta phenotypes (see Chapter 8, this volume). Differential diagnosis of MFS is hampered by three factors: the range of fibrillin-1 mutations in MFS and related conditions; the considerable prevalence of many phenotypic manifestations in the general population and in other connective tissue disorders; and the existence of a phenotypic continuum, which includes "healthy" people at one extreme and MFS at the other. Often, differential diagnosis in the clinic involves deciding not whether the consultant has MFS or another condition but whether any disease is present at all (Table 2).

Homocystinuria due to deficiency of cystathionine β-synthase (MIM 236200; see Chapter 13, this volume) was for decades lumped with MFS because of common features of tall, dolichostenomelic habitus, scoliosis, anterior chest deformity, and ectopia lentis. However, autosomal recessive inheritance, mental retardation, and vascular thromboses are not manifestations of MFS, and autosomal dominant inheritance, aortic dilatation and dissection, and joint hypermobility are not common in homocystinuria. Nonetheless, sporadic cases in a family, especially when evaluated at a young age, may be difficult to diagnose with certainty. When a person is the first in a family to be evaluated for MFS, a screening test for homocystinuria should be performed.

In some cases, the Stickler syndrome (hereditary arthroophthalmopathy, MIM 108300) shares with MFS autosomal dominant inheritance, tall stature, retrognathia, midfacial hypoplasia, joint problems, retinal detachment, myopia, and mitral valve prolapse. Stickler syndrome (see also Chapter 23, Part II, this volume) can usually be differentiated by the characteristic vitreoretinal degeneration, and by a family history of cleft palate or deafness segregating with the skeletal and ocular problems. Stickler syndrome shows locus heterogeneity, with mutations in the procollagen loci for type II and type XI collagens having been described [104,105]. As methods for detecting mutations in large genes evolve, diagnosis based on direct analysis of the basic defect will become feasible.

Aside from these two well-recognized syndromes that affect multiple organ systems, a host of other Mendelian conditions need to be considered because of the involvement of one of the major systems affected in MFS. The conditions that share skeletal manifestations include: classic and hypermobile Ehlers-Danlos syndrome (MIM 130010 and MIM 130020) (joint hypermobility and scoliosis) (see also Chapter 9, this volume); sickle-cell disease (MIM 141900.0243), multiple endocrine neoplasia type III (MIM 162300), trisomy 8 mosaicism, and Klinefelter syndrome (tall, dolichostenomelic habitus); and congenital contractural arachnodactyly (MIM 121050) (arachnodactyly, joint contractures, and scoliosis, *vide infra*). There is scant evidence that a "Marfanoid hypermobility syndrome" (MIM 154750) exists apart from MFS; rather, patients with marked joint flexibility either have MFS if they have ectopia lentis or aortic dilatation or else a form of Ehlers-Danlos syndrome.

With regard to ocular findings, retinal detachment occurs in Ehlers-Danlos syndrome type VI (MIM 225400), and ectopia lentis is common in Weill-Marchesani syndrome (MIM 277600) and occurs as isolated autosomal dominant (MIM 129600) and recessive traits, the latter with (MIM 225200) and without (MIM 225100) ectopic pupils.

The cardiovascular system poses the most difficult diagnostic dilemmas, not only because of overlapping hereditary and acquired phenotypes but also because inaccuracies can lead to improper counseling and management, with lethal consequences. First, with regard to the atrioventricular valves, a myxomatous valve apparatus is common in the population, is often associated with valvular prolapse and regurgitation, and may be inherited as an autosomal dominant trait (MIM 157700). Moreover, mitral valve prolapse occurs in many genetic conditions, including most of the heritable disorders of connective tissue [38]. The most difficult diagnostic problems arise in individuals with a spectrum of manifestations we have termed the "MASS phenotype" [64], where the M signifies involvement of the mitral valve, the A mild aortic dilatation (without much risk of progression or dissection), and the S's skin (striae atrophicae) and skeletal involvement. We chose the term "phenotype" to signify that there is unlikely to be a unitary cause among individuals with these findings; indeed, some but apparently not all have mild variations of the basic defect underlying MFS. The MASS phenotype accounts for only a small fraction of the ~5% of the general population found to have mitral valve prolapse, but it is important to avoid labeling these people with the Marfan diagnosis because of the prognostic, therapeutic, and psychosocial implications. Certain families are outliers from the MASS phenotype and constitute their own private syndromes, albeit similar to MFS in some respects [106].

Dilatation of the ascending aorta occurs in a number of acquired conditions (tertiary syphilis, ankylosing spondylitis, relapsing polychondritis, Reiter syndrome) that will not be confused with MFS. It occasionally occurs as an isolated problem, even in childhood. Here the causes include a sporadic Mendelian trait, a multifactorial trait, and one part of the triad of bicuspid aortic valve, coarctation of the aorta,

and medial degeneration of the ascending aorta. As with any congenital heart lesion, there is an increased prevalence of skeletal abnormalities in such patients; ascending aortic aneurysm is often accompanied by pectus excavatum, reduction of thoracic vertebral kyphosis ("straight back"), or scoliosis.

Among Mendelian disorders, the vascular type of Ehlers-Danlos syndrome (MIM 130050) causes rupture of large arteries due to defective type III collagen; rarely do aneurysms form, and usually the abdominal portion of the aorta is affected. Erdheim cystic medial necrosis (MIM 132900) is better called "familial aortic dilatation" (*vide infra*) because the histopathology is incorrectly described by "cystic medial necrosis" and there has been a tendency to lump families who primarily have dissection without much dilatation into the Erdheim category along with families in which aneurysms are prominent. At least until the underlying defects are described, the entities should be split. A study of 50 individuals with thoracic and abdominal aortic aneurysms found only one with a defect of type III collagen, and this family likely represented a mild form of vascular EDS [107]. Families in which dissection occurs in the absence of aneurysms pose considerable diagnostic and counseling challenges because of the lack of a clinical marker before tragedy strikes [108].

Prevalence and Demographics

The classic MFS, because of its variability, tends to be underdiagnosed. Current estimates of prevalence, based on the authors' personal experience, suggest a frequency of 1–3 per 10,000. Data from registries produce lower estimates, for example, of 1 per 20,000 for prevalence and 1 per 10,000 for incidence based on a survey of ectopia lentis in Denmark [109]. There does not appear to be any ethnic or geographic preference. Because the condition is a Mendelian dominant and there is a relatively high spontaneous mutation rate, there would be no *a priori* reason to suspect an ethnic predisposition.

Genetics

Weve [9] and Apert [15] were the first to suggest autosomal dominant inheritance of MFS. As the diverse manifestations of MFS were recognized, investigators struggled with the concept of pleiotropy and whether more than one locus was involved [110]. Indirect but convincing evidence of both unimodality of the distribution of intrafamilial phenotypic severity and lack of segregation of manifestations supported the unilocal hypothesis. Penetrance is extremely high, and we have never documented an example of nonpenetrance in hundreds of pedigrees. For example, in the largest pedigree yet described, each of 105 affected individuals had an affected parent [111].

At least one-quarter, and perhaps one-third, of patients have parents who do not have MFS, a revision of the earlier 15% estimate [3]. However, formal studies on this point are lacking and require detailed clinical assessment of the parents. Failure to scrutinize the parents of multiply affected sibs is the most likely explanation for purported autosomal recessive inheritance [112], although genetic heterogeneity of such an extent occurs in other disorders. Both somatic and germ-line mosaicism have been described in MFS [113–115].

Isolated cases are often more severely affected, an observation that reflects ascertainment bias, relatively higher reproductive fitness of patients with less severe phenotypes, and probably other factors. The paternal age at the time of conception of isolated cases is advanced (36 years versus 29 years), consistent with the notion that a new mutation in a spermatagonium is the cause of much sporadic MFS [116].

Several examples of purported "homozygous" MFS have been reported [48,117]. Only one case of compound heterozygosity at the *FBN1* locus has been confirmed at the molecular level, and the affected child had a severe phenotype that led to early demise [118].

Management and Clinical History

The cornerstones of management are prompt diagnosis, prospective assessment, prophylactic intervention, and genetic counseling. Because many patients first come to medical attention through referral to, or diagnosis by, specialists, it is vitally important that the orthopedic surgeon, ophthalmologist, or cardiologist recognizes the importance of multidisciplinary evaluation and follow-up and so advises the patient, parents, and referring physician.

The Skeletal System

Stature. Growth can be modulated by early induction of puberty [119–122]. The indications we have used are moderate scoliosis and markedly tall stature, both in girls. A conjugated estrogen tablet (Premarin), 10 mg daily, and, for 14 consecutive days each month, mederoxyprogesterone acetate (Provera), 10 mg, is one approach. The bone age must be ascertained before therapy and followed every 6 months during it. The endometrium should be biopsied after therapy is well along to ensure that unacceptable hyperplasia has not developed. Timing of therapy is important; beginning too early may reduce adult height unacceptably and may be difficult psychologically, whereas beginning after pubarche will have little impact. This approach has never been subjected to rigorous clinical investigation; however, no short- or long-term adverse effects are known [122]. There is little experience with induction of puberty in boys. The side effects of androgens compared to estrogens are more substantial, and tall stature is generally better tolerated by men.

Anterior chest deformity. Pectus excavatum is an important concern of children, parents, and primary care physicians but rarely causes symptoms or clinical consequences early in life. Repairs performed when substantial rib growth remains carry considerable risk of recurrence. Surgery delayed until midadolescence carries an excellent prognosis, provided a substernal strut is temporarily placed until the costal cartilages regenerate completely [123,124]. We currently recommend that severe pectus excavatum be repaired in all adolescents and adults with MFS. The advantages to respiratory mechanics are considerable, and exposure of the heart and aorta at the time of cardiovascular surgery is greatly facilitated. Combination of pectus repair and cardiovascular surgery is a considerable undertaking, with a risk of major hemorrhage from bone as a consequence of anticoagulation and platelet destruction during the cardiac phase of the procedure. Repair of pectus carinatum is usually performed for cosmetic reasons.

Vertebral column curvature. Abnormal curvature at any level of the vertebral column can develop and progress at any stage of growth (Fig. 7). Some infants have severe deformity and require early bracing or even instrumentation [23]. The hazard, of course, of early surgical manipulation is disruption of vertebral growth plates and reduction in thoracic height. In most patients, abnormal curves develop gradually during childhood and can be followed by both clinical and radiographic examination. Both abnormal kyphosis and

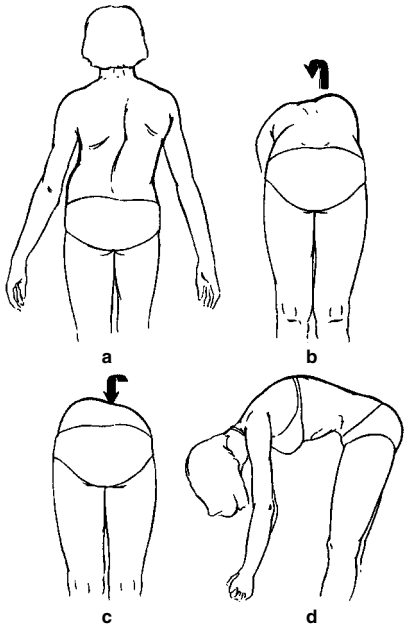

Figure 7. Clinical examination for abnormal vertebral column curvatures. **a**: The patient is first viewed standing upright to detect scoliosis. **b**: Bending forward to about one-half the extent possible will reveal abnormalities of vertebral column rotation of the thoracic spine as well as scoliosis. **c**: Bending forward to the extent possible will reveal abnormal rotation and scoliosis of the lumbar spine. **d**: Positioned as in c, the patient is viewed from the side to detect abnormal lordosis and kyphosis. (Figure courtesy of Paul Sponseller, M.D.)

scoliosis, if not severe, respond to bracing in most cases [21]. Upper thoracic lordosis is a relative contraindication to bracing lower thoracic scoliosis. We recommend bracing for children with scoliotic curves progressing beyond 15–20° and a strong consideration for surgical stabilization as curves increase beyond 40° [22]. Advances in orthopedic surgery obviate the need for prolonged casting or confinement to bed.

Progression of moderate and severe scoliosis in adults is becoming an increasing problem as these patients survive their cardiovascular manifestations. Indications for surgery include pain, neurologic signs, and thoracic curves over 45°, which could progress to cause restrictive lung disease.

The usual criteria for orthopedic management of spondylolisthesis apply [24].

Joints. Surgical stabilization of joints that dislocate repeatedly is occasionally necessary. The patella is the most common problem, followed by the ankle, wrist, and thumb. Every effort should be made at muscle strengthening to substitute for the lax ligaments.

Marked laxity of the ankle and foot produces calcaneoplanovalgus and distorts the normal lines of weight-bearing, which in turn places stress on the hips and knees. This problem is best approached in early childhood by prompt diagnosis and management with orthotics and bracing, followed by osteotomies or arthrodesis if necessary.

Children often have difficulty learning to draw and write because of difficulty holding and stabilizing pencils. The problems are multifaceted and include arachnodactyly, finger laxity or contractures, and wrist laxity. Occupational therapists, through braces, aids, and exercises, can often provide invaluable assistance.

The Ocular System

From the perspective of the primary caregiver, the most important responsibility is referral to an ophthalmologist experienced with MFS as soon as the diagnosis is suspected. Early, accurate detection of ocular problems coupled with aggressive therapy can prevent amblyopia in most patients and optimize development of visual acuity [34]. In practice, infants under 6 months are often easier to examine than toddlers.

The dislocated lens interferes with vision in a minority of people with MFS. In the first place, ectopia lentis is not present in about one-half of patients and in a substantial proportion of the rest is of mild degree. Visual difficulties result primarily from the edge of the lens bisecting the pupillary space. Typically, with a superiorly displaced lens, the patient has phakic vision when gazing upward and aphakic vision when looking downward. Some patients are helped by drugs that keep the pupil constricted (miotics), others by spectacles that incorporate phakic and aphakic correction, and still others by a combination of contact lenses and spectacles. Experience suggests that people with MFS who have serious visual difficulties on account of ectopia lentis respond well to lens extraction and placement of a lens prosthesis [125,126].

Highly myopic patients can generally receive adequate correction. Contact lenses are usually well-tolerated and are welcomed by those patients who must wear thick lenses in spectacles. More than 90% of patients, when treated from childhood, achieve visual acuity of better than or equal to 20/40 in both eyes. Because of the typically flat cornea, various procedures used to correct myopia by reshaping the cornea should be avoided.

There has long been concern about retinal damage, and Maumenee [34] clearly related lens extraction before the newer approaches of the 1990s to an increased risk of detachment. Furthermore, the globe is often elongated and, as in highly myopic patients in general, the retina is under abnormal stretch and more likely to detach. Retrospective evaluation of patients with MFS has found less risk of retinal detachment with exercise than had earlier been feared. Nonetheless, patients should avoid blows to the head (boxing, high-diving) and protect against blows to the globe (racquet sports) with cushioned spectacles.

The Cardiovascular System

As in the other body systems, cardiovascular diagnosis before clinical sequelae is key to the prevention of morbidity and mortality. Once detected, abnormalities must be followed by methods that are both sensitive and practical and interpreted by physicians familiar with both the natural and the clinical histories of the manifestations. Restriction of physical activity, reduction in emotional stress, pharmacologic reduction in hemodynamic stress, and cardiovascular surgery all contribute to improving the long-term function and life expectancy of patients.

Detection of abnormalities. Careful physical examination on a schedule dictated by the severity of the cardiovascular involvement is essential for management. The appearance of aortic regurgitation usually signals substantial aortic root dilatation. Mitral regurgitation may appear at any age as a result of progression of mitral valve prolapse, rupture of chordae tendineae, mitral annular dilatation, endocarditis, or a combination of these factors. Appearance of new systolic

ejection murmurs (especially those radiating to the neck), systolic bruits over the back or abdomen, loss of a peripheral pulse, or blood pressure differences between the arms all may signal aortic dissection. It is crucial to realize that aortic dissection in some patients with MFS is occult.

Transthoracic echocardiography is the mainstay of diagnosis and management of aortic root dilatation. Rarely does the maximum diameter of the undissected ascending aorta occur above the sinotubular ridge, which is the typical limit of visualization. Progression of aortic root dilatation is unpredictable, and a patient's dimensions should be charted regularly; the dimension should be normalized to body surface area for children to account for growth [62,127].

Mitral valve prolapse can also be diagnosed, followed, and defined qualitatively by echocardiography. In about one-quarter of patients with MFS, mitral valve prolapse or mitral regurgitation progress in severity [49]. With mitral regurgitation the most common indication for cardiac surgery in infants and children with MFS [43,47], it is especially important that children with mitral valve prolapse be studied at least annually. Most, but not all, patients with MFS with a prolapsed mitral valve have "floppy" leaflets, defined as thickened, redundant structures by echocardiography. Although not formally studied in MFS, based on studies of mitral valve prolapse in other people, it is likely that redundant valves are more likely associated with progressive mitral valve disease than are valve cusps of normal thickness and length, even if they do prolapse.

Echocardiography is also essential for assessing left ventricular size and function, left atrial size, and tricuspid valve function. Transesophageal echocardiography affords visualization of the distal ascending and the descending aorta and improves assessment of prosthetic valves.

Doppler echocardiography is useful for detecting and grading the severity of aortic regurgitation and mitral regurgitation. For mitral regurgitation in the absence of substantial aortic root dilatation, decisions about the need for surgical correction can be made solely on the clinical examination and the Doppler echocardiogram, and cardiac catheterization can be avoided.

Magnetic resonance imaging (MRI) is the best technique for regular assessment of chronic dissection of any region of the aorta (Fig. 8) and for following the potential for dilatation of any region of the aorta after repair of the root [128]. There is also an emerging role for MRI in the diagnosis of acute dissection when the clinical situation permits the 2 hours required to arrange and perform the scan. Aortography, once a mainstay of diagnosis and presurgical evaluation, is seldom required today (Fig. 9).

Ambulatory monitoring of the electrocardiogram should be performed on any patient with symptomatic palpitations, syncope or near-syncope, or a baseline electrocardiogram that shows major rhythm or conduction disturbance [41].

People with MFS should employ standard indications for routine antibiotic prophylaxis around dental and other procedures that carry a high risk of introducing bacteria into the blood stream [129,130]. Occasional patients may require parenteral antibiotic prophylaxis, but there is no evidence at this time that uncomplicated aortic root surgery alone is an indication.

The chief goal of management is prevention of aortic root dilatation. Acute aortic dissection (Fig. 9) is the major cause of sudden death, which may also be due to myocardial infarction, other organ ischemia, free rupture, pericardial tamponade, or a combination thereof. Because

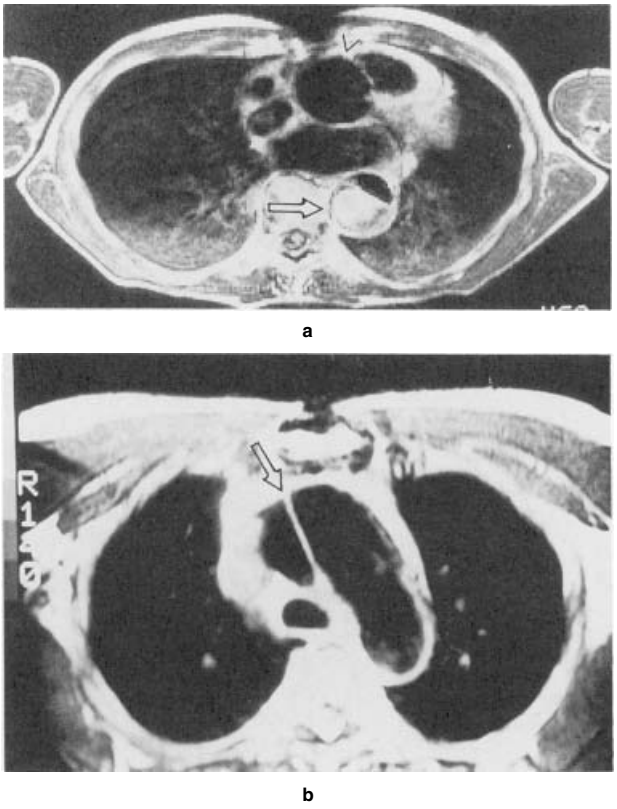

Figure 8. The Marfan aorta as revealed by magnetic resonance imaging. **a**: Axial image of the thorax through the level of the bifurcation of the main pulmonary artery. The aortic root is indicated by the arrowhead; it is moderately dilated. The arrow points to a dissected thoracic aorta that is minimally dilated. The scan is oriented with the dorsal surface down, and the patient's left to the right of the figure. **b**: Axial image of the thorax through the level of the aortic arch. The arrow points to the intimal flap of the dissected aorta. Orientation as in a.

both dissection and aortic regurgitation are associated with marked increase in aortic root diameter, it has long seemed logical that prevention of aortic dilatation would improve longevity [35,36].

Not every aortic dissection occurs during a moment of intense physical or emotional stress, but many do. In competitive athletes who die suddenly, MFS trails hypertrophic cardiomyopathy, congenital anomalies of the coronary arteries, and occlusive coronary disease [131]. Any situation that results in increased catecholamine release will heighten inotropy and, as a result, the rate of change of aortic pressure (dP/dt). Accordingly, we recommend that patients avoid situations of considerable emotional or physical stress. In practical terms, it is almost as difficult to counsel patients about what to avoid as it is for them to alter their lifestyles and personalities. Hence, this effort should not be undertaken lightly, and objective evidence of efficacy would clearly bolster the position and impact of the clinician. Moreover, the counseling should be individualized. A child with an aortic root dimension only slightly dilated is at considerably less risk of dissection than a young adult with an aortic root dimension of 53 mm. The child likely needs no outright restriction, only channeling of interests by parents and

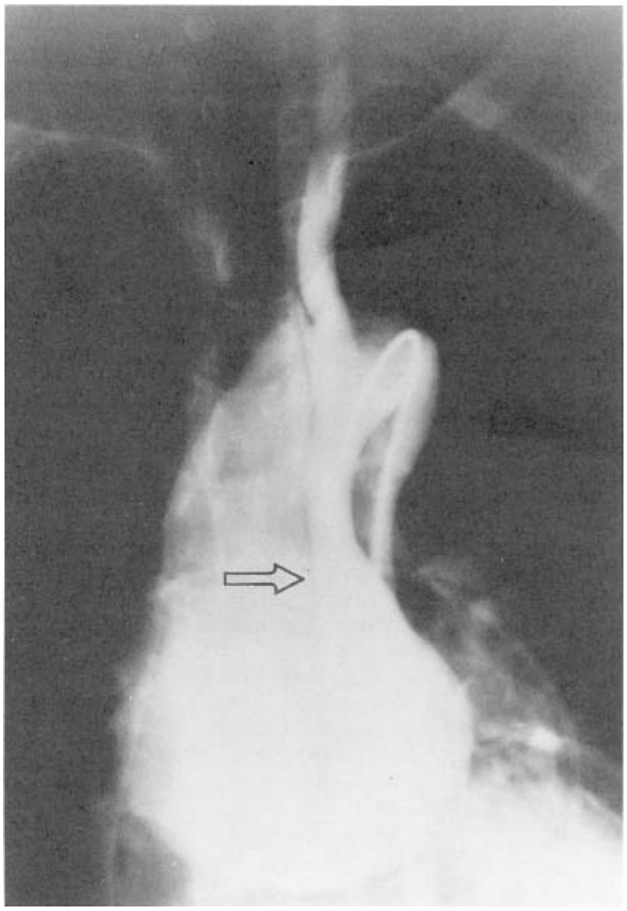

Figure 9. Aortography of a dissected ascending aortic root aneurysm. Anteroposterior radiograph after injection of contrast into the aortic root. The aortic root is markedly dilated, and the arrow points to the intimal flap of the dissection that extends from the valve to the great vessels of the arch. The patient's head is at the top, and the patient's left is to the right of the figure.

teachers away from competitive athletics, whereas the older patient should be told to avoid strenuous exertion of any sort, activities that carry a risk of sudden stops (especially collisions, as occur when playing basketball), or isometric exercises (which increase heart rate and blood pressure more than isokinetic work).

Inotropy of the left ventricle, and dP/dt in the aortic root, are increased by stimulation of myocardial β-adrenergic receptors. The first drug to block these receptors to be approved in the U.S. and to be used worldwide was propranolol. At maximal dosage (which varies widely among individuals), both the increase in heart rate and the more vigorous contraction that occur when isoproterenol is administered are prevented. Propranolol is widely used as an antihypertensive, but in a normotensive person, treatment with β-adrenergic blocking agents has minimal effect on blood pressure in the resting state.

In a randomized clinical trial using propranolol, 76 subjects with MFS were selected to receive either the drug or nothing [132]. The subjects were followed every 6–12 months with serum propranolol levels which averaged 135 ± 80 ng/mL in the treatment group), clinical examination, and echocardiography. Subjects who began the study with immature skeletons were analyzed separately from adults because the aortic diameter was increasing due to growth in the former. Dependent variables were clinical endpoints of the occurrence of aortic dissection or death and the appearance of aortic regurgitation or congestive heart failure, and the aortic root dimension. Follow-up continued for an average of seven years, by which time newer β-adrenergic blocking drugs had become available and a number of subjects in the study had reached a clinical endpoint. In the early years of the study, fewer subjects receiving treatment reached a clinical endpoint than did untreated ones. After nearly a decade, however, the gap between the groups narrowed. The rate of increase in aortic root dimension was significantly slower in both children and adults who took propranolol. In few cases was dilatation actually prevented. Adverse effects of propranolol occurred in one-third of subjects and limited attaining an optimal dose in one [132]. Other studies have confirmed these results [133] while suggesting that some patients may be more responsive than others [134]. The mechanical response of the aorta to acute administration of β-blockade appears variable, with an increase, decrease, or no change in aortic stiffness [134–137]. These differences have not been correlated with clinical outcome.

Because propranolol has unacceptable side effects in some patients, current studies are attempting both to confirm a beneficial effect of β-adrenergic blockade and to study alternative agents [127,136–139].

We currently recommend that all patients with MFS be considered for β-adrenergic blockade. Contraindications include severe diabetes, Raynaud phenomenon, and bronchospasm. The larger the aortic root, the greater the need for treatment. On the other hand, the less the relative dilatation, particularly in young children, the more willing we are to observe the aortic dimension before starting treatment. We start at a low dose and over several months gradually advance until the resting heart rate is in the low 60s and the heart rate after a minute of submaximal exercise does not rise above 100 beats per minute in adults and 110 in children.

Cardiovascular surgery. Techniques for repairing all aspects of cardiovascular disease in MFS have undergone rapid evolution, and today few patients, including children, carry unacceptably high risks of not surviving surgery [67,140]. Three areas of surgery deserve particular mention: the mitral valve, the nondissected aortic root, and the dissected aorta. Most patients with MFS have solid enough connective tissue away from dilatated or dissected areas in which to anchor sutures, and nearly all heal well, with scar tissue of normal substance.

Mitral regurgitation may demand surgical consideration long before the aorta is widely dilated. The surgeon is usually faced with patulous, redundant cusps, a dilated annulus, and occasionally ruptured chordae. Mitral valve repair, rather than replacement, has been developed and shown to be effective over a few years at least [141]. Repair preserves papillary muscle contribution to left ventricular shape and function and obviates the need for chronic anticoagulation (unless atrial fibrillation persists).

The dilated ascending aorta and the leaking aortic valve should be considered as a unit; either or both can be the major indication for repair. The development of the composite graft (Fig. 10), which came into widespread use in the mid-1970s, dramatically improved both operative and long-term survival in MFS [67,73,142,143]. More recently,

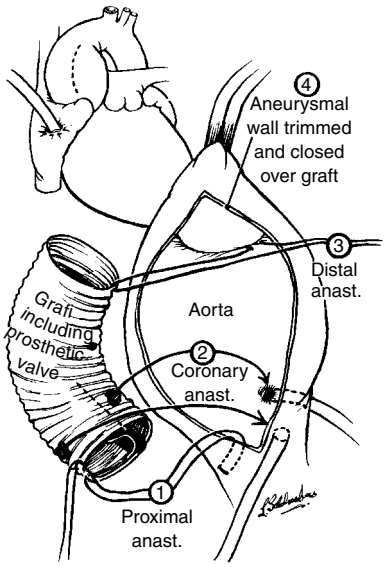

Figure 10. Depiction of the principal steps in composite graft repair of the ascending aorta. In step 1, the proximal end of the graft is sutured into the aortic annulus. In step 2, the coronary ostia are sutured to side holes cut into the graft. In step 3, the distal anastomosis is performed above the upper extent of aortic dilatation. Step 4 refers to a tight closure of the aneurysm wall around the graft, a procedure no longer employed. (Reprinted from [73] by permission of the New England Journal of Medicine.)

David [144,145] has advocated a valve-sparing procedure that has had good success over more than 5 years in some people with MFS [145–149]. This procedure may have limited application when the sinuses are widely dilated and the aortic valve is markedly stretched.

We recommend aortic root surgery when the sinuses dilate to 50–55 mm maximum diameter [150]. If the patient has a family history of sudden cardiovascular death, prophylactic repair should be considered when the diameter reaches 50 mm [151].

The dissected aorta poses the greatest challenge to the surgeon. There is general agreement that acute dissections of the ascending aorta are surgical emergencies; repair with a composite graft is the procedure of choice in MFS [143,146,152,153]. An acute dissection with origin beyond the left subclavian artery should be managed medically (reduction of blood pressure and dP/dt) until the clinical situation stabilizes. Ideally, dissection of the descending aorta will not need surgery but can be followed by MRI at regular intervals. However, progressive dilatation beyond 55 or 60 mm, recurrent pain or signs consistent with fresh dissection, and organ or limb ischemia are all indications for repair [154]. Williams [155] advanced an approach for repairing the chronically dissected abdominal aorta that does not involve a graft. Crawford and colleagues [156] pioneered the staged approach to aortic surgery and performed the first successful replacement of the entire Marfan aorta.

The Respiratory System

To protect against pneumothorax, patients should avoid rapid decompression, such as might occur with rapid ascents in elevators, scuba diving, and flying in unpressurized aircraft. When a clinically important pneumothorax occurs, a chest tube is the appropriate initial therapy; however, in MFS the ruptured bleb may not seal or may rupture again before healing is complete. This clinical history prompted Hall and colleagues [74] to advocate a more aggressive approach and to perform bleb resection and pleurodesis after one recurrence.

Some patients seem predisposed to emphysema, even in the absence of environmental exposures. Because susceptible patients cannot yet be identified, all patients with MFS and their household contacts should abstain from smoking.

The Integument

Striae atrophicae are of cosmetic concern only. An inguinal hernia should be repaired especially carefully because of the risk of recurrence. Similarly, any abdominal incision is at increased risk of ventral herniation. The majority of cases of dural ectasia detected among our clinic population have been asymptomatic [91]. The exceptions have been patients with intrapelvic anterior meningoceles or arachnoid cysts who had radicular pain, parasthesia, and weakness in lumbosacral root distribution. Conservative measures have helped only temporarily. However, attempts to marsupialize the cysts have had mixed success; the cysts may be loculated, and the nerve roots may be stretched across the wall of the cysts and severely limit surgical approaches.

Genetic Counseling

When anyone with MFS reaches reproductive age, he or she should be counseled about the risks of transmitting the mutant allele and of variable expression but not nonpenetrance.

The parents of the first individual diagnosed with MFS in a family should be counseled about the need for a thorough clinical evaluation of themselves to exclude MFS; this evaluation includes an ophthalmologic and an echocardiographic examination. Both somatic and germ-line mosaicism have been described in Marfan syndrome [113–115]. Germinal mosaicism is distinctly uncommon and of minor significance during the counseling of most patients and families.

Pregnancy. The third trimester, labor and delivery, and the first postpartum month carry an increased risk of aortic dissection in all women, especially those with MFS [157]. Most, but not all, cardiovascular complications in the pregnant woman with MFS occur in association with pre-existing problems, such as dilatation of the aortic root, aortic regurgitation, or mitral regurgitation [157–160]. Thus, a woman can be given some estimate of cardiovascular risk before conceiving. Risk of aortic root dissection is quite low when the aortic diameter is 40 mm or less [158] but undoubtedly higher than for women without MFS. However, some women with MFS suffer dissection of the descending thoracic aorta even in the absence of aortic root dilatation. At this time, this risk cannot be quantified but is also clearly greater than for the general population of pregnant women. Whether β-adrenergic blockade is completely harmless to the early fetus is still subject to debate, but such therapy is used routinely by obstetricians for other indications. β-blockade can be used in later stages of pregnancy should dysrhythmia or an increasing aortic root diameter develop.

Delivery should be by whatever method stresses the maternal circulation the least, with due regard to the health of the fetus. Individual assessment of these various risks should be started in advance of term but may not be finalized until early in labor. Epidural and spinal anesthesia should be performed only after consideration of the possibility of dural

ectasia; arachnoid cysts might result in considerable dilution of anesthetic.

Risks to family members. People affected with MFS transmit the condition to one-half of their offspring, on average. In counseling couples with such a risk, variability must be emphasized; an affected child may be more or less severely affected than the parent.

When unaffected parents have a child with MFS, the standard approach to counseling would be to advise of a minuscule risk of recurrence in a subsequent child.

Prenatal testing. Ultrasound is insensitive in the first two trimesters in detecting a fetus with MFS [161]. Prenatal diagnosis based on analysis of DNA obtained from chorionic villi or amniocytes is possible if the mutation is known in the affected parent or if linkage with markers in and around the FBN1 locus can be established [162,163]. Preimplantation diagnosis has been reported [164–166] but, as with all autosomal dominant conditions, is complicated by the potential for selective PCR amplification of the normal allele [167].

Psychosocial Aspects

How patients with MFS cope with the condition and its myriad implications is poorly studied, but in many respects they do not differ from people with other chronic, hereditary conditions. Concerns and emotional responses differ among patients on account of age and prior counseling, among numerous factors [168].

Patients and families often benefit from interacting with peers who have encountered similar problems. Volunteer support groups have been established in more than a dozen countries thus far. Contact details are obtainable from the Web site shown under "Patient Support Groups" at the end of this chapter.

Pathology

Although many reports of autopsies, histopathology, and ultrastructure have been published, no changes are entirely characteristic of MFS.

The gross pathologic alterations follow from the clinical phenotype [3,169–172]. One of the most striking sights in medicine is the massively dilated aortic root, filling the mediastinum of the chest opened by median sternotomy. The wall of the ascending aorta is often so thin as to be translucent, and blood can easily be seen pulsating literally a millimeter from disaster.

Histology of bone, cartilage, and the growth plate is unremarkable. Fascia, ligament, and skeletal muscle (with rare exceptions of possible myopathy [95,97]) appear normal. Skin shows fragmentation of elastic fibers, especially in areas of flexural stress where striae distensae are found; but the striae in MFS are not distinguishable from those occurring in the general population [85]. Immunohistopathology with a monoclonal antibody to fibrillin, a glycoprotein constituent of the microfibrillar component of elastic fibers (*vide infra*), shows considerably reduced binding in skin from patients with MFS [173,174].

The changes in the aortic wall, which are age-dependent, are primarily in the media and include fragmentation and disarray of elastic fibers, a paucity of smooth muscle cells, and the separation of muscle fibers by collagen and glycosaminoglycans [172,175]. The latter pools and resembles cysts, although no walled vesicles occur. Similarly, no necrosis is present, and the term "cystic medial necrosis" should be discarded [40,175] despite its long tradition [176]. These histopathologic changes are not specific for MFS; similar alterations occur in aortas subjected to hemodynamic stresses (e.g., bicuspid aortic valves, hypertension) and as a result of aging [175]. Moreover, the aorta in copper-deficient or lathyritic animals closely resembles that of MFS [177]. Medial degeneration is also present in other elastic arteries, even though dilatation is not common [178,179] except in the main pulmonary artery [180] (Zerhouni and Pyeritz, unpublished). The cardiac valves show myxomatous transformation, with thickening of the spongiosa layer and an increased amount of a cellular, nonfibrous matrix [40].

Studies of the ultrastructure of cells and matrix in the Marfan syndrome have not been particularly revealing. The chief finding is disorganization of constituents of the extracellular matrix [181], especially the elastic fibers [171,182].

Etiology and Pathogenesis

Historical Perspective — Implication of Microfibrils

Many clinical and histopathologic observations in Marfan syndrome suggested a primary defect in the extracellular matrix. Because of autosomal dominant inheritance, mutations in a structural protein that contributes to higher-order macroaggregates seemed likely. Over the years, preliminary evidence implicating most of the major classes of connective tissue components has been presented. One hypothesis held that aortic failure results from deficient tensile strength due to abnormal synthesis, matrix incorporation, and/or tissue homeostasis of fibrillar collagens, particularly type I [183,184]. Supporting evidence included sporadic demonstration of increased urinary excretion of hydroxyproline, increased solubility of dermal collagen, and decreased quantities of collagen cross-links [185–187]. Interest intensified after the reporting of an apparent mutation in the *COL1A2* gene in an affected individual [188]. None of the biochemical abnormalities were consistent findings; however, the putative pathogenic mutation was later shown to be a neutral polymorphism, and all of the genes encoding the major fibrillar procollagens were excluded by linkage analysis [189–194].

Speculation that the basic defect in Marfan syndrome localizes to the ground substance was fueled by the observation of excessive proteoglycans and glycosaminoglycans in affected tissues [171,172,195–197]. This histopathologic finding was later associated with increased hyaluronic acid synthetase activity in patient fibroblast cultures and decreased serum β-glucuronidase activity [198–200]. Others found evidence for decreased mRNA encoding decorin, a chondroitin-dermatan sulfate proteoglycan, in a subset of patients with Marfan syndrome [201–203]. It should be noted, however, that an accumulation of ground substance in the medial layer is a universal finding in aortic aneurysm, irrespective of etiology [204–207]. Moreover, perturbation of proteoglycan or glycosaminoglycan metabolism, including deficient expression of decorin, can be seen in heritable disorders of collagen metabolism and after experimental inhibition of elastin cross-links [208–212]. Thus, it appears that these findings represent nonspecific secondary pathology or compensatory changes that can be elicited by many primary disorders of connective tissue.

As early as 1955, it was suggested that the basic defect in Marfan syndrome localized to the elastic fiber [11]. Initial histopathologic studies demonstrated fragmentation of elastic fibers with a decrease in elastin content in both the skin and medial layer of the aorta [85,169,170,172,213,214]. These findings were later correlated with apparent abnormalities of elastin metabolism in Marfan patients, including decreased

urinary excretion of desmosines, increased elastin solubility, decreased desmosine and isodesmosine content of extracted protein, and increased elastin susceptibility to digestion by pancreatic elastase [213,215–218]. These results suggested a decrease in the density and/or quality of elastin cross-links.

Despite early speculation to the contrary [219], the gene encoding elastin was a poor candidate as the site of primary mutations causing Marfan syndrome. Most importantly, the Marfan phenotype involves tissues that are devoid of elastin, including the ciliary zonules and skeleton [120]. Ultrastructural, biochemical, and immunologic analyses of elastic fibers revealed that the amorphous component was surrounded by rod-like material with a distinct staining pattern and a distinguishable susceptibility to enzymatic digestion [220–223]. These so-called microfibrils have a diameter of 10–14 nm and are constituents of all elastic tissues. Microfibrils are also widely distributed in tissues devoid of amorphous elastin, including the oxytalan fibers intrinsic to the dermal-epidermal junction, tendons, fascia, periosteum, corneal stroma, and the adventitial layer of blood vessels, in numerous basement membranes, and in the mesangium of the renal glomerulus. Of particular interest was the observation that the ciliary zonules that suspend the ocular lens are composed of fibrillar aggregates that demonstrate an ultrastructural appearance and a biochemical profile that is reminiscent of elastin-associated microfibrils [224,225].

It stood to reason that if different tissues, particularly the elastic media of the aorta and the ciliary zonules, shared common mediators of microfibrillar structure, abundance, and/or functional integrity, then the genes encoding such factors would be attractive candidates as the site of primary mutations causing Marfan syndrome. Indeed, Streeten and colleagues [226] first formally proposed an etiologic link between microfibrillar components and the Marfan phenotype after observing that an antibody prepared against sonicated bovine zonules immunolabeled both zonular and elastin-associated microfibrils with an identical periodicity.

The Fundamental Defect — Fibrillin 1

Locus and gene identification. In 1986, Sakai and colleagues [227] first identified fibrillin-1, a 350 kDa glycoprotein component of extracellular microfibrils, and demonstrated that this protein is common to microfibrillar aggregates in all of the tissues altered in the Marfan phenotype, including the skin, vascular wall, hyaline cartilage, perichondrium, ciliary zonules, and alveolar wall. Subsequent immunohistochemical study of Marfan patient tissues showed that the vast majority of patients had diminished amounts of microfibrils in skin, fibroblast culture, or both [173,174]. Study of fibrillin-1 metabolism in cultured skin fibroblasts demonstrated reduced incorporation of fibrillin-1 into the extracellular matrix in about 85% of lines derived from individuals with MFS [228]. In addition, selected patient samples showed defects in protein synthesis or secretion. Subsequently, linkage studies mapped the Marfan locus to anonymous markers on the long arm of chromosome 15, allowing a tentative assignment to 15q15-q21.3 [111,229]. By this time, a portion of the cDNA encoding fibrillin-1 had been characterized [230,231], allowing the gene to be mapped to a position coincident with the Marfan locus at 15q21.1 [12,231,232]. Mutational analysis of fibrillin-1 cDNA from patients with MFS revealed an identical, *de novo* missense mutation in *FBN1*, the gene encoding fibrillin-1, in two unrelated individuals [12]. All linkage evidence suggests the absence of locus heterogeneity for the classic Marfan phenotype with a cumulative lod score in excess of 100 [12,102,111,229,231,233–237]. Although published reports suggest a second locus on chromosome 3p [238], this is based on the analysis of a single kindred that displays both similarities to MFS and differences from it [239]. Thus, it appears that *FBN1* gene mutations account for most, if not all, cases of the classic disorder.

Fibrillin-1 gene and protein. The 8616 bp coding sequence of the *FBN1* gene is contained within 65 exons that span approximately 200 kb of genomic DNA [240–242]. The presence of three alternatively used and in-frame exons upstream of the exon containing the putative initiating methionine in both the human and murine genes initially generated some ambiguity regarding the site of fibrillin-1 translation initiation [240,243]. The first coding exon of the porcine gene contains an in-frame termination codon upstream of the putative initiating methionine [242]. In view of the extreme amino acid conservation of fibrillin-1 among mammals, these data strongly suggest that this ATG methionine codon is the sole start site of translation for fibrillin-1.

Fibrillin-1 is a cysteine-rich 350 kDa glycoprotein with a complex modular organization that is shared within the fibrillin family of proteins [240,241,244,245]. Local differences in amino acid composition or domain content can be highlighted through the designation of five distinct regions of the protein (A–E; Fig. 11) [240,241]. A signal peptide is followed by a short N-terminal sequence encoded by exon 1 that does not show homology with other identified proteins (region A). The C-terminal sequence encoded by exons 64 and 65 (region E) is also unique, but selected features are conserved between fibrillin-1 and fibrillin-2, including a subsequence surrounding two cysteine residues and a high concentration of basic residues [240,241,245]. Both the N- and C-terminal regions contain propeptide cleavage sites that conform to the consensus sequence for furin-like proteases [246–250]. Region C is encoded by exon 10 and is extremely rich in proline residues (38%), in contrast to glycine residues in fibrillin-2 [240,241,245]. Rotary shadowing analysis of monomers reveals a characteristic bend at the corresponding region [227]. It has been postulated that this domain of fibrillin-1 may contribute to the inherent distensibility of microfibrils [240,241]. Further analysis has revealed that region C is sufficient to induce dimer formation of recombinant peptides, suggesting a role in nucleation during microfibrillar assembly [251,252]. This region has also been shown to contain sites susceptible to cleavage by matrix metalloproteinases [253,254]. We have come to appreciate that this domain contains ten perfect tandem repeats of PXXP, where P is proline and X is any residue (commonly also P):

P G R **P** E Y **P** P **P** L G **P** I P **P** V L **P** V P **P** G F **P** P G **P** Q I **P**

This structure is common in Src homology 3 (SH3) domain ligands, but the characteristic positions for interspersed and critical R and L residues are not seen in fibrillin-1 [255]. Rather, it would be predicted that this sequence would form a polyproline II (PPII) helix. PPII helices tend to be found connecting major structural parts of a molecule, where their high conformational mobility confers flexibility [256]. They are frequently located on the surface of proteins, where they can serve as sites of intermolecular interactions, often linking with other PPII helices. PPII helices are conserved in protein families and are found in all serine proteinases including

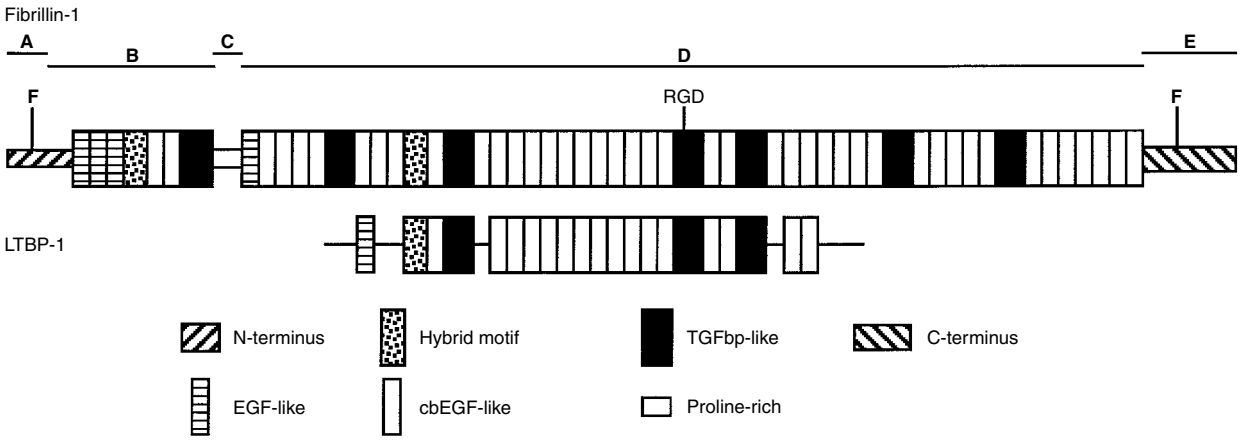

Figure 11. Domain organization of fibrillin-1 and LTBP-1 (latency-inducing transforming growth factor β-binding protein-1). The identity of each domain type is shown at the bottom of the figure. Fibrillin-1 is divided into five regions (A–E) for purposes of discussion (see the text). F, furin-like propeptide cleavage sites. The position of an RGD sequence in fibrillin-1 is indicated. The figure is modified from a previously published version [286].

neutrophil elastase, an enzyme known to cleave fibrillin-1 [256,257].

The bulk of the protein is contained within regions B and D, encoded largely by exons 2–9 and 11–63, respectively [241]. Both regions are characterized by the repetition of cysteine-rich domains, including those that were first identified in epidermal growth factor precursor (EGF-like) and latency-inducing transforming growth factor β-binding proteins (LTBP-like). EGF-like motifs are found in a wide variety of proteins, including many vitamin K-dependent clotting factors and components of the extracellular matrix. One consistent feature is the presence of six predictably spaced cysteine residues that interact via disulfide linkage (1–3,2–4,5–6) to form an antiparallel β-pleated sheet conformation [258] (Fig. 12). A subset of EGF-like domains, including 43 of 47 found in fibrillin-1, satisfy the consensus for calcium binding (cbEGF-like), an event that is required to maintain a rigid, rod-like structure for tandemly repeated motifs and to promote protein-protein interactions [240,259–265]. In brief, calcium binding is dependent on the maintenance of domain structure, as dictated by three intradomain disulfide bonds, and by a number of highly conserved residues that define the consensus sequence

$$(D / N)\ X\ (D / N)(E / Q)\ X_n(D^*/N^*)\ X_m(Y / F)$$

where *n* and *m* are variable and the asterisk indicates potential β-hydroxylation [266,267]. Other conserved residues stabilize the calcium-binding pocket at the N-terminus of an adjacent cbEGF-like domain by mediating interdomain hydrophobic packing interactions [268,269].

LTBP-like domains contain eight conserved cysteine residues that pair via intramolecular disulfide linkage (1–3,2–6,4–7,5–8) [270]. Although this domain contributes to the covalent association between LTBP-1 and TGF-β1 latency-associated propeptide (LAP) through disulfide bond exchange [271,272], the ability of the seven homologous domains in fibrillin-1 to participate in intermolecular interactions remains to be demonstrated. The RGD sequence within the fourth LTBP-like domain of fibrillin-1 has been shown to support cell adhesion through interaction with integrin αVβ3 [273–275]. Little is known regarding the function of other conserved residues in LTBP-like domains. A third cysteine-rich domain, termed the hybrid motif, occurs once in each of the regions B and D and likely manifests fusion between LTBP-like and EGF-like motifs during evolution [241]. The functional characteristics of this novel domain are entirely unexplored.

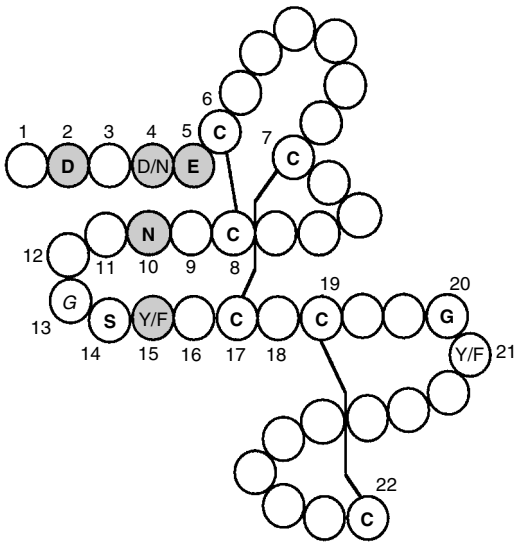

Figure 12. Schematic of a representative cbEGF-like domain from fibrillin-1. Highly conserved residues are indicated using the single-letter amino acid code. Intradomain disulfide linkage between cysteine residues is indicated by lines. Residues important for calcium binding are indicated by shading. Residues shown in italics (positions 13 and 21) are critical for interdomain hydrophobic packing. The figure is modified from a previously published version [290].

***FBN1* mutations imply pathogenetic mechanism.** To date, approximately 150 *FBN1* mutations in patients with MFS or related phenotypes have appeared in the literature or have

been reported to an international database [100,276–279]. More than 90% of mutations are unique to single individuals or families, and the remainder are only present in a handful of apparently unrelated probands. The low rate of recurrent mutations and the high degree of intrafamilial clinical variability has frustrated the development of firm phenotype-to-genotype correlations. The majority of mutations occur within the 47 tandemly repeated EGF-like domains of fibrillin-1, of which 43 satisfy the consensus for calcium binding [266,267]. Many of the mutations substitute one of the six predictably spaced cysteine residues that interact via intradomain disulfide linkage, highly conserved residues within the calcium-binding consensus sequence, or residues important for interdomain hydrophobic packing interactions [100,262,276–279] (Fig. 12). Solving for the solution structure of a pair of cbEGF-like domains demonstrated that calcium binding is required to maintain a rigid rod-like structure for tandemly repeated motifs [262,268]. Moreover, perturbation of domain folding or calcium binding has been shown to result in conformational changes and enhanced proteolytic degradation of single domains, recombinant peptides and intact monomers, and ultrastructural changes in microfibrils [259,263,280–283].

Few clear genotype-to-phenotype correlations have emerged. One exception is the clustering of mutations causing neonatal presentation of severe and rapidly progressive disease in exons 24–27 and 31–32 [284–287]. Interestingly, multiple mutational categories are represented and many mutations within these intervals cause classic or even mild forms of disease [100,276,278,286,288]. As a general rule, mutations causing the in-frame loss or gain of central coding sequence, either through genomic deletions or insertions or splicing errors, are associated with more severe disease [100,289]. In contrast, many mutations that create a premature termination codon and cause rapid degradation of mutant transcripts through the nonsense decay pathway are associated with mild conditions that may fail to meet diagnostic criteria for MFS [286,290–292]. Isolated skeletal abnormalities were observed in individuals harboring a mutation that prevented C-terminal propeptide processing [246]. The mutant gene product was synthesized and secreted but lacked the ability to interact with wild-type protein derived from the normal allele. Taken together, these data suggest a dominant-negative mechanism of disease [100,290]. This model has been validated by immunohistochemical studies and biochemical characterization of cultured patient dermal fibroblasts by pulse-chase analysis. Both methods show that the majority of patient lines exhibit a dramatic paucity of extracellular fibrillin-1, far below the 50% level that might be expected for an autosomal dominant disorder if the relevant mechanism were pure haploinsufficiency [173,228,293–295]. The degree of impairment of extracellular deposition of fibrillin-1 appears to correlate with disease severity and clinical outcome [295].

Although some mutations are associated with a mild phenotype, many that create premature termination codons (PTCs) and transcript instability are associated with the classic Marfan phenotype. As a general rule, the lower the residual mutant mRNA level the less severe the phenotype, but there are clear exceptions [113,296]. It has been hypothesized that N-terminal fragments of fibrillin-1 that are translated from nonsense alleles have dominant-negative activity (i.e., the ability to interfere with the utilization of protein derived from the normal allele through direct interaction). Alternatively, the apparent dominant-negative effect seen with heterozygous nonsense alleles may reflect a decrease in the efficiency of use of protein from the normal allele that is purely dependent on the less-than-normal expression levels [113]. In this scenario, low synthesis, secretion, or stability of mutant protein would all be expected to result in a severe decrease in the level of extracellular microfibrils, below 50% of normal.

The evolutionary conservation of the amino acid sequence of the fibrillins extends beyond the few residues in each EGF-like domain that are known to have functional significance. Indeed, nearly all of the intervening residues in human fibrillin-1 are identical in mouse, pig, and cow [242,243,297]. With few exceptions, missense mutations causing MFS that do not occur in cbEGF-like domains involve cysteine, proline, or glycine residues. The same is true for mutations within cbEGF-like domains that do not replace cysteine or calcium-binding consensus residues. These changes are predicted, or have been proved, to alter protein conformation and stability [262,282,298]. Selected mutations that occur within variable loop regions of EGF-like domains have been associated with very abbreviated forms of disease. Notably, mutation Arg1170His was found in two families with minor skeletal involvement with or without mitral valve prolapse but none of the major manifestations of MFS [113,299]. Such mutations may allow the deposition of stable microfibrils with an as yet undefined but restricted functional deficit.

***FBN1* gene mutations and higher-order pathogenesis of MFS.** The favored model of microfibrillar assembly suggests that fibrillin-1 monomers self-assemble into macroaggregates characterized by parallel arrays of linear extended structures with a head-to-tail orientation [300]. It is easy to imagine how the incorporation of relatively few mutant monomers would be sufficient to cause global perturbation of microfibrillar structure, stability, and function. The tight temporal and spatial link between the formation of microfibrillar aggregates and subsequent deposition of amorphous elastin prompted speculation that microfibrils represent an elastic fiber precursor or regulate elastin deposition during embryonic development [221,222]. In this scenario, the deficiency in elastic fiber abundance and architecture that is observed in mature vascular lesions in MFS predominantly reflects a primary failure of elastogenesis. The absence of organized elastic fibers would cause both a primary structural deficiency and a diminished capacity to modulate hemodynamic stress, perhaps predisposing to secondary damage. Elastic fiber formation is virtually complete after early postnatal life [221,222,301]. Fibrillin-1 and its close family member fibrillin-2 (mutated in congenital contractural arachnodactyly [302]) show divergent temporal and spatial patterns of expression, suggesting that they contribute to structurally and perhaps functionally distinct microfibrillar beds [243,245,303]. Under physiologic conditions, both proteins are no longer expressed after early childhood. Taken together, these data suggest a very limited window of opportunity to modulate the pathogenetic sequence in Marfan syndrome and bode poorly for the development of therapeutic strategies aimed at improving the integrity of elastic tissues including the aortic media. However, this perception may change on the basis of results obtained in studies of the mouse models of Marfan syndrome described below.

The lack of ability to follow the natural history of vascular histologic changes in patients with MFS precluded full appreciation of the pathogenetic sequence leading to aneurysm. New insight has now been provided through

the analysis of mice harboring targeted *Fbn1* alleles. One mutation created an in-frame deletion through the substitution of exons 19–25 with a neomycin resistance cassette (Neo^r) [304]. This so-called mgΔ allele expresses a centrally truncated monomer that was predicted to behave in a dominant-negative manner. However, due to transcriptional interference from Neo^r, very low levels of mutant mRNA and protein are synthesized, with only about 5–10% expression of the mutant allele when compared with its wild-type counterpart. Heterozygous mice were born at the expected frequency and showed only subtle skeletal abnormalities late in life [304]. There were no vascular abnormalities, and life expectancy was normal. Homozygous mgΔ/mgΔ animals were also born at the expected frequency. They died suddenly prior to weaning. Aortic dilatation and dissection were seen, with hemopericardium or hemothorax in all homozygous mutant animals that died naturally [304]. The aortic media showed disruption of elastic laminae and loss of elastin content with the accumulation of amorphous matrix elements, similar to the appearance of mature human lesions [207]. Surprisingly, these abnormalities were extremely focal. At the level of histologic resolution, the bulk of the aorta showed linear, uninterrupted, and parallel elastic fibers. These data demonstrate that minimal microfibrillar function is sufficient to support the deposition of extended elastic structures and highlight the prominent role of fibrillin-1 in elastic fiber homeostasis.

The early death of mgΔ/mgΔ animals prevents a full analysis of pathologic changes leading to aneurysm formation. Important new information, however, has come from the analysis of a second targeted allele (termed mgR) that results from the insertion of Neo^r into intron 18 without rearrangement in the coding sequence [305]. This allele is only expressed at a level approximating 15% of normal. However, protein derived from the mgR allele is normal in structure. Heterozygous mgR/+ animals show no abnormalities. Homozygous mgR/mgR mice show normal intrauterine development but die between 3 and 6 months of age [305]. In addition to vascular disease, these animals show bone overgrowth that is most prominent in the ribs and extremities. Focal calcification of intact elastic laminae is seen in the aortic media as early as six weeks of age. Calcified segments become more numerous and coalesce with advancing age. By 9 weeks of age, intimal hyperplasia with smooth muscle cell proliferation and excessive and disorganized deposition of matrix components is evident. Zones of adventitial inflammation tend to coincide with calcification and intimal thickening. Ultimately, mixed inflammatory cells infiltrate the media with associated expression of matrix metalloproteinases, intense elastolysis, and structural collapse of the vessel wall [305].

A review of autopsy specimens has revealed diffuse calcification and intimal hyperplasia in the aorta and other muscular arteries of young individuals with MFS (own unpublished data). In addition, Takebayashi previously reported the accumulation of an electron-dense and granular material on the surface of, and within, vascular elastic fibers in patients with MFS [171,182]. Although this finding, termed "osmiophilic elastolysis," had been attributed to the accumulation of a peculiar breakdown product of elastin, it is clear in retrospect that the authors were observing diffuse calcification of elastic structures. It is important to note that these findings in patients with MFS do not represent normal age-dependent changes. They were seen in all medium-to-large elastic arteries that were examined and were seen in children without correlation between distribution or severity and the age of the individual (own unpublished data). It is important to note that calcification and intimal hyperplasia occur in vessels that do not dilate or dissect in MFS. This suggests that these changes do not initiate aneurysm formation.

Ultrastructural analysis has defined cellular events that precede and occur coincidentally with destructive changes in the aortic media of fibrillin-1–deficient mice. Normally, elastic laminae connect to adjacent endothelial and smooth muscle cells through junctions composed of microfibrils [306–308], this perhaps being mediated by interaction between the RGD sequence of fibrillin-1 and integrin αVβ3 [273–275]. These connections may contribute to the structural integrity of the vessel wall through cell anchorage and coordination of contractile and elastic tensions [306,307]. Homozygous mgR mice show a striking deficiency of these connections, with direct abutment of smooth muscle cell processes onto elastic lamellae (own unpublished data). Loss of physical interactions, and hence signals that specify context, appears to induce alteration in the morphology and synthetic program of flanking cells that are characteristic of a dedifferentiated state. These cellular changes correlate with the onset of elastolysis (own unpublished data) and the observation of increased immunoreactivity for matrix metalloproteinases at the periphery of mature vascular lesions in patients with MFS [309]. Subsequent disruption of the internal or external elastic laminae in fibrillin-1–deficient mice allows infiltration of inflammatory cells into the media, resulting in accelerated elastolysis and progression to dissection [304,305].

Fibrillin-1–deficient mice and patients with MFS have partial but not complete loss of microfibrillar function due to different pathogenetic mechanisms. Although the *FBN1* genotype is a clear determinant of the degree of functional impairment, hemodynamic stress or other environmental factors may cause progressive loss of residual function. Figure 13 reviews the various molecular mechanisms that have been shown or proposed to result in Marfan syndrome or related phenotypes. Homozygosity for a wild-type allele (+/+) is not associated with abnormalities of the microfibrillar matrix or the development of phenotypic abnormalities. A functional haploinsufficiency state can result when the abnormal product from the mutant allele cannot interact with normal fibrillin-1 derived from the unaltered allele or when the mutant mRNA from heterozygous nonsense alleles is efficiently degraded. Both situations have been observed in association with mild phenotypes in which skeletal manifestations predominate [246,286,290–292]. Near-haploinsufficiency in mgR/+ or mgΔ/+ mice has been correlated with absence or abbreviation, respectively, of the disease phenotype [304,305]. The degree of loss of function that is achieved by the dominant-negative interaction in patients with missense mutations and classic MFS appears to be recapitulated in homozygous mgR mice [305]. Secondary events are required for this deficiency to become clinically manifest. The severe and rapidly progressive phenotype that presents in the neonatal period and is presumed due to alleles with extreme dominant-negative potential [286,289,310] can be recapitulated by homozygosity for an allele that expresses low levels of a centrally deleted monomer in mice (mgΔ/mgΔ) [304]. Even further loss of function is seen on inheritance of two mutant human *FBN1* alleles leading to prenatal fetal loss and absent postnatal viability [118]. An equally severe phenotype is seen

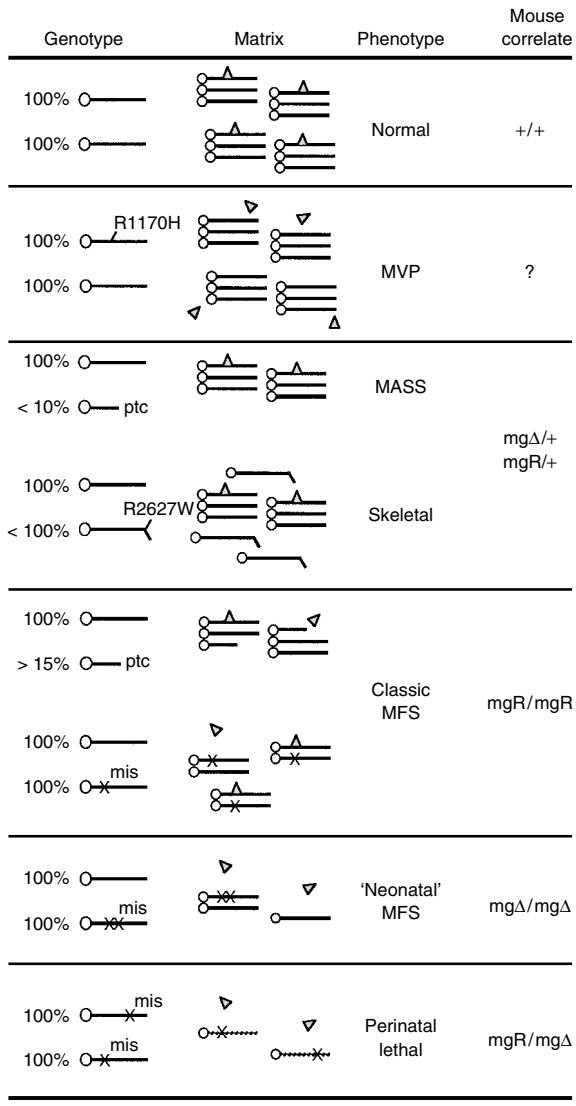

Figure 13. Genotypes and pathogenetic mechanisms in MFS and related disorders. Genotypes: +, wild-type allele; ptc, premature termination codon; X, missense mutation (mis) associated with classic MFS; XX, missense mutation associated with infantile presentation of severe disease (''neonatal MFS''); each horizontal line capped by a circle represents a fibrillin-1 monomer; percentage figure indicates expression level relative to a single + allele. Matrix cartoon indicates the abundance and character of microfibrils. A cluster of three aligned monomers indicates a microfibril; triangle indicates a putative interacting protein. Phenotype: MVP, mitral valve prolapse syndrome; MASS, mitral, aortic, skin, skeletal phenotype; Skeletal; isolated dolichostenomelia. Mouse correlate: genotypes of mice demonstrating a similar phenotype or proposed to manifest a similar pathogenetic mechanism. The figure is modified from a previously published version [390].

when the mgR and mgΔ alleles occur in compound heterozygosity. These data suggest that homopolymers of mutant monomers retain more function than heteropolymers and that the proper alignment of selected domains in adjacent monomers is critical for the assembly or integrity of microfibrils.

The extracellular matrix is an important mediator of many cellular events, including differentiation, proliferation, and migration. The phenotype of smooth muscle cells in developing blood vessels and myofibroblasts in the developing lung may be linked to elastin expression [311–313]. To a large extent, this hypothesis is based on there being an association between alterations in the quantity or quality of extracellular elastin and abnormalities in cellular composition and behavior. Both supravalvular aortic stenosis (SVAS) patients hemizygous for the elastin gene (see also Chapter 10, this volume) and elastin knockout mice show an abnormal proliferative and synthetic smooth muscle cell phenotype, perhaps manifesting a loss of matrix-cellular signaling [313,314]. It is also possible that phenotypic changes simply reflect the loss of a structural constraint to cellular proliferation that is normally imposed by neighboring elastic laminae. The basis for the different vascular phenotypes seen in primary and secondary elastin deficiency in SVAS and MFS, respectively, is not fully understood. Microfibrils may play a structural role in the aorta that is independent of their association with elastin, as seen in the suspensory ligament of the ocular lens. Alternatively, there may be a narrow developmental window for the formation of the obstructive changes characteristic of SVAS. Finally, it is possible that microfibrils have nonstructural functions that are critical in the development and/or homeostasis of the blood vessel wall. For example, it has been proposed that microfibrils may be active in the regulation of the local activity of TGF-β [315]. To an extent, this hypothesis stems from the homology between a repeated 8-cysteine domain in fibrillin-1 and a motif first observed in latency-inducing transforming growth factor β-binding proteins (LTBPs) [271]. TGF-β is secreted in a latent form resulting from its association with its processed propeptide dimer, called the latency-associated peptide (LAP) [316]. This small latent complex binds to LTBPs via a disulfide linkage to form the large latent complex [316,317]. The extracellular matrix regulates TGF-β activity by sequestering the large latent complex from cell-surface–associated activators, including plasmin and integrins αVβ6 and αVβ1 [316,318,319]. There is considerable speculation, and emerging direct evidence, that complexed TGF-β interacts with microfibrils [315,320-323]. In this scenario, a genetically determined deficiency of microfibrils (MFS) would allow excessive processing of the large latent complex, resulting in an increase in TGF-β activity. The effects of TGF-β vary among tissues. In general, this multipotential cytokine inhibits cell proliferation and migration and promotes cellular differentiation and matrix deposition. Interestingly, the embryologic origin of vascular smooth muscle cells determines their response to TGF-β [311,324–327]. Mesoderm-derived vascular smooth muscle cells that populate the descending aorta are inhibited in proliferation and migration and have a neutral response in collagen production. Neural-crest–derived vascular smooth muscle cells that populate the proximal ascending aorta show opposite effects, with the induction of cellular proliferation, migration, and collagen production. Thus, the histologic abnormalities that would be predicted for a pathologic up-regulation of TGF-β activity would occur at the aortic segment that is uniquely predisposed to aneurysm formation in the Marfan syndrome and would closely mimic pathologic findings in fibrillin-1–deficient mice.

Analysis of the tight skin (*Tsk*) mouse provides further evidence for a regulatory role for microfibrils. Homozygosity for this naturally occurring mutation causes early developmental failure. Heterozygosity is associated with a phenotype that

includes progressive tissue fibrosis, obstructive lung changes, and autoimmunity [328,329]. It has now been demonstrated that Tsk mice harbor a large genomic duplication within the *Fbn1* gene, resulting in the tandem repeat of exons 17–40 in the mRNA [330,331]. The fate of the mutant protein is a topic of ongoing investigation [332,333]. Earlier evidence suggested that mutant monomers interact with each other but lack the ability to polymerize with normal fibrillin-1 [332]. More recent results suggest that the abnormal protein is degraded near the cell surface (F. Ramirez, personal communication). It is difficult to reconcile the *Tsk* phenotype with a pure deficiency of the structural functions of microfibrils. It is interesting to note that the duplication within the mutant protein spans the region of fibrillin-1 that shows greatest homology to the LTBPs [330]. There is also preliminary evidence that TGF-β shows increased binding affinity for the *Tsk* gene product in an *in vitro* binding assay [333]. The lack of significant overlap between the *Tsk* and MFS phenotypes suggests that the internally duplicated product may result in excessive fibrosis through a gain-of-function mechanism. In addition, autoantibodies to fibrillin-1 have been identified in *Tsk* mice and patients with scleroderma and other related connective tissue disorders [334,335]. The functional significance of this finding remains to be elucidated.

The study of genetic perturbation of the elastin-microfibrillar array in mice has resulted in an enhanced understanding of multiple disease processes. Although the events and molecules that mediate the clinical expression of a deficiency in this specialized matrix have yet to be fully defined, it is important to note that selected vascular smooth muscle cell and connective tissue abnormalities observed in MFS are also seen in common disease processes, including nonsyndromic aortic aneurysm, hypertension, and atherosclerosis [336–338]. Elastic fiber calcification and degradation and intimal hyperplasia are also a component of the normal aging process. It is therefore possible that an acquired loss of elastic matrix-cell attachments, and consequent destructive changes, are integral to the pathogenesis of disorders resulting from a wide variety of genetic predispositions.

PHENOTYPES THAT OVERLAP WITH MARFAN SYNDROME

Mass Phenotype and Familial Mitral Valve Prolapse Syndrome

Historical Introduction

The clinical signs of mitral valve prolapse (MVP), most typically an apical systolic click, a late-systolic apical murmur, or both, were associated with the angiographic findings of systolic billowing of the mitral leaflets in the early 1960s. Since then, MVP has been associated, whether through chance or true pathophysiology, with a variety of clinical signs and symptoms [339]. It has become clear that in some cases MVP is associated with clear evidence of one or another heritable abnormality in connective tissue other than MFS. In this section, the most common presentations are described. Mitral valve prolapse is also a feature of some forms of the Ehlers-Danlos syndrome (Chapter 9, this volume), pseudoxanthoma elasticum (Chapter 11, this volume), and osteogenesis imperfecta (Chapter 8, this volume).

Phenotype, Natural History, and Prognosis

The skeletal system. Tall, asthenic individuals are more prone to having MVP in the general population, but whether this represents an underlying variation in the extracellular matrix is unknown. Women with MVP in families in which relatives have MVP are more likely to have a relatively long arm span [340]. Deformity of the anterior chest, especially pectus excavatum, scoliosis, and reduced thoracic kyphosis (“straight back”), have been found with relatively high frequency in people with MVP [341]. In our study of probands referred for evaluation of possible MFS, we found a substantial proportion had multisystem involvement but not enough criteria to warrant a diagnosis of MFS. To distinguish these people from those with MFS and from those in the general population with MVP and little else, we coined the term “MASS phenotype” based on the association of mitral valve prolapse, myopia, mild aortic root dilatation, striae, and mild skeletal changes [64]. The word “phenotype” rather than “syndrome” was carefully selected so as not to imply a common pathogenesis in people with MASS. The skeletal features include the mild ones of MFS, namely, tall stature, mild dolichostenomelia, joint hypermobility (but rarely contractures), pectus excavatum, reduced thoracic kyphosis, mild or moderate scoliosis, and pes planus. Occasionally, an individual with MASS will have sufficient skeletal involvement to warrant the skeletal system being a “major criterion” according to the MFS criteria (*vide supra*), but they will not otherwise qualify for MFS.

The ocular system. No specific ocular abnormalities have been described. Myopia may be more common in MASS because it is a diagnostic criterion.

The cardiovascular system. The clinical spectrum of MVP is quite broad, from nonpenetrant (deduced from families in which an autosomal dominant pattern is clear) to causing severe mitral regurgitation in childhood. The occurrence and severity of MVP depend on age and sex; MVP is always more common in women and in both sexes reaches peak prevalence in adolescence and early adulthood [342]. Mitral regurgitation is associated with the severity of the prolapse. When MVP is mild, no regurgitation occurs. As the prolapse increases, leakage first occurs in late systole (hence, the late-systolic murmur), and with severe prolapse, the valve leaks throughout systole. Some factors, such as position, afterload, and medications, affect the tension on the chordae tendineae and the severity of the prolapse and hence the clinical findings. For that reason, failure to detect MVP on an echocardiogram (usually obtained with the subject supine and quiet) does not exclude MVP when the subject is upright and active. Thus, a variety of bedside maneuvers are useful to evoke the click, murmur, or both, even in the face of a negative echocardiogram.

The complications of MVP, in addition to mitral regurgitation, include nonanginal (“atypical”) chest pain, palpitations, bacterial endocarditis, systemic embolization (likely from platelet thrombi that form on the patulous leaflets), and dysrhythmia (including sudden death). Women seem to be predisposed to chest pain and palpitations, whereas men seem more prone to developing mitral regurgitation later in life, which is associated with pronounced thickening and stiffening of the leaflets [339,343]. Complications of MVP have replaced rheumatic disease as the leading cause of surgery on the mitral valve [344].

In one family, MVP was an early sign of severe myxomatous degeneration, with involvement of the aortic and tricuspid valves as well as the mitral. Multiple relatives had required valve replacement. Anatomic inspection showed gross and microscopic calcification of the valves and valve

annuli along with extensive fibrosis and glycosaminoglycan deposition [106].

The tricuspid valve often prolapses if the mitral valve does. Because the right-sided circulation is of lower pressure, the clinical signs and relevance are generally less. The click and murmur of tricuspid valve prolapse are heard at the lower sternal border rather than the apex.

In familial MVP, the aortic root is not dilated. In the MASS phenotype, the aortic root is often at the upper limit of normal, based on body surface area [64]. Although detailed follow-up of subjects has not been performed, the impression is that subjects with MASS do not show progressive dilatation or unusual susceptibility to dissection.

The respiratory system, the integument, and the central nervous system. People who are tall and asthenic are more prone to have MVP and to suffer pneumothorax. People with the MASS phenotype (by definition) are more prone to have striae distensae (atrophicae) at sites of flexural stress. There is no evidence that the CNS is involved in either MVP or MASS.

Diagnosis and Differential Diagnosis

No stringent criteria have yet been established. When multiple individuals in a family have MVP but little in the way of involvement of other systems, the label "familial mitral valve prolapse" seems warranted. When the skeletal, ocular, skin, and cardiovascular systems are involved but no one in the family meets the criteria for MFS, then the label "MASS phenotype" seems reasonable. The entry in MIM 157700, familial mitral valve prolapse, subsumes all of these variants. Marfan syndrome is the major issue in differential diagnosis.

Prevalence, Demographics, and Genetics

All ethnic groups seem to harbor MVP and MASS. Several percent of the population of the U.S. have MVP [345,346].

The familial occurrence of MVP has been noted since the late 1960s [347–349], and the pattern of inheritance is consistent with autosomal dominance with reduced penetrance. The inheritance of the MASS phenotype has not been studied formally, and given that the etiology is undoubtedly heterogeneous, multiple modes of inheritance are possible. In a few families defined both phenotypically and genetically, inheritance was autosomal dominant [100,286].

One family with "familial myxomatous valvular disease" showed autosomal dominant inheritance [106].

Management and Clinical History

The cardiovascular system. People with MVP should be followed by both clinical examination and echocardiography; the frequency of each depends on the severity of the cardiovascular involvement. No therapy has been found to alter the natural history of MVP. Once mitral regurgitation appears, the progression to clinically important leakage can take years, even decades. During this time, the left ventricle gradually dilates and compensates in other ways so that symptoms are a late occurrence. Occasionally, a chorda tendineae ruptures, and the occurrence of acute mitral regurgitation can produce sudden left heart failure, manifested by pulmonary edema, because there has been no time for compensation. In the case of a ruptured chorda, valve repair or replacement is usually necessary. Chronic mitral regurgitation can be managed medically. Complications include left atrial enlargement, supraventricular dysrhythmia, especially atrial fibrillation, left ventricular failure, and embolization [350]. The goal is to protect the circulation, with afterload reduction, while following the size and function of the left ventricle by echocardiography. Mitral valve repair, or, if the valve cannot be repaired, replacement should be performed before irreversible left ventricular enlargement occurs. Chronic or recurrent atrial fibrillation should prompt systemic anticoagulation.

Other systems. Occasional individuals will require repair of pectus excavatum or bracing for scoliosis. The approach to management should follow that described above for MFS. Pneumothorax should be managed as in MFS. Anxiety neurosis and panic disorder have been associated with MVP, but careful studies have cast doubt on any causal link [351,352].

Genetic counseling. People with familial MVP and the MASS phenotype should be counseled about likely autosomal dominant inheritance. To some extent, the complications of MVP tend to recur in a family [347].

Pathology, Etiology, and Pathogenesis

The mitral and tricuspid valve leaflets are often patulous and thickened and, in essence, too large for the opening. The mitral annulus can be dilated, as in MFS, or may be normal. The histology of the valve leaflets shows "myxomatous" degeneration; the excess extracellular matrix is proteoglycan of one sort or another. The gross pathology of the annulus may play a role [353].

In a few patients, the MASS phenotype has been determined to be due to mutations in *FBN1*, the gene coding for fibrillin-1 [286,290–292]. As a general rule, these mutations have created premature termination codons and cause efficient and selective degradation of the mutant transcript. One premature termination codon that was associated with the MASS phenotype occurred in the last exon and therefore did not induce transcript instability [286]. Too few mutations have been described to determine whether the mild character of the disease phenotype is determined by *FBN1* genotype or the influence of modifiers. The fact that the phenotype holds true in extended kindreds is supportive of the former hypothesis. Mutation Arg1170His has been associated with dolichostenomelia with [113] and without [299] MVP. No individual in either family exhibited lens dislocation or aortic enlargement. Finally, an individual who exhibited somatic mosaicism for an *FBN1* mutation showed MVP with subtle skeletal manifestations. The same mutation caused classic and severe MFS when inherited through the germline [113].

It is clear that familial MVP is a genetically heterogeneous disorder. We have excluded linkage to *FBN1* in a number of large pedigrees (unpublished data). Furthermore, a form of familial myxomatous valvular dystrophy has been mapped to Xq28 [354], and a locus for autosomal dominant MVP maps to 16p11.2-p12.1 [355].

Familial Aortic Aneurysm, Dissection, or Both

Relatively little has been written about familial occurrences of aneurysm, dissection, or both, of the ascending aorta. That both can occur in an apparent autosomal dominant mode is certain [108,356–358].

Phenotype, Natural History, and Prognosis

As in the MASS phenotype (*vide supra*), subjects with familial aortic aneurysm have deformity of the thoracic cage at an increased prevalence in relation to the general population. Other subtle signs of connective tissue involvement, such as joint hypermobility and dolichostenomelia, do occur

in some, but overall the diagnostic criteria do not rise to the level of MFS.

In some families, the aortic root dilates in a fashion similar to that of MFS, with the risk of dissection being most dependent on the diameter. In other families, little or no dilatation precedes the dissection. Although these two ends of the spectrum may breed true in some families, others show all gradations [108,357,358]. Mitral valve prolapse occurs with increased frequency in both conditions.

The frequency of myopia may be increased, but if ectopia lentis is present, the person likely has MFS. Striae atrophicae are common.

Diagnosis

No formal criteria have been proposed. Familial aortic aneurysm can be defined as an autosomal dominant condition in which the ascending aorta, beginning in the sinuses of Valsalva, dilates. This condition excludes the ascending aortic aneurysm that is often associated with coarctation of the aorta and/or bicuspid aortic valve [359]; in this association, the aorta may be enlarged when the aortic valve is functionally normal [360]. Familial aortic dissection is an autosomal dominant condition in which the aorta, in the absence of substantial dilatation, dissects, usually first in the ascending segment. This condition excludes the vascular form of Ehlers-Danlos syndrome (see also Chapter 9, this volume).

Prevalence, Demographics, and Genetics

Unsuspected dilatation of the aortic root is the most common cause of isolated aortic regurgitation, having supplanted rheumatic valvular disease [361]. Our suspicion is that many of the cases of "idiopathic" aortic root dilatation represent either familial aortic aneurysm or the developmental defect associated with coarctation and bicuspid aortic valve (all parts of this triad need not be present). Thus, the true prevalences of these two heritable diseases of the aorta, which may be part of the same pathophysiologic spectrum, are unknown. Ascending aortic dissection in the absence of dilatation appears to be exceedingly rare after exclusion of the vascular variant of Ehlers-Danlos syndrome (type IV).

Both familial aortic aneurysm and familial aortic dissection tend to show autosomal dominant segregation, with some families demonstrating high penetrance.

Management and Clinical History

No studies have been conducted on either of these two conditions. For familial aortic aneurysm, affected individuals should be managed in the same way as for the cardiovascular features of MFS (*vide supra*). For familial aortic dissection, the major challenge is identifying those with the predisposition before dissection occurs.

Relatives in families with either of these conditions should undergo transthoracic echocardiography to attempt to document whether disease of the ascending aorta is present. Relatives of an affected individual in a family in which two or more individuals have been affected should be counseled about the risk of autosomal dominant inheritance.

For familial aortic aneurysms, the issues are similar to MFS, and the National Marfan Foundation welcomes such families into full membership. For familial aortic dissection, the anxiety and sense of helplessness in patients can be heightened because of the difficulty in assigning affected status.

Pathology, Etiology, and Pathogenesis

The aortic wall pathology is identical to that of MFS [108]. A few *FBN1* mutations have been reported in families with predominant involvement of the ascending aorta. Mutation Gly1127Ser was found in a large kindred exhibiting late-onset dilatation of the aortic root [362]. Selected individuals in this family showed fragile zonules and/or skeletal manifestations, but no individual satisfied the diagnostic criteria for MFS. This glycine residue is conserved in cbEGF-like domains, but it has not been implicated in calcium binding or hydrophobic packing interactions. NMR analysis revealed defective domain folding [298]. Interestingly, an analogous mutation in a cbEGF-like domain of factor IX is associated with mild hemophilia [363].

Mutations Asp1155Asn and Pro1837Ser were also found in individuals with ascending aortic aneurysms and skeletal involvement that failed to meet diagnostic criteria for MFS [364]. No affected family members were available for analysis. Interestingly, many mutations in cbEGF-like domains at positions analogous to Asp1155Asn have been associated with classic MFS. Also, cells harboring mutations Gly1127Ser and Asp1155Asn showed diminished fibrillin-1 deposition in the extracellular matrix by pulse-chase analysis [362,364]. It is therefore difficult to postulate pathogenetic mechanisms that distinguish MFS from these cases of familial ascending aortic aneurysm.

Many families with familial ascending aortic aneurysm do not show linkage to *FBN1* or the chromosome 3p locus described for a Marfan-like condition [238,357]. The relevant genes have not yet been localized or identified.

Familial Ectopia Lentis

Phenotype, Natural History, and Prognosis

Familial ectopia lentis is another "overlap" phenotype with MFS. The most appropriate MIM number, 129600, isolated ectopia lentis, subsumes autosomal dominant conditions, some with and some seeming to lack involvement of other organ systems. "Isolated" ectopia lentis clearly occurs in an autosomal dominant pattern in some families, although in some the displacement is nonprogressive and the ocular problems relatively benign [365]. In other families, progressive displacement occurs and long-term ophthalmologic management is necessary. Management of the genetic forms of familial ectopia lentis should follow that of MFS.

A mild systemic phenotype (joint stiffness, dolichostenomelia, thoracic cage deformity, and even mild aortic root enlargement) has occurred in a few pedigrees that suggests a generalized connective tissue abnormality [284,366].

Diagnosis

No formal criteria have been established. The major differential diagnosis when inheritance is autosomal dominant is MFS. When only a sibship has affected cases, or in the isolated case, a wide differential diagnosis exists, including autosomal recessive ectopia lentis (MIM 225100) or ectopia lentis with ectopic pupils (MIM 225200), homocystinuria (MIM 236200) (see also Chapter 13, this volume), Weill-Marchesani syndrome (MIM 277600), sulfocysteinuria (sulfite oxidase deficiency) (MIM 272300) (see also Chapter 13, this volume), and hyperlysinemia (MIM 238700).

Prevalence and Genetics

Little formal data exist, but most congenital ectopia lentis is genetic, while that which occurs after adolescence is often traumatic. In a Danish registry, the incidence of congenital

ectopia lentis was 0.83 per 10,000 live births. A cause was evident in about two-thirds, with MFS accounting for two-thirds of those. Ectopia lentis et pupillae was present in 21% of those with a defined cause, and simple dominant ectopia lentis accounted for 8%. Other causes were Weill-Marchesani syndrome, homocystinuria, and sulfite oxidase deficiency in about 1% of those with defined cause [109].

The majority of cases with no or mild systemic manifestations, in the absence of trauma to the head, are autosomal dominant.

Pathology, Etiology, and Pathogenesis

The zonules, which are rich in microfibrils, either stretch or break. The former usually leads to superior displacement; the latter invariably leads to inferior displacement.

The so-called isolated forms of familial ectopia lentis have varied causation. Some forms, including those in families with mild signs of a systemic connective tissue disorder, have shown linkage to *FBN1* [235,367]. To date, however, only a single fibrillin-1 mutation causing dominant ectopia lentis has been identified, and selected members of this family had significant skeletal and skin involvement but none had aortic enlargement. The mutation (Glu2447Lys) resulted in the substitution of lysine for a glutamic acid residue that is highly conserved in cbEGF-like domains and is part of the calcium-binding consensus sequence. Interestingly, mutations at the corresponding residue in other cbEGF-like domains in fibrillin-1 cause typical MFS [100,276,286].

Familial Tall Stature

In one family demonstrating familial tall stature, the proband had mild skeletal features of the MFS (mild dolichostenomelia, pectus carinatum, scoliosis) without involvement of other systems [246]. A mutation in *FBN1* was detected in the proband, and other relatives were examined phenotypically and for the mutation. Those with the mutation were tall without other skeletal manifestations.

No formal criteria have been proposed for diagnosis. Based on this one family, features should be limited to the skeletal system.

The role of mutations in either fibrillin gene in causing "idiopathic" skeletal problems such as scoliosis, anterior chest deformity, congenital joint laxity or contracture, and tall stature is unknown.

Based on the one family and the fact that heterozygosity for mutations in *FBN1* causes other connective tissue conditions, autosomal dominant inheritance should be assumed.

No studies have been done, but the assumption should be that the natural and clinical histories follow those of the skeletal features of the MFS and that their management should be the same.

Mutation Arg2627Trp in fibrillin-1 substitutes a residue that lies immediately adjacent to the consensus site for cleavage by PACE/furin-like proteases [246]. Indeed, this mutation prevents C-terminal processing. The abnormal fibrillin-1 monomer is synthesized and secreted but fails to participate in microfibrillar assembly [246,250]. These data suggest that the initiation of multimerization is negatively regulated by the presence of the C-terminal propeptide. Because the proline-rich domain near the N-terminus can support dimerization [251,252], it is appealing to suggest that this regulation involves intramolecular interactions. This may explain why mutations that truncate or substitute fibrillin-1 beyond the cleavage site can impair microfibrillar assembly and cause MFS.

Shprintzen-Goldberg Syndrome (MIM 182212)

This rare condition was first codified in 1982 [368] on the basis of two unrelated boys with craniosynostosis producing exophthalmos, hypoplasia of the mandible and maxilla, deformed ears, a narrow palate due to hypertrophy of the palatal shelves, anterior chest deformity, arachnodactyly, and camptodactyly. Subsequent cases have broadened the phenotype to include ptosis, hypotonia, developmental delay or moderate mental retardation, osteopenia, and cranial asymmetry [369]. Some have had aortic root dilatation, marked skeletal changes, and a course consistent with MFS [370–372].

The phenotype seems heterogeneous, based on its genetics and diversity of features. Greally and colleagues [373] suggest that in addition to craniosynostosis and other craniofacial features, the presence of dysplasia of the first and second cervical vertebrae, dilatation of the lateral ventricles, and a Chiari I malformation define Shprintzen-Goldberg syndrome. Megarbane and Hokayem suggested that the term Shprintzen-Goldberg syndrome type I be applied to those patients with mental retardation, craniosynostosis, and skeletal changes of MFS, while Shprintzen-Goldberg syndrome type II should include those with normal intelligence and aortic root dilatation [372].

Most cases have been sporadic, occasionally with advanced paternal age. One patient has been shown to be heterozygous for a point mutation in *FBN1*, with neither of the parents having the mutation in somatic tissue [371]. The report of Ades and colleagues described three affected sisters with apparently unaffected parents, suggesting an autosomal recessive variety [374]. Germline mosaicism or nonpenetrance could not be excluded.

All patients suspected of Shprintzen-Goldberg syndrome should have radiologic evaluation of the skull, cervical spine, and brain and echocardiography. Craniosynostosis in an infant and child should be managed by standard techniques to prevent constriction of brain growth and asymmetry of the skull. If aortic root dilatation is present, management should proceed as for MFS.

A fibrillin-1 mutation (Cys1223Tyr) was identified in one patient with Shprintzen-Goldberg syndrome. This mutation involved the substitution of a cysteine residue in a cbEGF-like domain, the most common mutational class causing MFS. This mutation was also identified in a mother and daughter who had been diagnosed with MFS [375]. Despite suggestive data, the diagnosis of Shprintzen-Goldberg syndrome could not be confirmed. Mutation Pro1148Ala was initially identified in a patient with Shprintzen-Goldberg syndrome and in individuals with ascending aortic aneurysm and their family members [371]. This mutation was subsequently also identified in people without apparent connective tissue abnormalities, with an increased population frequency in those of Hispanic or Asian descent [376–378]. In view of the highly variable clinical presentations of individuals diagnosed with Shprintzen-Goldberg syndrome, genetic heterogeneity seems assured.

Congenital Contractural Arachnodactyly

Historical Introduction

Some have argued that Marfan's original patient, Gabrielle P., did not have what we now call MFS but congenital contractural arachnodactyly (CCA) (MIM 121050) [14]. The first cases of CCA were defined in the late 1960s [19]. Beals and Hecht [13] subsequently described two two-generation pedigrees of affected parent and two

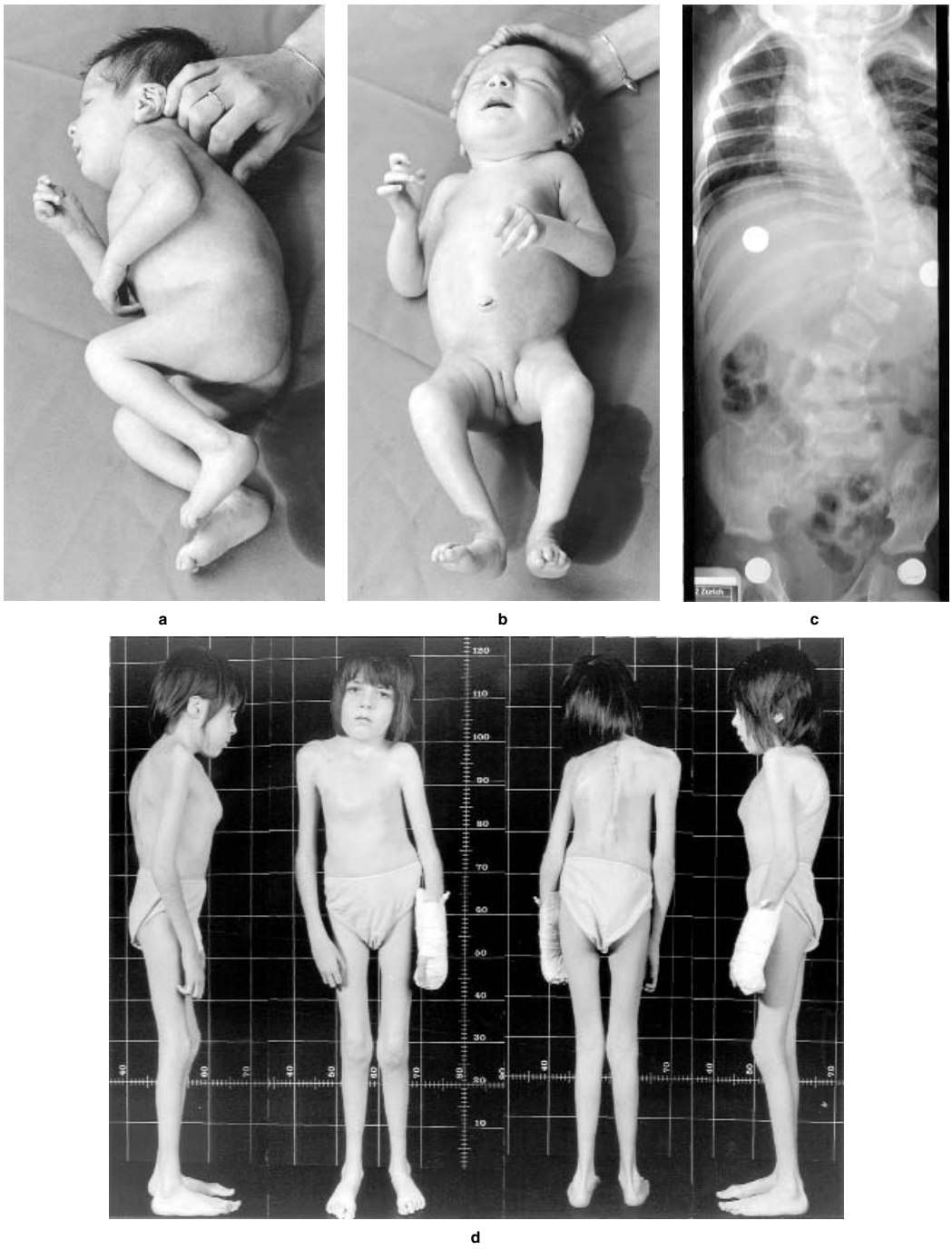

Figure 14. Congenital contractural arachnodactyly. R.R. is the third child of healthy, unrelated parents, born after a normal pregnancy and delivery. Her weight was 3070 g (25th centile), length 55 cm (>95th centile), and head circumference 34 cm (50th centile). Crumpled ears, joint contractures, arachnodactyly, and scoliosis were noted at birth. Numerous procedures had been performed to correct her severe scoliosis, beginning with bracing at 2 months, followed by halo traction and rod insertion at age 2.3 years. This was followed by repeated rod elongation, removal, and replacement. Although her initial growth curve was above the 97th centile, this flattened due to the severity of the scoliosis until it was only at the 3rd centile at age 5 years. Her pulmonary function was restricted. She had a normal 46,XX karyotype. Repeated echocardiographic examinations of her heart were normal, and the results of ophthalmologic examinations were normal. Her defect is due to a *de novo* missense mutation leading to skipping of exon 26. **a** and **b**: R.R. (at age 10 days) presents with contractures of the knees, hips, elbows, and fingers (arachnodactyly), dolichostenomelia, retrognathia, and crumpled ears. **c**: Radiograph at age 4 months shows severe and progressing scoliosis despite bracing. **d**: Severe kyphoscoliosis at age $6\frac{1}{2}$ years despite numerous orthopedic interventions such as bracing at 2 months, halo traction with rodding at 2.3 years, removal of rod at 3.5 years, insertion of rod and partial spondylodesis at 3.7 years, removal of halo at 3.8 years, elongation of broken rod, repair and then removal of broken rod at 4 years, insertions of many rods at 6.3 years, and spondylodesis at 6.5 years. She has severe restrictive pulmonary dysfunction. (Courtesy of Dr. B. Steinmann.)

offspring with arachnodactyly, congenital contractures of peripheral joints that tended to improve with physical therapy, progressive scoliosis, deformed ears ("crumpled" helices), and an apparent absence of cardiovascular and eye manifestations of MFS. Many cases have been reported since, some with clear cardiovascular features consistent with MFS. Given that congenital contractures (especially of the digits and elbows) and some crumpling of the scapha helix are relatively common in MFS, since the mid-1970s there has been much debate whether CCA and MFS are distinct disorders [379–381]. The discovery that CCA is due to mutations in *FBN2*, the gene encoding fibrillin-2, has put to rest the issue of genetic heterogeneity [231,302]. However, given that relatively few subjects of literature reports have been genotyped, the following description of the phenotype may be based on a sample that includes subjects with MFS.

Phenotype, Natural History, and Prognosis

The joints most prone to congenital contractures in CCA are the fingers, elbows, and knees (Fig. 1d). Kyphoscoliosis often appears in childhood and can progress despite orthopedic management (Fig. 14). People with CCA do not have ectopia lentis or any other consistent ocular features. Whether aortic root dilatation occurs in true CCA has not been resolved; this issue will remain uncertain until all patients with congenital contractures and aortic dilatation are shown to have mutations in *FBN1* and not in *FBN2*. Several patients and families with what was called CCA have had MVP [382] and even severe mitral regurgitation due to MVP [383]. Atresia of the gastrointestinal tract (duodenal, esophageal) and intestinal malrotation have been described [384].

Diagnosis

No formal criteria have been established. A minimal set would seem to include congenital contractures, dolichostenomelia including arachnodactyly, crumpled ears, absence of ectopia lentis, and absence of a history of MFS in a close relative.

The principal syndrome overlapping phenotypically is MFS. Congenital contractures are uncommon in the other conditions described in this chapter. Two unrelated Indian females had congenital contractures and a characteristic facies consisting of blepharophimosis, a beaked nose, everted lips, and large ears; the authors suggest that this is a distinct syndrome [384].

Prevalence, Demographics, and Genetics

No ethnic or geographic predilection seems evident. Well over 100 cases have been reported, but the incidence is unknown.

All cases have been sporadic or autosomal dominant. Given that subjects with a defined molecular defect are heterozygous for mutations in *FBN2*, autosomal dominance is secure. In one pedigree, one of the parents of two affected sisters was a somatic mosaic for a mutation in *FBN2* [385].

Management and Clinical History

Aggressive physical therapy, starting in infancy, seems to have a beneficial impact on the congenital contractures and may result in nearly normal joint extension. The spine should be examined regularly, starting in infancy, with aggressive bracing at a relatively early stage, because in some reported examples of CCA, kyphoscoliosis can progress rapidly.

All patients suspected of having CCA should, at several points during growth, have detailed ocular examinations by an ophthalmologist experienced in connective tissue disorders to ensure that ectopia lentis has not appeared. Similarly, a transthoracic echocardiogram should be performed in childhood and early adolescence to ensure that neither MVP nor aortic root dilatation is present. If either is present, management should follow the description in the section on MFS.

Affected subjects should be counseled about autosomal dominant inheritance and variable expression.

Etiology and pathogenesis. All families that have been studied show linkage to the *FBN2* gene on chromosome 5 [231,235]. All of the mutations that have been identified thus far occur in a defined region of fibrillin-2 flanked by exons 24–34. Mutations causing the most severe variant of MFS cluster in the corresponding region of fibrillin-1. The character of mutations seen in *FBN2* closely resembles that of those seen in *FBN1*, including cysteine substitutions in cbEGF-like domains and splicing mutations [302,384–388]. Multiple mutations that have a less obvious functional consequence exactly correspond to *FBN1* mutations causing MFS [387]. In view of the small number of mutations identified to date, it remains unclear whether this clustering of mutations is influenced by any selection bias. It seems likely that mutations outside this region are either phenotypically neutral or result in a milder variant of disease. Interestingly, a fetus with a defined interstitial deletion encompassing the *FBN2* gene showed selected features of CCA [389]. These data suggest that loss of function may be an important pathogenetic mechanism.

PATIENT SUPPORT GROUPS

Contact details of support organizations in a number of countries can be found at the National Marfan Foundation Web site: http://www.marfan.org.

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Chapter 13

The Homocystinurias

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SUMMARY

- **Homocysteine occupies a central position in the transsulfuration pathway. A product of the essential amino acid methionine, homocysteine can either be remethylated to methionine or irreversibly converted to cystathionine. The normal concentration of reduced homocysteine moieties in plasma is 5–15 μmol/L. Several autosomal recessively inherited enzymatic defects cause hyperhomocysteinemia and excretion of homocystine in the urine, called homocystinuria.**
- **Deficiency of cystathionine β-synthase (CBS) is the most common cause of homocystinuria. Major clinical manifestations include dislocated lenses of the eyes, mental retardation, osteoporosis, and thromboembolism. More than 100 mutations have been documented in the *CBS* gene, but the pathophysiological mechanisms are poorly clarified. Increased tissue levels of homocysteine may interfere with disulfide bonding of proteins and cross-linking of collagen. Treatment with a methionine-restricted diet, pyridoxine, and/or betaine will prevent or reduce symptoms, especially if started very early in life.**
- **Deficiency of methylenetetrahydrofolate reductase impairs the remethylation of homocysteine to methionine. Most patients have neurologic dysfunction, failure to thrive, or psychiatric abnormalities, but they have no connective tissue abnormalities. Treatment including betaine may be helpful if started early.**
- **Methylcobalamin is a cofactor of methionine synthase, one of two enzymes responsible for the remethylation of homocysteine, and various genetic defects in the biosynthesis of methylcobalamin may therefore cause hyperhomocysteinemia. Symptoms include neurologic dysfunction, psychiatric abnormalities, and megaloblastic changes in the blood. Some patients respond to betaine and intramuscular hydroxycobalamin, at least biochemically. The long-term prognosis is poor.**
- **Deficiency of sulfite oxidase, due to either defective apoenzyme or deficient biosynthesis of molybdenum-containing cofactor, causes severe neurologic dysfunction and dislocated lenses but no connective tissue abnormalities or homocystinuria. Effective therapy is currently not available, and many affected infants die.**
- **Prenatal diagnosis is feasible for all of the above-mentioned disorders.**

INTRODUCTION

Homocystinuria is an important laboratory finding in several inborn errors of metabolism, some of which have connective tissue manifestations. Disturbances of homocysteine metabolism may also be found in patients with thromboembolic vascular disease. This chapter focuses on the clinical and connective tissue abnormalities in patients with homocystinuria due to deficiency of cystathionine β-synthase and inherited defects of folate and cobalamin metabolism as well as in patients with other disorders of the transsulfuration pathway. Reviews of these disorders with a more comprehensive scope have been published [1–4].

HISTORICAL PERSPECTIVE

Homocystinuria was first discovered by Carson and Neill in 1962 in the course of screening mentally retarded individuals for abnormal urinary amino acids [5,6]. Independently, Gerritsen and colleagues [7,8] found homocystine in the urine of a child with lens abnormalities, mental retardation, and thromboembolism. These and subsequent reports rapidly documented the clinical diversity of "classical "homocystinuria. The biochemical findings of hypermethioninemia, homocyst(e)inemia, and decreased plasma cyst(e)ine enabled Mudd and his co-workers to predict an underlying defect in the transsulfuration pathway. Their hypothesis was soon confirmed by the demonstration of a deficient activity of cystathionine β-synthase (CBS) in liver tissue from a typical patient [9]. The unexpected presence of CBS activity in cultured fibroblasts from normal individuals [10] led to the characterization of a large number of mutant fibroblast lines with respect to catalytic activity. Following the purification of human CBS [11,12] and the cloning of human *CBS* cDNA [13], over 100 mutations have been identified in the *CBS* gene [14].

Applying the lessons learned from phenylketonuria, a low-methionine diet was the first treatment devised for homocystinuria [15]. Shortly thereafter, some patients were found to respond biochemically to oral treatment with pyridoxine (vitamin B_6) by normalizing or improving to varying degrees their plasma and urine levels of sulfur amino acids [16]. An international survey done in 1982–1983

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of more than 600 patients with CBS deficiency clarified parts of its natural history and demonstrated the efficacy of a low-methionine diet and pyridoxine in preventing or delaying some of the major manifestations of classical homocystinuria [17]. More recent studies have validated the long-term use of betaine in this disorder [18,19].

Before the recognition of homocystinuria as a clinical entity, several patients were "lumped" into the category of Marfan syndrome. Heterogeneity became evident when deficient remethylation of homocysteine was also found to cause homocystinuria. Discoveries of defects in the metabolism of cobalamins have continued the "splitting" of homocystinuria.

METABOLISM OF HOMOCYSTEINE

Homocystinuria due to inborn errors of metabolism is a consequence of elevated levels of homocysteine in the blood, where it circulates as the free thiol, as part of disulfides, and bound to proteins by disulfide bonds.

The pivotal location of homocysteine in the transsulfuration pathway and the methionine-homocysteine cycle is illustrated in Figure 1. The sulfur-containing essential amino acid, methionine, is supplied through catabolism of proteins. The average Western male consumes about 2.4 g methionine per day, or approximately 35 mg/kg [20]. Minimal daily requirements for methionine vary with age and are not well-established; values of 20–40 mg/kg and 10 mg/kg have been suggested for infants and young adults, respectively [21,22].

The vast majority of methionine not used for protein anabolism is converted to S-adenosylmethionine by transfer of the adenosyl moiety of ATP to methionine. The reaction is catalyzed by methionine adenosyltransferase (EC 2.5.1.6). S-adenosylmethionine participates in methylation reactions, polyamine synthesis, and aminobutyryl side-chain donation. It is also an important allosteric enzyme regulator. Transmethylation is the most frequent metabolic fate of S-adenosylmethionine, resulting in S-adenosylhomocysteine and, ultimately, homocysteine.

The rate-limiting enzyme of the transsulfuration pathway, cystathionine β-synthase (CBS, EC 4.2.1.22), condenses homocysteine with serine to form cystathionine in an irreversible reaction requiring pyridoxal 5′-phosphate (reaction 1, Fig. 1). Cystathionine is cleaved to cysteine and α-ketobutyrate by cystathionine γ-lyase (EC 4.4.1.1). The net result of these reactions is the transfer of the sulfur atom of methionine to cysteine, which takes part in several metabolic reactions, such as synthesis of glutathione and proteins. Cysteine may also be converted through intermediates to sulfite, which, after oxidation by sulfite oxidase (EC 1.8.3.1; reaction 2, Fig. 1), is eventually excreted as urinary sulfate.

Instead of being converted to cystathionine, homocysteine can be remethylated to methionine by either of two reactions. One is catalyzed by the vitamin B_{12}-dependent enzyme, methionine synthase (5-methyltetrahydrofolate-homocysteine *S*-methyltransferase; EC 2.1.1.13; reaction 3, Fig. 1), and requires 5-methyltetrahydrofolate, which is produced from 5,10-methylenetetrahydrofolate by methylenetetrahydrofolate reductase ($NADPH_2$) (EC 1.5.1.20; reaction 5, Fig. 1). The other possible remethylation of homocysteine is catalyzed by betaine-homocysteine *S*-methyltransferase (EC 2.1.1.5) and is an obligatory step in the catabolism of choline (reaction 4, Fig. 1).

As an alternative to transsulfuration, methionine may be catabolized by transamination. This pathway has been shown

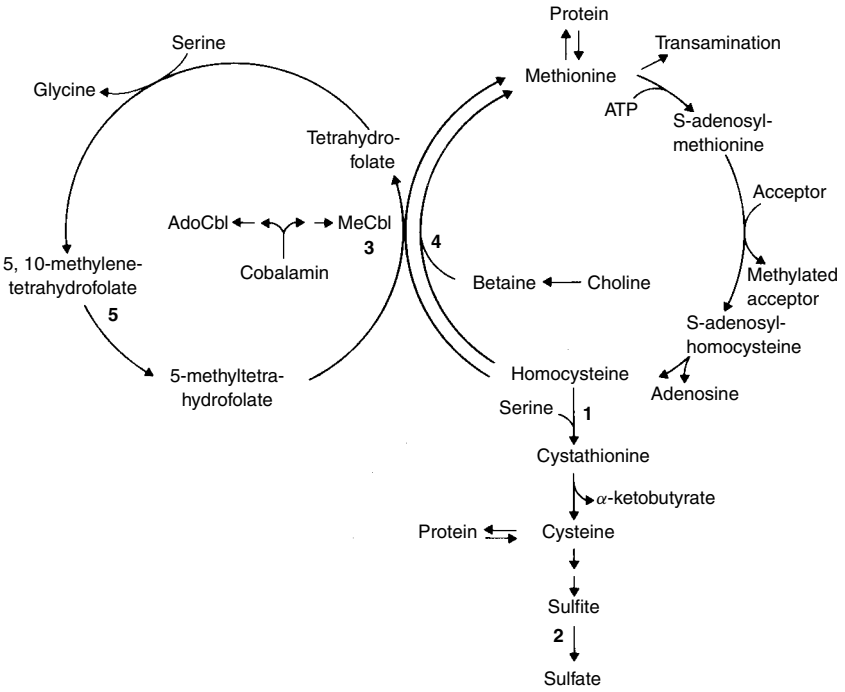


Figure 1. The metabolism of methionine, homocysteine, and tetrahydrofolates. **1**, cystathionine β-synthase, CBS; **2**, sulfite oxidase; **3**, methionine synthase (5-methyltetrahydrofolate-homocysteine *S*-methyltransferase); **4**, betaine-homocysteine *S*-methyltransferase; **5**, methylenetetrahydrofolate reductase. AdoCbl = adenosylcobalamin; MeCbl = methylcobalamin. (Adapted from [2].)

to metabolize at least 20% of the methionine in a patient with very high plasma levels due to deficiency of hepatic methionine adenosyltransferase [23]. In control subjects, however, and even in patients with hypermethioninemia due to deficient CBS, transamination of methionine is of no quantitative significance [24,25].

Molecular Biology and Biochemistry of Cystathionine β-Synthase

CBS catalyzes the replacement of the β-hydroxyl group of serine by homocysteine to form cystathionine in the transsulfuration pathway (reaction 1, Fig. 1). The locus for human *CBS* was mapped to chromosome 21 by study of Chinese hamster-human somatic cell hybrids [26], an assignment corroborated by *in situ* hybridization studies using a cDNA probe for CBS [27]. The gene was subsequently localized more precisely to the subtelomeric region of band 21q22.3 of chromosome 21 [28].

To clone the cDNA for *CBS*, a plasmid library was prepared using rat liver CBS mRNA purified to homogeneity by immunopurification [27]. A partial *CBS* cDNA clone was isolated and used, in turn, to isolate full-length cDNA clones [29,30]. The cDNA with the longest continuous reading frame, type I, was used as a probe to screen a rat genomic library, and genomic inserts were identified that together permitted reconstruction of the organization of the entire rat *CBS* gene. The gene is approximately 25 kbp long, with the coding sequence broken into 18 exons [30,31]. Using the rat *CBS* cDNA as a probe, several human *CBS* cDNAs were isolated from liver and skin fibroblast libraries [13]. The full-length human cDNA is 2,554 nucleotides long and encodes a polypeptide of 551 amino acids. More recently, additional human *CBS* cDNAs have been isolated encoding the most upstream portions of the 5′-untranslated region. The 5′-untranslated region of human CBS mRNA is formed by one of five alternatively used exons, designated −1a to −1e, and one that is invariably present, exon 0, while the 3′-untranslated region is encoded by exons 16 and 17 [32]. Interestingly, intron 16 appears to be retained in the 3′-untranslated region of most of the fibroblast and liver mRNA of every individual [13].

The entire human *CBS* gene was cloned and sequenced in 1998 [33]. In addition to its 23 exons, the 28,046 nucleotides contain approximately 5 kbp of 5′-flanking sequence. The CBS polypeptide is encoded by exons 1–16. Exon 15, the human homolog of rat exon 16, is alternatively spliced and appears to be incorporated in relatively few mature human CBS mRNA molecules. While the exon-intron organization of the human *CBS* gene in the protein-coding region is perfectly conserved with respect to the rat and mouse *CBS* genes, this is not the case for the genomic organization of the untranslated regions [30–33].

There are at least two alternatively used promoters in the human *CBS* gene upstream of exons −1a and −1b. They are GC-rich and contain numerous putative binding sites for proteins known to interact with other promoters such as Sp1, Ap1, Ap2, and c-myb, but they lack the classical TATA box. To evaluate the relative levels of the −1a and −1b promoter activities, reporter constructs were generated by cloning the promoter regions of approximately 400 bp into a luciferase reporter vector. Following transfection of the reporter constructs into COS 7 and HepG cells, analysis of luciferase activity in these cells indicated that both regions contain all of the sequences essential for promoter activity, with the −1b promoter activity being 7–10-fold stronger than that of −1a [33].

An unusually high number of *Alu* repeats may predispose the *CBS* locus to deleterious rearrangements. Additionally, a number of DNA sequence repeats and single base variations that are polymorphic in Caucasians have been reported [13,33]. An insertion of 68 bp in exon 8 (844ins68) was first observed in an allele from a CBS-deficient patient, which also carried the frequent Ile278Thr mutation [34]. The insertion was shown subsequently to be a polymorphism occurring in about 5–10% of Caucasian alleles [35–37]. It duplicates the intron 7 acceptor splice site and may lead to two alternatively spliced transcripts. If the upstream splice site is used, the resulting mRNA will contain the Ile278Thr mutation and a stop codon. If, on the other hand, the downstream site is used, the Ile278Thr mutation and part of the insertion are spliced out, leaving a completely normal *CBS* transcript. Only normal CBS mRNA is detectable in the cytoplasm, whereas both forms are present in the nuclear RNA fraction, suggesting that the abnormal transcript is degraded before it leaves the nucleus [35].

The primary translational product of both the human and the rat *CBS* genes is a polypeptide with a molecular weight of 63 kDa [38]. In fresh liver extracts, the enzyme is found predominantly as a tetramer of this subunit, whereas after a procedure in which the enzyme is first "aged" at 4 °C for 7 days and then purified, CBS is isolated as a dimer of 48 kDa subunits [12,38]. The reduction in size, caused by limited proteolysis, is accompanied by a significant increase in catalytic activity at physiologic concentrations of homocysteine.

CBS has been purified from various vertebrate livers [11,12]. Human *CBS* cDNA has been used in various vector systems to express the human recombinant enzyme in *Escherichia coli* [39], yeast [40], and Chinese hamster ovary cells [13]. Large amounts of recombinant human CBS have been purified from *E. coli* and further characterized [39,41]. Each subunit of CBS binds, in addition to the two substrates, three ligands: pyridoxal 5′-phosphate, S-adenosylmethionine (an allosteric activator), and, surprisingly, heme [41]. It has been suggested that the role of heme in CBS may be catalytic by activating homocysteine [42], similar to the action of zinc in the enzymes involved in remethylation of homocysteine [43,44]. CBS depends fully on the presence of pyridoxal 5′-phosphate for activity [11,45,46], which correlates with the heme saturation. The presence of heme is required for the binding of pyridoxal 5′-phosphate, and the amount of pyridoxal 5′-phosphate bound is limited by the heme content [41]. It has now been demonstrated unequivocally that the residue in CBS forming a Schiff base with pyridoxal 5′-phosphate is Lys 119 [47].

The active core of CBS has been generated by limited digestion with trypsin from the full-length enzyme or, alternatively, by expression of a truncated *CBS* cDNA in *E. coli* [48]. This core, extending from Glu 37 to Arg 413, forms a dimer of 45 kDa subunits. The dimer is about twice as active as the tetramer. It binds both pyridoxal 5′-phosphate and heme cofactors but is no longer activated by S-adenosylmethionine [48]. The 45 kDa active core is the portion of CBS most homologous to the related enzymes isolated from plants, yeast, and bacteria. Similar results were obtained when human *CBS* cDNA containing a premature stop codon at position 409 was expressed in CBS-deficient yeast [49]. The enzyme lacking the carboxyl-terminal 143 amino acids was twice as active as the wild-type enzyme.

Surprisingly, when any one of several human inactivating pathogenic mutations was expressed in yeast in *cis* position with this truncation, CBS activity was restored sufficiently to permit growth of the yeast on medium lacking cysteine. For some mutations, enzyme activities expressed *in vitro* were near wild-type levels.

The displacement of the hydroxyl group of serine by homocysteine proceeds with the retention of configuration [50]. CBS can catalyze alternative β-replacement reactions in which sulfide is a substrate or a product according to the general scheme

$$XCH_2CH(NH_2)COOH + YH \longrightarrow XH + YCH_2CH(NH_2)COOH$$

where X = OH or SH, and Y = SH or S-alkyl [51].

The active core of human CBS shares a high degree of structural similarity (52% if conservative replacements are counted) with the related O-acetylserine sulfhydrases (cysteine synthases) from plants and bacteria [30,52]. These enzymes catalyze the synthesis of cysteine from sulfide and acetylserine. During evolution, the capacity to synthesize cystathionine may have been acquired by broadening the substrate specificity of such enzymes to include homocysteine as well as inorganic sulfide [30].

CYSTATHIONINE β-SYNTHASE DEFICIENCY (MIM 236200)

With more than 700 patients known through an international survey made in 1981–1982 [17], CBS deficiency is by far the most frequent cause of homocystinuria.

Clinical Features and Natural History

The Pyridoxine Response

Based on their biochemical responses in relation to sulfur amino acids in plasma and urine, the majority of patients in the 1981–1982 international survey were classified into two roughly equal groups as being nonresponsive or responsive to pyridoxine [17]. Given our current knowledge, especially about the distribution of homocysteine moieties in plasma, such a classification is an oversimplification. There is a continuum between the two extremes, and the manifestations listed in Table 1 may occur regardless of the extent of the response. In general, so-called pyridoxine-nonresponsive patients present earlier and have more severe phenotypes than patients with a clear response to pyridoxine. The correlation between *CBS* genotype and pyridoxine responsiveness is discussed below.

The Central Nervous System

CBS deficiency often leads to mental retardation. If not detected and treated from early infancy, pyridoxine-responsive and nonresponsive patients have mean intelligence quotients of 79 and 57, respectively [17]. A considerable number of homocystinuric individuals do, however, have normal mental abilities. Epilepsy, most often generalized tonic-clonic seizures, occurs in about 21% of patients, and extrapyramidal symptoms have been noted in several instances [53]. Dystonia, which develops in a high proportion of patients [54], has been mistaken for mental illness [55].

The Eye

Ectopia lentis — that is, subluxation or dislocation of the ocular lens (usually downward, in contrast to Marfan syndrome) due to disruption of its suspensory apparatus — is a highly distinctive feature of CBS deficiency (Fig. 2). It is the clinical sign that most often leads to detection of homocystinuria [17]. Although ectopia lentis has been discovered as early as 4 weeks of age, it is rare in the first two years of life. The risk of ectopia lentis increases with age (Fig. 3). Our current estimate of this risk is probably influenced by ascertainment bias, and the absence of ectopia

TABLE 1. Clinical Abnormalities in Homocystinuria Due to Cystathionine β-Synthase Deficiency

Connective Tissue Involvement
Eye
Frequent
Ectopia lentis
Myopia
Less frequent
Glaucoma
Optic atrophy
Retinal degeneration
Retinal detachment
Cataracts
Corneal abnormalities
Vascular system
Frequent
Vascular occlusions
Malar flush
Livido reticularis
Skeleton
Frequent
Osteoporosis
Scoliosis
Increased length of long bones
Skeletal disproportion
Restricted joint mobility
Pes cavus
Less frequent
Arachnodactyly
Pectus carinatum or exacavatum
Genu valgum
Kyphosis
Other manifestations
Frequent
Thin skin
Fair hair
Less frequent
Inguinal hernia
Other System Involvement
Central nervous system
Frequent
Mental retardation
Psychiatric disturbances
Seizures
Less frequent
Extrapyramidal signs
Other manifestations
Less frequent
Asthma
Liver dysfunction

Adapted from [2], where references to individual symptoms can be found.

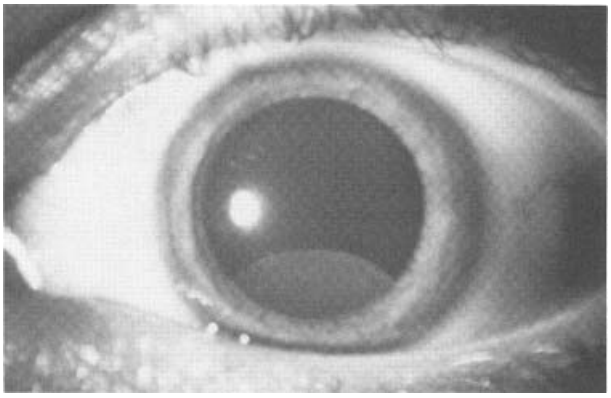


Figure 2. Ectopia lentis. Downward dislocation of the left lens in a 13-year-old girl with pyridoxine-responsive cystathionine β-synthase deficiency

lentis at any age should not deter a clinician from screening a patient's urine for abnormal metabolites. Increased curvature of the lens secondary to gradual disintegration of the zonular fibers gives rise to myopia, which usually precedes complete dislocation. On physical examination, ectopia lentis is reflected by iridodonesis, a tremulousness of the iris due to lack of its usual support. Dislocation of the lens into the anterior chamber of the eye may lead to acute angle glaucoma that, if untreated, results in loss of vision of the affected eye. Some of the other ocular abnormalities listed in Table 1 may also be secondary to ectopia lentis.

The Skeleton

Bony abnormalities have been noted in many patients with homocystinuria. Skeletal disproportion due to excessive growth of the long bones is a cardinal feature [56] and one of the causes of confusion with Marfan syndrome. In contrast to the joint laxity of this latter disorder, however, homocystinuric patients often have reduced joint mobility. Other points of differential diagnosis are discussed below. The decreased upper/lower segment ratio of homocystinuric patients may be exaggerated by a scoliosis that is frequently severe enough to require surgical correction (Fig. 4). It is unknown to what extent scoliosis is due to osteoporosis (Fig. 5). In a group of 364 untreated homocystinuric patients all of whom were examined with a lateral radiograph of the spine, the overall risk of having osteoporosis was 50% by age 16 [17] (Fig. 6). The risk is age-dependent and greater in pyridoxine-nonresponsive patients than in

Figure 3. Time-to-event graphs for lens dislocation in untreated patients with cystathionine β-synthase deficiency. Probabilities were calculated from data on the following numbers of patients: all categories, 628; pyridoxine-responsive, 231; pyridoxine-nonresponsive, 231; intermediate response and unclassified, 166. (Reprinted from [17] with permission.)

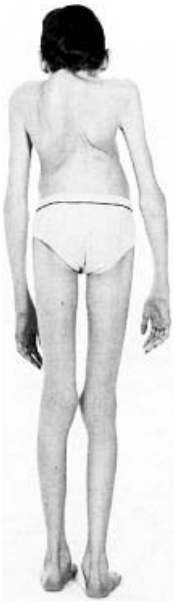


Figure 4. Kyphoscoliosis and Marfanoid skeletal disproportion in a 15-year-old boy with pyridoxine-nonresponsive cystathionine β-synthase deficiency

responsive patients of the same age. Compared with conventional radiography, determination of bone density by computerized tomography provides a more quantitative measure of the significantly reduced bone mineralization in homocystinuric patients [57]. Osteoporosis may lead to pathological fractures of the long bones and the spine (Fig. 5).

The Skin

The classical description of a patient with homocystinuria includes coarse, wide-pored facial skin with malar flushing, whereas the skin on other body parts is thin, atrophic, or even papyraceous [58–60]. It is not known how frequently these abnormalities occur or how they vary with age. A darkening of the coarse and often fair hair has been observed in some patients during treatment with pyridoxine and betaine [61–63].

The Vascular System

Thromboembolic events in both arterial and venous blood vessels are a major cause of morbidity and mortality in CBS deficiency. The risk of thromboembolism in untreated patients has been estimated from information on 629 individuals with proven or presumed enzyme deficiency out of whom 158 had a total number of 253 events [17]. The predominant locations for thromboemboli were peripheral veins (51%) and cerebral blood vessels (32%); myocardial infarction occurred less often (4%). Vascular events seem to be equally frequent in pyridoxine-responsive and nonresponsive patients, but they generally happen earlier in the latter. The maximal risk of about 0.04 events per year, or one event per 25 years, is attained after age 10. Approximately 50% of all untreated CBS-deficient patients have had a thromboembolic episode by age 25. Endothelial dysfunction and early signs of arterial disease in the absence of ischemic symptoms may be detected by means of ultrasonography [64,65].

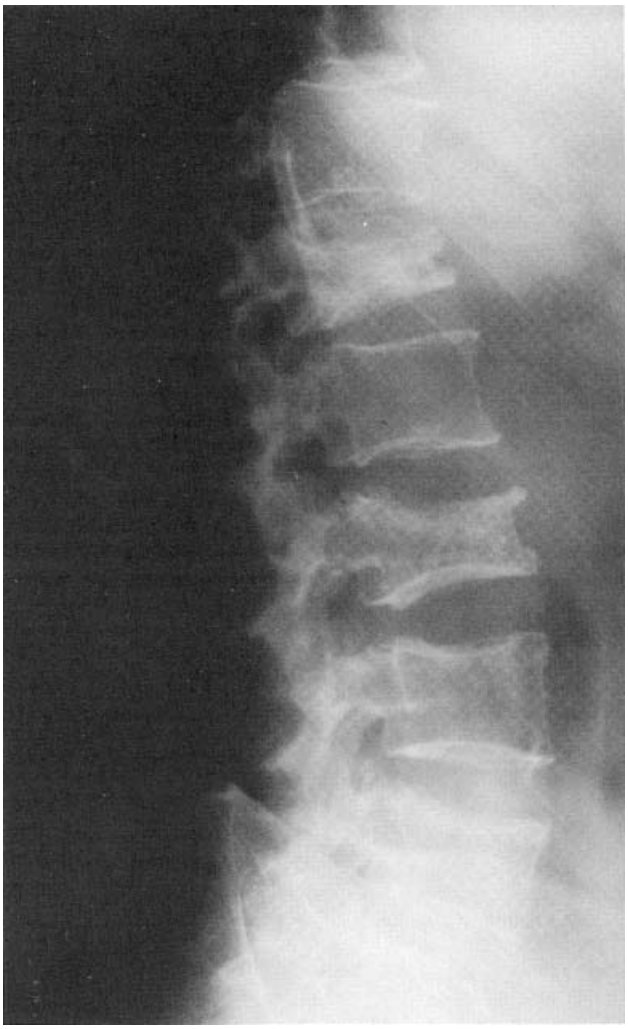


Figure 5. Osteoporosis and compression fractures of the lumbar spine. X-ray of 30-year-old woman with pyridoxine-responsive cystathionine β-synthase deficiency

Other Clinical Features

Table 1 lists a variety of other symptoms and signs noted in homocystinuric patients. The frequency with which each of these abnormalities occurs has not been determined, and their individual pathophysiological significance is uncertain.

Laboratory Findings

Patients with CBS deficiency are characterized biochemically by a combination of homocystinuria and hypermethioninemia. Screening for homocystinuria can be done easily by use of a modified cyanide-nitroprusside test [2]. A positive result should be followed by quantitative analysis of amino acids in urine and plasma. Most, but not all, patients with CBS deficiency have plasma concentrations of methionine above the normal range of about 20–40 μmol/L. Hypermethioninemia develops earlier in pyridoxine-nonresponsive patients than in those responsive to pyridoxine. This difference may partly explain the preferential detection of pyridoxine-nonresponsive patients in programs screening neonates for hypermethioninemia [17]. These observations

Figure 6. Time-to-event graphs for radiological spinal osteoporosis in untreated patients with cystathionine β-synthase deficiency. Probabilities were calculated from data on the following numbers of patients: all categories, 364; pyridoxine-responsive, 154; pyridoxine-nonresponsive, 137; intermediate response and unclassified, 73. (Reprinted from [17] with permission.)

and the fact that some patients respond to very small oral doses of pyridoxine make it clear that normal amino acid analyses of blood and urine do not completely rule out deficiency of CBS in a clinically suspect individual. It may be necessary to repeat the studies after a period of no vitamin intake or to measure the catalytic activity of the enzyme directly.

The deficient transsulfuration is responsible for a 10–20-fold increase of free and protein-bound homocysteine as well as for the presence of homocysteine-containing disulfides in plasma. The time- and temperature-dependent protein binding of sulfhydryl moieties complicates measurements of these compounds. If plasma is not deproteinized immediately after sampling, the fraction of protein-bound homocysteine will increase over time and thereby result in lower values of nonprotein-bound (''free'') homocysteine than are found *in vivo* [66]. Total homocyst(e)ine, determined after the release of homocysteine moieties from plasma proteins and disulfides by treatment with reducing reagents, such as 2-mercaptoethanol or dithiothreitol, is a more reliable measure of hyperhomocyst(e)inemia. Untreated CBS-deficient patients may have fasting plasma total homocyst(e)ine levels that are 10–40-fold above the normal adult range of 5–15 μmol/L [67]. Plasma total homocyst(e)ine in healthy individuals is influenced by a number of factors, including age, sex, pre- or post-menopausal status, pregnancy, renal function, lifestyle, and genetic determinants [67].

Cultured skin fibroblasts have been used most often for confirmation of classical homocystinuria, but deficient CBS activity may also be demonstrated in liver tissue, lymphocytes, brain tissue, cultured amniotic fluid cells, and cultured chorionic villi.

Molecular Genetics

CBS Mutations

Work in more than a dozen laboratories around the world has identified 102 different disease-associated mutations in the *CBS* gene among 324 alleles from homocystinuric patients [14]. Most are ''private'' missense mutations. Only four nonsense mutations have been observed; the remainder are various deletions, insertions, and splicing mutations. An up-to-date list of *CBS* mutations is maintained on the CBS Web site at http://www.uchsc.edu/sm/cbs.

Of the 72 missense mutations found in CBS-deficient patients, nearly one-third have been expressed in *E. coli*. All of these had significantly decreased levels of CBS activity. About one-fourth of the missense mutations are found in exon 3 (Fig. 7), which is, evolutionarily, the most conserved part of the *CBS* gene [52,68]. The two most frequent mutations, Ile278Thr and Gly307Ser, are found in exon 8. The Ile278Thr mutation is panethnic and, overall, accounts for almost one-fourth of homocystinuric alleles. In some countries (e.g., the Netherlands), it accounts for more than half of affected alleles [69]. The Gly307Ser mutation is undoubtedly the leading cause of homocystinuria in Ireland (71% of affected alleles) [70]. It has also been detected frequently in U.S. and Australian patients of ''Celtic'' origin, including families with Irish, Scottish, English, French, and Portuguese ancestry. In contrast, the Gly307Ser mutation has not been detected among a large number of alleles in Italy, the Netherlands, Germany, and the Czech Republic. The finding of Gly307Ser in Norway may indicate that it originated in Scandinavia and spread elsewhere [71].

The third most frequent alteration is a splice mutation in intron 11, IVS 11-2 A>C, which results in skipping of the entire exon 12 (Fig. 7). It has been found in Germany in about 20% of affected chromosomes of German and Turkish origin [72]. Surprisingly, it has never been detected in Italy or the Netherlands among nearly 70 alleles studied. Together with the Ile278Thr mutation, IVS 11-2 A>C is the most prevalent mutation in patients of Czech and Slovak origin.

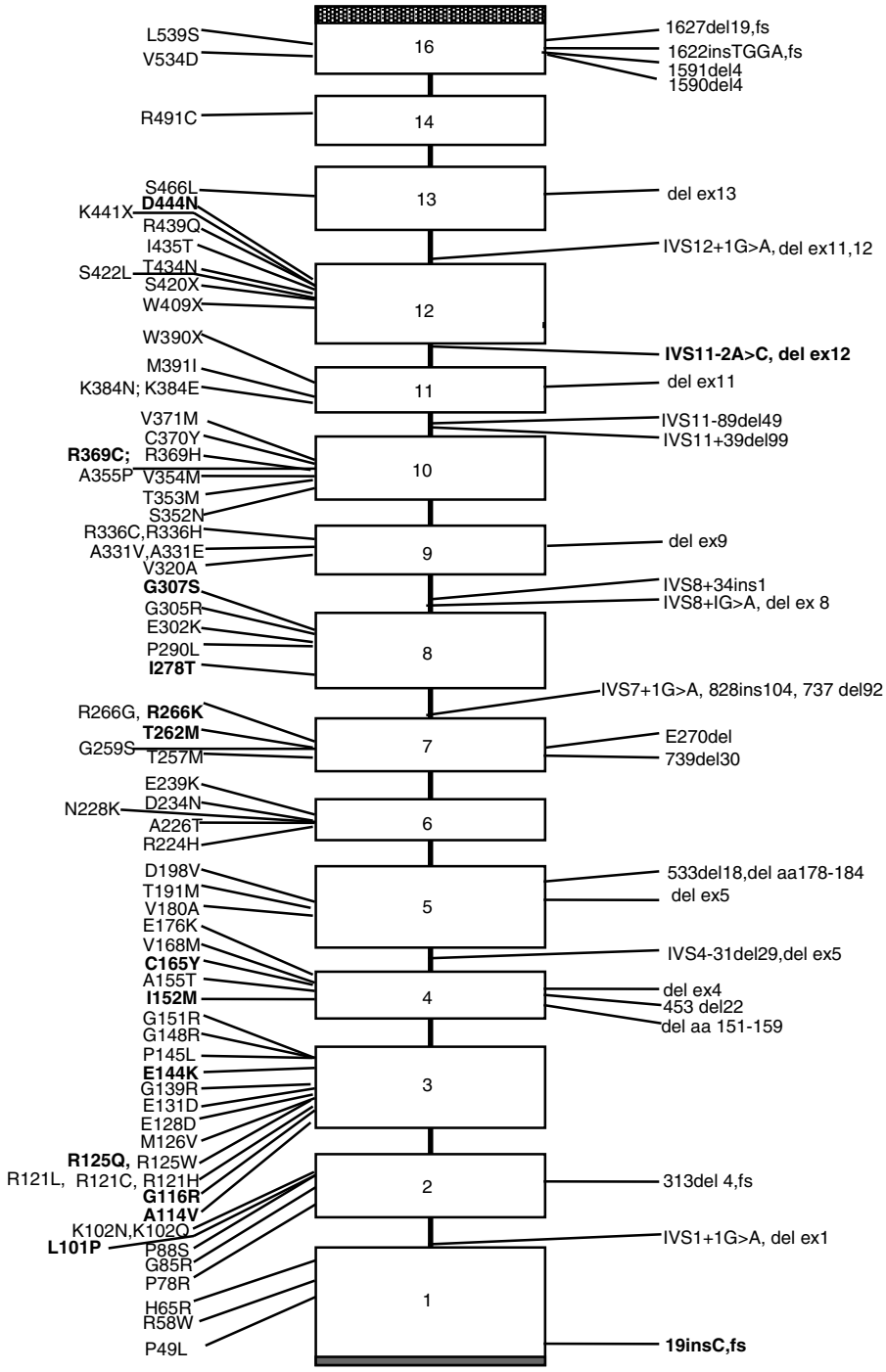


Figure 7. Location of human *CBS* mutations. The exons in the coding region are drawn to scale; the introns are not. The shaded areas are parts of the 5′- and 3′-untranslated regions of CBS mRNA found in exon 1 and exon 16, respectively. The mutations shown on the left are missense and nonsense; the mutations on the right are deletions, insertions, and splicing errors. The mutations shown in bold have been detected in three or more alleles.

Several other, less common splicing mutations as well as insertions and deletions have been found. Some of these mutations result in a frame shift, which, in turn, introduces a premature termination codon.

In nine isolated cases, the mutation has been linked to another mutation on the same allele [14,73]. Most of these mutations have been reproduced separately by *in vitro* mutagenesis, and each has been found to be deleterious to CBS activity. Cystic fibrosis and Gaucher disease (see Chapter 22, this volume) are other examples of diseases with double mutations in *cis* position [74,75]. In Gaucher disease, such alleles are thought to arise from recombination between

the glucocerebrosidase gene and its adjacent pseudogene. The origin of double mutant alleles in the *CBS* gene is not clear because there is no evidence of a *CBS* pseudogene in the human genome. Homologous recombination between the numerous *Alu* sequences or other repetitive elements within the *CBS* gene is an alternative explanation for some of the *CBS* double mutations found in *cis* position [33].

Genotype and Pyridoxine Responsiveness

The majority of homocystinuric patients are compound heterozygotes, with the exception of Ile278Thr/Ile278Thr and Gly307Ser/Gly307Ser homozygotes. The Ile278Thr mutation usually confers pyridoxine responsiveness, whether in the homozygous or the compound heterozygous state (Table 2). With the exception of a group of Dutch patients [69], the clinical phenotype in many of these patients appears to be mild [76]. Although the genotype by itself does not predict the phenotype, several general statements can be drawn from Table 2. In addition to the Ile278Thr mutation, the Ala114Val, Arg266Lys, Ile278Thr, Arg336His, Lys384Glu, and Leu539Ser mutations appear to correlate with pyridoxine responsiveness *in vivo*. On the other hand, the Arg121Leu, Arg125Gln, Cys165Tyr, Glu176Lys, Thr191Met, Thr257Met, Thr262Met, and the frequent Gly307Ser mutation, in one or two copies, appear to be incompatible with pyridoxine responsiveness. Accordingly, patients carrying the Gly307Ser mutation seem to have moderate to severe phenotypes, except for those who have been treated since birth [70]. While pyridoxine responsiveness is constant within sibships, the clinical phenotype often is not [14].

TABLE 2. Correlation Between Genotype and Pyridoxine (Vitamin B_6) Responsiveness *In Vivo* in Cystathionine β-Synthase Deficiency

Cell Line	Genotype		B6**
419	19insC (fs,K36X)	19insC (fs,K36X)	−
LT	A114V	A114V	+
R600	R121L	R121L	−
SGo	R125Q	R125Q	−
AP	C165Y	C165Y	−
428	E176K	E176K	−
S	T191M	T191M	−
NO	T257M	T257M	−
N2	T262M	T262M	−
N1,N4a*,N4b,N9	R266K	R266K	+
RB,C110,MP, 426,PA,CG,M	I278T	I278T	+
AB, B-H, H.B/G, JU,GE,W.S/J	I278T	I278T	+
L209, L188	I278T	I278T	+
NM, MW	I278T	I278T	+
NM	I278T	I278T	+
426	I278T	I278T	+
RS*,SS	I278T	P88S	+
LM*,AM	I278T	G116R	+
L264	I278T	G139R	+
L265	I278T	E144K	+
JC*, MC	I278T	I152M	+
403	I278T	A155T	+
HvE(RD55)	I278T	C165Y	+
GC	I278T	G305R	+
IWa	I278T	T353M	−
N6a*, N6b	I278T	R369C	+
AC	I278T	C370Y	+
JM, JR	I278T	V371M	+
DS	I278T	R439Q	+
366	I278T	IVS11-2A>C, del ex12	+
427	I278T	IVS11-2A>C, del ex12	+/−
TD	I278T	IVS11-2A>C, del ex12	+
ST,FH	G307S	G307S	−
N10	G307S	G307S	−
MGL166, MGL246	G307S	G307S	−
AP,DA	G307S	G307S	−
7215	R336H	R336H	+
P465	K384E	K384E	+
P325	L539S	L539S	+

*An asterisk indicates sib pairs.

** + /− indicates some response to pyridoxine treatment with plasma total homocyst(e)ine concentrations remaining above 50 μmol/L.

Additional information, including original references, can be found at the CBS Web site http://www.uchsc.edu/sm/cbs.

Inheritance and Prevalence

Analysis of pedigrees, enzyme studies of family members, and molecular genetic investigations have firmly documented the autosomal recessive inheritance of CBS deficiency.

The frequency of infants detected by neonatal screening programs varies greatly among different geographic locations, from 1:65,000 in Ireland to 1:900,000 in Japan [77]. The worldwide frequency is 1:344,000 [2]. The reported frequencies may be falsely low due to the failure of amino-acid–based screening programs to detect the majority of pyridoxine-responsive patients. Thus, DNA-based screening of 500 newborns in Denmark showed 1.4% of them to be heterozygous for the Ile278Thr mutation [78]. This corresponds to a homozygote frequency of at least 1:20,500, or 3–4 children per year. Since the authors are not aware of any children diagnosed with classical homocystinuria in Denmark over the past decade, most of these predicted patients have failed so far to reach clinical recognition.

Pathology and Pathophysiology

Despite many studies performed since the 1970s, satisfactory pathophysiological explanations are lacking for most of the clinical manifestations of CBS deficiency. The slow progress in this area of our knowledge has, to a large extent, been due to the absence of a suitable animal model for experimental studies.

Excision of exons 3 and 4 from the mouse *CBS* gene by homologous recombination has generated a CBS knockout mouse [79]. Surprisingly, total CBS deficiency in mice is highly deleterious, with more than 80% of animals dying at a postnatal age of eight weeks. Although the levels of plasma total homocyst(e)ine measured in these mice are comparable to those of human patients, the mice suffer from severe growth retardation and liver damage, characterized by enlarged and multinucleated hepatocytes with lipid droplets and by extramedullary hematopoiesis. Heterozygote mice had liver CBS activity equal to 42% of that of wild-type animals, and plasma total homocyst(e)ine increased twofold above normal for mice. Clinically, the heterozygotes did not have vascular disease or other abnormalities. Further experiments will show the extent to which this animal model

will help to elucidate the pathophysiology of human CBS deficiency.

The Central Nervous System

Ischemic changes due to vascular thromboses or emboli are the only neuropathological abnormalities consistently reported. White matter vacuolar degeneration, hippocampal neuronal loss, and gliosis have been observed in the absence of vascular changes [80,81]. A pathological correlation with dystonic clinical features has suggested a functional disturbance [53]. Indeed, most patients with neurological dysfunction have no focal signs or neuroimaging changes compatible with cerebrovascular disease [82]. Homocysteine, but not methionine or S-adenosylmethionine, induces seizures and epileptiform electroencephalographic activity in rodents after intraperitoneal administration [83]. The neurochemical pathways through which homocysteine or related metabolites cause seizures, extrapyramidal symptoms, and mental retardation are open to speculation. Proposed mechanisms include impairment of brain homeostasis by trapping of adenosine by homocysteine, thereby forming S-adenosylhomocysteine [84], and overproduction of homocysteic acid and homocysteine sulfinic acid, which are excitotoxic (i.e., they cause neuronal damage due to excessive activation of glutamate receptors) [85,86]. CBS is highly expressed in the hippocampus, and CBS deficiency may impair the endogenous production of hydrogen sulfide from L-cysteine and its potential role as a neuromodulator [87]. Dystonia could result from reduced levels of taurine in the basal ganglia or from an effect of folate deficiency on dopaminergic neurotransmission [53].

The Eye

Pathological examination of eyes from CBS-deficient patients with ectopia lentis reveals disrupted zonular fibers attached to either the ciliary body or the lens itself [88,89]. The masses of disrupted fibers contain microfilaments structurally similar to those found in normal zonular fibers but with a degree of disorganization proportional to the age of the patient [90]. The biochemical events behind the disruption are not clear, but several observations of the composition of zonular fibers relate to sulfur amino acid metabolism and connective tissue pathology. Zonular fibers contain the glycoprotein fibrillin, which has a high content of cysteine but lacks the hydroxyproline and hydroxylysine typical of collagen. In this regard, as in their susceptibility to proteases, zonular fibers are like microfibrils of elastin [91–96]. Isolated human zonules are remarkable for their very high cysteine content and no significant amounts of hydroxyproline or hydroxylysine. This implies an important role for intermolecular disulfide bonds in the structure of zonular fibers. Biochemical studies of isolated zonular fibers or fibrillin from homocystinuric individuals, however, have not been reported, and the absence of an appropriate animal model limits the source of material available for laboratory studies. A decreased number of disulfide bonds in zonular proteins of CBS-deficient patients, resulting in impaired functional ability, could be due to interference with the process of disulfide bonding by any of a number of sulfur-containing compounds, including homocysteine, present in increased amounts in homocystinuric individuals. Deficiency of cysteine during formation of the zonular fibers might impair total protein synthesis or diminish glutathione concentrations, thereby altering the redox environment of the cell. The pathophysiological mechanism, yet to be identified, that produces ectopia lentis in CBS deficiency may operate in the same manner in sulfite-oxidase–deficient patients who also have ectopia lentis and an abnormal sulfur amino acid metabolism (but no hyperhomocysteinemia).

The Skeleton

Homocysteic acid, an oxidation product of homocysteine, is found in the urine of homocystinuric patients [97] and in normal human serum [98]. Homocysteic acid was reported to promote growth of hypophysectomized rats, thereby providing an explanation for the excessive length of the long bones in homocystinuria [99]. Intraperitoneal administration of homocysteic acid caused increased tail growth and increased thickness of the epiphyseal cartilage of the tibia, but other workers using larger numbers of animals could not reproduce these observations [100].

In an *in vitro* study using a clonogenic assay, homocysteine, but not homocysteic acid, correlated strongly with the growth-promoting activity of plasma cyclin–dependent kinase [101]. This observation supports earlier theoretical considerations as well as experimental data, which pointed toward a role for homocysteine in the pathophysiology of osteoporosis in homocystinuric patients. McKusick first proposed that excess homocysteine might interfere with the normal synthesis of collagen cross-links [102]. An increased proportion of collagen extractable with 0.5 mol/L acetic acid from skin biopsies was subsequently reported in two of four patients with homocystinuria [103]. Three additional homocystinuric patients with decreased amounts of precursor aldehydes and cross-link compounds in dermal collagen have also been described [104]. Two of these patients, unresponsive to pyridoxine, also had significantly increased solubility of dermal collagen under nondenaturing conditions when compared with controls and one pyridoxine-responsive patient.

Homocysteine may exert its effect on collagen by a mechanism analogous to that of D-penicillamine, which is structurally similar to homocysteine. D-penicillamine has been shown to increase the solubility of collagen, particularly in soft tissues, either by reacting with the lysyl, and to a lesser extent hydroxylysyl, aldehydic groups required for cross-linking [105] or by interfering with polyfunctional cross-link formation from Schiff-base bifunctional cross-links [106]. Support for this idea came from laboratory studies of purified skin collagen in solution in which homocysteine was able to inhibit the synthesis of cross-links [104]. Insoluble collagen fibrils failed to form at high concentrations of homocysteine (10^{-2}–10^{-1} mol/L), and much smaller amounts of cross-link compounds were demonstrated in the presence of homocysteine than in preparations containing similar concentrations of methionine or homocystine.

Lubec et al. [107] have reported measurements in plasma of the terminal propeptides of type I and type III procollagens, which reflect collagen synthesis, and in serum of the carboxyl-terminal telopeptide of collagen I, which reflects collagen cross-linking. Only in the latter measurement was there a significant reduction in the group of nine patients with CBS deficiency compared with the control group. These results support McKusick's hypothesis of deficient collagen cross-linking as an explanation for the osteoporosis in homocystinuria. Alternatively, the effect of homocysteine on connective tissue could be due to direct inhibition of lysyl oxidase. As discussed in Chapter 2 of this volume, this pyrroloquinoline quinone and copper-dependent enzyme catalyzes the cross-linking of collagen

and elastin. *In vitro* studies of lysyl oxidase purified from embryonic chick cartilage showed inhibition of catalytic activity at concentrations of homocysteine greater than 10^{-3} mol/L, whereas little inhibition occurred at concentrations lower than 10^{-4} mol/L or with methionine or homocystine [106,108,109]. In a study of chicks with vitamin B_6 deficiency, which results in reduced lysyl oxidase activity, light microscopy showed abnormalities in the extracellular matrix of the connective tissues, but cross-links and aldehydic content were not significantly lower in cartilage and tendon collagens than in age-matched controls [110]. The activity of lysyl oxidase in cells or tissue from CBS-deficient patients has not been explored.

Homocystinuric patients have abnormally high levels of plasma total copper due to an unexplained increase in ceruloplasmin [111]. Any possible limitation of lysyl oxidase activity is therefore unlikely to be due to shortage of copper. Deficiency of pyridoxine and the use of pyridoxine antagonists have been shown experimentally to decrease cross-linking of collagen, possibly by reducing the activity of lysyl oxidase [112,113], and to decrease cross-linking in lung elastin [114]. However, homocystinuric patients usually show no evidence of pyridoxine deficiency. Asthma has been noted in several patients [60], but neither lung elastin nor the incidence of pulmonary disorders of CBS-deficient patients has been studied in any detail.

The Skin

Because the thin or papyraceous skin of some homocystinuric individuals resembles that of patients after prolonged use of penicillamine, a homocysteine-induced defect of collagen cross-linking, as discussed above, has been proposed to account for the cutaneous pathology in homocystinuria. In addition to the biochemical evidence mentioned previously, a limited number of histological observations point to defects in the connective tissue elements of the skin. Light microscopy of skin from an 8-year-old boy revealed an uneven, granular distribution of collagen fibers in the superficial dermis and too few fibers in the deeper dermis; their ultrastructure was normal [115]. Fragmented and thickened elastic fibers have been observed by both light and electron microscopy of skin from homocystinuric patients [115,116].

Some patients have a hypopigmentation of hair and skin that is reversible after treatment for hyperhomocysteinemia. Experimental evidence has been presented to suggest that homocysteine at concentrations of 110 μmol/L or above inhibits the activity of tyrosinase, the major pigment enzyme [63]. The probable mechanism of this inhibition is an interaction of homocysteine with copper at the active site of tyrosinase.

The Vascular System

Early reports on the clinical manifestations of homocystinuria documented remarkable abnormalities of the arterial walls of patients who had suffered thromboembolic events [117–119]. The gross and microscopic appearance of the blood vessels resembled that of advanced atherosclerosis, which, for the age of the patients, was greatly premature. Following the discovery of vascular pathology in young individuals with homocystinuria due to defects of remethylation, and therefore no elevation of plasma methionine, McCully formulated "the homocysteine theory of arteriosclerosis" [120]. The vascular lesion typical of homocystinuria, irrespective of the responsible enzymatic deficiency, is a fibrous plaque with smooth muscle hyperplasia, deposition of extracellular matrix and collagen, and degeneration and destruction of elastic fibers [121]. Although this description suggests abnormalities of collagen and/or elastin, these changes do not seem to result in vascular complications from weakness of the vascular wall, nor have such changes been shown to cause the observed, widespread endothelial damage. In addition, venous thromboemboli are the most frequent vascular manifestation of homocystinuria [17], yet little pathology of the veins has been reported.

The association of early atherosclerosis and thromboembolism with hyperhomocysteinemia has attracted the interest of many investigators. Detailed reviews of pertinent data can be found elsewhere [1,67,122]. Hypotheses have included endothelial injury [123–127], increased thrombogenesis [128–131], abnormal clotting [132–134], enhanced collagen production [135], decreased availability of nitric oxide [136], and abnormal metabolism of lipoproteins [137,138]. Lipids, however, are notably absent from the vascular plaques seen postmortem in homocystinuric patients [121].

Despite these and other investigations to clarify the pathogenic potential of homocysteine, the exact sequence of pathophysiological events remains obscure. Perhaps other predispositions to cardiovascular disease are required for hyperhomocysteinemia to trigger thrombosis. Thus, in a study of 11 patients with classical homocystinuria, thromboses were observed in only six patients, who also carried the factor V Leiden mutation [139]. Screening for a wide range of cardiovascular risk factors therefore is important in patients with hyperhomocysteinemia.

Mild Hyperhomocysteinemia and Vascular Disease

Wilcken and Wilcken [140] reported in 1976 that seven of 25 patients with coronary heart disease, but only one of 22 control subjects, had elevations of plasma nonprotein-bound homocysteine following a standardized methionine load. Data from about 80 clinical and epidemiological studies, including more than 10,000 patients, have confirmed since that mild hyperhomocysteinemia is a risk factor for vascular disease [67].

Hyperhomocysteinemia without homocystinuria may have several explanations. The degree of homocysteinemia observed in some patients with vascular disease is of the magnitude seen in heterozygotes for CBS deficiency, and such heterozygosity was initially thought to account for about 30% of patients with premature occlusive arterial disease [141]. Subsequent molecular analysis, however, failed to reveal any pathogenic mutations in patients with vascular disease and mild hyperhomocysteinemia [142].

Mild hyperhomocysteinemia is observed also in individuals deficient in cobalamin (vitamin B_{12}) and folate [143–145] as well as in some patients with renal disease, cancer, or psoriasis [66]. Supplements of B vitamins, especially folic acid, can safely reduce plasma total homocyst(e)ine [67]. Deficiencies of cobalamin or folate are not known to be associated with premature atherosclerosis, and there is as yet no evidence that lowering mildly elevated homocysteine with supplements of these vitamins can reduce the risk of vascular disease.

It is conceivable that mild hyperhomocysteinemia is an epiphenomenon of vascular disease. In a study of endothelial dysfunction and acute hyperhomocysteinemia induced by a methionine load, flow-mediated vasodilatation was completely prevented by folic acid supplementation, which did not attenuate the hyperhomocysteinemia [146]. Any explanation of vascular disease due to mild hyperhomocysteinemia will also have to contend with the apparent paucity of vascular episodes in patients with homocystinuria who, despite

dietary and drug therapy, maintain severely elevated plasma total homocyst(e)ine [18,19,147].

Differential Diagnosis

Isolated bilateral ectopia lentis (MIM 129600) may occur as an autosomal dominantly inherited disorder of either congenital or late spontaneous onset. Ectopia lentis and arachnodactyly are frequent findings in Marfan syndrome (MIM 154700) (Chapter 12, this volume), but patients with this dominant disorder have no increased incidence of mental retardation, and their vascular symptoms are usually due to dilatation or dissecting aneurysm of the aorta rather than to thromboembolism. Infants with ectopia lentis should also be examined for deficiency of sulfite oxidase, as discussed at the end of this chapter. Affected children have neurological symptoms and failure to thrive but no manifestations in the skeletal or vascular systems. Furthermore, ectopia lentis is also seen in Weill-Marchesani syndrome (MIM 277600), of which short stature and brachydactyly are additional features, and in occasional patients with Ehlers-Danlos syndrome (Chapter 9, this volume) [148]. The presence of homocystinuria distinguishes deficiency of CBS from all of these disorders.

Homocystinuria, on the other hand, may be caused also by deficient methylenetetrahydrofolate reductase activity or by inherited or acquired states of cobalamin deficiency. These disorders, discussed in more detail below, may be accompanied by mental retardation and thromboembolism but not by ectopia lentis or skeletal abnormalities. Urine should routinely be examined by gas chromatography for the presence of methylmalonic acid due to deficient cobalamin cofactor. Patients with deficient remethylation of homocysteine have low or low-normal concentrations of plasma methionine in contrast to the hypermethioninemia usually seen in CBS deficiency. Definitive diagnosis of the latter can, of course, be established by an absent or significantly reduced enzymatic activity in extracts of cells from a suspected patient.

Management and Prognosis

Treatment of CBS-deficient patients includes methionine restriction to decrease the homocysteine load, cystine supplementation to avoid cysteine deficiency, pyridoxine to increase the residual activity of the enzyme, folate and hydroxycobalamin to correct and prevent respective deficiencies, betaine to increase remethylation of homocysteine to methionine, and antithrombotic agents.

Methionine Restriction

Most homocystinuric individuals diagnosed in early infancy have been managed with methionine restriction. The synthetic formulas with no or low content of methionine that are available for use in children are supplemented with cystine to avoid cysteine deficiency. This is especially important in early life, when cystine may be an essential amino acid [149]. In addition to the formula, the child must receive sufficient methionine from natural proteins to sustain linear growth. The immediate goal of management is to keep blood and urine homocyst(e)ine as low as possible. The long-term results of therapy with methionine restriction have been very encouraging [17,19]. Most children treated since early infancy have normal intelligence, and significantly fewer suffer from ectopia lentis than would have been expected without treatment. In addition, results on pyridoxine-nonresponsive patients diagnosed by neonatal screening in Ireland suggest a significant reduction in the incidence of thromboembolic events [19]. Early treatment of homocystinuria also reduces the incidence of osteoporosis [150].

The unpalatability of a methionine-restricted diet, however, especially in the form of a synthetic formula, makes its use less easily acceptable when introduced after infancy.

Pyridoxine

Identification of an individual with homocystinuria due to deficiency of CBS should prompt a determination of the patient's biochemical responses to oral administration of pyridoxine. Occasional patients improve or normalize their abnormal levels of sulfur amino acids in plasma and urine in response to only a few milligrams of pyridoxine, but most require daily doses of more than 100–200 mg; some patients need 500–1,000 mg daily in order to respond. The classification of many patients as responsive to pyridoxine has probably not taken into account their levels of total plasma homocyst(e)ine, and it is therefore uncertain how many patients have a complete or only a partial biochemical response to these doses of pyridoxine. When exceeding a daily dose of 500 mg, the possibility of pyridoxine-induced neuropathy must be kept in mind [151–153]. Treatment with pyridoxal phosphate at 1,000 mg/day caused hepatic dysfunction in one pyridoxine-nonresponsive patient who subsequently had no untoward reaction to pyridoxine hydrochloride at 1,800 mg/day [154].

Depletion of folate has been reported to obscure the response to pyridoxine in patients otherwise responsive [155]. To optimize the folate-dependent remethylation of homocysteine, most patients therefore have routinely received folate supplements of the order of 5 mg daily, along with pyridoxine. Care should be taken also to ensure that patients are not vitamin B_{12}-deficient.

Determination of time-to-event graphs (e.g., Figs. 3 and 6) for the various major manifestations of untreated CBS deficiency has permitted comparisons with patients treated with pyridoxine (with or without folate supplements) [17]. Because the mainstay of therapy of patients detected as newborns has been methionine restriction, an evaluation of the effect of pyridoxine has been possible only in late-detected pyridoxine-responsive patients, in whom such treatment significantly reduces the rate of occurrence of thromboembolic events.

Betaine

Early interest in the treatment of homocystinuric patients with the methyl donor betaine (trimethylglycine), or its precursor choline, abated due to the high concentration of methionine caused by the treatment [15,156]. As more experience with hypermethioninemic patients has accumulated, and such concern has lessened, several groups have reported the beneficial effects of prolonged treatment with betaine [18,19,62,157,158]. Anhydrous betaine in daily doses of 100 mg/kg is well-tolerated, and treatment of adult patients with 12–15 g/day seems to be without side effects. Even larger doses have been used in children with defects of homocysteine remethylation [159]. Pyridoxine-nonresponsive patients unable to comply with methionine restriction are the prime candidates for betaine treatment. Betaine reduces total homocyst(e)ine in both plasma and cerebrospinal fluid [160]. Reported clinical effects include darkening of new hair growth, improved behavior, and better control of seizures [15,62]. Importantly, among 15 pyridoxine-nonresponsive homocystinurics during 258 patient-years of treatment, betaine contributed to a highly

significant reduction in the number of thromboembolic episodes [18].

Treatment of pyridoxine-responsive patients with pyridoxine and folate does not prevent an abnormal rise of plasma homocyst(e)ine following a methionine load [161,162]. Addition of betaine to the standard regimen of pyridoxine and folate virtually normalizes the homocysteine response in pyridoxine-responsive patients who therefore also may benefit from this form of therapy [158].

Antithrombotic Agents

Aspirin and dipyridamole have been used in many CBS-deficient patients on the assumption that these agents reduce the risk of thrombosis. No studies have evaluated their efficacy, however, in hyperhomocysteinemic patients. Because the pathophysiological mechanisms behind the vascular events are unknown, as discussed above, no recommendations can currently be made as to the use of antithrombotic agents in homocystinuria.

Mortality

Early survivorship data suggested 50% mortality by age 20 [163], but subsequent analysis of more than 600 patients, including many with mild clinical manifestations of homocystinuria, gave evidence of a much better prognosis [17]. The mortality at age 20 was less than 5% among pyridoxine-responsive patients and approximately 20% in those not responsive to pyridoxine. Early dietary intervention and treatment with pyridoxine and betaine seem to further improve these numbers [18,19].

Prenatal Diagnosis

Prenatal diagnosis of CBS deficiency has been performed in both the first and the second trimesters of pregnancies at risk. Fowler and colleagues [164] were first to report the diagnosis of an affected fetus by enzyme assay of an extract of cultured amniotic fluid cells. The range of catalytic activity in cultured cells from heterozygotes overlaps the ranges of both homozygous normal and homozygous abnormal cells. Deficiency of CBS has also been diagnosed, as well as excluded, in the first trimester of several pregnancies by assay of extracts of cells grown from chorionic villi. Chorionic villus biopsies themselves contain insufficient activity for direct assay of CBS.

DNA analysis for first-trimester prenatal diagnosis of CBS deficiency is possible in the few families in which the mutant alleles have been studied previously. The genetic heterogeneity mentioned previously complicates mutation analysis of the majority of probands with CBS deficiency.

DISORDERS OF FOLATE AND COBALAMIN METABOLISM AFFECTING HOMOCYSTEINE REMETHYLATION

A growing number of inborn errors other than CBS deficiency have been found to cause homocystinuria. The common denominator for these metabolic defects is an impaired remethylation of homocysteine to methionine, a reaction catalyzed by methionine synthase (reaction 3, Fig. 1). This enzyme uses 5-methyltetrahydrofolate and methylcobalamin as cosubstrate and cofactor, respectively. Deficient remethylation of homocysteine may therefore result from an inadequate supply of 5-methyltetrahydrofolate due to deficiency of methylenetetrahydrofolate reductase (reaction 5, Fig. 1). It may also be caused by a number of different defects in the biosynthesis of cobalamins. Genetic heterogeneity explains some of the marked clinical variability of these disorders that do not, in general, have connective tissue manifestations. All pedigrees reported so far are compatible with autosomal recessive inheritance.

Methylenetetrahydrofolate Reductase Deficiency (MIM 236250)

Since the original description by Mudd and his colleagues [165], information has been published on approximately 50 patients with severe deficiency of methylenetetrahydrofolate reductase ($NADPH_2$) [3,166–168].

Clinical Features

The age at presentation varies greatly. Table 3 lists some of the clinical abnormalities observed in affected individuals. Most patients present in early childhood or adolescence with neurological dysfunction, psychiatric abnormalities, or failure to thrive. Respiratory difficulties are frequent in the terminal phase of patients with severe neurological damage. Symptoms or signs directly referable to the vascular system have not been observed in living patients. Anemia is unusual, and the lack of megaloblastic anemia is an important point in the differential diagnosis against other inborn errors of homocysteine remethylation.

Biochemical Abnormalities and Molecular Genetics

The homocystinuria and hyperhomocysteinemia of methylenetetrahydrofolate reductase-deficient patients are usually milder than those seen in CBS deficiency. The concentrations of methionine in plasma and cerebrospinal fluid are often low and result in correspondingly low levels of S-adenosylmethionine [169]. The concentrations of folates are reduced in serum and red cells. In addition to low folates, several patients have also had reduced concentrations of neurotransmitter amine metabolites in the cerebrospinal fluid [169–171].

Deficient activity of methylenetetrahydrofolate reductase can be demonstrated in liver, leukocytes, lymphoblasts, and cultured skin fibroblasts from affected patients. The presence of activity in cultured amniotic fluid cells and in chorionic villi allows prenatal diagnosis in the first or second trimester of at-risk pregnancies [172].

Molecular studies have revealed genetic heterogeneity and a good correlation between the genotype, residual enzyme activity, and age at onset and severity of symptoms [166,168]. Five unrelated patients with the lowest levels of enzyme activity (0–3% of control) were each homozygous for one of three mutations. These patients had had onset of symptoms in the first year of life, with a severe form of the disorder and, in some cases, an early demise [166].

Deficiency of methylenetetrahydrofolate reductase due to a thermolabile variant of the enzyme can cause

TABLE 3. Clinical Abnormalities in Homocystinuria Due to Methylenetetrahydrofolate Reductase Deficiency

Developmental delay	Seizures
Mental retardation	Peripheral neuropathy
Motor abnormalities	Hypotonia
Psychiatric abnormalities	Ataxia
Failure to thrive	Parkinsonism
Microcephaly	Dementia
Breathing abnormalities	Vascular disease?
Lethargy	

mild hyperhomocysteinemia. A single C677T nucleotide exchange resulting in an alanine-to-valine substitution appears to account for most, if not all, such cases [173]. The allele carrying C677T has a frequency of 35–40% in some populations [166,173], and its association with cardiovascular disease in initial reports of homozygosity generated considerable interest. More recent analyses seem to document, however, that although this variant of methylenetetrahydrofolate reductase is associated with hyperhomocysteinemia, it does not increase the risk of vascular disease [174,175].

Pathology and Pathophysiology

Postmortem investigations have been performed in at least 11 children or adolescents with proven or presumed methylenetetrahydrofolate reductase deficiency [171,176,177]. In six of these individuals, vascular occlusions were thought to have contributed significantly to the cause of death. On gross and microscopic examination, the vascular lesions were similar to those observed in CBS deficiency, albeit more severe than one would have expected from the milder aminoacidemia. The accumulated evidence from the two disorders strongly invokes homocysteine as the causative agent of the vascular disease, but the observations made on methylenetetrahydrofolate reductase-deficient patients do not improve our understanding of the vascular pathophysiology of hyperhomocysteinemia.

The neurological manifestations of methylenetetrahydrofolate reductase deficiency can, in contrast to those seen in CBS deficiency, be correlated with consistent neuropathological findings, including cerebral atrophy, gliosis, and demyelination in the brain [177]. Subacute combined degeneration of the spinal cord, a well-known complication of cobalamin deficiency, has been observed in two affected children with fatal courses of progressive demyelination [171,177]. An inadequate supply of methionine or S-adenosylmethionine to the brain, a low concentration of folate in the cerebrospinal fluid, and low levels of amine neurotransmitters could, alone or in combination, play a role in the pathogenesis. There is good evidence for an association between deficient S-adenosylmethionine and demyelination [169,178,179].

There is no animal model that corresponds to the enzyme deficiency in humans. Nitrous oxide (N_2O) inactivates methionine synthase by oxidation of the cobalamin cofactor, and anesthesia with nitrous oxide has been used in monkeys to produce a neuropathy that resembles subacute combined degeneration [180]. The use of nitrous oxide may have precipitated developmental regression and fatal respiratory symptoms in one infant with deficient methylenetetrahydrofolate reductase [171].

Management and Prognosis

Treatment of patients with methylenetetrahydrofolate reductase deficiency has included folic acid, methyltetrahydrofolate, methionine, pyridoxine, cobalamin, carnitine, and betaine [3]. Most patients have received a combination of two or more agents. Administration of folic acid to a 2-year-old girl caused convulsions and acute deterioration of her Parkinsonism [171]. Pyridoxine further aggravated the neurological dysfunction of another patient [181]. Betaine, on the other hand, has had a beneficial effect in all patients in whom it has been used. When started in the neonatal period, therapy including betaine may prevent or improve neurological symptoms. Concentrations of methionine in the cerebrospinal fluid and, probably, of S-adenosylmethionine in the brain appear to be proportional to the daily dose of betaine [169]. Doses of up to 1–1.5 g/kg/day or 20 g/day have been used without side effects [159,168]. Nevertheless, the available data indicate that most patients with methylenetetrahydrofolate reductase deficiency have a poor prognosis. However, ascertainment bias toward the reporting of more severely affected patients, the limited number of patients known, and their genetic heterogeneity should be taken into account.

Inherited Disorders of Cobalamin Metabolism

Only two reactions in mammalian tissues require derivatives of vitamin B_{12}, or cobalamin (Cbl), as cofactors (Fig. 8). One is the conversion of methylmalonyl-CoA to succinyl-CoA by methylmalonyl-CoA mutase (EC 5.4.99.2), which depends on adenosylcobalamin (AdoCbl) for activity. The other is the methylation of homocysteine to methionine by methionine synthase, which requires methylcobalamin (MeCbl). Patients with inborn errors of metabolism causing reduced availability of AdoCbl and/or MeCbl may have methylmalonic aciduria (cbl A and B diseases), homocystinuria (cbl E and G diseases), or both (transcobalamin II deficiency, cbl C, D, and F diseases). Classification into separate genetic entities has been achieved by complementation analysis. Inheritance of all of these disorders is autosomal recessive. Figure 8 shows where the defects are localized in the cobalamin pathway; only those involving homocysteine are mentioned below. Clinically, neurological dysfunction, psychiatric signs, and hematological abnormalities characterize disorders with homocystinuria due to defects of cobalamin biosynthesis; they do not involve connective tissue.

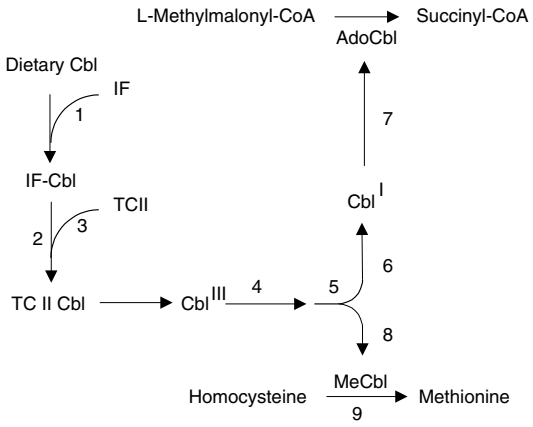
Transcobalamin II Deficiency (MIM 275350)

Transcobalamin II is the major protein carrier of cobalamin in plasma. After recognition by a specific receptor in the cellular membrane, the transcobalamin II-cobalamin complex is endocytosed, transported into lysosomes, and degraded. Cobalamin is then released and used as a cofactor in the cytosol or in mitochondria. Findings in the approximately 40 known infants with genetic deficiency of transcobalamin II include megaloblastic anemia, neurologic abnormalities, immune deficiency, methylmalonic aciduria, and homocystinuria [182]. Deletions in the transcobalamin II gene have been detected in one patient [183]. Patients respond clinically to cobalamin given intramuscularly.

cbl C (MIM 277400), cbl D (MIM 277410), and cbl F (MIM 277380) Diseases

More than 100 patients are known with cbl C disease [182]. Of the 50 patients reported in a survey by Rosenblatt and co-workers, 44 had onset in infancy with feeding difficulties, hypotonia, seizures, ophthalmologic abnormalities, and megaloblastic changes [184]. One-quarter of these children died, and all but one survivor had psychomotor retardation. Onset of cbl C disease later in childhood or adolescence was associated with less severe hematological abnormalities, extrapyramidal signs, dementia, delirium, or psychosis. Some, but not all, of these late-onset patients had mild to moderate neurologic impairment at follow-up [184]. One of the two brothers reported to have cbl D disease was diagnosed at age 14 during admission for acute psychosis; he was mildly retarded and had had a total of four thromboembolic episodes [185]. The pathogenic mechanisms behind cbl C and D diseases are thought to involve the reduction of cobalamin in the cytosol (reaction 5, Fig. 8).

Six patients have been diagnosed with cbl F disease [3,182]. Clinical manifestations include developmental



SITE	MUTANT CLASS	LOCALIZATION OF CONGENITAL DEFECT	MIM NUMBER(S)
1		Intrinsic Factor	193090, 243320, 261000
2		Intestinal Cbl Absorption	261100
3		Transcobalamin II	275350
4	cbl F	Lysosomal Cbl Efflux	277380
5	cbl C, cbl D	Cytosolic Cbl Metabolism	277400, 277410
6	cbl A	Mitochondrial Cbl Reduction	251100
7	cbl B	Mitochondrial Cbl(I) Adenosylation	251110
8	cbl E	Methionine Synthase-associated Cbl Utilization	236270
9	cbl G	Methionine Synthase	250940

Figure 8. Inherited defects of cobalamin metabolism. Cbl = cobalamin; AdoCbl = adenosylcobalamin; MeCbl = methylcobalamin. (Adapted from [3].)

delay, minor facial anomalies, stomatitis, arthritis, pigmentary skin abnormality, and sudden death in one infant. The defect in cbl F mutant cells appears to reside in the release of cobalamin from the lysosome (reaction 4, Fig. 8), analogous to the defect in nephropathic cystinosis.

Pathological examination of two infants with cbl C disease revealed cerebral lesions typical of those seen in subacute degeneration of the cord in one case [186] and, in the other, vascular abnormalities similar to those of CBS deficiency [187]. Autopsy of a girl with cbl F disease did not explain her sudden death at age 5 months [188]. Focal areas of aortic intimal thickening in this patient were thought to reflect hyperhomocysteinemia; the degree of myelination of the central nervous system was appropriate for age.

Most experience with management has been gained from cbl C patients, in whom partly successful attempts at therapy have included intramuscular doses of hydroxycobalamin, administration of folic acid and betaine, protein restriction, and carnitine supplementation [182,184,189,190]. The poor outcome, with high mortality and residual neurological damage in most survivors, may reflect the duration of the disease prior to diagnosis and treatment, the nature of the disease, or both.

cbl E (MIM 236270) and cbl G (MIM 250940) Diseases

The combination of neurological dysfunction, failure to thrive, megaloblastic anemia, hypomethioninemia, homocystinuria, and low levels of methylcobalamin in cultured cells has been recognized in a group of about 30 patients who fall into one of two complementation groups [2,182,191,192]. Patients with cbl G disease have mutations in the gene for methionine synthase [193–195], while those with cbl E disease have a defect in the regeneration of active methionine synthase, including methionine synthase reductase [196–198]. Most of these individuals have come to medical attention in infancy or early childhood, but delay in onset of neurological symptoms until adult life has been documented in cbl G disease [192,199].

The hematological and biochemical, but not the neurological, abnormalities of patients with cbl E and G diseases fully respond to intramuscular hydroxycobalamin [3,182,192]. The long-term prognosis is unknown. Prenatal diagnosis of cbl E disease has been accomplished by analysis of radiolabeled vitamin B_{12} incorporation by cultured amniotic fluid cells [200].

INHERITED DISORDERS OF SULFITE OXIDASE (MIM 272300) AND MOLYBDENUM METABOLISM (MIM 252150 AND MIM 252160)

Deficiency of sulfite oxidase (reaction 2, Fig. 1) was discovered in 1967 by Mudd and co-workers, who investigated an infant with neurologic symptoms and dislocated lenses but no homocystinuria [201,202]. Sulfite oxidase contains a molybdenum cofactor, and defects in its biosynthesis have subsequently been found to be a more frequent cause of sulfite oxidase deficiency than primary deficiency of the apoenzyme [4]. Regardless of the nature of the basic defect, patients present with devastating neurological dysfunction. They have no connective tissue

manifestations except for dislocated lenses. Effective therapy is currently not available. Inheritance is autosomal recessive, and pathogenic mutations have been identified in patients with isolated sulfite oxidase deficiency [203,204] as well as in those with deficient molybdenum-containing cofactor [205]. First-trimester prenatal diagnosis is feasible by direct assay of sulfite oxidase activity in samples of chorionic villi.

Clinical Features

Neurologic dysfunction and feeding difficulties start shortly after birth. The neonate may have axial hypotonia, increased muscle tone peripherally, and major motor fits that typically are unresponsive to therapy. Some affected infants are claimed to have dysmorphic facial features [206,207]. Survivors have profound mental retardation and dislocated lenses, and neuroimaging shows brain atrophy. A milder course into the third decade with learning disability and moderate hemiplegia has been described [208].

Biochemical Abnormalities and Pathophysiology

Deficiency of sulfite oxidase leads to accumulation of sulfite, and increased levels of sulfite, S-sulfocysteine, thiosulfate, and taurine may be detected in urine. Because sulfite is rapidly oxidized to sulfate by atmospheric oxygen at room temperature, the urine specimen must be fresh to avoid false-negative results [4]. Measurement of the more stable S-sulfocysteine is more reliable for diagnosis. Deficiency of molybdenum cofactor also leads to decreased activity of xanthine dehydrogenase and aldehyde oxidase, which causes a low level of uric acid and xanthinuria but no significant clinical pathology.

The pathophysiology of sulfite oxidase deficiency is unclear. The culprit seems to be excess sulfite, which may disrupt the disulfide bonds of lenticular fibers in the same manner as does excess homocysteine. The steps leading to the severe neuropathology have not been clarified.

RECENT DEVELOPMENTS

Molecular Biology and Biochemistry of Cystathionine β-Synthase

Elucidation of the x-ray crystal structure of the active core of CBS has shown it to share a fold with O-acetylserine sulfhydrylase [209]. More significantly, the location of the heme moiety in the polypeptide chain was revealed, and it was shown to bind in the N-terminal part of the subunit to a region with no secondary structure. This heme binding motif together with a spatially adjacent oxidoreductase active site motif could explain the regulation of CBS enzyme activity by redox changes.

Native western blot analysis of 14 mutant fibroblast lines showed a paucity of CBS antigen, which was detectable only in aggregates [210]. Five mutant polypeptides produced by recombinant expression in *E. coli* predominantly formed aggregates devoid of heme. These findings suggest that abnormal folding, impaired heme binding, and aggregation of mutant polypeptides may be a common pathogenic mechanism in CBS deficiency.

A functional assay in yeast has been used to characterize *CBS* mutations, many of which are functionally suppressed by deletion of the C-terminal 142 amino acids containing a 53 amino acid motif known as the CBS domain [211]. This domain is found in a wide variety of proteins of diverse biological function. When screening for C-terminal missense mutations that can suppress the common Ile278Thr mutation, the assay identified seven suppressor mutations, four of which mapped to the CBS domain. Combined in *cis* position with Val168Met, another pathogenic mutation, six of the seven mutations rescued the yeast phenotype to as much as 64% of wild-type enzymatic activity. These results suggest that subtle changes within the C-terminus can restore mutant CBS activity, and provide a rationale for screening for compounds that can activate mutant CBS alleles.

Purification of two patient-derived C-terminal mutant forms of CBS, Ser466Leu and Ile435Thr, has provided further support for a regulatory function of the CBS domain [212]. The Ile435Thr protein is inducible by S-adenosyl-L-methionine (AdoMet) but the response is 10-fold less than for wild-type CBS. The Ser466Leu form does not respond to AdoMet but is constitutively activated to a level intermediate between that of wild type CBS in the presence and in the absence of AdoMet. Both proteins are able to bind AdoMet, indicating that their impairment is related to their ability to assume the fully activated conformation that AdoMet induces in wild-type CBS. It was possible to activate the Ile325Thr and wild-type CBS by partial thermal denaturation, but neither Ser466Leu CBS nor a truncated form of CBS lacking the C-terminal region could be activated by this treatment. Activation by AdoMet, limited proteolysis, and thermal denaturation share a mechanism involving the displacement of an autoinhibitory domain located in the C-terminus of CBS.

Pathophysiology of Cystathionine β-Synthase Deficiency

Neither the incidence nor the cause of the increased skeletal growth or tall stature of homocystinuric patients is known. One study has demonstrated a direct correlation between plasma free homocyst(e)ine and growth velocity SD scores [213]. Both growth velocity and height SD scores were lower during optimal metabolic control, but growth hormone and growth hormone-related peptides did not deviate significantly from the reference ranges. These findings suggest that overgrowth is directly mediated by homocysteine, and that it may be prevented by optimal metabolic control. The retrospective nature of the study presumably precluded measurements of plasma total homocysteine.

Management of Cystathionine β-Synthase Deficiency

A study involving several large clinical centers has further documented the beneficial effects of long-term treatment on vascular outcome [214]. During 1314 patient-years, 53 vascular events would have been expected without treatment, but only five events were observed with appropriate homocysteine-lowering therapy. The high statistical significance of these data, and of additional data published in abstract form [215], is based on the risk of 0.04 events per year observed in untreated patients [17]. This risk was calculated from data collected in 1981–1982, since which time a greater clinical variability of the disease has been appreciated, especially at the mild end of the spectrum. The likely bias of the 1981–1982 survey should be kept in mind when interpreting recent and future observational studies.

Molybdenum Cofactor Deficiency

Gephyrin is a polypeptide required for the clustering of inhibitory glycine receptors in postsynaptic membranes in the rat central nervous system [216]. It also catalyzes the insertion of molybdenum into molybdopterin, and a

deletion in the gephyrin gene (*GEPH*) has been described in a child with symptoms typical of molybdenum cofactor deficiency [217].

ELECTRONIC DATABASE

Information about *CBS* mutations can be obtained at the following Web site: http://www.uchsc.edu/sm/cbs.

The authors are not aware of any support groups for patients with homocystinuria.

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Chapter 14

Menkes Disease and the Occipital Horn Syndrome

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SUMMARY

- **Menkes disease (MD) is an inborn error of copper metabolism affecting several body systems, including the connective tissues. The clinical manifestations of MD can be quite variable, and both severe and milder forms exist. In the milder forms, the neurological symptoms are less severe, while connective tissue findings remain prominent. This group is likely underdiagnosed.**
- **The classical, severe form of MD comprises 90–95% of all known cases, and its clinical manifestations include progressive neurological degeneration, seizures, growth failure, arterial aneurysms, and skeletal defects, as well as characteristic hair changes (pili torti) and hypopigmentation, and death results in early childhood.**
- **The occipital horn syndrome is the mildest form of MD. Remarkable changes are inguinal herniae, bladder diverticula, skin laxity, and skeletal abnormalities, including a narrow trunk, deformed elbows, and genu valgum. A diagnostically important feature is the occurrence of bony protuberances on the occiput, the so-called occipital horns. Chronic diarrhea and orthostatic hypotension are other characteristic features.**
- **The defect in MD is in the transport of copper into the intracellular sorting station for proteins, the Golgi complex. The affected enzyme is a P-type ATPase, ATP7A, that requires energy for copper transport across the membrane. At basal copper levels, the protein is located in the *trans*-Golgi network, where it transports copper into the lumen for incorporation into secreted and vesicular copper-requiring enzymes. When exposed to excessive copper levels, the protein rapidly moves to the plasma membrane, where it functions in copper efflux. Deficient activity of ATP7A results in intracellular copper accumulation.**
- **The Menkes gene (*ATP7A*) has been cloned, and mutations have been identified in both classical and milder forms of MD. The mutations identified show great variability, but there is as yet no good genotype-phenotype correlation, although more mildly affected patients have been shown to have mutations that are predicted to result in ATP7A with residual enzyme activity.**

Abbreviations: ABP1, amiloride binding protein 1, amine oxidase, copper-containing (MIM 104610); AOC2, amine oxidase, copper-containing (MIM 602268); AOC3, amine oxidase, copper-containing (MIM 603735); ATP, adenosine triphosphate; ATP7A, Cu-transporting ATPase that is deficient in Menkes disease (MIM 300011); *ATP7A*, gene symbol for the Menkes gene; *Atp7a*, the mouse gene homologous to *ATP7A*; *Atp7a*^{Mo}, the mottled mouse locus, previously called *Mo*; ATP7B, Cu-transporting ATPase that is deficient in Wilson disease (see MIM 277900); *ATP7B*, gene symbol for the Wilson gene; Atx1, antioxidant 1, Cu chaperone for Ccc2p, the yeast homolog of HAH1; BAEP, brain stem auditory evoked potential; Ccc2p, yeast homolog of ATP7A/ATP7B; CCS, Cu chaperone for superoxide dismutase 1 (MIM 603864); CP, ceruloplasmin, plasma ferroxidase needed for iron mobilization (MIM 117700); CT, computed tomography; CTR1, Cu transporter 1 that is necessary for cellular uptake of copper, also named COPT1 (MIM 603085); CTR2, Cu transporter 2, possibly involved in cellular copper uptake; also named COPT2 (MIM 603088); COX, cytochrome c oxidase (MTCO1: MIM 516030; MTCO2: MIM 516040); COX17, a mitochondrial copper shuttle or Cu chaperone for delivery to mitochondria and cytochrome c oxidase; DBH, dopamine β-hydroxylase (MIM 223360); ddF, dideoxy fingerprinting; DHPG, dihydroxyphenylglycol; DOPA, dihydroxyphenylalanine; L-DOPS, L-threo-3,4-dihydroxyphenylserine; EDS, Ehlers-Danlos syndrome; EEG, electroencephalogram; ERG, electroretinogram; Fre1, ferrireductase 1 in yeast; Fre2, ferrireductase 2 in yeast; GSH, glutamyl-cysteinyl-glycine, also named glutathione; HAH1, human ATX homolog 1, Cu chaperone for incorporation of copper into ATP7A/ATP7B, also named antioxidant protein 1, ATOX1 (MIM 602270); HEPH, hephaestin, membrane-bound ferroxidase involved in iron absorption; LOX, lysyl oxidase (MIM 153455); LTQ, lysine tyrosylquinone, cofactor of lysyl oxidase; MBD, metal-binding domain; MD, Menkes disease (MIM 309400); MR, magnetic resonance; MTCO1, mitochondrial cytochrome c oxidase subunit 1 (MIM 516030); MTCO2, mitochondrial cytochrome c oxidase subunit 2 (MIM 516040); OHS, occipital horn syndrome (MIM 304150); PAM, peptidyl α-amidating enzyme (MIM 170270); TGN, *trans*-Golgi network; TMD, transmembrane domain; SCO1, *Saccharomyces cerevisiae* COX-deficient homolog 1 (MIM 603644); SOD1, cytoplasmic Cu/Zn superoxide dismutase (MIM 147450); SOD2, mitochondrial Mn superoxide dismutase that is located in the matrix (MIM 147460); SOD3, extracellular Cu/Zn superoxide dismutase (MIM 185490); SSCP, single-strand conformation polymorphism; VEP, visual evoked potential.

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- **MD shows progressive deterioration. Administration of copper histidine before brain damage may ameliorate the disease progression and result in less severe neurological symptoms. However, connective tissue signs and symptoms remain prominent.**

INTRODUCTION

Copper is a trace element necessary for the normal activity of numerous metalloenzymes. Lack of copper influences vital metabolic processes in the body and causes characteristic disturbances among which connective tissue abnormalities are prominent. Through its role as a cofactor for lysyl oxidase, copper has a profound influence on the formation of the extracellular matrix.

Two hereditary disorders, Menkes disease (MIM 309400) and the occipital horn syndrome (MIM 304150), mimic the effects of copper deficiency. Since the original description of Menkes disease (MD), numerous reports on patients with similar and dissimilar clinical and radiographic features and biochemical findings have been published, the mildest symptoms representing the occipital horn syndrome (OHS). It took a number of years before it was accepted on clinical and biochemical grounds that the spectrum of severity was due not to genetic heterogeneity of similar phenotypes but to allelic variability. It has now been confirmed that these two disorders are caused by mutations in the same gene, *ATP7A*, differing in severity only in relation to the extent to which copper-dependent processes are affected.

ATP7A codes for a copper pump that is responsible for the extrusion of the metal from most types of cells, and cloning of its gene initiated a new interest in copper metabolism. Since then, significant new knowledge about the cellular handling of this metal has been gained, and we therefore start the discussion of the two diseases by reviewing current knowledge about copper homeostasis. In the following, we use Menkes disease as a generic term and only distinguish between the two forms when it is necessary for comparison of, say, the clinical features.

BIOLOGICAL ROLE OF COPPER

Copper Biochemistry

Copper belongs to the transition group of elements and can exist as Cu(I) or Cu(II). Under physiological conditions, the metal is readily convertible between the two oxidation states. This redox property is used in a number of copper-requiring enzymes that are typically involved in electron transfer reactions, and the metal is crucial for the normal activity of these enzymes [1].

However, this same property also makes copper a very toxic element when present at high levels [2]. Consequently, special molecular mechanisms for handling the metal are required. If the copper-specific complexing molecules become saturated, the free ion concentration will increase and copper will bind to other molecules and catalyze unspecific oxidation. Eventually, copper may cause adverse reactions, as has been documented in Wilson disease (MIM 277900), which is caused by a defect in the homologous gene, *ATP7B*. The Wilson gene product is primarily expressed in the liver, and its main function is in excretion of copper via the bile and delivery of copper to ceruloplasmin. Dysfunction of ATP7B leads to hepatic accumulation of copper and subsequent overflow to other organs, especially the brain, kidney, and cornea. Wilson disease is an autosomal recessive disorder that usually presents in late childhood or adolescence with hepatic cirrhosis and neurological or psychiatric problems and the typical Kayser-Fleischer rings in the cornea [3].

Copper Homeostasis

Whole Body Transport

In the body, the balance between requirement and toxicity of copper is tightly controlled [3–7]. Homeostatic regulation of copper levels in the whole body and individual cells appears to be achieved primarily via an efficient export mechanism combined with detoxification of surplus of the metal.

Total body stores of copper are dependent on a balance between intestinal absorption, distribution to organs and tissues, and biliary excretion. Absorption occurs primarily in the upper small intestine, and gut transfer contains an energy-dependent step (probably the release from the enterocytes by ATP7A). Once in the bloodstream, copper is bound to albumin and amino acids and is then rapidly taken up by tissues, mainly the liver, where it is either stored or secreted. Hepatic release occurs by two different routes, namely secretion to the circulation bound to ceruloplasmin or excretion via bile. Biliary excretion is the principal route for elimination of excess copper, and ATP7B is involved in export from the hepatocytes. Thereby, the liver plays a central role in the regulation of whole-body copper homeostasis. Urinary loss is negligible due to an efficient reabsorption of copper filtered in the glomeruli, but there is minimal enterohepatic recirculation of biliary excreted metal. Copper transport into the brain is also a regulated process but is not yet fully understood [3].

Copper ions remain tightly bound to specific carrier molecules, from absorption in the gut to export via the bile, and are carefully transferred from one carrier to another in blood and extracellular fluid, across cell membranes, and even within cells.

Ceruloplasmin. Ceruloplasmin is a quantitatively important copper-binding protein in the circulation. It binds about 90% of serum copper and has long been regarded as a tissue copper donor. Its role in copper transport has been disputed, and it is no longer regarded as a specific transport protein for copper [8]. However, the role of ceruloplasmin as a transport vehicle has been discussed thoroughly [7], and it still remains plausible that ceruloplasmin participates in whole-body copper homeostasis.

Ceruloplasmin appears primarily to be important for iron mobilization through its role as a ferroxidase (see below). Individuals with an inherited deficiency of ceruloplasmin, aceruloplasminemia (MIM 604290), suffer from specific disturbances of iron metabolism with accumulation of the metal in certain tissues, primarily the brain, whereas copper metabolism appears to be normal [8,9].

Albumin. Another important copper carrier in blood is albumin, which possesses a copper-specific binding site in its amino-terminal portion [10]. It is also a carrier for several other molecules, such as steroids, fatty acids, and thyroid hormone. Only about 5% of the total serum copper content is complexed with albumin, but this form is still thought to constitute the major transport form in blood. Albumin appears, however, not to be a prerequisite for delivery of the metal to tissues and organs because individuals with analbuminemia (MIM 103600) due to a genetic defect do not show symptoms of copper deficiency [11].

Copper bound to albumin is in dynamic equilibrium with copper bound to amino acids, especially histidine. This dissociable pool is in rapid equilibrium with copper

in the tissues and may be regarded as a buffer securing the availability of sufficient copper for various organs and cells and also protecting the organism against copper toxicity. Dogs lack the terminal copper-binding imidazole group of albumin, and this species appears to be highly susceptible to copper toxicosis.

Cellular Transport

Also at the cellular level, uptake, export, and intracellular compartmentalization, as well as the buffering of copper ions, appears to be strictly regulated [6,12–13]. Several of the components participating in copper transport have now been identified, and significant advances have been made toward understanding cellular copper homeostasis. Cells have developed specific mechanisms to ensure a unidirectional flow of copper ions from when they are taken up by the cell until they are pumped out again. In individual cells, copper is taken up across the plasma membrane by specific carriers, and once inside the cell, the metal is distributed to various enzymes and compartments bound to other carriers. Surplus of copper is either neutralized by a specific storage protein or pumped out of the cell (Fig. 1).

Knowledge of how copper is handled at the cellular level has broadened dramatically since the identification of the export mechanism, ATP7A. This protein belongs to a unique family of energy-requiring metal pumps and possesses metal-binding sites that are highly homologous to sites in a yeast protein with a similar function, Ccc2p. The discovery of a strong evolutionary conservation of protein sites used for metal coordination led to the rapid identification of several other specific copper carriers, isolated by functional complementation of yeast strains deficient in specific known transport mechanisms.

Uptake. Uptake of copper across the plasma membrane seems to occur via facilitated transfer using an energy-independent membrane carrier, CTR1 (Fig. 1). CTR1 was identified by functional complementation of a yeast mutant (ctr1) defective in high-affinity copper uptake [14], and a second, homologous membrane protein, CTR2, was identified by database searching. Both proteins contain three potential membrane-spanning regions. In addition, CTR1 contains repeated metal-binding sequences rich in methionine and histidine. CTR2 has a lower abundance of these residues and lacks obvious metal-binding motifs, and it has therefore been proposed to be a low-affinity uptake carrier, but this needs to be demonstrated. Both proteins were expressed in all tissues examined, suggesting a ubiquitous role for the proteins in copper transport in humans [14].

By analogy with yeast, the metal is most likely taken up as Cu(I) and therefore needs to be reduced from the Cu(II) state prior to uptake. Two plasma membrane proteins (Fre1p, Fre2p) with iron and copper reductase activity have been identified in yeast [15]. In human liver, a similar reductase activity has been identified, but the gene remains to be isolated [16]. Copper reductases are probably operating in other cell types as well.

Buffering. On entering the cell, copper is immediately bound to intracellular ligands. The cytoplasm constitutes a reducing environment, and glutathione, a tripeptide thiol (glutamyl-cysteinyl-glycine; GSH) that is found ubiquitously in all living cells, is effective in stabilizing copper ions in the Cu(I) state [17]. Free ionic copper is thereby maintained at very low concentrations, and if the buffering capacity of this system is exceeded, the metal will induce synthesis of metallothionein, to which copper is bound efficiently

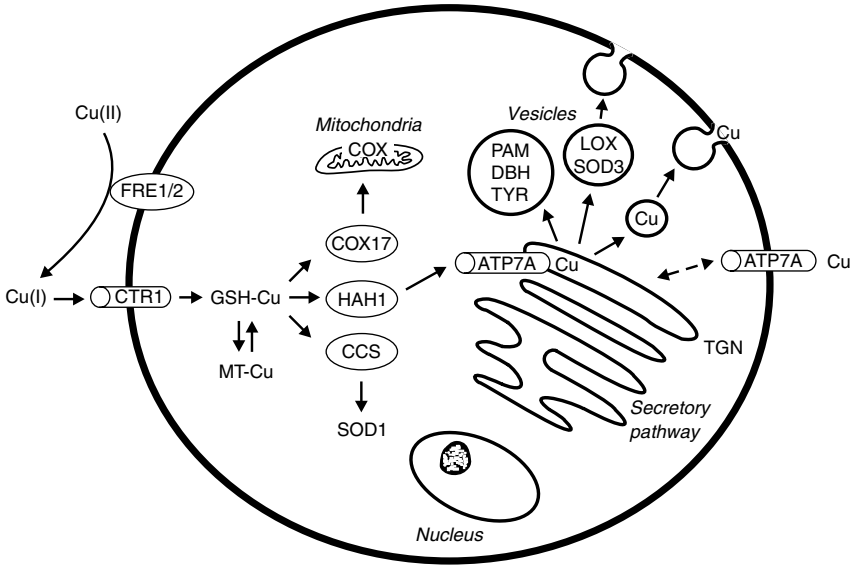

Figure 1. Schematic illustration of cellular copper transport. Analogously to the liver in respect of the whole body, the Golgi network is the organelle controlling cellular copper metabolism. Copper is taken up across the plasma membrane by the copper uptake transporter (CTR1) after it has been reduced from cupric ions to cuprous ions by ferrireductase 1 and 2 (FRE1/2). Within the cell, copper is first attached to glutathione (GSH), and surplus is scavenged by metallothionein (MT). Specific chaperones deliver copper to enzymes and compartments: COX17 is the copper chaperone for cytochrome c oxidase (COX), CCS is the copper chaperone for the cytoplasmic superoxide dismutase (SOD1), and HAH1 is the copper chaperone for ATP7A that delivers copper to peptidyl α-amidating enzyme (PAM), dopamine β-hydroxylase (DBH), tyrosinase (TYR), lysyl oxidase (LOX), and extracellular superoxide dismutase (SOD3). ATP7A is also responsible for copper export from cells. At low copper concentrations, the localization of the protein is at the *trans*-Golgi network (TGN), but at high copper concentrations it is relocated to the plasma membrane.

in a strong thiolate complex and thereby neutralized. Metallothionein therefore scavenges the cell, protecting against toxic accumulations of copper [18]. Metallothionein has also been proposed as a physiological storage protein for copper, but the mechanism by which copper is mobilized from metallothionein is not fully understood. Evidence indicates that glutathione interacts with the thiolate ligands to release the metal [19].

Chaperoning. Because copper cannot exist in its free form, metal delivery to various compartments for incorporation into enzymes also needs to be carrier-mediated. ''Chaperoning'' is now the accepted term for this process, whereby a copper ion is directed and donated to a specific acceptor molecule by a small soluble carrier molecule that binds and escorts the metal to its destination (Fig. 1). Copper-requiring enzymes are thereby loaded without other cellular components being damaged.

To date, three genes coding for cytosolic copper chaperones have been identified by functional complementation cloning, and each protein delivers copper to a specific target molecule. The first cytoplasmic copper carrier to be identified was HAH1 (human ATX homolog; also named ATOX1), which delivers copper ions to the Golgi complex. By analogy with yeast, it was suggested that HAH1 binds and donates cytosolic copper to ATP7A and ATP7B in the *trans*-Golgi network [20–23]. Another protein that delivers copper intracellularly is CCS, the copper chaperone for superoxide dismutase (SOD1) [24]. Direct chaperoning of copper from CCS to its target enzyme, SOD1 (cytoplasmic superoxide dismutase), has been demonstrated [25]. A third chaperone, COX17, delivers copper to the periplasmic space of the mitochondrion (Fig. 1) [26–28]. Before the copper can be incorporated into the two copper-containing subunits of cytochrome c oxidase, a second mitochondrial-specific factor SCO1 (*Saccharomyces cerevisiae* COX-deficient homolog 1) seems to be required for the transfer of copper across the inner mitochondrial membrane into the matrix [29,30]. SCO1 contains a mitochondrial leader peptide, a putative transmembrane domain, and a copper-containing domain.

These chaperones are small cytosolic proteins containing one metal-binding site. The chaperones CCS and HAH1 share a copper-binding site (MTCXXC), that is very similar to those found in ATP7A and ATP7B and probably has a similar affinity for copper. The motif of COX17 is slightly different (CCXC) but also implies a strong metal-binding capacity [31]. Studies on the homologous yeast proteins indicate that copper binds as Cu(I) to these chaperones [26,31].

Besides the copper-binding motif, other unique sequences secure an efficient metal delivery from the chaperone to the target enzyme. Thus, copper-mediated protein-protein interactions have been demonstrated for Atx1 (antioxidant 1), the yeast homolog of HAH1 (human ATX homolog 1) [21]. Sequence similarities between CCS and SOD1 have been demonstrated [32], suggesting that copper exchange occurs through specific interaction between the donor and acceptor molecules. These features ensure a unidirectional flow of copper from one protein to another, from weaker to stronger binding, until the metal is pumped out of the cell. Copper is pumped out as Cu(I), but before the metal is bound as histidine or albumin complexes for return to the liver, it needs to be oxidized to Cu(II). Nothing is known about enzymes or proteins involved in this step.

It is conceivable that additional copper-specific chaperones exist in the cell. Chaperone-like mechanisms are responsible for activation of the copper uptake and export genes in *Enterococcus hirae*. This regulation occurs through a copper-sensitive gene repressor, and direct chaperoning of copper to the repressor has been demonstrated [33].

Export. In the individual cell, copper homeostasis is controlled by excretion of the metal, and ATP7A plays a key role in cellular copper metabolism by controlling the export of copper from nonhepatic tissues. In the liver, this function is taken over by the homologous ATP7B, which is defective in Wilson disease.

ATP7A pumps copper from the cytoplasm into the secretory pathway for incorporation into secreted enzymes or export. Several lines of evidence suggest that it is located in the *trans*-Golgi network [34–36], the same compartment in which sorting between secretory and constitutive proteins takes place. It is conceivable that ATP7A is responsible for the copper loading of most, if not all, secreted copper enzymes.

When the cell is exposed to high copper levels, ATP7A is reversibly translocated to the plasma membrane, indicating that it also plays a role in the exocytosis of copper. This occurs either by the metal being pumped into secretory vesicles, which are subsequently sorted to the plasma membrane for release of their contents, or by direct extrusion by ATP7A located at the plasma membrane. ATP7A cycles between the *trans*-Golgi network and the plasma membrane, depending on the concentration of copper within the cell [34,37]. This copper-dependent shuttling between the plasma membrane and the Golgi complex seems to be a physiologically important mechanism for the cellular extrusion of surplus copper. The gene sequences necessary for localization to the Golgi complex and for recycling between the two membranes are beginning to be defined [12,38–42]. Gene regulation of the export of excess copper is, however, not well-understood. Although copper regulates its efflux, this does not occur at the transcriptional level since ATP7A mRNA is not responsive to copper [43]. In yeast, the expression of the homologous gene (Ccc2p) is controlled by iron and not copper [44].

Apparently, the membranes of other cellular organelles also use energy-dependent transport mechanisms for translocating heavy metals. Thus, copper translocation across the inner mitochondrial membrane and incorporation into the metallocenter of COX (cytochrome c oxidase) also seems to require a specific membrane carrier [29]. Used copper also needs to be exported again from this organelle, and the homologous protein ATP7B may be implicated in this step [45]. In the lysosomal membrane, an energy-dependent transporter that binds the heavy metals silver, copper, and cadmium has been characterized [46]. This protein was proposed to be a P-type ATPase, but the gene remains to be isolated. It is probable that other organelles such as peroxisomes may also possess a specific copper pump.

THE CLINICAL SPECTRUM OF MENKES DISEASE AND THE OCCIPITAL HORN SYNDROME

Menkes disease (MD) is a multisystemic disorder resulting from a disturbed copper metabolism. The phenotype of Menkes disease includes a wide range of symptoms in many body systems, all of which can be explained by the disturbed copper metabolism, but there is considerable variability in the severity of clinical expression. In the most common, classic, severe form, symptoms may appear during the neonatal period, but milder forms, which first present with symptoms much later, have also been described. Today, it is clear that the different phenotypes form a clinical continuum from

severe to mild disease with variable somatic and mental problems. In this chapter, we discuss the clinical features of the disease under four categories: classical Menkes disease, the occipital horn syndrome as the mildest form, intermediate phenotypes, and treated patients who show a considerable divergence from the classical severe form.

Classical Menkes Disease

Clinical Characteristics

In 1962, Menkes and his co-workers reported on five male members of a family who were similarly affected with developmental regression starting from the age of four months. Clinical symptoms comprised peculiar facial features and unusual hair, and there were cerebral and cerebellar degenerative symptoms, including hypotonia, spasticity, and convulsions, that eventually led to death by ages between 7 months and 3 years. The children were related through female members in the family, suggesting an X-linked recessive inheritance [47]. This initial description has been followed by numerous additional observations, which, over the past more than 35 years, have defined the clinical phenotype of MD [5,48–52].

The early clinical features of classical MD are quite uniform, whereas the long-term course seems to be more variable. The evidence indicates that there is a remarkable clinical phenotypic spectrum from a severe, early lethal form to milder forms of MD, the mildest form being classified separately as the occipital horn syndrome [5,48,49,53].

During pregnancy, there are no predictive symptoms, and the results of current procedures for maternal health care, including maternal serum studies for α-fetoprotein, and repeated routine fetal ultrasound examinations are normal. There may be premature labor and delivery, but most male patients are born at term with appropriate birth measurements. Postnatal cephalohematomas and spontaneous fractures have occasionally been observed. In the early neonatal period, patients may present with hypothermia, hypoglycemia, and prolonged jaundice, and feeding problems, pectus excavatum, and umbilical and inguinal hernias have been reported [50]. However, the first sign may be unusual sparse and lusterless scalp hair that becomes tangled on the top of the head at 1–2 months of age. Initial psychomotor development is usually unremarkable, with normal babbling and smiling up to about 2–4 months of age, whereafter hypotonia, failure to thrive, poor eating, vomiting, diarrhea, and gradual loss of psychomotor milestones become evident. Most patients will also develop seizures that are difficult to control. These problems combined with characteristic physical findings, such as pudgy cheeks, lack of facial expressions, and lax skin, usually bring the child to pediatric attention early (Figs. 2 and 3). Hair and eyebrows become steely, hypopigmented, and breakable, causing a stubble on the occipital and temporal areas (Fig. 4). Weight gain, growth in length and head circumference, and psychomotor development gradually come to a complete halt. There is a marked truncal hypotonia, inability to lift the head, and spasticity of the extremities, and gradual loss of reactivity. Recurrent urinary tract infections are associated with bladder diverticula, which occasionally may cause spontaneous bladder ruptures, ureteropelvic obstruction, and hydronephrosis [54,55]. The occasional intracranial hemorrhage and massive bleeding in other sites are associated with evolving intimal thickening and aneurysms of the arteries. Optic atrophy, abnormal ERG (electroretinogram), and loss of VEP (visual evoked potential) and BAEP (brain stem auditory evoked potential) have been observed, suggesting progressive deterioration of vision and hearing [56,57]. Initially, microscopic examination of hair may be normal, but later typical changes and loss of pigmentation become evident (Fig. 4). The diagnosis of MD is usually suspected by the age of 3 to 6 months and can be confirmed by laboratory investigations. In the classical form, the clinical course of MD is a gradual neurodegenerative deterioration with subdural hematomas, and affected children usually die of neurological and respiratory failure by the age of 3 years. However, careful medical care and possibly copper therapy may extend life up to 13 years or even more [58–60].

Radiographic Observations

Radiographs at the time of suggestive clinical signs and symptoms show a number of specific changes that are reminiscent of acquired copper deficiency and scurvy. These abnormalities include a generalized osteoporosis, metaphyseal flaring and spurs in the tubular bones (Fig. 5), multiple rib fractures, and Wormian bones in the skull [61,62]. Cystourethrography and ultrasound may demonstrate bladder diverticula and, occasionally, ureter diverticula. By arteriography there may be widespread elongation, tortuosity, and variation in caliber of the abdominal and cerebral arteries (Fig. 6). Head CT (computed tomography) and MR (magnetic resonance) imaging reveal progressive patchy areas of white matter abnormalities, suggesting disordered myelinization and diffuse cerebral

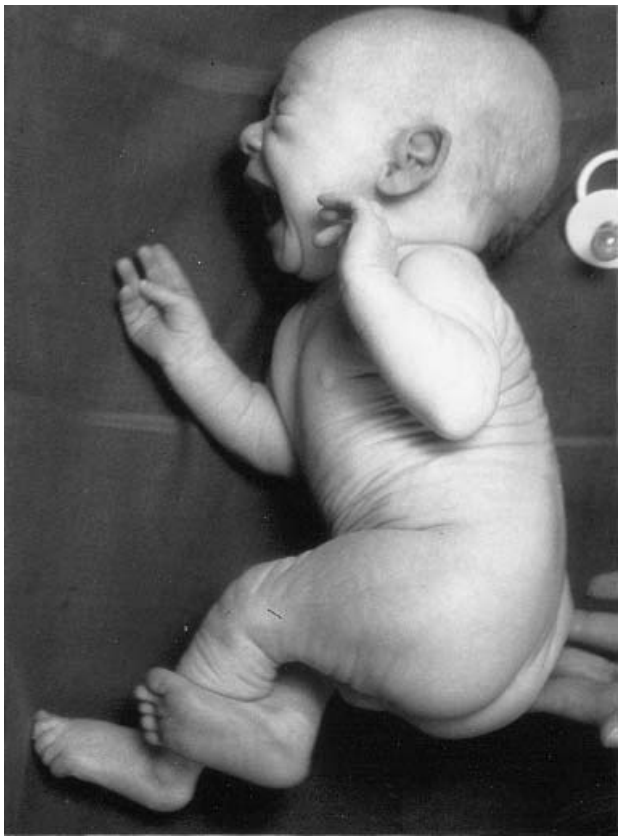

Figure 2. Clinical appearance at age 3 weeks of a patient with classical Menkes disease. Note the lax skin.

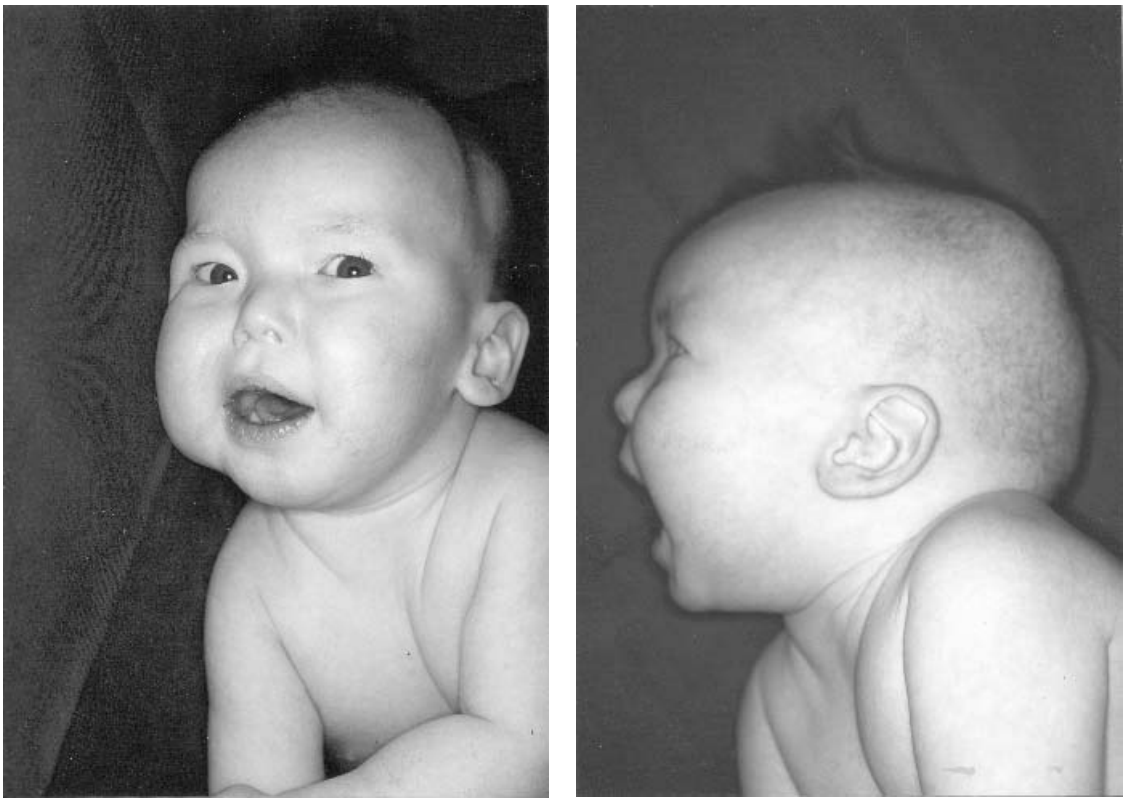

Figure 3. An 8-month-old patient with classical Menkes disease. Close-up of the head shows pudgy cheeks (**a**) and short hair that is rubbed off at the back of the head (**b**). Note the pale skin. (Courtesy of L. Skov, Glostrup University Hospital, DK-2600 Glostrup, Denmark.)

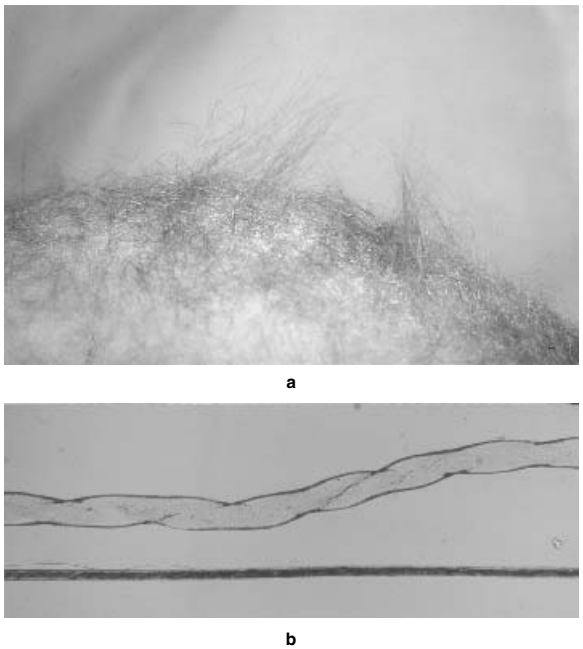

Figure 4. Hair in a patient with classical Menkes disease. **a:** Stubby appearance of depigmented scalp hair. **b:** Hair microscopy (×100) of twisted hair shaft (pili torti) (**above**) and a normal hair (**below**).

atrophy with dilatations of the ventricles. Cerebellar inferior vermian hypoplasia has also been described [57].

Occipital Horn Syndrome

Clinical Characteristics

The phenotypic manifestations of the occipital horn syndrome (OHS) [5,49,63,64] were first described by Lazoff et al. [65] in an 11-year-old boy and his two maternal uncles. The child presented with diarrhea, recurrent urinary tract infections, bladder diverticula, inguinal hernia, and peculiar occipital horns. All three had educational problems but were not mentally retarded. One year later, a new form of X-linked cutis laxa with bladder diverticula and skeletal abnormalities was reported [66]. The phenotype was associated with a deficiency of lysyl oxidase that was later inferred to result from disordered copper metabolism [67]. The condition was later renamed Ehlers-Danlos (EDS) type IX [63], but because of the skin laxity it was withdrawn from the EDS nosology [53] and the accepted term is now the occipital horn syndrome. The proper term should be mild Menkes disease with occipital horns.

Since the first description, more than 20 OHS case reports have been published with the essential observation of the radiographic occipital horns [49,64]. The diagnostic criteria differentiating classical MD, mild MD, variant MD, and OHS are artificial. The principal clinical features in the mild end of the spectrum, OHS, are related to the connective tissues and the skeleton and the extended prognosis for life.

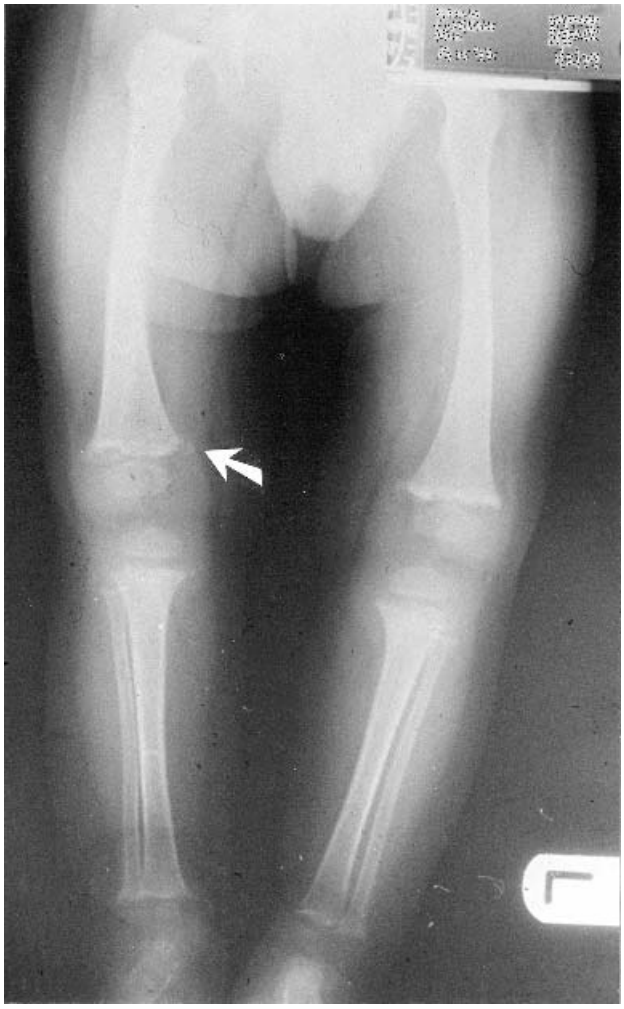

Figure 5. Characteristic bone changes in classical Menkes disease. Generalized osteopenia with metaphyseal irregularities at both distal femoral and proximal tibial metaphyses and a corner spur on the medial aspect of the right femur (arrow). (Courtesy of D. C. Davidson, Alder Hey Children's Hospital, Liverpool L12 2AP, England.)

Pregnancy and delivery of an OHS child are usually normal. The skin may appear wrinkled and lax at birth, and the child may have umbilical and inguinal herniae. Within days, hypothermia, jaundice, hypotonia, and feeding problems may transiently ensue. Over the years, the clinical problems gradually become obvious, and the first signs that bring the child to medical attention may be intractable diarrhea and recurrent urinary tract infections. Cystography usually reveals an atonic bladder with diverticula suggesting obstructive urethropathy (Fig. 7). Motor development is delayed due to muscular hypotonia and is associated with unusual clumsiness. Prepubertal growth in height is usually normal, while mild disproportion with a long trunk and the development of flat feet, knock-knees, thoracolumbar kyphosis or scoliosis, and pectus deformity with narrow chest and shoulders are common (Fig. 8). The joints are hypermobile, and operations may be required to stabilize the ankle joints. Gradually, flexion and supination-pronation limitations at the elbows ensue due to hyperostosis of the proximal ulna and radius. There is also a tendency toward dislocation of the elbows (Figs. 9b and 9c). The face becomes narrow, often with a high forehead, downslanting palpebral fissures, a hooked nose and long philtrum, and dental alveolar hyperplasia. Skin laxity is variable and may increase with age, resulting in droopy wrinkles around the trunk (Fig. 8a). However, there is no particular bruisability or fragility of the skin, and surgical incisions either heal normally or may form atrophic scars. Hair is not usually reported as abnormal. However, some patients may have lusterless and unusually coarse hair. Recurrence of the inguinal hernia is common. Vascular anomalies, such as varicose and tortuous veins, are common, and arterial aneurysms have also been described [68]. Gradually, full extension of the head becomes limited. A particular problem is syncopic orthostatic episodes resembling convulsions. Intellectual development is normal or mildly retarded. Pubertal development is normal.

Despite the many clinical problems, the diagnosis of OHS is usually arrived at only around 5 to 10 years of age. The clinical course is characterized by chronic diarrhea, recurrent urinary tract infections with bladder diverticula and occasional spontaneous bladder ruptures, orthostatic syncopes, and joint instabilities in the inferior extremities and limitations at the elbows. Severe progressive thoracolumbar kyphosis, spontaneous retinal ablation, and mitral valve insufficiency, all requiring operative procedures, have also been encountered (I. Kaitila, personal communication). Based on family histories of case descriptions, life expectancy in OHS is variable. There are adult patients aged up to 50 years who have maternal male relatives dying in early childhood, usually of infections. One Finnish patient died at 32 years of age of fulminant peritonitis caused by rupture of an intestinal diverticulum [69], and his brother later died at 26 years of age from subdural hemorrhage (I. Kaitila, personal communication).

Radiographic Findings

There is little information on skeletal radiographic abnormalities in infancy and early childhood. In the skull, there may be Wormian bones, which disappear within a few years. An atonic, often diverticulous bladder is observed in investigations into recurrent urinary tract infections in early childhood by cystography. In the skeleton, osteoporosis is found in later childhood. There are coxa valga and horizontal, flattened acetabula. Chest radiographs show a narrow thorax, somewhat thickened ribs, and short, thick clavicles with hammer-shaped distal ends (Fig. 9a). There is characteristic progressive deforming hyperostosis at the proximal ends of the bones in the forearm. This results in the appearance of dislocation of the proximal radial head. Kyphoscoliosis is common, but there is no consensus as to whether vertebrae are normal or abnormal. The most peculiar and characteristic radiographic finding is the symmetric parasagittal bony projections extending down the extensor musculature of the neck (i.e., occipital horns) (Fig. 10). The mastoid processes similarly extend along the sternocleidomastoid musculature. The horns may be found around 1–2 years of age if looked for [70], but are usually detected only around 5–10 years of age and continue to grow up to early adulthood.

Intermediate Phenotypes

Clinical Characteristics

In several case reports, the description of the symptoms and signs indicates a later onset and milder problems than in typical classical Menkes patients, and such an

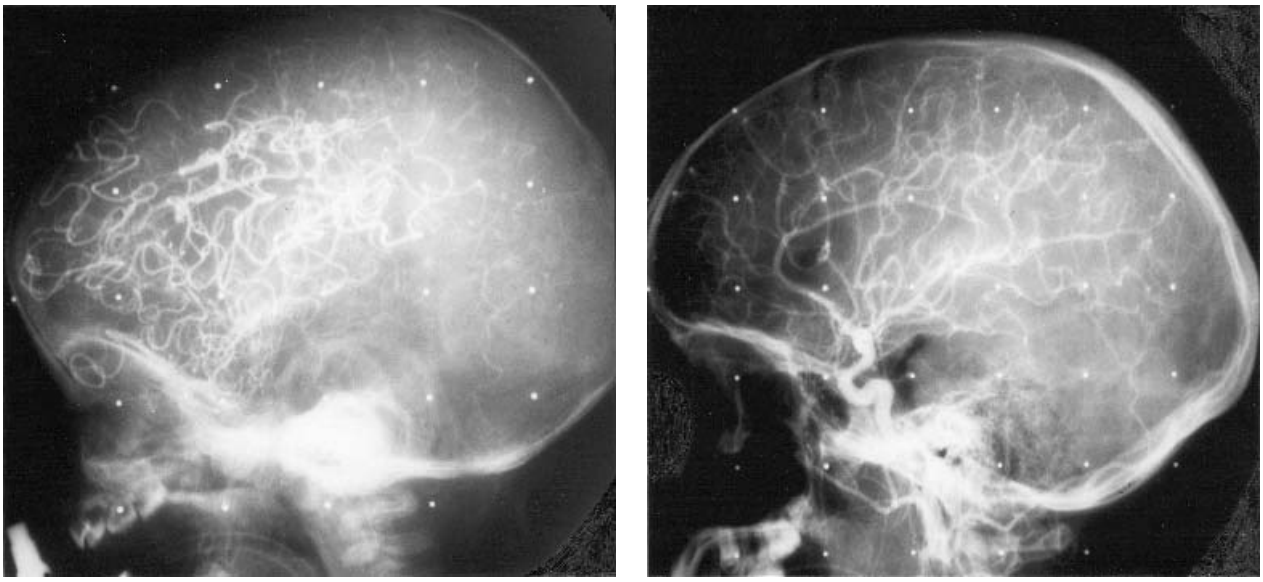

Figure 6. Vascular abnormalities in Menkes disease **a:** Carotid angiography in a classical MD patient showing hairpin bends of the artery in the neck and elongated, twisted, and numerous intracranial branches. **b:** Normal carotid arterial system in a normal child, shown for comparison. (Courtesy of S. Westermark, Glostrup Hospital, DK-2600 Glostrup, Denmark.)

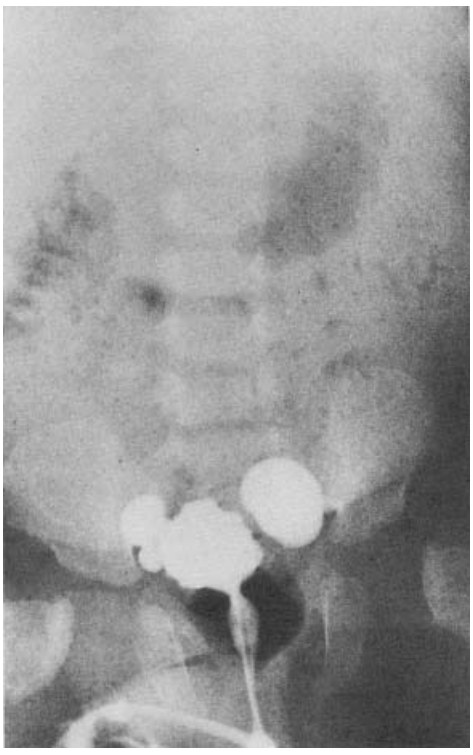

Figure 7. Bladder voiding cystogram demonstrating multiple bladder diverticula in a patient with OHS. (Courtesy of E. Gautier, Centre Hôspitalier Universitaire Vaudois, Lausanne, Switzerland.)

occurrence may result in delayed diagnosis of the condition. In several of these cases, typical hair changes led to the suspicion of a clinical variant of MD [49,71–76], and occipital horns have also been the diagnostic pointer in some cases [74,77]. Initial symptoms may include ataxia, mild mental retardation [49,71,72], and extrapyramidal movement disorder [78,79]. Intellectual capacity varies from severe retardation, although less striking than that in classical MD [74–76,78], to only mild mental deficit [49,73]. One of the mildly affected patients later developed occipital horns and has therefore been reclassified as having OHS [80].

In addition, a number of long-surviving though severely affected Menkes patients have also been reported [58–60]. There seems to be a consensus that modern clinical patient care *per se* might alleviate the clinical problems and extend life span, independent of medical treatment (i.e., copper therapy).

Because OHS and classical MD share many clinical features, reduced concentration of copper, and low lysyl oxidase activity, allelism of the two conditions was suggested quite early [81]. Mutation analysis of *ATP7A* in patients with classical MD, OHS, and intermediate phenotypes has definitively proven this to be the case, thus confirming the phenotypic diversity in the clinical spectrum of Menkes disease.

Radiographic Findings

There are limited detailed follow-up descriptions of radiographic findings in the variants of Menkes disease. Bone x-rays were reported to be unremarkable at 10 years of age in the mild MD patient reported by David Danks [49]. The American patient originally classified as mildly affected was later reported to have developed changes specific for the occipital horn syndrome [80]. One family with a variant form of MD showed a number of radiographic features very similar to those described in the occipital horn syndrome, and the findings have been thoroughly documented [74].

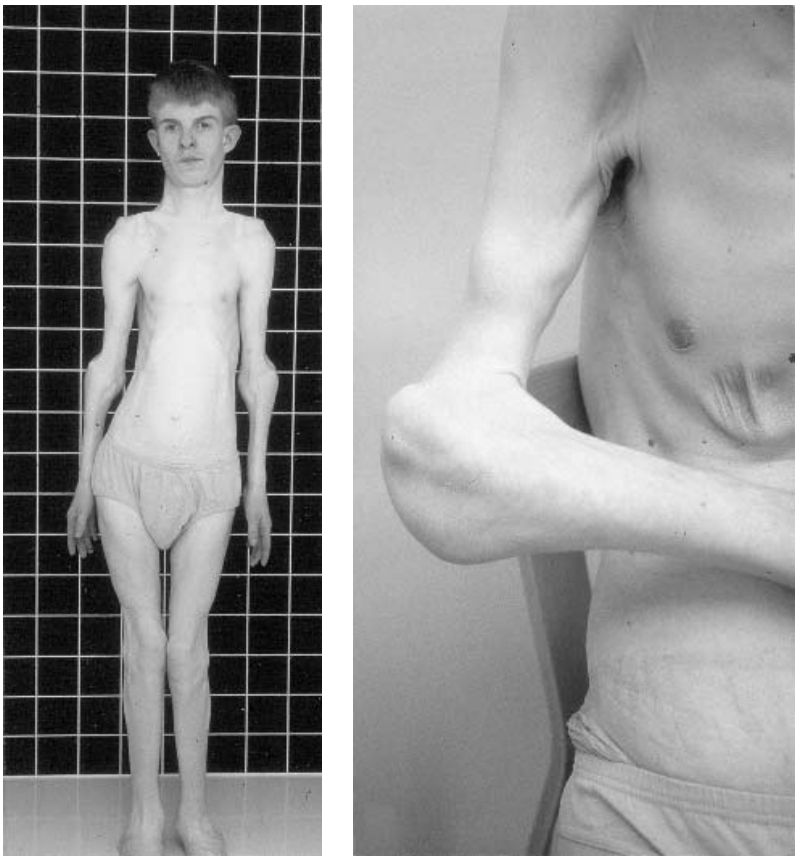

Figure 8. Patient with the occipital horn syndrome. Clinical appearance at age 23 years. He has a narrow chest and knock-knee as well as deformity of the elbow (**a**). Close-up of the elbow and the chest demonstrates loose skin and an atrophic scar on the trunk (**b**). (Courtesy of I. Kaitila, Helsinki University Hospital, SF-00290 Helsinki, Finland.)

Treated Patients

Copper therapy initiated after the development of brain abnormalities will not change the clinical course significantly, although survival may be prolonged [60]. If initiated early, copper therapy may, however, modify disease progression substantially. Four classical MD patients have received long-term parenteral treatment with complexed copper, and it is now possible to predict the phenotypic expression after successful copper therapy. All four treated patients have exhibited a clearly milder clinical course [82]. They have limited brain involvement with near-normal intellectual development, no seizures, and mild to moderate ataxia. Some problems related to autonomic failure, such as postural hypotension and chronic diarrhea, are, however, of concern in these well-treated patients. They have all developed skeletal dysplasias similar to the OHS patients. Poor wound healing with atrophic, stretched scars has also been described [83].

PATHOLOGY

The pathological changes observed in Menkes disease (MD) are diverse, reflecting the multisystemic involvement, and connective tissue and central nervous system (CNS) disturbances are the most prominent. In classical MD, due to the severe neurological involvement, the main interest has been in pathological changes in the brain. In OHS, the neurological involvement is milder, making the connective tissue manifestations more conspicuous and the main focus of investigation, while CNS changes have received limited attention. Below we describe the connective tissue changes in detail and summarize CNS changes, referring to reviews of this issue.

Connective Tissue

All patients exhibit connective tissue symptoms to varying degrees as a metabolic consequence of the restricted availability of copper for synthesis of the extracellular matrix. The fiber-forming proteins, elastin and collagen, are secreted as soluble monomers, which are organized into polymers and stabilized by intermolecular cross-linking. The cross-linking process requires the copper-dependent enzyme lysyl oxidase (see below and also Chapter 2, this volume). Consequently, a compromised copper metabolism will interfere with normal collagen and elastin fibrillogenesis and affect the stability of these two proteins. In humans, elastogenesis seems to be particularly sensitive to disturbance [84], and marked changes in the elastic fibers are evident in Menkes disease. Collagen synthesis seems to be affected to a lesser extent, although collagen fibril diameter can be variable, and collagen fibrils are often more prominent, which probably represents a repair response. Microfibrillar structures remain unaffected because their biosynthesis is not copper-dependent. In the light of this

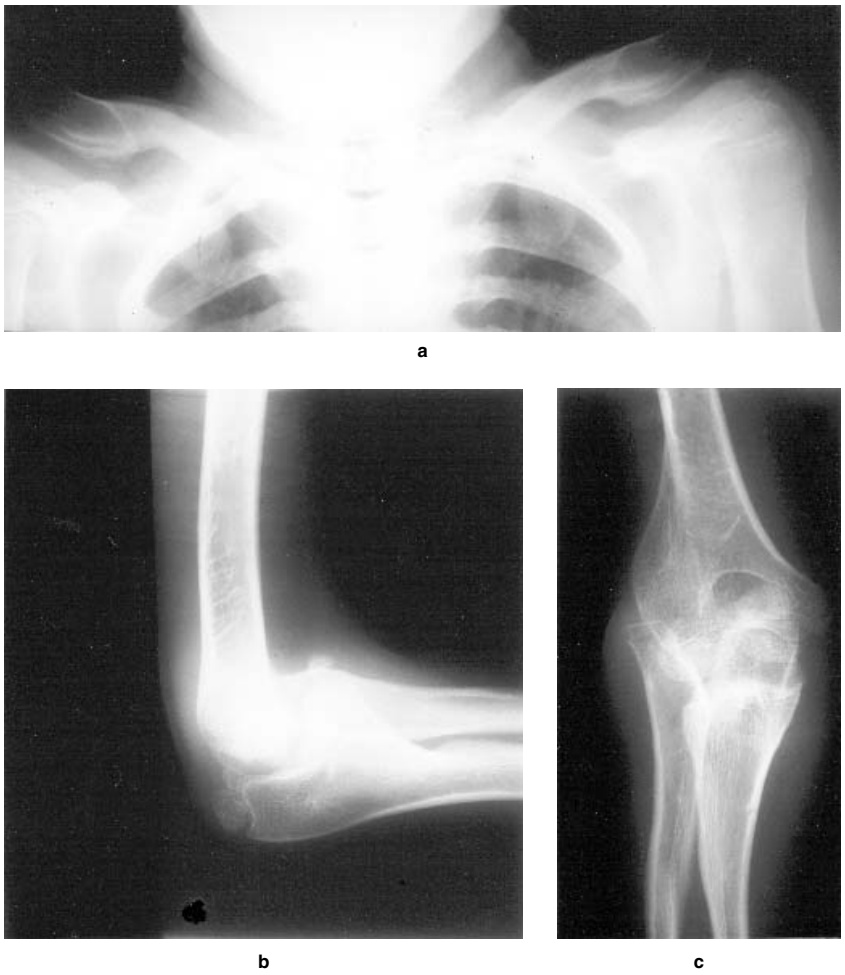

Figure 9. Radiograph of the upper chest at age nine years (same patient as in Fig. 8) showing the thick and short clavicles with hammer-shaped ends. The humerus and the scapula also appear dysmorphic (**a**). Radiographs of the elbow show abnormal-shaped joints, dislocation of the head of the radius, and metaphyseal spurring (**b**,**c**). (Courtesy of I. Kaitila, Helsinki University Hospital, SF-00290 Helsinki, Finland.)

knowledge, it is obvious that blood vessels, skin, bones, and lungs will display profound pathological changes, which are discussed below.

Blood Vessels

In classical MD, profound changes have been documented in the elastic-walled arteries, both systemically and within the brain [49]. Major arteries are elongated and dilated, leading to rupture and hemorrhage and subintimal thickening with partial occlusion. The arteries are tortuous, with an irregular lumen and a frayed and split intimal lining. The aorta and larger arteries may be grossly tortuous, with multiple areas of localized narrowing, while smaller vessels that contain more smooth muscle in their walls may display this feature to a lesser extent [51]. Histological cross sections of the arteries show intimal thickening and fragmentation and reduplication of the internal elastic lamina (Fig. 11). In severely affected arteries, fragmentation of elastic tissue is seen throughout the media, extending into a ''heaped-up'' intima that can completely occlude the lumen. Major arteries may show intimal fibromuscular proliferation [85].

Ultrastructural studies show that the elastin that is present is electron-dense and surrounded by a prominent sheath of microfibrils. Collagen is much less disturbed and shows a proliferation, apparently as a healing response. The cells present in the arterial wall show accumulations of metachromatic material thought to be proteoglycans [49,86] that play a role in the assembly of the fibrous matrix proteins. Arterial disease is less severe in OHS but can be observed. Degeneration of elastic fibers of the vessel walls has also been described in a moderately affected case [77].

Skin

The skin also shows specific alterations of elastic tissue [87]. The elastin fibers may be granular and fragmented in the lower dermis, while a normal microfibrillar structure is preserved. The diminished formation of insoluble elastin may give an empty impression that has been described as a ''moth-eaten'' appearance of the fibers. An overactive proteolytic degradation of the improperly organized elastic fibers may explain the histological appearance of the elastic tissues. Collagen fibers are less affected. They may be widely spaced and usually have an irregular thickness and contour. Histological changes in the skin, with a decreased number of elastic fibers in addition to degeneration and splitting of elastic fibers in the dermis,

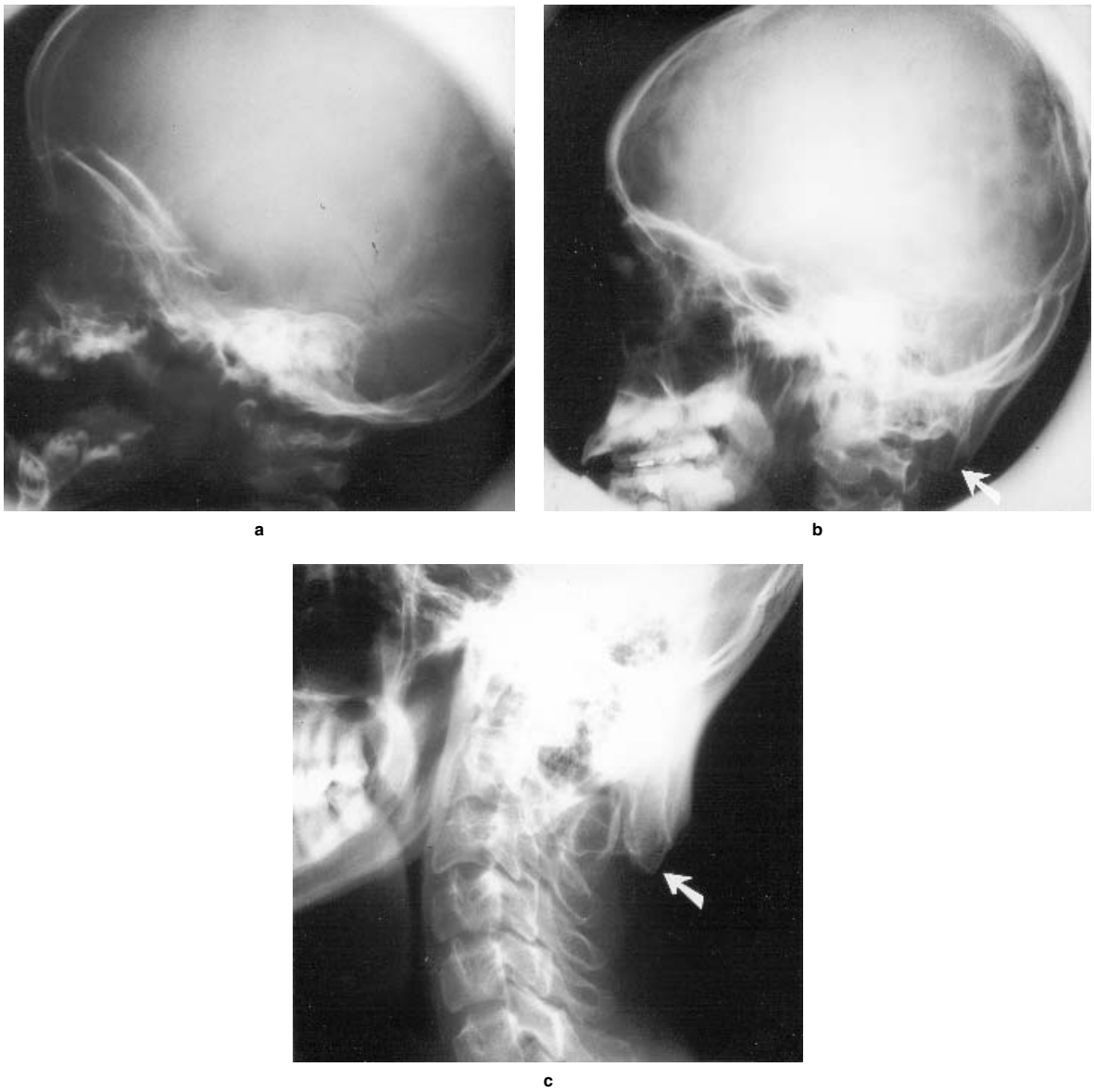

Figure 10. Lateral skull radiographs of the same patient as in Figure 8, demonstrating occipital horns. The skull at age one year and four months shows no signs of occipital exostoses (**a**). The skull at nine years of age demonstrates typical occipital exostoses (arrow) (**b**). At age 23 years, the occipital exostoses become more prominent (arrow) (**c**). (Courtesy of I. Kaitila, Helsinki University Hospital, SF-00290 Helsinki, Finland.)

have also been demonstrated in a more mildly affected case [77]. In OHS, hypertrophic collagen fibers with dense fiber bundles and excessive diffuse matrix have been observed [67]. The cross-sectional appearance of the fibers has been described as "collagen flowers" because of their irregular contours [88]. In contrast, elastin fibers appear normal.

Lung

In MD, elastin in the pulmonary alveoli may also be affected, leading to emphysema [89]. The lung contains abundant elastin in the walls of alveoli, alveolar ducts, bronchioles, and bronchi, as well as in lung vessels. It is thus reasonable to expect that any quantitative or qualitative abnormality of elastin would result in emphysema.

Bones

Skeletal changes are prevalent and may be observed in both severe and milder cases. Normal cross-linking of collagen seems to play an important role in the proper mineralization of bone tissue [90], and poor cross-linking may explain the numerous skeletal changes, including the occurrence of osteoporosis. Polarized light microscopy of bone showed a "woven" pattern rather than the normal lamellar rib pattern [51]. Focal hyperostosis observed at sites of tendinous and ligamentous insertions into bones in milder Menkes cases may represent traction phenomena secondary to abnormal bone collagen [91]. This is manifested as occipital horns in OHS patients, which may also be observed in classical MD patients who have received long-term copper treatment.

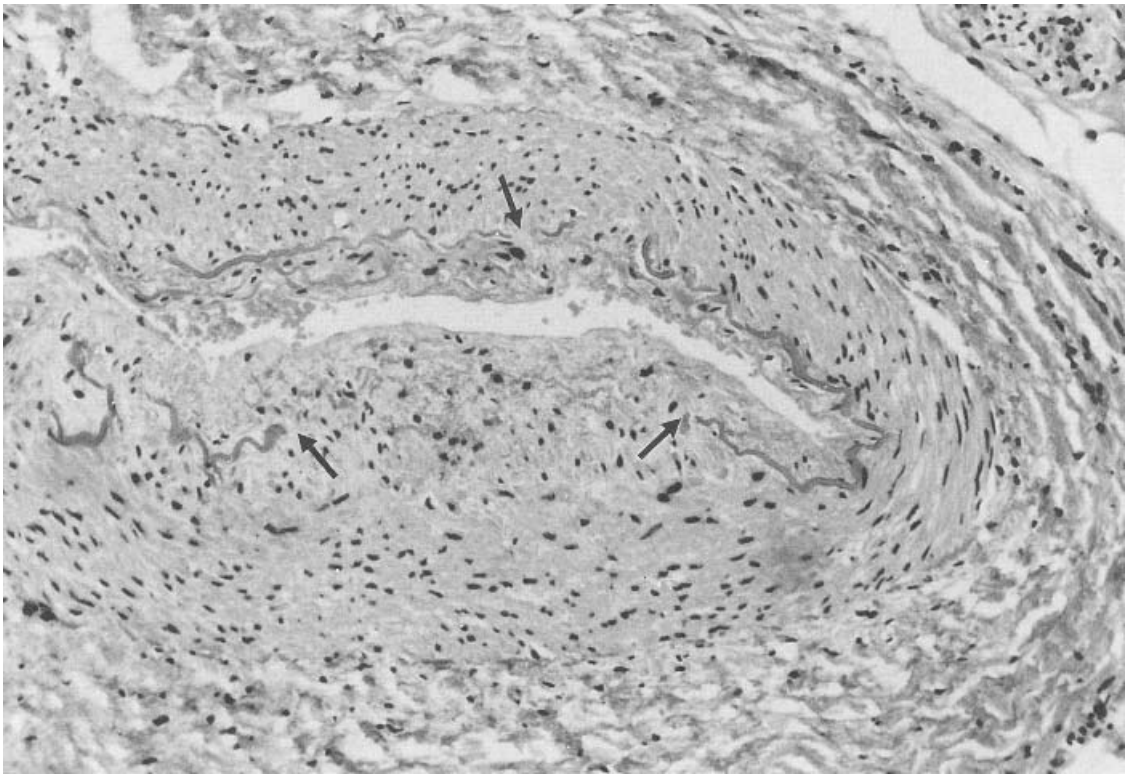

Figure 11. Disruption of internal elastic lamina of a large artery (arrowed), with proliferation of bundles of poorly organized elastic fibers and intimal thickening. (Courtesy of D. Danks, Royal Children's Hospital, Melbourne, Victoria 3052, Australia.)

Hair

Pathognomonic hair changes are observed in classical MD. The hair is typically hypopigmented, and its color may be almost white or grayish [92]. A decreased melanin content of hypopigmented hair has been demonstrated [93]. Light microscopy shows individual hairs that are twisted about their own axes (pili torti), which may show fraying and splitting at the ends (trichorrhexis nodosa) and show varying diameters of the shafts (monilethrix). There seems to be a gross reduction in number of disulfide bonds and an increase in free sulfhydryl groups in the hair [94]. Some more mildly affected cases may also show hair changes, but they are usually less pronounced and the color is darker.

Central Nervous System

Central nervous system pathology observed in classical MD has been reviewed extensively in the medical literature [48,49] and is only summarized in this chapter. Pathological alterations within the CNS are mainly confined to the cerebral hemispheres and the cerebellum. The brain shows evidence of a progressive and extensive degeneration of gray matter with resultant neuronal loss, gliosis, and secondary white-matter degeneration. A generalized atrophy and secondary subdural fluid collections accompany the neuronal loss. Areas of infarction secondary to vascular ischemia are also common. At the microscopic level, severe neuronal loss is evident, with replacement by proliferating astrocytic glial cells and macrophages. The white matter is usually markedly demyelinated and shows evidence of a glial reaction. Severe demyelination in the cerebellum, midbrain, pons, and medulla is frequently observed. Cerebellar sections show a substantially reduced number of Purkinje cells, which are irregularly distributed, some of them deep in the granular layer. Their shape is highly pathological, with abnormal dendritic arborization (weeping willow) and perisomatic processes (somal sprouts), and focal axonal swellings (torpedoes) are also observed.

More mildly affected cases may suffer from ataxia, which is likely caused by cerebellar dysfunction, because this feature persisted in a well-treated MD patient despite administration of L-DOPS (L-threo-3,4-dihydroxyphenylserine) to circumvent the metabolic block in noradrenaline biosynthesis [82]. This may be due to poor development of the adrenergic innervation secondary to low transmitter levels. Investigation of Purkinje cells in milder forms of the disease will therefore be of great interest.

Mitochondrial disorganization and accumulation of glycogen has been observed in brain tissue and retina, as well as in skeletal muscle [95]. Subsarcolemmal clustering of mitochondria (''ragged-red'' fibers), which is a specific sign of mitochondrial dysfunction, has also been reported [96].

Tissue Copper

Abnormal cellular sequestration of copper leading to organ-specific accumulation and depletion is a characteristic feature of classical MD, and maldistribution is already evident at the fetal stage. High placental levels indicate a compromised maternal-fetal transfer of copper, but the supply is sufficient to allow the accumulation of abnormally high levels in most nonhepatic tissues. Copper storage in the placentae of affected males is of diagnostic value. In contrast to the situation in postnatal life, fetal brain copper

levels are not significantly reduced, indicating that poor brain function is primarily the result of copper deprivation after birth. However, the fetal hepatic stores fail to build up, indicating a disturbance in copper availability already before birth [1,48].

In affected children, accumulation of copper in nonhepatic tissues, except the brain, is well-documented, and the levels are especially high in the intestine, kidneys, muscles, and pancreas. This accumulation is augmented further by copper therapy, but brain levels still remain subnormal. Hepatic copper stores are low in untreated patients, but copper injections can restore the levels to normal [1].

Tissue copper stores also appear to be elevated in the milder forms of the disorder, but tissue levels have been reported for only one OHS patient [69]. When compared with age-matched controls, the overall picture is very similar to that in classical MD patients. Increased storage of copper is documented in most tissues, except for the liver and brain, where low levels are found. The spleen level appears to be normal.

As a result of the basic disturbance in extrusion of copper from cells, nearly all tissues except liver and brain will accumulate copper to abnormal levels. The transport disturbance also affects the intestinal mucosa, leading to copper malabsorption. Nevertheless, the absorption of copper is sufficient to build up large copper stores in the bodies of affected males. At the whole-body level, the problem associated with dysfunction of ATP7A is a maldistribution of copper between organs combined with abnormal compartmentalization within cells. This makes the surplus copper unavailable for incorporation into several enzymes, resulting in a functional copper deficiency rather than a gross deficiency in the body. The low brain levels indicate a disturbance in copper uptake into the central nervous system that will probably aggravate the severity of the functional copper deficiency in this organ. In the liver, the role of ATP7A is taken over by the Wilson disease homolog, ATP7B, and no intracellular copper disturbance is present, but sequestration of copper elsewhere results in depletion of the metal in this organ. The low ceruloplasmin activity in plasma underscores that MD babies are indeed copper-deficient despite the increased stores in extrahepatic tissues.

PHYSIOPATHOLOGY

General Cellular Physiopathology

On entering the cell by the normal uptake mechanisms, copper is attached to the cytoplasmic chaperones, COX17, CCS, and HAH1, which deliver copper to cellular enzymes and compartments (Fig. 1). In Menkes disease, the defect in ATP7A results in an increase in intracellular free copper ion levels. This, in turn, induces metallothionein synthesis in an attempt to detoxify surplus metal. Metallothionein has been known and well-studied for decades, whereas little is known about the more recently identified chaperones. HAH1, the chaperone for ATP7A, is likely to be saturated as the next step in the pathway is blocked. On the other hand, COX17 and CCS are not expected to be deficient in copper, and the delivery of copper to COX and SOD1 should, in theory, not be diminished. However, because a mitochondrial disturbance is evident in MD, this suggests that COX may not receive sufficient copper even in organs with surplus copper [96]. SOD1 is deficient in the copper-deprived brain, while normal levels have been found in red blood cells.

ATP7A is necessary for the delivery of copper to the secretory pathway; hence, all secreted and vesicular copper-requiring enzymes may not receive sufficient metal for their normal function. The secretory pathway consists of stacks of disk-shaped membranous sacs (cisternae), the Golgi apparatus, through which proteins migrate during their biosynthesis. Nascent proteins are synthesized in the endoplasmic reticulum and transported in vesicles to the *cis*-Golgi cisternae (entry face) and gradually moved to the *trans*-Golgi cisternae (exit face) while the enzyme is post-translationally modified and the metallic cofactor is incorporated. Several of the copper-requiring enzymes can only incorporate copper during their passage through the Golgi apparatus. Information about the copper loading of various secreted enzymes is included in the general descriptions of the copper-requiring enzymes below.

Copper Enzymes

Copper enzymes are widely distributed in the body and are found both intracellularly and extracellularly. The intracellular enzymes are located in various cellular compartments, and biosynthesis of the active enzymes as well as their site of function is reviewed in the following.

Copper enzymes are involved in a number of reactions, and according to their main functions they can roughly be categorized as being involved in (1) energy formation, (2) protection against free radicals, (3) communication between cells and organs, and (4) formation of extracellular structure (Table 1).

Energy Formation

A prerequisite for life is the existence of mechanisms that can capture and store energy, and the respiratory chain

TABLE 1. Mammalian Copper Enzymes

Function	Enzyme	Biological Activity
Energy Capture	Cytochrome c oxidase	Cellular respiration
Protection	Superoxide dismutase	Free radical scavenging
	Ceruloplasmin	Ferroxidase
	Tyrosinase	Pigment formation
Communication	Dopamine β-hydroxylase	Catecholamine production
	Peptidyl α-amidating enzyme	Activation of peptide hormones
	Amine oxidases	Mono-, di- and poly-amine catabolism
Structural	Lysyl oxidase	Collagen and elastin cross-linking
	Sulfhydryl oxidase	Cross-linking of keratin

constitutes such a mechanism. In the respiratory chain, electrons are passed from one respiratory complex to another with the concomitant formation of energy-rich phosphate molecules (ATP), and the terminal oxidase reacting with molecular oxygen, cytochrome c oxidase, is a copper-dependent enzyme. Thus, copper plays an important role in cellular respiration. ATP is used as the energy source in numerous reactions in the body, and copper deficiency is therefore likely to result in widespread effects. Especially tissues with a high-energy requirement, such as muscles and brain, will likely be affected [97].

Cytochrome c oxidase. Cytochrome c oxidase (COX) [EC 1.9.3.1] is a large enzyme complex located in the inner mitochondrial membrane. It consists of 13 polypeptides, and the two copper-containing subunits (MTCO1 and MTCO2), which form the catalytic center of the enzyme, are encoded by the mitochondrial genome [31]. Hence, copper needs to be translocated across the mitochondrial membranes for incorporation into COX. Two genes involved in this translocation have been identified, namely the chaperone COX17, which transports copper to the periplasmic space [26–28], and SCO1, which is involved in incorporation of the metal into the metallocenter of the COX complex [29].

Protection Against Free Radicals

In several metabolic processes, highly reactive superoxide anions (O_2^-), also described as free radicals, are produced by partial reduction of molecular oxygen [98]. Because of their very toxic nature, free radicals need to be eliminated immediately, and numerous systems to protect the body against oxidative stress have been developed. An important class of protective enzymes is the superoxide dismutases, but other copper enzymes, such as ceruloplasmin and tyrosinase, also serve a scavenging role. Free radicals will attack and oxidize various molecular structures, such as lipids in the brain, and are believed to be involved in aging [99] and the development of Alzheimer disease [100].

Superoxide dismutase. Superoxide dismutase (SOD) [EC 1.15.1.1] converts superoxide anions into oxygen and hydrogen peroxide and thereby prevents the accumulation of toxic intermediates. Superoxide dismutase exists as three forms, encoded by three different genes. Two of these forms, SOD1 and SOD3, contain copper and zinc. SOD1 is the best-studied of the two enzymes. It is localized mainly in the cytoplasm, but evidence suggests that it is also located in peroxisomes and the nucleus [101]. SOD3 is secreted from the cell and has often been referred to as extracellular SOD (EC-SOD). SOD3 is found attached to the vascular wall and is the major superoxide dismutase in such extracellular fluids as plasma, lymph, and synovial fluid. Because it is secreted, the enzyme is most likely copper-loaded in the secretory pathway [102].

On the other hand, SOD2 (Mn-SOD) is manganese-dependent and is found in the matrix of mitochondria. There seems to be some interaction between SOD1 and SOD2 in their protective function. If one is low, perhaps due to a low body content of the catalytic metal, the amount of the other will increase [103]. However, the cytoplasmic SOD1 is present in greater quantities and may therefore be the physiologically most important form.

Ceruloplasmin. Besides SOD, ceruloplasmin (CP) [EC 1.16.3.1] is also involved in free radical scavenging. CP has ferroxidase activity and catalyzes the oxidation of highly reactive ferrous ions, Fe(II), to less toxic ferric ions, Fe(III), which, in turn, are bound to transferrin. During this oxidation process, ferrous ions can react with molecular oxygen and form superoxide radicals [98]. Rapid removal of free ferrous ions is therefore necessary. Findings in patients with aceruloplasminemia (MIM 604290) indicate that the main function of CP is indeed in iron metabolism. Thus, CP may play an important function in providing protection against superoxide radicals. Deficient iron mobilization will also affect heme biosynthesis and may cause anemia if the CP concentration is below 5% [104].

Circulating CP is synthesized primarily in the liver, and copper ions are incorporated during its synthesis by means of ATP7B. Copper does not influence the rate of synthesis or secretion of apoceruloplasmin, but a failure to incorporate copper during biosynthesis results in the secretion of an apoprotein that is devoid of oxidase activity and is easily degradable [105].

Hephaestin. Hephaestin (HEPH) is a protein identified in the late 1990s that plays an important role in intestinal iron absorption [106]. It is homologous to CP and contains similar copper-binding sites. In addition, it contains a transmembrane domain, indicating that the CP-like domain is located extracellularly. Based on its homology to CP, it has been proposed that HEPH is a ferroxidase necessary for iron release from intestinal epithelial cells. It has been suggested that ATP7A is involved in the copper loading of HEPH (C.D. Vulpe, personal communication). Deficiency of HEPH may also be speculated to result in hypochromic anemia. Thus, both CP and HEPH represent links between copper and iron metabolism and may explain iron-deficiency anemia associated with copper deficiency.

Tyrosinase. The production of free radicals may also be induced by photons, and the harmful effect of the sun is neutralized by the presence of melanin pigment in the skin. One of the enzymes involved in the production of melanin is the copper-requiring tyrosinase [EC 1.10.3.1; EC 1.14.18.1] that catalyzes several steps in the biosynthesis of melanin pigment. Dysfunction of the enzyme will consequently lead to hypopigmentation of the skin and hair. Tyrosinase is stored in pigment granules and thus is probably copper-loaded during its passage through the endoplasmic reticulum or Golgi apparatus.

Communication Between Cells and Organs

In any multicellular organism, communication between cells and organs is necessary. Copper participates in a number of reactions that have evolved to ensure the production and removal of various neurohormones and transmitters, and copper is necessary for the normal function of important enzymes such as dopamine β-hydroxylase, peptidyl α-amidating monooxygenase, and amine oxidases.

Dopamine β-hydroxylase. Dopamine β-hydroxylase (DBH) [EC 1.14.17.1] participates in the formation of adrenaline and noradrenaline from dopamine, and deficient activity of DBH will likely lead to autonomic failure. Besides copper, the enzyme requires ascorbate as a cofactor [107]. DBH is stored in secretory vesicles in nerve terminals and the adrenal medulla. Because of its vesicular location, the enzyme has to pass through the secretory pathway of the cells, and it is likely that copper is added at this stage. Low brain levels of copper will reduce the activity of DBH [108], but addition of copper after the enzyme has been synthesized can restore enzymatic activity [109].

Peptidyl α-amidating monooxygenase. Peptidyl α-amidating monooxygenase (PAM) [EC 1.14.17.3] is structurally similar to DBH, and it also requires copper

and ascorbic acid as cofactors [107]. PAM catalyzes the post-translational amidation of a number of essential peptide hormones involved in hypothalamic-pituitary regulation, as well as intestinal function, and such modification is required for full biological activity. The functional significance of PAM is not well-understood, but its deficiency will most probably result in widespread effects. Like DBH, PAM is found in both secreted and membrane-associated forms, and the biosynthesis of the enzyme is likely to be similar to that of DBH, with copper loading occurring in the secretory pathway. By analogy with DBH, the enzymatic activity of copper-deficient PAM can probably be restored after its biosynthesis by addition of the metal.

Amine oxidases. The copper-containing amine oxidases (AOCs) [EC 1.4.3.6] comprise a heterogeneous group of enzymes that are found in various tissues, including vascular smooth muscle, lung, eye, adipose tissue, placenta, and, in particular, intestine and plasma [110,111]. AOCs catabolize a variety of substrates, including monoamines, diamines, and polyamines, and thus regulate the intracellular concentrations of putrescine, spermine, and spermidine [112]. These polyamines exhibit a wide variety of functions and have been recognized to be vitally important in numerous fundamental cellular processes, including wound healing, tissue differentiation, cellular proliferation, and possibly apoptosis [110]. Modulation of ion channels has also been implicated [113]. One of the AOCs, histaminase, has long been recognized to play a role in inflammation, allergy, and ischemia. However, the exact physiological functions of AOCs are still unresolved, and because of the diversity of the substrates, it is difficult to delineate the precise clinical importance of this enzyme group.

Human genes for kidney (ABP1), retina (AOC2), and placenta (AOC3) specific oxidases have been identified [114–116], and the structural determinants of the proteins are beginning to be defined. A novel quinone cofactor has been identified in several amine oxidases, and these enzymes are now called quinoenzymes. The cofactor is formed by a copper-dependent post-translational modification of a tyrosine residue in the backbone of the enzyme [117]. Lysyl oxidase, which is discussed below, is also a quinoenzyme. Studies on a copper-free form of histaminase indicate that its enzymatic activity can be restored by the addition of copper after the protein has been synthesized and excreted [117], although this is not the case for lysyl oxidase.

Formation of Extracellular Matrix

Multicellular organisms obviously also have a need for intercellular stability, and copper enzymes play a major role in this function. Copper plays a key role in the biosynthesis of the extracellular matrix proteins, collagen and elastin, through its function as a cofactor for the enzyme lysyl oxidase.

Lysyl oxidase. Lysyl oxidase (LOX) [EC 1.4.3.13] is a secreted enzyme, which catalyzes the initial cross-linking of collagen and elastin and is found extracellularly attached to its substrates. Inhibition of LOX has profound effects on the strength of bone and elastic tissues. Collagen type I comprises 90% of the organic matrix of bone, and a decrease in the number of cross-links is accompanied by reduced mechanical strength of the bone, which may lead to spontaneous fractures [90]. Dysfunction of LOX will also affect other types of collagen, resulting in several connective tissue defects. When cross-linking is inhibited, the tensile strength of the collagen fibrils is reduced and they become fragile. Together with low elasticity due to defective cross-linking of elastin, this affects structures such as blood vessels, which become friable and may eventually rupture. Elastic tissues in skin become less stretchable and will not restore shape after extension, leading to premature wrinkling. Copper deficiency may therefore mimic heritable disorders of collagen and elastin (see also Chapter 9, this volume).

As mentioned above, lysyl oxidase is a quinoenzyme using a quinone cofactor named lysine tyrosylquinone (LTQ) as a transient electron carrier in the enzymatic reaction cycle [118–120]. After LOX has been synthesized, the enzyme is copper-loaded and the quinone cofactor is formed as part of the Golgi or *trans*-Golgi processing. In contrast to other quinoenzymes, a copper-catalyzed intrachain cross-linking of two amino acids in the LOX molecule is a prerequisite for a functional cofactor.

The LTQ cofactor is an integral part of the enzyme molecule and is formed by a copper-catalyzed cross-linking of the ε-amino group of a peptidyl lysine with the modified side chain of a tyrosyl residue before the enzyme is secreted. The formation of the quinone cofactor of LOX is clearly distinct from that of other quinoenzymes in the requirement of copper first for modification of the tyrosine residue and subsequently for the cross-linking of this modified residue to the lysine residue. This knowledge is important for the understanding of why LOX in particular is susceptible to copper deficiency.

Copper deficiency will affect the formation of the cofactor and hence the function of LOX, but it does not appear to affect the biosynthesis of the apoenzyme [121]. Copper added after secretion of the enzyme is unable to catalyze the intramolecular cross-linking and restore the enzymatic activity of LOX, in contrast to other quinoenzymes for which only a copper-dependent modification of a tyrosine residue is required. Thus, LOX activity in the body is solely dependent on the amount of copper available during the biosynthesis of the enzyme.

Keratinization. The skin constitutes a mechanical protective barrier to the body. Keratinization is also a copper-dependent process, although the precise enzymatic reaction has not yet been characterized. Lack of copper results in deficient cross-linking of keratin in hair and skin by an as yet unidentified mechanism [94]. Copper therapy may, however, ameliorate the structural defects seen in Menkes disease patients.

Clotting factors V and VIII. The blood-clotting factors V and VIII both contain copper, and failure of incorporation of the metal might impair the synthesis or function of the proteins. The copper-binding site is structurally related to that of ceruloplasmin, and this suggests a role for copper in these coagulation factors, the specific nature of which is not yet known. It has been suggested that copper may play a role in the correct folding of the proteins [122]. Lack of copper could be speculated to result in a bleeding tendency, and hematomas are frequently observed in MD.

Whole Body Physiopathology

The clinical and pathological features of MD are the result of a complex interplay of several copper-dependent processes. Certain features can be clearly related to dysfunction of specific copper-requiring enzymes, but for other symptoms it can be difficult to identify the role of each enzyme in the overall picture (Table 2). Biochemical defects in one

TABLE 2. Relationship Between Symptoms and Copper Enzymes in Menkes Disease and the Occipital Horn Syndrome

Symptom	Enzyme Involved
Premature rupture of fetal membranes Cephalohematoma Abnormal facies High-arched palate Emphysema Hernias Bladder diverticula Arterial aneurysms Loose skin and joints Osteoporosis	Lysyl oxidase
Petechial hemorrhage	Lysyl oxidase, clotting factors V+VIII
Poor wound healing	Lysyl oxidase, amine oxidase?
Abnormal hair Dry skin	Sulfhydryl oxidase
Hypopigmentation	Tyrosinase
CNS degeneration	Superoxide dismutase, cytochrome c oxidase, lysyl oxidase
Ataxia	Dopamine β-hydroxylase, cytochrome c oxidase
Muscle weakness	Cytochrome c oxidase
Hypothermia Hypotension Diarrhea	Dopamine β-hydroxylase
Anemia	Ceruloplasmin, hephaestin

metabolic pathway can be influenced by the concurrent failure of other copper-dependent systems. These processes may act synergistically and aggravate disease progression.

Changes in enzymes necessary for the formation of body structures such as the connective tissues and skin and hair will cause profound visible effects. Defects in other enzyme systems may add to these changes or primarily affect the way in which the body reacts to stress of different types. In the following, we discuss the defects at a structural and functional level, still knowing that these categories overlap.

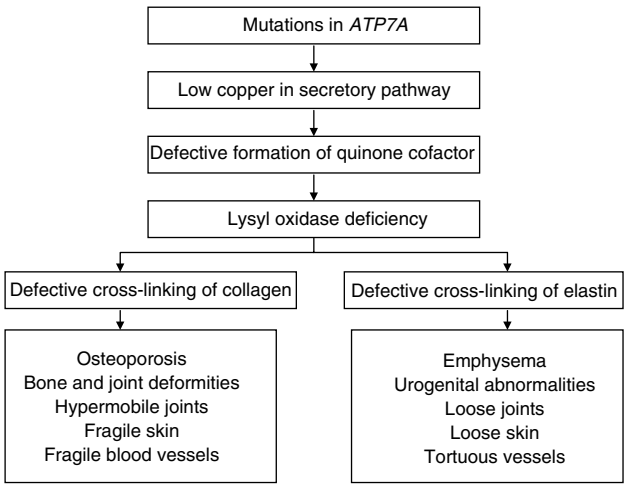

Figure 12. Physiopathological mechanisms of the connective tissue disturbances in Menkes disease.

Structural Defects

Copper is involved in the formation of collagen and elastin, and in MD connective tissues, and hence organs rich in this type of tissue, are severely affected (Fig. 12). Collagen and elastic fiber pathology is the result of deficient LOX activity secondary to a defect in the cellular copper pump, ATP7A, resulting in the faulty delivery of copper for activation of LOX. A defect in ATP7A will result in a reduced availability of copper and affect the formation of the quinocofactor in LOX and hence the activity of the enzyme. After secretion, this activity cannot be restored by copper supplementation, pointing to a direct link between the availability of copper in the Golgi complex and LOX activity [120,121]. This is also reflected by the inefficiency of copper-histidine treatment in correcting the connective tissue manifestations (see "Treatment" below).

The cross-linking defect may affect a number of types of collagen. Thus, changes that are specific to collagen defects of type I (bone fractures as in osteogenesis imperfecta — Chapter 8, this volume), type II (bone and joint deformities as in chondrodysplasia — Chapter 23, part II, this volume), and type III (fragile skin and blood vessels as in Ehlers-Danlos syndrome type IV — Chapter 9, this volume) can be seen in MD. Classical MD patients exhibit a wide range of connective tissue defects ranging from lax skin and joints to osteoporosis. In severe cases, bone fractures and vascular rupture may even raise the suspicion of child abuse. Milder forms of MD manifest progressive connective tissue defects as a result of long-term effects of poor connective tissue cross-linking.

Deficient LOX activity also interferes with the cross-linking of elastin, affecting connective tissues with a high content of elastic fibers, including ligaments, arterial walls,

and elastic cartilage. Humans appear to be extremely sensitive to reduced elastin levels and may develop profound symptoms, as can be seen in various inherited disorders such as supravalvular aortic stenosis (MIM 185500), Williams syndrome (MIM 194050), and certain forms of cutis laxa (MIM 123700) [123,124] (see also Chapter 10, this volume).

The elastic fiber is responsible for restoring shape after stretch in a variety of tissues, and symptoms related to improper function of elastic tissues are prominent in both severe and milder cases of MD. These symptoms comprise inguinal and umbilical hernias, diverticula of the bowel and bladder, gastric polyps [125], skin laxity, hyperextensible joints, and, later, joint contractures and lordosis, or kyphosis.

The striking hair changes seen in MD are probably the result of defective keratin cross-linking. This process appears to be copper-dependent because copper deficiency in sheep leads to the formation of abnormal wool with defective cross-linking of disulfide bonds. This led Danks and his co-workers in 1972 to detect the abnormal copper metabolism [51]. They recognized the similarity of patients' hair to wool from sheep grazing on copper-deficient pastures and subsequently demonstrated copper deficiency in the brain, liver, and blood of MD patients. In MD patients, the content of free sulfhydryl groups in hair is increased, and the diminished cross-linking of the hair results in an abnormal structure with easily recognizable changes. In fact, the hair changes are one of the key symptoms in identifying patients with classical Menkes disease. Patients with a milder clinical course have less severe hair changes, and it also appears that the hair tends to normalize with increasing age and with copper therapy. Defective keratinization can also explain the high prevalence of seborrheic dermatitis in Menkes patients. The hypopigmentation of skin and hair is most likely due to decreased activity of tyrosinase, which is involved in melanogenesis.

Functional Defects

When functional defects in MD are discussed, the body tissues and organs can be viewed in three categories: liver, brain, and other tissues. In liver, ATP7A is not expressed, underscoring the fact that its role is taken over by ATP7B. Liver copper levels are low in Menkes disease, but this can be corrected by copper supplementation, indicating that it is a secondary effect. Brain is unique in that it is the only organ deprived of copper due to the blood-brain barrier, whereas for the other organs and tissues copper deficiency is functional. Therefore, the pathological mechanisms are likely to be different in the peripheral and central nervous systems.

Although LOX is the enzyme responsible for most of the connective tissue disturbances, the other main group of symptoms, the neurological abnormalities, is probably the consequence of a combined deficiency of several copper-dependent enzymes. Low amounts of COX will result in deficient energy production, affecting nerve conduction. Defects in the respiratory chain will also lead to the production of free radicals and, in combination with deficient activity of superoxide dismutase, this will lead to further accumulation of superoxide anions. This in turn, will result in peroxidation of unsaturated lipids and cause irreversible damage to myelin sheaths and result in neuronal degeneration. Reduced activity of DBH will affect the production of neurotransmitters, resulting in increased levels of dopamine and low levels of catecholamines and noradrenaline. Low levels of neurotransmitters in early life will lead to deficient brain development, and this cannot be corrected later by restoration of transmitter function. PAM is involved in the maturation of neuropeptide hormones, and low levels of this enzyme may therefore also contribute to the neurological defects. Vascular abnormalities as a result of defective LOX will cause intracranial bleeding and further aggravate the neurological dysfunction.

Another way of discussing the relative contributions of the copper enzymes to the neurological symptoms is to consider the possible effect of each individual enzyme, such as COX, SOD, and DBH. Cytochrome c oxidase is one of the enzymes in the respiratory chain, deficiency of any of which will affect energy production and hence all energy-requiring (ATP-dependent) processes in the body, including nerve transmission. MD may therefore mimic a mitochondrial disorder in which multiorgan involvement may also be present and complicate the clinical picture. Low activity of COX could result in hypotonia besides being partly responsible for the complex neurological picture seen in MD patients. Patients with mitochondrial myopathy due to a specific COX deficiency often suffer from neurological disturbances, with mental retardation and seizures, and cardiac and skeletal muscle weakness [97], but deficient thermoregulation is not a common feature. The claimed effect of COX on thermoregulation [49] is therefore disputable.

Superoxide dismutase may be affected differently in the peripheral and central nervous systems. In the brain, there is a well-documented lack of copper and the activity of SOD1 is decreased as expected, but there appears to be overexpression of the manganese form of superoxide dismutase (SOD2) [103]. This form of the enzyme is localized in the mitochondria, and the production of superoxide radicals in the respiratory chain may hence be partly neutralized. The full contribution of superoxide dismutase deficiency to the neurodegenerative processes in MD is therefore difficult to determine. In the peripheral nervous system, the cellular free-copper pool is increased and there may therefore be no lack of copper for incorporation into SOD1. The activity of SOD1 in red blood cells has been reported to be normal in several patients [1].

DBH is important for the biosynthesis of catecholamines, and deficient production in MD is documented by an abnormal pattern of catecholamine metabolites. Several clinical features seen in MD and OHS can be explained by an autonomic dysfunction, and orthostatic hypotension is a common problem in OHS and well-treated MD patients, which can be corrected by ingestion of L-DOPS [82]. The sympathetic nervous system is also important for thermoregulation, and hypothermia is a recurrent problem in MD patients. Mice deficient in DBH due to a targeted gene disruption show poor thermogenesis, leading to low body temperature when exposed to cold [126]. Like MD babies, neonates with congenital DBH deficiency suffer from episodic hypothermia [127]. Chronic diarrhea also seems to be caused by autonomic dysfunction because the administration of L-DOPS can control this problem [82]. Ataxia is yet another symptom that can be aggravated by lack of DBH. Mice deficient in DBH have fewer noradrenergic terminals in cerebellum compared with controls [128], and this region of the brain appears particularly resistant to the restoration of noradrenaline by administration of L-DOPS. This could explain the persistent occurrence of ataxia in a well-treated MD patient receiving supplementary treatment with this drug [82].

PAM is required for the activation of numerous peptide hormones (e.g., corticotrophin-releasing factor, thyrotropin-releasing hormone, growth hormone–releasing hormone, gastrin, vasopressin, calcitonin, and cholecystokinin) involved in hypothalamic-pituitary regulation as

well as in gut function. Low activity of PAM might be expected to cause a wide range of effects, although the precise consequences are difficult to predict. Both central nervous system effects and systemic problems are likely. The activity of this enzyme has been investigated in serum samples from moderately affected MD patients and found not to be diminished [129]. However, when copper was added to the serum samples, the enzyme activity increased. This might indicate the presence of extra apoenzyme released as a compensatory secretion secondary to copper deficiency at the site of loading of the PAM molecule.

Low tyrosinase activity can easily explain the hypopigmentation of skin and hair [1], but it may also affect visual function. A hypopigmented fundus has been described in MD patients [130], and it is well-known that albinism (MIM 203100) due to a genetic defect of tyrosinase is accompanied by visual problems.

Low levels of ceruloplasmin are well-documented in classical MD patients, but on the whole the ferroxidase activity appears to be sufficient for iron mobilization. However, some MD patients may show moderate hypochromic anemia. The newly discovered iron transporter, hephaestin, may also be speculated to play a role in the development of anemia. The low availability of hepatic copper could also be speculated to affect clotting factors V and VIII, interfering with the normal function of the coagulation cascade. This could add to the bleeding tendency that is caused by the fragile blood vessels secondary to LOX deficiency.

MOLECULAR PATHOLOGY

Structure of the Protein (ATP7A)

ATP7A is a member of the copper ATPase protein family, which is a subgroup of a large family of P-type ATPases that are energy-using membrane proteins functioning as cation pumps. They are called "P-type" ATPases because they form a phosphorylated intermediate during the transport of cations across a membrane.

ATP7A has functional domains with highly conserved motifs that are common to all P-type ATPases (Fig. 13). The so-called phosphorylation domain contains an invariant cytoplasmic DKTGT motif within which the aspartate

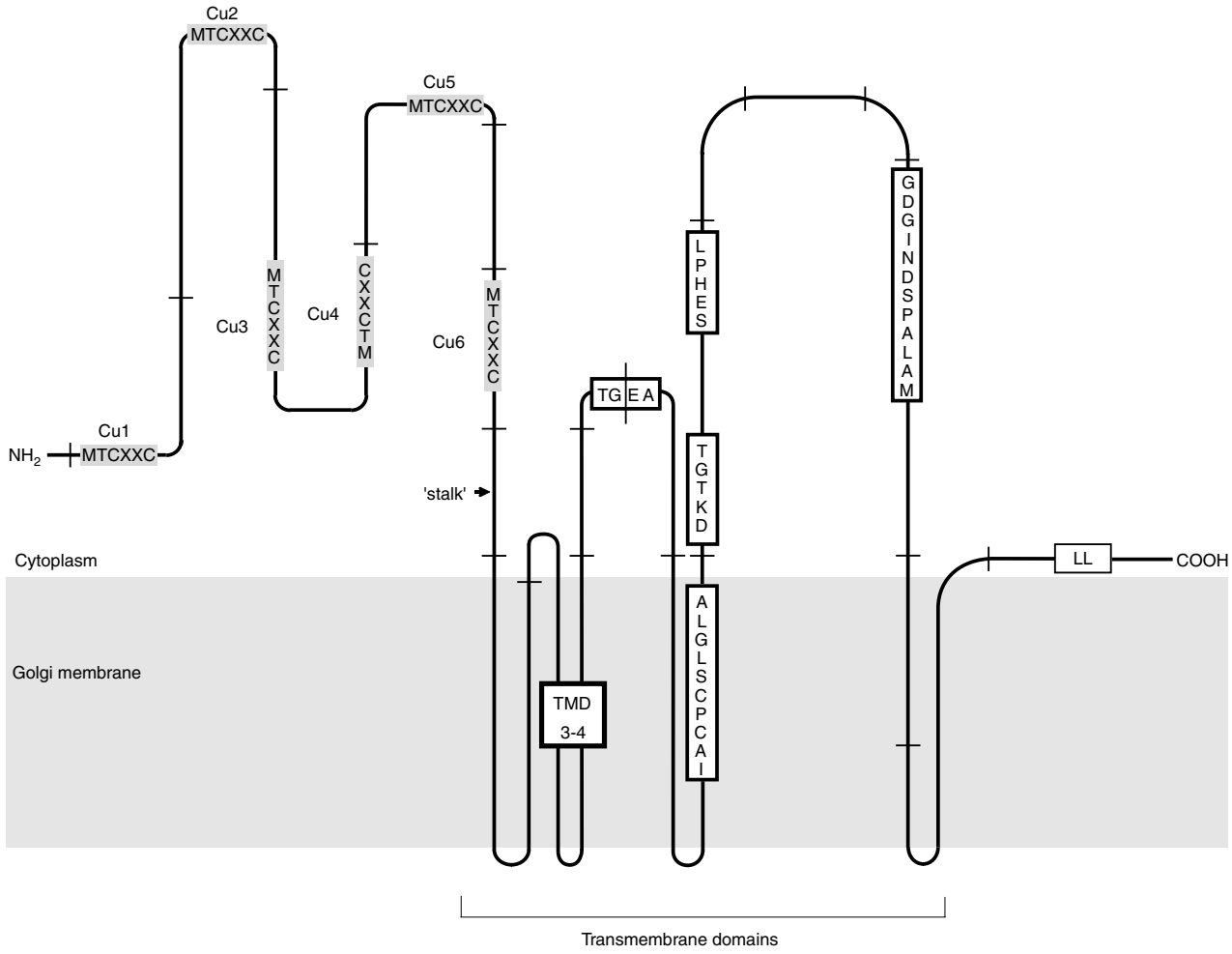

Figure 13. Protein structure of ATP7A. Cross lines indicate the positions of exon/intron boundaries. The copper-binding motifs (MTCXXC) are numbered Cu1–6; transmembrane domains 3 and 4 (TMD 3-4) contain a Golgi localization signal; phosphatase domain (TGEA); transduction domain (IACPCSLGLA); phosphorylation domain (DKTGT); loop motif (SEHPL); ATP-binding domain (GDGINDSPALAM); recycling signal (LL). "Stalk" indicates the region between the last metal-binding domain and the ATPase core.

residue is phosphorylated by the terminal phosphate of ATP in the transport cycle. The ATP-binding domain with a conserved GDGXNDXP motif is responsible for the binding of ATP. The phosphatase domain contains the consensus TGEA/S sequence, which removes the phosphate from the phosphorylated aspartic acid during cation transport. These proteins also possess hydrophobic transmembrane domains (TMD), but their numbers differ among the ATPases. ATP7A, like the other Cu-ATPases, has eight TMDs that anchor the protein into the Golgi membrane [131].

Besides having features common to all P-type ATPases, ATP7A and other copper ATPases also contain some motifs specific for this subgroup [131,132]. Interestingly, these motifs are also conserved in P-type ATPases transporting other heavy metals, such as mercury or cadmium. A remarkable feature of Cu-ATPases is a characteristic intramembranous motif, CPC, unique to this subgroup. This motif is likely to constitute the cation channel involved in transduction of the metal through the Golgi membrane. A highly conserved HP motif (SEHPL/I) situated in the second cytoplasmic loop C-terminal to the phosphorylation domain is present in all known copper-transporting ATPases characterized so far. This motif is likely to have a regulatory role in copper translocation because its position is analogous to that of the regions that modulate the affinity of ion binding in calcium-transporting P-type ATPases [131].

At the amino terminus, ATP7A has six domains of approximately 100 amino acids, each containing a consensus GMXCXXC motif, where the two cysteine residues play an important role in copper binding. Studies suggest that this region is directly involved in the selective binding of heavy metals, the highest affinity being toward copper, followed by zinc, nickel, and cobalt [133–135]. The whole region binds six copper atoms, one atom per metal-binding repeat [136,137]. NMR studies on the fourth metal-binding domain of ATP7A demonstrated that Ag(I) could bind to the two cysteine residues of the conserved GMXCXXC motif [138]. Different site-directed mutagenesis studies suggest that not all of the metal-binding domains are equally important for the normal function of the protein, but a consensus has not yet been reached on which is most essential [139,140]. The functional significance of the repetition of these metal-binding sites is not yet clearly understood either. Both a regulatory role and direct involvement in transfer of the metal have been suggested [132]. Analysis of the whole copper-binding region indicates an allosteric control of metal binding [137,141]. It has also been suggested that binding of copper to the repeated motifs is the signal required for the reallocation of the protein from the Golgi apparatus to the plasma membrane [41,42,141].

Structure of the Gene (*ATP7A*)

The 8.5 kb *ATP7A* mRNA consists of 23 exons and spans approximately 150 kb of genomic DNA [142,143]. The 5′-untranslated region of the gene is rich in GC sequences, which is a feature of housekeeping genes required by all or most tissues. This is in line with the multisystemic character of Menkes disease (MD). An interesting feature of the promoter region of *ATP7A* is the presence of three 98 bp tandem repeats. The function of these sequences is still unknown, but deletion of one of these repeats has been observed in one OHS patient (see below) [144]. The 134 bp first exon of *ATP7A* contains only untranslated sequences, and the first base pair of this exon has been suggested as the transcription start site [142]. Levinson et al. [144] have also identified other possible transcription start sites. The ATG translation start codon is within the 141 bp second exon, which also contains part of the 5′-untranslated region and the first metal-binding domain. The last exon is 4,120 bp in length and contains a 274 bp translated sequence, a TAA translation termination site, a large 3′-untranslated region, and a polyadenylation site. Apart from the 23 exons mentioned, the large first intron of *ATP7A* includes a 192 bp exon (exon 1b), which is alternatively spliced and expressed in low amounts in several tissues examined (T. W. Glover, personal communication).

In *ATP7A*, the exons seem to correspond to the predicted functional/structural domains of the protein product, and this may be of importance in the case of in-frame mutations [142]. The predicted metal-binding domains (Cu1–6) and the transmembrane domains (TMD) are encoded by separate uninterrupted exons, with some exceptions: Cu3 and Cu4 are together encoded by a single exon, and the exons coding for TMD2 and TMD7 are interrupted by introns. The phosphorylation and ATP-binding domains are also encoded by a single exon, while the exon coding for the phosphatase domain is interrupted by an intron.

Several exons in *ATP7A* are alternatively spliced [143]. One of these is exon 10, encoding TMD3 and TMD4. Exon 14 coding for TMD5 and/or exon 15 coding for TMD6 and the putative cation channel with the highly conserved CPC motif are also alternatively spliced. Exon 1b identified within intron 1 is another alternatively spliced exon (T. W. Glover, personal communication).

Mutation Spectrum of *ATP7A*

To date, a total of 191 mutations affecting *ATP7A* have been identified in unrelated MD patients with the classical severe form or with one of the atypical phenotypes [145].

Chromosomal abnormalities affecting *ATP7A* have been detected in seven patients. One of these patients was a male with a unique abnormality whereby the segment Xq13.3-q21.1 was inserted into the short arm of the X chromosome [146]. One female patient was mosaic for the Turner karyotype [147] and the remainder had X;autosome translocations [148–150] (D. Wattana and S. Mohammed, personal communication).

Of the remaining 184 gene mutations identified, 35 have been partial gene deletions of different sizes and locations. The largest defect observed has been the deletion of the whole gene except for the first two exons, and the smallest one detectable by Southern blot hybridization has been a deletion of the first exon.

The total number of point mutations identified within *ATP7A* is 149, comprising 78% of all the mutations. Eighteen of these mutations have been reported by other groups [80,144,151–155,159] and 44 by ours [156–158], while 87 mutations have not yet been published. Of the 149 point mutations, 132 were novel. A common mutation observed is a missense mutation within exon 10 [151], which has been detected in nine unrelated families.

The four types of point mutation in *ATP7A* (deletions/insertions, missense and nonsense mutations, and splice site mutations) are represented in almost equal proportions (Fig. 14). About half of the point mutations are frame-shift or nonsense mutations (22% and 14% of all *ATP7A* mutations, respectively), which are predicted to result in a nonfunctional truncated protein. These truncating mutations are distributed almost equally throughout the gene, although none has been observed in exons 1, 2, 5, or 23. A clustering of mutations

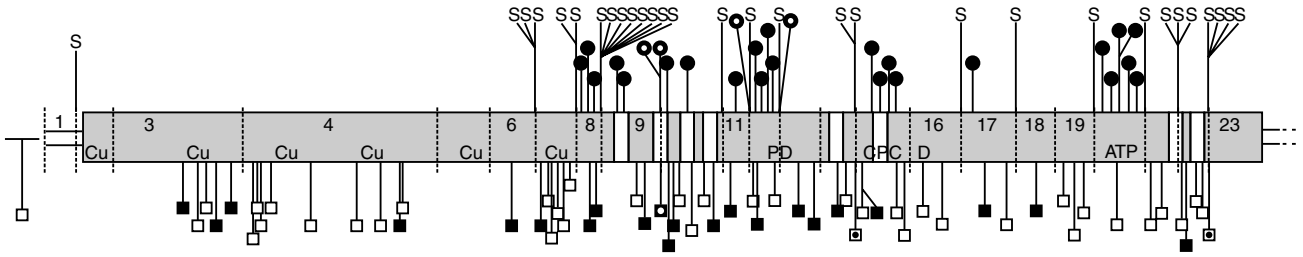

Figure 14. Mutation spectrum of Menkes disease. Localization of 118 different point mutations identified in *ATP7A*. The copper-binding domains are indicated by Cu and the transmembrane domains by vertical white bars. PD: the phosphatase domain; CPC: the cation channel; D: phosphorylation domain; ATP: ATP-binding domain. The coding region of *ATP7A* is shown as a gray horizontal box. The vertical dotted lines indicate the positions of the introns, and the exons are numbered from 1 up to 23. The 3′- and 5′-untranslated regions are shown as horizontal empty boxes, and a horizontal line indicates the flanking genomic sequence of the 5′ untranslated region. Empty squares: insertion/deletion mutations; filled squares: nonsense mutations; filled circles: missense mutations; S: splice site mutations. Mutations at the coding sequences that are predicted to affect normal splicing are marked with a small dot.

can be observed in those exons that represent the functionally important and highly conserved regions of the protein; these are 12, 15, and 20, coding for the phosphatase, transduction, and ATP-binding domains of the protein, respectively. An exception to this is exon 8, in which a total of 15 point mutations (including acceptor and donor site mutations) have been identified [151,155,158]. Exon 8 encodes a region between the last metal-binding domain and the first transmembrane domain of ATP7A. Although a specific function has not yet been ascribed to this region, it may play an important role in the folding of the protein and serve as a "stalk" joining the metal-binding domains and the ATPase core.

No missense mutations have been observed in the copper-binding domains of ATP7A encoded by exons 2–7. This lack is in line with the amino acid diversity allowed between species. In contrast, missense mutations are frequent in exons 8 to 23, encoding the ATPase core, especially in the conserved motifs, underscoring the importance of these domains for the normal function of the protein.

Relationship Between Type of *ATP7A* Mutation and Severity of Menkes Disease

Naturally occurring mutations are invaluable in assessing the importance of the functional domains of a protein, and the nearly 200 mutations of the *ATP7A* gene identified in MD patients give important clues. However, spontaneous mutations are limited, and site-directed mutation analysis using a yeast system has been applied to *ATP7A* (and *ATP7B*). These studies may, at the same time, give a better understanding of the mechanisms that may lead to differences in the severity of symptoms among MD patients. We will therefore discuss the mutations found in OHS patients in more detail in this part. In general, MD patients with milder phenotypes seem to have a higher proportion of mutations that would result in a protein with residual activity.

To date, seven point mutations have been reported in patients diagnosed with OHS [80,144,152,155,159]. In all of these, except for that described by Levinson et al. [144], the DNA mutation has led to RNA splicing defects. In general, splice mutations comprise approximately 30% of the point mutations and 20% of all mutations found in MD patients.

In five OHS patients [80,152,159], the mutations have been at the consensus splice site sequences affecting normal RNA splicing. In three of these patients [80,152], in addition to the abnormal size transcripts, a normal transcript was always present in low amounts. The milder phenotype of the patients could thus be explained by the presence of the normal transcript, albeit in low amounts.

In one patient, the mutation was a single base-pair substitution within the splice site sequence of exon 11 (Ser833Gly). In addition to two abnormal size transcripts, a normal size transcript was present, but this transcript included the mutation [80]. This missense mutation was not within a region coding for a conserved motif, and its effect on the function of the protein probably was not severe.

In the patient described by Ronce et al. [155], the RNA-splicing defect was due to a missense mutation in exon 8. In this patient, both a normal size transcript bearing the mutation and abnormal size transcripts were detected. A possible explanation for the mild phenotype is the presence of an at least partially functional protein coded by the normal size transcript bearing the missense mutation. Previously, a missense mutation within exon 8 was detected in a patient with the classical phenotype, but this mutation has not yet been analyzed at the transcriptional level [158].

In the OHS patient described by Qi and Byers [159], a normal size transcript was not present. The abnormal transcript lacked exon 10 which is alternatively spliced out in normal individuals [143]. The protein product missing transmembrane domains 3 and 4 encoded by exon 10 was localized to the endoplasmic reticulum [159] instead of the Golgi complex, where normal ATP7A resides [34–36]. Part of the amino acid sequence encoded by exon 10 was suggested to be responsible for the Golgi localization of ATP7A [40]. The milder phenotype of the OHS patient suggests that at least some of the enzymes, other than LOX and DBH, could receive copper, even though ATP7A was mislocalized.

The only mutation not to affect RNA splicing in an OHS patient was described by Levinson et al. [144]. One of the three repeated elements in the regulatory region of *ATP7A* was deleted [144], but the level of transcription was normal. In this patient, other *ATP7A* mutations could not be detected, and the relationship of this deletion to OHS remains to be investigated.

ANIMAL MODEL

The mottled mouse has long been considered an animal model of MD on the basis of phenotypic and biochemical evidence, but its status as a true animal model was only proven in the late 1990s by the demonstration of mutations in the homologous murine gene, *Atp7a*, in several mottled alleles [160].

The first mutant, the mottled mouse, arose spontaneously in 1953 [161]. Mutations at the X-linked mottled locus (*Atp7a*^{Mo}) lead to a characteristic variegated coat color in female heterozygotes. In 1974, a defect in copper metabolism was demonstrated in mice bearing the mottled brindled allele [162]. Since then, several similarities between the biochemical pathology and phenotypic features of the mottled mouse and Menkes disease (MD) have been delineated [160,163,164]. Another indication of homology between MD and the mottled phenotype is that both loci belong to a region of genes on the X-chromosome that are highly conserved in both man and mouse.

Following the isolation of *ATP7A*, the mouse gene (*Atp7a*) was cloned using the human sequences [165,166]. The predicted protein product shows great sequence homology to ATP7A, and the tissue expression profile is also similar [167,168]. The genomic structure of the mouse gene is highly homologous to that of the human gene. The 8.0 kb transcript sequence consists of 23 exons spanning about 120 kb, and all the introns interrupt the aligned coding regions at the same positions [169].

There are about 30 independent mottled mutants, which show considerable variability of neurological and connective tissue abnormalities ranging in severity from allowing viable and fertile males with coat hypopigmentation to causing death *in utero* of affected male pups [170] (Boyd, personal communication). Today, *Atp7a* mutations have been identified in nine of the mottled alleles [152,166,171–177] (Cunliffe et al., personal communication).

Two of the most well-characterized mottled mutants, the mottled brindled and mottled macular, present with severe neurological impairment and death about day 14 and are suggested as murine models for the classical form of MD [160,162,163,177]. Mottled brindled has a 6 bp deletion in the cytoplasmic loop containing the phosphatase domain of *Atp7a*, and mottled macular has a missense mutation in the eighth transmembrane domain (TMD). On the other hand, the mottled blotchy phenotype resembles OHS by showing predominantly connective tissue manifestations [178]. Mottled blotchy has a splice site mutation affecting normal RNA splicing, similar to that observed in several OHS patients [80,152,159]. Interestingly, Kaler and co-workers have described an OHS patient with a mutation occurring at the same splice site (IVS, DS 11) as in mottled blotchy [80]. The mottled viable brindled phenotype is similar to that of mild MD [160], and this allele has a missense mutation in a conserved domain of *Atp7a* [174]. A missense mutation in the seventh TMD has now been identified in a patient with mild MD [179].

In general, the mutations in *Atp7a* are rather similar to those in *ATP7A* [160]. Small insertions or nonsense mutations have not been identified in mottled mice, but the presently elucidated nine mutations are not sufficient to define the mutation spectrum of *Atp7a*.

DIAGNOSIS

The initial diagnosis of Menkes disease (MD) and its milder variants is based on the clinical symptoms. In classical cases, the typical hair changes are a valuable diagnostic pointer. Low serum copper and ceruloplasmin levels will support the clinical suspicion, but biochemical confirmation in tissue culture is needed, especially in milder cases (Table 3). The ultimate diagnostic proof is the demonstration of a molecular defect in *ATP7A*.

Clinical Diagnosis

All MD patients exhibit connective tissue symptoms to varying degrees. Classical MD patients also have severe neurodegeneration, whereas the neurological symptoms of OHS are mild, leading to a clinical picture characterized

TABLE 3. Postnatal Diagnostic Markers for Menkes Disease and the Occipital Horn Syndrome

	Menkes	OHS	Comments
Pathognomonic features	Pili torti	Occipital horns	Clinical continuum
Blood markers			
Copper	Severely decreased	Moderately decreased	Not diagnostic in neonates
Ceruloplasmin	Severely decreased	Moderately decreased	Not diagnostic in neonates
DOPA/DHPG[1]	Significantly increased	Significantly increased	Diagnostic in neonates
Tissue copper			
Placenta[2]	Significantly increased	Probably increased	Diagnostic in neonates, noninvasive
Liver	Significantly decreased	Decreased	Invasive, not routine
Cultured fibroblasts			
Lysyl oxidase	Decreased	Decreased	Great interlaboratory variation, not routine
Radiolabeled Cu/Ag[2]	Increased uptake and retention[2]	Increased uptake and retention[2]	Not discriminatory between severe and mild forms
Mutation[2]			
DNA	Broad spectrum of mutations; all types represented	Splice mutations prevalent	Large gene (23 exons); clustering of mutations in central portion of the gene
RNA/protein	80% leading to translation stops	Residual protein activity likely	

[1] DOPA: dihydroxyphenylalanine; DHPG: dihydroxyphenylglycol.

[2] Definitive diagnosis.

mainly by connective tissue manifestations. Intermediate variants show less pronounced CNS involvement, although usually marked connective tissue symptoms.

Physical Examination

Clinical diagnosis of classical MD is often fairly easy once a few cases have been seen, but the diagnosis of milder cases may still pose a problem and they are probably underdiagnosed. The cloning of the gene has increased interest in the variant phenotypes, including OHS. The X-linked inheritance may help to direct attention to the correct diagnosis in familial cases.

In classical MD, the most striking and diagnostically important symptom is the abnormal hair structure. The hair appears colorless and friable. There may be a good reason for keeping the eponymic name, Menkes steely (kinky) hair disease, to direct attention to this important diagnostic feature. There is marked hypotonia, poor head control, and the facies is often characteristic, with frontal bossing and an expressionless appearance due to facial hypotonia. The skin is pale and loose, and the joints are hypermobile. Umbilical and inguinal hernias are frequent. Other manifestations are hypothermia, poor feeding, impaired weight gain, delayed developmental milestones, and the occurrence of intractable seizures.

Physical findings in OHS comprise craniofacial abnormalities, with a long face and neck, a high forehead and a hooked nose, and skeletal dysplasia with a narrow chest and shoulders, deformed elbows, genu valgum, and pes planus, as well as other connective tissue abnormalities that are reminiscent of Ehlers-Danlos syndrome (EDS) (see Chapter 9, this volume). Chronic diarrhea with malabsorption and orthostatic hypotension are two other common problems associated with OHS. Seizures may be observed, but intellectual development is usually borderline normal. There may also be muscular atrophy with muscle weakness. Demonstration of the bony protuberances on the occiput will clinch the diagnosis, and these can be palpated in some patients, usually around puberty.

Clinical Diagnostic Tests

Clinical tests are often distinctive and of diagnostic value. Electroencephalograms (EEGs) are usually moderately to severely abnormal in classical MD patients, with multifocal spike and wave discharges, but may be normal in young MD babies [180,181]. Unspecific EEG changes have also been reported in a few OHS patients [182]. Electrocardiography may be helpful in detecting cardiac murmur [64,68].

Magnetic resonance (MR) angiography and computed tomography (CT) are helpful for imaging the brain and thorax [183,184]. Brain (MR) imaging [185] readily detects diagnostically significant features such as white matter abnormalities due to impaired myelination, diffuse atrophy with ventriculomegaly, cerebral blood vessels with a corkscrew-like appearance, and subdural collections and hematomas. MR angiography is a noninvasive method for the study of the vasculature. Ultrasonography with color-flow Doppler imaging is another powerful tool for screening for aneurysms [68], while invasive diagnostic techniques such as arteriography should preferably be avoided. Urogenital abnormalities [50] can be detected by pelvic ultrasonography (with or without color-flow Doppler imaging) or cystography.

Radiographs are distinctive in both classical MD and milder forms. In classical MD, they often disclose mild diffuse osteoporosis and abnormalities of bone formation in the posterior region of the skull (Wormian bones), long bones (metaphyseal spurring), and ribs (anterior flaring and multiple fractures). The bone abnormalities may mimic changes observed in scurvy and nonaccidental injury but are symmetrical. Bilateral calcified projections from the occipital calvarium (occipital horns) and short, broad clavicles with hammer-shaped distal ends are pathognomonic skeletal findings in OHS. Additional findings are dislocation of the radial head, an undulating cortical configuration of the long bones, and hyperostosis at sites of ligamentous insertions.

Differential Diagnosis

Because of the pronounced involvement of the connective tissues, MD and occipital horn syndrome patients share phenotypic traits with a number of other connective tissue disturbances (see "Physiopathology" above). Some of these are important to keep in mind when evaluating a patient with an as yet undiagnosed condition.

Ehlers-Danlos Syndrome

Several clinical features are shared with those of the Ehlers-Danlos syndrome (EDS), which is described in detail in Chapter 9 of this volume. These include joint laxity, easy bruisability, and atrophic scarring. Vascular abnormalities such as varicose veins, tortuous peripheral veins, and aneurysms are also features that may be shared with several types of EDS [68]. The presence of occipital horns, narrow shoulders and chest, and deformed elbows may help to differentiate OHS from EDS.

The term Ehlers-Danlos syndrome type V (MIM 305200) has been used to describe an apparently X-linked recessive trait. Skin hyperextensibility, joint mobility, bruising, and intramuscular hemorrhage characterize the disease. Deficient lysyl oxidase activity has been reported in one family [186], but this could not be confirmed in later investigations [49,187]. The molecular etiology of EDS V remains unknown and indeed the existence of EDS V as a separate entity is questionable (Chapter 9, this volume).

Marfan Syndrome

Marfan syndrome (MIM 154700) (Chapter 12, this volume) is an autosomal dominant disease caused by a defect in a structural protein, fibrillin. Fibrillin is necessary for the proper organization of matrix proteins including elastin, and defects in fibrillin may therefore produce some of the features seen in OHS [188]. A significant proportion of Marfan cases are sporadic and, like OHS patients, they exhibit a marked variability in skeletal features, including a highly arched palate, pectus deformities (excavatum and carinatum), inguinal hernias, joint hypermobility, and scoliosis.

Cutis Laxa

A number of MD patients present with unusually lax skin [189,190] that may lead to a diagnosis of cutis laxa. In one of the first reports of OHS, the condition was named X-linked cutis laxa, and this eponym is still widely used although no longer officially promulgated [53].

Cutis laxa due to a defect in the elastin gene [123,124] (see also Chapter 10, this volume) may lead to symptoms similar to those seen in classical Menkes disease and OHS. Thus, angiographic abnormalities of the aorta and great vessels, lax inelastic skin, hernias, and genital prolapse are common features.

Osteogenesis Imperfecta

A significant proportion of patients are born with fractures of the skull and long bones, and a suspicion of osteogenesis imperfecta (Chapter 8, this volume) was raised in a number of cases before the correct diagnosis was reached [189,190].

Child Abuse

Because of the metaphyseal spurs, periosteal reaction, and cerebrovascular abnormalities, MD can also be confused with child abuse. The symmetrical involvement should, however, alert the physician, and other clinical manifestations such as mental retardation, seizures, and abnormal hair should direct attention to a specific metabolic disorder. In one case, a family was brought into court for child abuse before the correct diagnosis was established [191].

Mitochondrial Disorders

Most classical MD patients are hypotonic and floppy, and milder cases may show a myopathy that may lead to the suspicion of a mitochondrial disturbance. The finding of high lactate levels in blood and cerebrospinal fluid due to a deficiency of COX may further strengthen this suspicion. Measurement of respiratory enzymes in a muscle biopsy may show low activities, and "ragged-red" fibers, symptomatic of mitochondrial abnormalities, have also been reported in an MD patient [96].

Biochemical Diagnosis

Body Fluids

Serum copper and ceruloplasmin levels are decreased in both classical MD and OHS. Although levels in OHS may be only moderately reduced, this cannot be used in the differential diagnosis of these two forms. Furthermore, these markers are unreliable during the first months of life due to low levels in all newborn babies [192].

Deficiency of dopamine β-hydroxylase (DBH) in MD patients is reflected by an abnormal pattern of catecholamine metabolites in blood and cerebrospinal fluid. Kaler and his colleagues have demonstrated that the ratio of the catecholamine precursor, DOPA (dihydroxyphenylalanine), to the metabolite DHPG (dihydroxyphenylglycol) is a good indicator of intraneuronal DBH deficiency [50,193]. This ratio also has a diagnostic value in MD, especially in the newborn period, where low copper and ceruloplasmin levels are not reliable.

Tissue Culture

The abnormal accumulation of copper by cells is reflected in tissue culture (fibroblasts), and this can be used as a very reliable marker for confirmation of a clinical suspicion. In contrast to the use of serum copper levels, both MD and OHS can be safely diagnosed this way, but the test cannot distinguish between classical cases and milder forms [49,60].

The test is based on two parameters, increased accumulation and retention of copper in cultured cells, both of which are consequences of impaired efflux [194]. Accumulation is usually measured in cultured fibroblasts after a 20 hour pulse using radioactive copper, followed by a 24 hour chase period. Evaluation of the results needs expertise, and tissue culture diagnosis has therefore been confined to a few specialized centers worldwide. The short half-lives and low specific activities of the copper isotopes available (^{64}Cu: $T_{1/2}$ 12.8 hours; ^{67}Cu: $T_{1/2}$ 61.8 hours) have further limited the use of these diagnostic methods.

An additional test has been developed based on the uptake and retention of silver. The tissue culture phenotype observed using silver is very similar to that obtained with copper [195]. Silver is also a heavy metal with many biological properties similar to those of copper, but its radioisotope has a much longer half-life ($^{110\ m}$Ag: $T_{1/2}$ 255 days). It is, however, more radiotoxic than the short-lived copper isotopes and requires high biological containment as a safety precaution, which limits its widespread use.

Molecular Diagnosis

Molecular diagnosis of MD is established by identifying the disease-causing mutation in the *ATP7A* gene. The extreme heterogeneity observed among *ATP7A* mutations and the length of the *ATP7A* mRNA may, however, diminish the immediate prospects of developing a rapid genetic test for MD. Therefore, in a family in which the mutation is unknown, the coding sequences and the splice sites of noncoding sequences are screened by a mutation screening technique, such as SSCP (single-strand conformation polymorphism) or ddF (dideoxy fingerprinting), followed by direct sequencing of the regions showing an abnormal band pattern. Because a large proportion of the known mutations have been found in exons 7–15, it is appropriate to start screening from these exons and subsequently to expand the analyses to other exons and untranslated regions. When the mutation within a family is known, this mutation is directly analyzed on the referred specimen (skin fibroblasts, leukocytes, chorionic villi, or amniotic fluid cells). Genetic testing is currently only performed in a few laboratories worldwide.

PRENATAL AND CARRIER DIAGNOSIS

Both severe and mild forms of Menkes disease (MD) are devastating diseases without effective treatment, and their occurrence may impose a severe psychosocial stress on the family. Prenatal diagnosis and carrier detection therefore become an important preventive aspect (Table 4).

Prenatal Diagnosis

Because only limited success has been achieved with copper therapy, prenatal detection is of importance in at-risk families, and both biochemical and molecular studies can be applied to prevent the birth of another affected child. Only male fetuses need to be evaluated, and rapid sexing can be achieved using Y-chromosome-specific DNA sequences.

Prenatal diagnosis using biochemical methods has been an option for many years [196]. Like most other cell types, amniotic fluid cells and chorionic villi sequester large amounts of copper, making both first-trimester and second-trimester diagnosis a possibility [197–199].

In the first trimester, the total copper content in chorionic villus samples can be measured directly by neutron activation analysis. However, due to the limited amount of tissue used (about 20 mg), a significant risk of false-positive results through contamination with exogenous copper exists. Copper may originate from the utensils used to handle the tissue specimen as well as from the solutions used. Handling of chorionic villus samples therefore requires special caution to prevent contamination [199].

The method for second-trimester prenatal diagnosis is similar to that described for diagnosis using cultured fibroblasts (see "Tissue Culture"). However, the incorporation of copper into cultured amniotic fluid cells is less pronounced than in fibroblasts, and this problem tends to get worse with increasing gestational age. Early amniocentesis is therefore recommended.

TABLE 4. Carrier Diagnosis and Prenatal Diagnosis

	Prenatal	Carrier	Comments
Copper accumulation			
Tissue copper	Chorionic villi	Not used	CAUTION! Risk of false-positive results*
Radiolabeled Cu/Ag	Amniocytes	Not discriminatory	Carrier diagnosis complicated by X-inactivation
DNA analysis for known mutations			
Point mutation	Applicable	Applicable	
Partial gene deletion	Applicable	Not applicable**	Contamination with maternal tissue deleterious in PD
Linkage analysis in familial cases			
Val767Leu polymorphism	Applicable	Applicable	Only in a core family***
Intragenic CA repeats	Not applicable	Applicable	Too many ''shadow'' bands for safe reading in PD

*Contamination by exogenous copper in chorionic villi.

**Occasionally carriers can be detected (Southern blot; deletion spanning).

***High gene frequency in the normal population.

PD: prenatal diagnosis.

With the isolation of *ATP7A* and the determination of its exon/intron structure, the use of routine DNA-based methods has now become possible [194]. Due to the size of *ATP7A* and the diversity of mutations observed, the family-specific mutation must be identified before prenatal testing. In first-trimester diagnosis, incomplete separation of maternal deciduum can be a significant source of error. Due to the sensitivity of PCR-based methods, contamination of fetal DNA by maternal DNA may be deleterious. Genotyping with several autosomal markers may minimize this risk.

Carrier Diagnosis

Reliable determination of carrier status in a female is important for clarification of the true risk of having an affected child. Heterozygous females may show phenotypic signs such as hypopigmented areas of the skin [200] and mild hair changes [201], but generally female carriers show no obvious clinical manifestations and, as expected, serum copper and ceruloplasmin levels are normal.

Some female carriers show abnormal handling of copper in tissue culture, indicating carriership. The absence of a mutant cellular phenotype in a woman at risk will, however, not exclude heterozygosity. Due to random X-inactivation, a significant proportion of female carriers will go undetected when applying a method dependent on gene expression.

The situation has changed dramatically with the cloning of the gene, and the availability of direct testing for mutations in *ATP7A* constitutes a major advance in carrier diagnosis. It is now possible, in most cases, to define a woman's carrier status unequivocally. It will even allow carrier detection in families with only a single affected male.

In families where the mutation has not been detected, indirect testing for carrier status by linkage analysis is possible. However, carrier detection of this type is limited to familial cases and cannot be used in families with single affected males in whom the disorder may be the result of a new mutation. In addition, successful linkage analysis is dependent on the availability of informative family members for study [202].

Polymorphic markers within the gene constitute the optimal basis for a reliable linkage analysis, and *ATP7A* contains a common amino acid polymorphism, Val767Leu [151] in exon 10, that can be used for linkage analysis in a core family. Due to a high frequency in the normal population, this marker cannot safely be applied for carrier diagnosis in more distant female relatives. Two highly polymorphic intragenic ''microsatellite'' markers comprising dinucleotide (CA) repeats are present in introns 2 and 5, respectively [203], and can be applied for carrier diagnosis in families with a positive family history.

As with any lethal X-linked disease, two-thirds of new cases of MD can be predicted to have inherited the disorder from their carrier mother, while one-third represent sporadic cases [204]. In the one-third of cases for which the mother cannot be shown to be a carrier, gonadal mosaicism may result in the birth of another affected child [205]. Prenatal monitoring may therefore still be indicated in these mothers.

TREATMENT

The treatment of Menkes disease (MD) is mainly symptomatic. Clinical reports suggest that care is an important factor in elongating survival. Copper treatment has been useful in correcting the neurological symptoms in a few cases, when given at a very early stage, but it cannot yet be accepted as a definitive cure.

Symptomatic Treatment

Seizures are usually resistant to antiepileptic drugs but can be controlled in some cases. Chronic diarrhea is a lifelong problem that is difficult to control. Evidence indicates that it may be secondary to dopaminergic imbalance and treatable by administration of L-DOPS (see below) [82]. The urogenital abnormalities with incomplete bladder emptying may cause frequent urinary tract infections that may need prophylactic antibiotic treatment [64,206]. In some cases, surgical correction is necessary. Hernias are also correctable by surgery. There are a limited number of reports describing adverse events in MD patients following surgery, but the high prevalence of seizure disorders, gastroesophageal reflux with the risk of aspiration, and airway complications related to poor pharyngeal muscle control are of anesthetic concern [206]. Postoperative complications include the risk of respiratory failure and precipitation of neurological problems. In addition, stretchable scars, poor wound healing,

a poor immune response, and a bleeding tendency may cause complications [83].

Specific Treatment

Copper Therapy

With the level of current knowledge about the widespread and fundamental disturbance of cellular copper transport in MD, it is not surprising that administration of various copper compounds has been disappointingly ineffective in most cases. However, encouraging long-term clinical outcomes with four early-treated MD patients have been reviewed [82], showing that in some cases copper treatment can moderate the progression of the neurological disease substantially.

A therapeutic trial with copper necessitates consideration of some fundamental issues. First and most importantly, the treatment must be started very early. Once brain damage has developed, copper therapy will not alter the course of the disease significantly. Secondly, the copper compound should be chosen carefully to allow the metal to reach the target sites for incorporation into metalloenzymes. Crossing of the blood-brain barrier is very important, and the intestinal block must also be bypassed by applying copper parenterally. Furthermore, the success of any copper therapy may depend partly on the gene mutation, but this issue requires further research. Finally, treatment should still be regarded as experimental and not as a serious alternative to prevention through prenatal diagnosis.

Several copper compounds have been tried over the years, but copper-histidine has proven most successful, and there are good reasons for choosing it. Copper-histidine is one of the forms in which copper is found in plasma and is possibly of physiological importance. Histidine facilitates copper uptake into liver and brain, suggesting that this may be the form recognized by specific membrane carriers in tissues [49,207].

If started early, parenteral copper-histidine administration can prevent the severe neurodegenerative problems, but the patients may still develop substantial residual abnormalities. The four patients mentioned above who received parenteral copper histidine from an early age have had significantly milder clinical courses with less severe neurological symptoms. All of them have, however, developed connective tissue symptoms to varying degrees, emphasizing that LOX is particularly sensitive to lack of copper.

Parenteral copper-histidine therapy can restore plasma copper and ceruloplasmin levels to normal. Copper therapy will not, however, restore normal copper brain levels [1,208]. Nevertheless, significant cerebral uptake of the metal could be demonstrated at the functional level [208]. Thus, catecholamine metabolism can be modified, although even in well-treated patients symptoms of autonomic failure persist, such as ataxia, orthostatic hypotension, and chronic diarrhea [82].

The efficacy of the copper dose needs to be monitored, mainly by serum copper and ceruloplasmin measurements. Monitoring of liver stores has rarely been used because it involves an invasive procedure. The catecholamine pattern in cerebrospinal fluid may also be a useful indicator for monitoring the effect of copper supplementation [193].

Copper therapy carries the risk of systemic overload. D-penicillamine is a potent copper chelator that is successfully used in Wilson disease [3] and has also been applied in MD to control the body stores [208,209].

Metabolite Substitution

The frequent observation of orthostatic hypotension in MD patients successfully treated with copper-histidine [82] suggests that dopamine β-hydroxylase (DBH) may not be receiving sufficient copper to allow restoration of normal activity. In one such patient, hypotension could be corrected by the administration of L-DOPS (L-threo-3,4-dihydroxyphenylserine), which has been used successfully in patients with autonomic failure due to DBH deficiency [210]. Moreover, this compound could also control chronic diarrhea. L-DOPS is a synthetic amino acid that is converted to noradrenaline by aromatic L-amino acid decarboxylase, which is present in all adrenergic cells. In this way, the requirement for DBH can be bypassed. L-DOPS is efficiently absorbed in the small intestine and readily crosses the blood-brain barrier. Oral administration may therefore be used to restore normal central and peripheral noradrenaline levels.

Cerebellar ataxia may also be partly due to deficient DBH activity, but the administration of L-DOPS may not be very beneficial. The cerebellum of DBH knockout mice has fewer noradrenergic terminals [128], and this region appears to be very resistant to the restoration of normal noradrenaline levels using the L-DOPS precursor. The persistent occurrence of ataxia in a well-treated MD patient receiving L-DOPS [82] is in line with this observation.

Antioxidant Supplementation

Protection against oxidative stress is defective in MD due to the malfunctioning of several enzymes involved in free radical detoxification. The administration of antioxidants such as oral vitamin C and vitamin E may constitute important adjunctive therapy, although this has attracted little attention. Vitamin C has, however, been given with variable results to a few classical cases because of the similarity between the skeletal changes observed in MD and scurvy [211,212].

RECENT DEVELOPMENTS

Several reviews have recently been published on copper metabolism [213–217].

Copper Pathway

CTR1 (Cu transporter 1) has been demonstrated to promote copper uptake into human fibroblasts [218] and mouse cells [219], and targeted gene disruption in the mouse has shown it to have an essential role in early embryogenesis [219, 220] and to affect copper accumulation in certain tissues in heterozygous animals. Defective embryogenesis in these animals is probably due to the cumulative lack of several copper-dependent enzymes. Deficient development of the mesoderm and mesoderm-derived structures, and the severe basement membrane defects observed in CTR1 knockout mice [220], may thus be the result of deficient lysyl oxidase activity.

In mice, disruption of *Atox1* (*Hah1* in the earlier nosology) leads to a phenotype similar to that of the mottled mouse, although less severe than observed in some of the allelic variants [221]. This indicates that it may still be possible to deliver copper to ATP7A by means other than ATOX1, perhaps as a Cu-GSH complex. The intra-peritoneal administration of copper leads to improved survival of affected mice, and the normalization of pigmentation that occurs supports this hypothesis. Cultured cells from *Atox1* knockout mice show increased copper retention similar to that observed in Menkes fibroblasts. The phenotype of the

Atox1 knockout mouse can thus be attributed to deficiency of copper-dependent enzymes by analogy with Menkes disease. In support of this presumption, decreased activities of tyrosinase, in skin, and cytochrome c oxidase, in brain, are demonstrable. The mice also suffer from severe hemorrhage, which may be explained by defective delivery of copper to factors V and VIII in the liver. These results emphasize that ATOX1 is the chaperone for copper delivery to both ATP7A and ATP7B.

The crystal structure of copper-containing ATOX1 has been established, and provides structural support for direct metal ion exchange between the conserved copper-binding motif of the chaperone and its target protein [222]. The interaction between two such molecules requires copper [223–225]. This is in line with the metal transfer mechanism proposed for the exchange of copper between ATOX1 and ATP7A (or ATP7B) [226], which is also potentially applicable to intramolecular copper transfer between individual metal-binding repeats in ATP7A. The vectorial delivery of copper is not based on a higher affinity of the target molecule, but rather on rapid metal transfer to the recipient partner. Thus, chaperones possess a catalytic function similar to that of enzymes [227].

The repetitive metal-binding domains in ATP7A seem to act as sensors of low concentrations of copper and to activate the protein, but seem not to be essential for overall ATPase catalytic activity at basal copper levels [228–230]. Mutation of the six metal-binding motifs resulted in a mutant transport molecule with a lower affinity for copper than the wild-type protein, indicating that these motifs may be internal regulators of the pump function [229]. Binding of copper to these high affinity copper sensors facilitates catalysis, probably by altering the conformation of the molecule and thereby initiating phosphorylation and transport through the channel, as well as trafficking of the protein [230,231]. The six metal-binding repeats thus possess a dual role, regulating both the catalytic activity and the intracellular localization of the protein by controlling its phosphorylation. Cycling between the plasma membrane and the *trans*-Golgi network occurs at both basal and elevated copper levels [232].

Copper Pathway in Mitochondria

Mutations in *SCO1* and *SCO2* have been shown to result in two distinct disease phenotypes, early onset hepatic failure with encephalomyopathy [233] and fatal hypertrophic cardiomyopathy with encephalopathy (MIM 604377) [234,235], respectively.

Copper Activation of Enzymes

Direct evidence for the activation of tyrosinase by ATP7A has been obtained by transfection studies [236]. In Menkes fibroblast cell lines, tyrosinase is inactive, whereas in normal fibroblasts its activity is substantial. The co-expression of ATP7A and tyrosinase in Menkes fibroblasts was shown to lead to the activation of tyrosinase and subsequent melanogenesis.

Sulfhydryl oxidases contribute to disulfide bridge formation by catalyzing the oxidation of sulfhydryl groups in disulfides. The role of copper in activation of a subset of this group of enzymes has mainly been deduced indirectly, and we have recently reviewed the current state of knowledge [237]. Sulfhydryl oxidases have been purified from a number of mammalian sources, but a possible copper-dependent form has only been described in skin [238].

The *LOX* Gene Family

Besides its well-known role in extracellular matrix formation, several new biological functions have been attributed to LOX, ranging from developmental regulation to tumor suppression and cell growth control [239]. Apart from *LOX*, four distinct genes belonging to the expanding LOX gene family have been isolated: lysyl oxidase-like genes 1,2 and 3 (*LOXL1*, *LOXL2*, and *LOXL3*, respectively) [240–244] and lysyl oxidase-related gene, *LOR* [245]. The products of these new members may contribute to the diverse functions attributed to LOX. The predicted amino acid sequences of the products of all members of this gene family contain a copper-binding site and the two conserved amino acids (Lys and Tyr) that are known to participate in the formation of the intramolecular quinone cofactor (LQT). All these enzymes, except for LOXL2, which lacks a hydrophobic export signal sequence necessary for extracellular transport, are likely or known to act extracellularly. LOXL1 apparently cross-links type III collagen [246], and it has been suggested that LOR might be involved in cell adhesion and senescence [245]. High expression levels of LOXL2 in reproductive tissues suggest a role in the reproductive system. LOXL3 is expressed in several tissues, but no specific function has yet been attributed to it [243,244]. Lysyl oxidase (and hence also each other member of this gene family) is particularly sensitive to impaired delivery of copper, because the metal has an essential role in biogenesis of the cofactor [247]. Although the mechanisms by which copper is delivered to lysyl oxidase are still not well studied, it is likely that ATP7A is involved in this process, as LOX secretion and copper efflux use the same pathway. This hypothesis is supported by studies in rat which showed that the relative levels of LOX and ATP7A mRNA transcripts were quantitatively similar throughout embryonic and early fetal life [248].

Mutation Update

Thirty six additional mutations in *ATP7A* have been reported. These include mutations in three OHS patients, one mildly affected patient, and one early-treated patient [249–256]. Two of the OHS patients had the same splicing mutation, but showed some difference in its phenotypic expression [250,252]. The milder case had normal serum copper and ceruloplasmin levels, and about 19% normally spliced mRNA transcript [252]. The more severe case, however, had only 2–5% correctly spliced *ATP7A* and also showed decreased serum copper and ceruloplasmin levels [250]. This suggests that minute amounts of ATP7A are sufficient to prevent the development of a severe phenotype and will lead to milder symptoms

The third OHS patient had a small deletion in the last exon of the gene, leading to a truncated protein lacking the di-leucine motif that is important for retrieval of the protein from the plasma membrane [254]. When ATP7A is located principally at the plasma membrane, lysyl oxidase will receive insufficient copper for normal activity, explaining the development of connective tissue symptoms, whereas other secreted enzymes, such as peptidyl α-amidating enzyme and dopamine β-hydroxylase, may still receive sufficient copper for near normal function.

The mildly affected patient previously described clinically [71,72] was found to have an amino acid substitution in the highly conserved seventh transmembrane domain (TM7) of ATP7A [249]. Its Golgi localization was preserved, suggesting that the defective protein may deliver sufficient copper

to lysyl oxidase to prevent the development of severe connective tissue symptoms.

The early-treated case (GV) [82] was found to have an insertion of a single base in exon 22 leading to a truncated protein lacking the last two transmembrane domains [249]. This suggests that when excess copper is provided as Cu-histidine, the truncated protein can still translocate copper across the membrane, as reflected by the patient's mild symptoms [249].

A study showing partial gene deletions in *Atp7a* has expanded the mutation spectrum of mottled alleles [257]. In mottled mice, three distinct groups of phenotypic severity can be delineated, partial gene deletions occurring in the most severely affected group with embryonic lethality [257]. In contrast, Menkes patients with similar gene deletions survive beyond infancy, and caution should therefore be applied when extrapolating from mottled mice to the human disease [257].

Early Symptoms in Menkes Disease

Congenital skull fractures acquired during or around the time of delivery represent a rare perinatal complication of Menkes disease, and their occurrence should raise the suspicion of this severe disorder in newborns [258]. Small occipital horns have led to the biochemically confirmed diagnosis of the mildest form of Menkes disease in a 2-year-old boy [259].

Follow-up on Treated Patients

Two patients treated early with copper histidine [82] have died suddenly. MF died at age 20, but autopsy that could have established the cause of death was not performed (J. Clarke, personal communication, 2000). RH died at 10 years of age [260]. He suffered from recurrent, and ultimately fatal, peritonitis secondary to a ruptured bladder diverticulum. At autopsy, occipital horns not present at eight years of age were observed. The third patient described by Christodoulou et al. [82], (GV), had developed occipital horns at eight years of age and suffered from marked connective tissue abnormalities, but was otherwise reported to be doing well at 11 years of age [249].

PATIENT SUPPORT GROUPS

Friends of Alexander Deihl — www.friendsofalex.org

Climb — www.climb.org.uk

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5720 Buckfield Ct.
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Support eGroup
http://groups.yahoo.com/group/Menkes_kinky_hair

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Chapter 15

Epidermolysis Bullosa

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SUMMARY

- **Epidermolysis bullosa (EB) is a clinically and genetically heterogeneous group of inherited disorders that are characterized by blistering of the skin and certain other tissues. In all subtypes, the blisters result from minor trauma, although the severity of expression ranges from mild occasional blistering to severe bulla formation, erosions, scarring, and mutilation. The group contains many distinct disorders and, to date, defects in ten different genes are known to lead to the clinical abnormalities.**
- **Classification of this heterogeneous group is problematic because of the multitude of clinical presentations and the number of genetic and molecular abnormalities. The currently used practical classification is based on categorization according to the precise level of blister formation in the dermal-epidermal junction (DEJ) zone and subsequent clinical presentation. Three major categories are defined.**
- **EB simplex (EBS) exhibits trauma-induced skin blistering as a consequence of cytolysis of basal keratinocytes. Keratin intermediate filament scaffolding, which sustains the three-dimensional structure of the cells, is weakened due to defects of the keratins or their linker proteins and collapses as a result of external shearing forces. The blistering is intraepidermal, and healing takes place without scarring. In general, the prognosis is favorable, and blistering activity diminishes with age. Defects in the genes for keratins 5 and 14 and plectin have been shown to underlie several of the subtypes.**
- **Junctional EB (JEB) is a heterogeneous group with extreme clinical variation and defects in at least six distinct genes. In the most severe subtype, JEB Herlitz, the course is usually lethal within two years after birth. In contrast, JEB progressiva only manifests at school age and progresses slowly. Healing takes place without scarring. Nail and dental problems as well as alopecia are often associated with this category. The hemidesmosome-anchoring filament complex is weakened by genetic defects of one of the structural proteins, resulting in epidermal detachment. Mutations in the genes for laminin 5, α6β4 integrin, and collagen XVII cause different JEB subtypes.**
- **Dystrophic EB (DEB) is characterized by dermal blistering below the basement membrane and subsequent scarring. Within this category, the clinical presentation varies from localized mild acral involvement to generalized blistering, scarring, and disabling mutilation of hands and feet. Mucous membranes are often involved, nails shed, or dystrophic and dental problems common. Abnormalities of the anchoring fibrils and mutations in *COL7A1*, the gene encoding collagen VII, the major anchoring fibril protein, underlie all subtypes. Traditionally, DEB contained two dominantly and four recessively inherited subtypes, but molecular genetic studies have shown that dominant and recessive mutation combinations also exist.**
- **Extracutaneous abnormalities can be associated with EB because the basement membrane zones of the eye, the mucous membranes of the orifices, the gastrointestinal, respiratory, and urogenital tracts, and even of muscles are similar to that in the skin.**
- **For the diagnosis of EB, a combination of the family history, clinical examination of the index patient and family members, a skin biopsy for antigen mapping and eventually for electron microscopy and cell cultures, and blood samples for mutation analysis are required.**
- **At present, the management of EB is mainly supportive and therapy symptomatic because no specific cure exists. A high standard of personal hygiene and daily skin care, protection from trauma, and avoidance of infection are fundamental for optimal management. In some cases, surgical release of contractures and skin grafting have been successful, and secondary growth retardation and anemia in some affected children have been reversed using additional feeding via a gastrostomy tube. For the future treatment of EB, the development of successful gene therapy approaches is required. Although the application of gene therapy for EB may still be years away, the rapid development of new technologies holds**

[1] *Abbreviations used are*: EB, epidermolysis bullosa; EBS, EB simplex; EBS-L, EB simplex localisata; EBS-MD, EB simplex with muscular dystrophy; JEB, junctional EB; JEB-PA, junctional EB with pyloric atresia; DEB, dystrophic EB; GABEB, generalized atrophic benign EB; DEJ, dermal-epidermal junction; PTC, premature termination codon; CSGE, conformation sensitive gel electrophoresis; PGD, preimplantation genetic diagnosis; TBDN, transient bullous dermolysis of the newborn.

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promise for individually designed and biologically valid curative treatments for different EB subtypes.

INTRODUCTION

Definition

The term epidermolysis bullosa (EB)[1] refers to a clinically, genetically, and biologically heterogeneous group of inherited disorders that are characterized by blistering of the skin and certain other tissues with stratified epithelia. The blisters usually result from minor trauma but may appear spontaneously. This characteristic is common to the whole group, although the severity of expression ranges from mild occasional blistering to severe extensive bulla formation, erosions, scarring, and mutilation [1,2]. The group contains many distinct disorders and, to date, defects in ten different genes are known to lead to the clinical abnormalities [3].

Enormous progress has been made since the mid-1990s in understanding the molecular basis of EB, and some of the old concepts about this disease have had to be modified. Classification of this heterogeneous group is problematic because of the multitude of clinical presentations and the number of genetic, biological, biochemical, and morphological abnormalities continually being detected. The currently used practical classification was suggested by Gedde-Dahl Jr. [2] and is based on categorization according to the precise level of blister formation in the dermal-epidermal junction (DEJ) zone and subsequent clinical presentation. Three major categories are defined: in EB simplex (EBS), the separation occurs within the basal keratinocytes, as a consequence of cytolysis of the cells; in junctional EB (JEB), the cleavage occurs along the lamina lucida; and in dystrophic EB (DEB), the cleavage occurs below the basement membrane, within the uppermost dermis. Predictably, abnormality of any molecular component of the dermal-epidermal junction zone can lead to diminished adhesion of the skin layers and blistering. The multitude of pathologic alterations is alluded to by the extensive clinical heterogeneity of EB, and more than 20 genetic and clinical subtypes are known [3–5]. In Table 1, the clinical subtypes of EB and their underlying molecular defects are summarized. The present classification may have to be modified after more complete data on the abnormal molecules and the genes coding for them become available.

Investigations into EB by ultrastructural analysis, determination of candidate genes, and isolation and characterization of target proteins have also had a major impact on our understanding of the normal biology of the dermal-epidermal junction, of epithelial-mesenchymal interactions, and of epithelial cell adhesion in general. Data on the molecular basis of blistering diseases have already disclosed ample new information about the physiological functions of many of the molecular components of the dermal-epidermal junction, and a rapid expansion of knowledge in the field can be anticipated when genotype-phenotype correlations of more patients are discerned. Further, these new data have allowed the characterization of autoantigens in acquired autoimmune blistering skin diseases, making better diagnostic procedures and the design of novel therapeutic strategies possible.

History

First descriptions of EB appeared in the medical literature more than 100 years ago [6,7]. The name epidermolysis bullosa refers to the mechanically induced detachment of the epidermis from the dermis, into the blister roof, and was coined for these conditions by Köbner [6]. Despite modern knowledge about the genetic and biological heterogeneity of the disease group, the name has stayed.

EB subtypes that have long been characterized have been assigned numbers in McKusick's *Mendelian Inheritance in Man* [8], the on-line version of which, OMIM, can be found under http://www.ncbi.nlm.nih.gov/Omim. The MIM numbers do not exactly correspond to the present clinical classification, and some confusion results, for example from the fact that two separate dominant disorders, the localized EBS and the localized DEB, have received the same number (MIM 131800).

Other Names and Synonyms

Many of the EB subtypes have been called by the name of their first describer. In this chapter, in the subheadings, following the names of the EB subgroups, synonyms used to denote the disorder and the abbreviations commonly used, as well as the MIM numbers, are written in parentheses. The first letters of each abbreviation describe the mode of inheritance (i.e., AD for autosomal dominant, AR for autosomal recessive, and XL for X-linked).

GENERAL ASPECTS OF EPIDERMOLYSIS BULLOSA

Dermatological Features

Since the first description of EB, the group of clinical entities has grown to contain three major categories and more than 20 clinically and genetically defined subtypes (Table 1). The clinical symptoms in the major categories were first believed to be constant and pathognomonic. However, in recent years, many such clinical concepts have had to be modified. Based on reports of mutations and examination of a growing number of patients, it has become clear that phenotypic variability is more extensive than thought before. Traditional characteristic signs, such as milia, scarring, and albulopapuloid lesions, have been described as secondary changes in "nontypical" EB subgroups, and children with different EB subtypes can have indistinguishable cutaneous features during the neonatal period. Thus, clinical diagnosis alone is no longer sufficient, and immunopathological, and eventually ultrastructural, investigations are necessary for classification. Mutation analysis is required for ultimate confirmation of a diagnosis. In the following, the typical clinical signs of the different EB categories are commented on.

Blistering

Blisters usually result from minor trauma but may appear spontaneously. This characteristic is common to the whole group, although the severity of expression ranges from mild occasional blistering to the formation of severe extensive bullae in the skin and mucous membranes. The blisters are filled with a clear serous liquid but can occasionally be hemorrhagic. If a fresh blister is left unopened, it has a tendency to enlarge centrifugally. It is not possible to differentiate clinically by naked eye between blisters within the epidermis, in the basement membrane, or below it (i.e., between the different EB categories).

Scarring and Milia

Scars and milia are typical secondary phenomena in DEB but occur in other subtypes as well, often as a result of secondary traumatization and chronic inflammation. Any wound that reaches into the dermis, below the basement membrane, heals with scarring. Most EB children, regardless of subtype, have temporary milia at trauma-prone sites. In the most severe forms of recessive dystrophic EB, DEB

TABLE 1. Subtypes of Epidermolysis Bullosa

Subtype	Gene	Chromosomal Locus	Inheritance	Predilection Sites	Remarks, Synonyms
EB Simplex (EBS)					
EBS generalisata	*KRT5*	12q11–q13	AD (AR)*	generalized	Koebner disease
	KRT14	17q12–q21			
EBS localisata	*KRT5*	12q11–q13	AD (AR)	acral	Most common EB subtype, often very mild, Weber-Cockayne disease
	KRT14	17q12–q21			
EBS herpetiformis	*KRT5*	12q11–q13	AD	generalized	Dowling-Meara subtype
	KRT14	17q12–q21			
EBS with ''mottled pigmentation''	*KRT5*	12q11–q13	AD	generalized	Pigment anomalies
EBS with muscular dystrophy	*PLEC1*	8q24	AR	generalized	Late-onset muscular dystrophy
EBS Ogna	*PLEC1*	8q24	AD	acral	Bruising, common in Norway
EBS herpetiformis with ''mottled pigmentation''	Not known		AD	generalized	Pigment anomalies
EBS Kallin	Not known		AR	acral	Anodontia, hair and nail anomalies
EBS Mendes da Costa	Not known		XL	acral	Alopecia, hyperpigmentation
Junctional EB (JEB)					
JEB Herlitz	*LAMA3*	18q11.2	AR	generalized	Lethal within 1–2 years, JEB gravis
	LAMB3	1q32			
	LAMC2	1q25–31			
Generalized atrophic benign EB	*COL17A1*	10q24	AR	generalized	GABEB, JEB atrophicans mitis, alopecia
	LAMB3	1q32			
JEB localisata	*COL17A1*	10q24	AR	acral	No alopecia, nail dystrophy
JEB with pyloric atresia	*ITGA6*	17q25	AR	generalized	Pyloric atresia at birth
	ITGB4	2q24–q31		acral	
JEB inversa	Not known		AR	major flexures	
JEB progressiva	Not known		AR	acral	Late onset
JEB cicatricans	Not known		AR	generalized	Unusual scarring
Dystrophic EB (DEB)					
DEB generalisata	*COL7A1*	3p21.1	AD	generalized	Pasini disease, albulopapuloid papules
DEB localisata	*COL7A1*	3p21.1	AD	acral	Cockayne-Touraine disease
DEB generalisata mutilans	*COL7A1*	3p21.1	AR	generalized	Hallopeau-Siemens subtype, mutilation
DEB generalisata nonmutilans	*COL7A1*	3p21.1	AR	generalized	No synechia
DEB localisata	*COL7A1*	3p21.1	AR	acral	
DEB inversa	*COL7A1*	3p21.1	AR	major flexures	
Transient bullous dermolysis of the newborn	*COL7A1*	3p21.1	AR/AD	generalized at birth	TBDN, self-limiting course

*AD: autosomal dominant; AR: autosomal recessive; XL: X-linked.

generalisata mutilans and DEB generalisata nonmutilans, significant scarring is a constant feature of the disease, often also causing joint contractures. In the mutilans subtype, the fingers and toes are fused together and covered with an atrophic, scarred, and nailless skin, a phenomenon for which the term "mitten hands" has been coined. In certain generalized JEB subtypes, such as generalized atrophic benign EB, a moderate generalized atrophy of the skin develops in the course of the disease.

Nail Changes

Nail changes in EB are characteristic and sometimes highly suggestive, but not pathognomonic [9]. They result from abnormalities of the nail matrix and nail bed due to the genetic defects underlying EB. In addition, secondary traumatization in areas of epidermal-dermal separation and chronic inflammation of the nail matrix contribute to the development of nail dystrophy in nonscarring forms of EB also. This sometimes creates unexpected and confusing phenotypes. In EBS, the nails usually remain normal. Regrowth and regeneration follow nail loss after blistering. Onychodystrophy may represent pathologic signs induced by injury to the nail matrix or scarring of the nail bed. The fact that big toe nails are often affected supports this hypothesis. In the most severe JEB subtype, JEB Herlitz, nail involvement typically begins early as paronychia-like periungual lesions. These are followed by loss of nails due to extensive blistering and the development of large plaques of granulation tissue that cover most of the dorsal finger tip. Most JEB subtypes are associated with generalized nail dystrophy. The nails are often lost after subungual blistering and regrow normally at the beginning, but they become thickened, dome-shaped, or sometimes onychogryphotic in the course of the disease. The rare JEB progressiva can show dystrophy and loss of nails as first symptoms in the teens without any other signs of skin disease, and acral blistering may follow years later. In the most severe forms of recessive DEB, loss of all nails is a constant feature. Repeated, extensive blistering and scarring lead to permanent loss of the germinative nail matrix. In other DEB subtypes, dystrophy and loss of some nails due to scarring, atrophy, and hyperkeratosis of the germinative matrix and the nail bed are also common. In individuals with localized DEB with mild expression, some or all nails may remain normal or, on the contrary, nail dystrophy may be the only symptom of DEB.

Hair Anomalies

The most dramatic hair anomalies are seen in the JEB subtype called generalized atrophic benign EB, GABEB. A nonscarring, diffuse, or parietal alopecia develops at school age, often leading to total alopecia by the teenage years or early adulthood. Here again, clinical heterogeneity is evident, with not all patients showing a similar degree of alopecia. The reason for the loss of hair is not known. Mild to moderate scarring alopecia is typical in DEB.

Dental Anomalies

Dental anomalies are common in EB (e.g., enamel defects and dystrophy of the teeth). Because the tooth anlage during development contains an epithelial-mesenchymal interface and a basement membrane very similar to that of the skin, anomalies of the structural components lead to abnormal development of the teeth, with enamel hypoplasia as the most common symptom [3,4,10,11]. In addition, gingival erosions, bleeding, and pain impede dental hygiene, with insufficient dental care and extensive caries as consequences [11]. More recent studies on heterozygous carriers suggest that enamel defects may represent a minimal symptom of mutations in the genes coding for structural proteins of the DEJ.

Pigment Anomalies

Hyperpigmented maculae can be associated with several EB subtypes. EBS with mottled hyperpigmentation and EBS herpetiformis with mottled hyperpigmentation present with light brown, ephelis-like spots with bizarre configurations on the trunk. The hyperpigmentation seems to be associated with the blistering, but its biological basis remains elusive. In GABEB, dark brown pigmented maculae or nevi are sometimes seen. Early reports on this condition described hyperpigmentation as an obligatory sign of the subtype [12], but observations of more patients have shown that many lack such maculae. As an unspecific sign, reversible diffuse, postlesional hyperpigmentation can be associated with many EB subtypes.

Hyperkeratosis

In EBS, hyperkeratosis of the palms and soles is a typical sign and a common problem. Pressure-induced hyperkeratosis is often a consequence of repeated blistering and can be very painful. In contrast to normal skin, which reacts to strong mechanical stimulus such as walking or manual labor with hyperkeratosis, EBS-associated hyperkeratosis is induced by minimal mechanical trauma.

Skin Cancer

Statistical analysis has revealed a high incidence of squamous cell carcinoma in patients with generalized DEB in early adulthood [13]. Repeated blistering and scarring contributes to malignant transformation of the epithelial cells, and even today many patients develop metastasizing carcinomas before the 40th year of life. DEB patients seem to be most at risk, although one report describes multiple squamous cell carcinomas also in two patients with GABEB [14].

Extracutaneous Features

General Aspects

Because the basement membrane zone at the dermal-epidermal junction is similar to that of the eye, the mucous membranes of the orifices, the gastrointestinal, respiratory, and urogenital tracts, and even muscles to some extent, abnormalities of these organs can be associated with EB. Extracutaneous involvement of all types of epithelia has been observed in EB despite the fact that this disorder is commonly considered to affect only the stratified squamous epithelia of the skin and related mucous membranes [15]. It is possible that deeper epithelia are more extensively involved than is currently recognized, thus leading to the different extracutaneous symptoms of EB [3–5,15–18].

Gastrointestinal Complications

In both JEB and DEB, affection of at least some parts of the gastrointestinal tract is common. Blistering of the oral or laryngopharyngeal mucosa causes hoarseness and pain and disturbs feeding. Scarring of the oral mucosa and the lingual band restricts opening of the mouth and movement of the tongue and may lead to speech difficulties. Esophageal involvement, in particular in DEB in the form of strictures, and occasionally in JEB, causes difficulties in swallowing. Anal blisters, fissures, and strictures lead to painful and difficult defecation. If these symptoms are strong, they result in intake of liquid, low-fiber foods with insufficient nutritional content. Dietary problems, together with protein

loss through the blisters and erosions, can lead to anemia and growth retardation, typically seen in DEB generalisata mutilans and nonmutilans.

In a rare JEB form, pyloric atresia (PA) is associated with skin blistering [19]. In severe cases of this subtype, JEB-PA, the association is evident and the diagnosis clear. In milder phenotypes, only pyloric atresia, and not skin blistering, is present at birth. The skin phenotype may develop later, during the first months or even years of life [20].

Neuromuscular Features

A distinct EBS variant, EBS with muscular dystrophy (EBS-MD), manifests relatively mild blistering at birth and late-onset muscular dystrophy, which is progressive and leads eventually to patients being wheelchair-bound [21]. This phenotypic constellation was initially puzzling, and it was suggested that the occurrence of these two relatively rare clinical conditions, EB and muscular dystrophy, may be coincidental because no clear explanation was apparent [21]. However, it is now known that mutations in the gene for plectin, which is expressed in both skin and muscle, underlie this variant of EB [22].

Ophthalmological Aspects

Blistering of the corneal and conjunctival epithelium manifests with strong pain. Patients typically cannot open the eyes, and they keep them closed for a day or two until reepithelialization has taken place. The ophthalmologist observes erosions, occasionally a blister, and a conjunctival redness. The erosions usually heal well, without affecting visual acuity [16].

Abnormalities of the Urogenital Tract

In different JEB subtypes, erosions and/or subepithelial cleavage may be present in the urinary tract. Dysuria and secondary urinary tract obstruction caused by meatal or other stenosis, as well as dilatation of the proximal urogenital tract, are sometimes seen. Certain facultative features are unique to JEB with pyloric atresia (e.g., obstruction of the ureterovesical junction) [23].

The Dermal-Epidermal Junction

Before discussion of ultrastructural, immunopathological, and molecular abnormalities in EB, a short review of the normal dermal-epidermal junction (DEJ) [24–26] is useful. This zone is the site of attachment of the epithelium to the dermis and is pathologically altered in EB. Figure 1 summarizes in a schematic way the microanatomy of the zone, as it appears in the electron microscope, and the location of some of its structural macromolecules. The basal epithelial cells are attached to a bilaminar basement membrane consisting of the 25–50 nm in wide, optically translucent lamina lucida and, below this, the optically dense

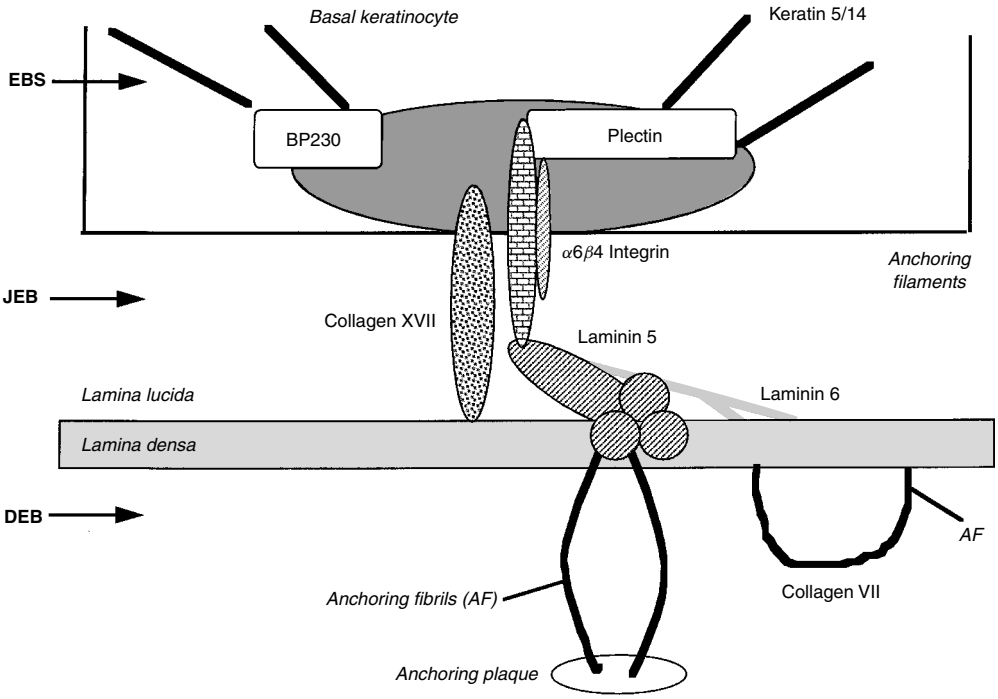

Figure 1. A schematic representation of the anchoring complex at the dermal-epidermal junction. The anchoring complex is an ultrastructurally recognizable unit, comprising hemidesmosomes, anchoring filaments, and anchoring fibrils. Hemidesmosomes (gray oval) at the basal surfaces of the basal keratinocytes connect the intracellular cytoskeleton to anchoring filaments that traverse the lamina lucida and connect with the anchoring fibrils (AF), which originate in the lamina densa and either end in anchoring plaques (morphologically defined electron-dense, plaque-like structures in the papillary dermis) or loop back to the lamina densa. Keratins 5 and 14 are the major components of the intermediate filaments in basal keratinocytes. Plectin and BP230 (bullous pemphigoid antigen-1, BPAG-1) are structural proteins of the hemidesmosomal plaque together with the intracellular domains of collagen XVII (BP180, bullous pemphigoid antigen-2, BPAG-2) and α6β4 integrin. The extracellular domains of these two transmembrane proteins, together with laminins 5 and 6, form the anchoring filaments. Collagen VII is the major structural component of the anchoring fibrils, which often enmesh dermal collagen fibrils, thus ensuring the connection between the anchoring complex and the dermal extracellular matrix. Integrin α3β1, also a component of the basal keratinocyte surface, is located outside the anchoring complex and is not shown in this figure.

lamina densa, 20–50 nm in width. The attachment of the basal keratinocytes to the basement membrane is mediated by the so-called anchoring complex, which consists of the hemidesmosome and the underlying anchoring filaments that traverse the lamina lucida and insert into the lamina densa. The basement membrane is joined to the dermal connective tissue by a three-dimensional network of anchoring fibrils, which is intersected by interstitial collagen fibers and other microfibrillar structures. The anchoring fibrils are fibrous aggregates, 20–75 nm in diameter, with a nonperiodic cross-striated banding pattern and frayed ends [27]. The upper ends insert into the lamina densa and, at their lower ends, the fibrils either insert into so-called anchoring plaques or loop back to the lamina densa [28–30]. The distinct ultrastructural units of the DEJ result from highly specific protein interactions of the structural macromolecules of the zone.

Many of the molecular components of the DEJ (Table 2) have been characterized at the protein and cDNA levels (for reviews, see [5,24–26,28,30]). Important hemidesmosomal proteins are the intracellular bullous pemphigoid antigen-1, or BP230, and plectin, and the transmembrane components α6β4 integrin and BP180/collagen XVII, which together form the intracellular hemidesmosomal plaque. Together with laminins 5 and 6, the extracellular domains of collagen XVII and α6β4 integrin are likely to participate in the formation of the anchoring filaments. α3β1 integrin is located in the plasma membrane between the hemidesmosomes. The lamina densa contains a number of macromolecules [26]. Collagen IV is the major structural protein in all basement membranes and provides a basic three-dimensional scaffold through the association of the individual molecules through their globular N- and C-terminal domains. Within the mesh of the network, perlecan, nidogen/entactin, and in the skin also the N-terminal globular domain of collagen VII may be intertwined. The main structural component of the anchoring fibrils, collagen VII, has an extended triple helix and amino- and carboxyl-terminal globular domains. It forms antiparallel dimers with the N-termini pointing outward, and several dimers aggregate laterally to form the anchoring fibrils [30] (see also Chapter 2, this volume). A functionally important affinity between collagen VII and laminin 5 has been described [31,32]. The papillary dermal connective tissue contains a variety of collagens: types I, III, V, VI, XII, XIV, and XVI are built into interstitial collagen fibrils or other fibrillar-filamentous structures. Together with elastin, fibrillins, fibulins, vitronectin, proteoglycans, and other macromolecules, they form the stroma of the dermis [33].

All DEJ molecules have a modular structure, with typical domains of adhesive extracellular matrix components, and are strongly interactive, with multiple binding sites through which they contribute to dermal-epidermal cohesion by chemical and physical interactions. Experimental evidence exists for the following interactions: β4 integrin-plectin [34,35], α6β4 integrin-laminin 5 [36], α6 integrin-collagen XVII [37], β4 integrin-collagen XVII [34], laminin 5-laminin 6 [38], laminin 6-nidogen, nidogen-collagen IV and nidogen-perlecan [26,39], laminin 5-collagen VII, and collagen IV-collagen VII [31,32].

Both keratinocytes and fibroblasts contribute to the synthesis of the DEJ. Ample evidence points to active epithelial-mesenchymal crosstalk in the regulation of DEJ

TABLE 2. Molecular Components of the Dermal-Epidermal Junction

Molecule	Synthezised by	Special Features	Ligands
Intermediate filament components			
Keratin 5	K	Dimer → intermediate filaments	Plectin, BP230
Keratin 14	K	Dimer → intermediate filaments	Plectin, BP230
Hemidesmosomal plaque components			
BP230	K	Cytoskeleton linker, isoforms	Keratins 5 and 14
Plectin	K	Cytoskeleton linker, isoforms	Keratins 5 and 14, β4 integrin
Transmembrane components			
α6β4 Integrin	K	Heterodimer	Plectin, collagen XVII, laminin 5
Collagen XVII	K	Homotrimer, ectodomain proteolytically shed	α6β4 integrin, laminin 5?
α3β1 Integrin	K	Plasma membrane between hemidesmosomes	Laminin 5
Lamina lucida components			
Laminin 5	K	Heterotrimer, major anchoring protein	α6β4 integrin, collagen VII
Laminin 6	K	Heterotrimer, linked to laminin 5	Laminin 5
Lamina densa components			
Collagen IV	K/F	Heterotrimer, forms basic basement membrane scaffold	Nidogen, perlecan
Nidogen	F	Forms a network	Collagen IV, perlecan, laminin 6
BM-40/SPARC	K/F	Calcium binding	
Perlecan	F	Heparan sulfate proteoglycan	Collagen IV, nidogen
Anchoring fibril components			
Collagen VII	K	Proteolytically processed, dimer → fibril	Laminin 5, collagen IV

K: keratinocytes; F: fibroblasts.

formation, the biosynthesis of molecules in one cell type being modulated by soluble signals from neighboring cells or from the extracellular matrix. *In vitro*, depending on experimental conditions, one or another cell type plays a more active role in the production of certain molecules [40–42]. Epithelial cells manufacture collagen VII and most laminin chains, whereas mesenchymal cells synthesize collagen IV, nidogen, perlecan, and the laminin α2 chain [43]. In skin equivalent cultures [42] and developing mouse organs [44], fibroblasts are the only source of nidogen at the epithelial-mesenchymal interface.

An interesting additional regulatory step in the supramolecular assembly of the DEJ is provided by the post-translational proteolytic processing of some components. Such trimming is thought to contribute to the correct association and folding of the polypeptide subunits, because propeptides can recognize binding sites for the accurate alignment of subunits or prevent premature aggregation of the molecules. Two important components of the anchoring complex are subject to such processing, laminin 5 and collagen VII. The 200 kDa α3 chain of laminin 5 is processed to a 145 kDa mature chain by cleavage in the C-terminal G domain, and the 155 kDa γ2 chain is cleaved within the N-terminal short arm to yield a 105 kDa polypeptide [40]. The carboxyl-terminal NC-2 domain of procollagen VII is removed before stabilization of the anchoring fibrils [45]. BMP-1 (bone morphogenetic protein 1), also known as procollagen C-proteinase [46], processes the γ2 chain of laminin 5 [47], and possibly procollagen VII, in the extracellular space. Interestingly, BMP-1 is synthesized mainly by fibroblasts, whereas both laminin 5 and collagen VII are epithelial products, suggesting that epithelial-mesenchymal interactions play an important regulatory role at this step. The significance of proteolytic processing in assembly of the DEJ was demonstrated in BMP-1 knockout mice, which showed a disorganized basement membrane structure [48]. This is further corroborated by the finding that a deletion of the BMP-1 consensus sequence in human procollagen VII is associated with DEB [49].

Yet another form of regulation may be proteolytic cleavage of the extracellular domains of integral transmembrane proteins in type 1 or type 2 orientation. The ectodomains of collagen XVII and syndecans are shed from the keratinocyte surface by selective proteolysis [50–52]. The process is catalyzed by a group of enzymes collectively referred to as secretases, or sheddases, which have been only partially characterized but of which many can be grouped as metalloproteinases and/or serine proteinases [53]. At least in some instances, such as in the processing of the collagen XVII ectodomain, proprotein convertases of the furin/PACE family are involved. Furin-mediated proteolysis is also required to yield a heavy and a light chain of the α6 integrin polypeptide, a necessary step for activation of the integrin [54]. The proteolytic cleavage of the ectodomains of keratinocyte surface proteins may provide a means of regulating cell adhesion, differentiation, and migration at the DEJ; however, the details of these events still remain unclear.

Current evidence indicates that the DEJ contains additional physiologically and pathologically relevant components. For example, syndecans, collagen XIII, and β-dystroglycan are transmembrane proteins in basal keratinocytes, laminins 2 and 10 [25,43] are localized in the lamina lucida of the DEJ, and several distinct proteins colocalized with the anchoring fibrils, such as the GDA-J/F3 antigen [55] and collagen XVIII [56], are known to exist. Future research must show whether these molecules and their genes are involved in the pathogenesis of EB.

Classification of EB

On Clinical Grounds

The clinical classification of EB only becomes reliable when the full clinical picture has developed. Apart from certain obvious associations, such as skin blistering and pyloric atresia, a purely clinical diagnosis is not possible at birth. The differential diagnosis of EB and other bullous affections in the newborn is listed in Table 3. Corresponding microbiological, histological, ultrastructural, serological, and other laboratory tests help discern these conditions. Within the first months and years of life, the secondary signs of EB begin to develop, allowing clinical determination of the major EB categories. In EBS, intraepidermal blisters heal without atrophy or scarring. The prognosis is mostly good, and the blistering activity decreases with age. In JEB, blistering occurs at the basement membrane through the

TABLE 3. Differential Diagnosis of Epidermolysis Bullosa and Neonatal Vesicobullous Disorders

Condition	Characteristic Features
Genetic	
Bullous ichthyosiform erythroderma (MIM 113800)	Red, scaly skin; flaccid bullae at trauma-exposed sites; hyperkeratosis, AD*
Incontinentia pigmenti (MIM 308300)	Streaky blistering, resulting in swirly pigmentation, mainly on trunk, XL
Aplasia cutis congenita (MIM 107600,207700)	Focal absence of skin on scalp, rarely elsewhere, AD or AR
Kindler's disease (MIM 173650)	Poikiloderma, acral blistering, cutaneous atrophy
Infectious	
Congenital herpes simplex	Herpetiform blisters and erosions, generalized
Staphylococcal scalded skin syndrome	Generalized erythema, fever, and necrotic peeling of the epidermis
Candidiasis	Erythematous vesicular or pustular eruption in diaper area or generalized
Impetigo neonatorum	Vesicles, bullae, or pustules on erythematous base, crusts
Autoimmune	
Bullous autoimmune dermatoses	Maternal disease? Immune deposits in skin
Mechanical	
Suction blisters	Noninflammatory blisters on thumb or radial aspects of the hand

*AD: autosomal dominant; AR: autosomal recessive; XL: X-linked.

lamina lucida. Healing takes place without scarring, but minor skin atrophy follows repeated blister formation. This category is clinically heterogeneous, ranging from the very severe lethal subtype to mild disease with late onset. DEB is characterized by dermal blistering below the basement membrane and subsequent scarring and milia formation. Clinical expression varies from a localized acral blistering to a severe mutilating disease with extensive extracutaneous involvement and poor prognosis. Traditionally, DEB has been believed to comprise two dominant and four recessive subtypes; however, molecular genetic analyses have revealed combinations of dominant and recessive mutations in several cases. Consequently, it is not possible to distinguish the DEB subtypes on clinical grounds except maybe for the severe DEB generalisata mutilans with typical early formation of synechia. DEB may be the first EB category to undergo reclassification on molecular and genetic grounds.

On Biochemical and Molecular Grounds

The classification of EB on biochemical and molecular grounds is based on the knowledge of the ultrastructural location of the molecular components of the DEJ. Specific monoclonal and polyclonal antibodies are useful for antigen mapping (Fig. 2), a technique employing marker antibodies with restricted, well-characterized binding sites at the dermal-epidermal junction for immunofluorescence staining [57]. When spontaneously blistered skin is stained, the level of dermal-epidermal separation can be deduced from the disposition of fluorescent markers on the blister roof or base. This fast technique has become a routine supplement and an alternative to electron microscopy in defining the precise splitting level in EB skin (Fig. 2) and with it the major EB category. Another important diagnostic sign is the lack of an indicator molecule in certain EB subtypes: plectin in EBS with muscular dystrophy, laminin 5 in JEB Herlitz, α6 or β4 integrin in JEB with pyloric atresia, collagen XVII in generalized atrophic benign EB (Fig. 2C), and collagen VII in DEB mutilans [22,58–62]. Correspondingly, mutations in the genes encoding these proteins cause these and related EB subtypes, and mutation analysis finally confirms the diagnosis.

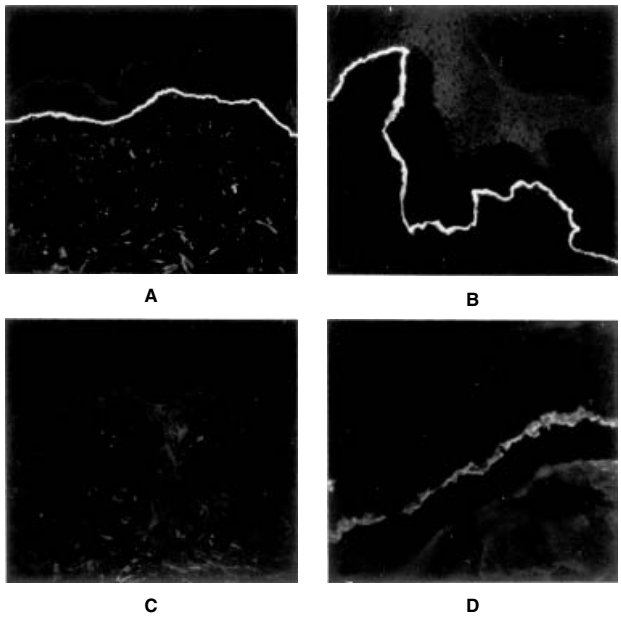

Figure 2. Antigen mapping. Indirect immunofluorescence staining of components of the dermal-epidermal junction in spontaneously separated EB skin. **A**: Laminin 5 shows a linear fluorescence in normal dermal-epidermal junction. **B**: Laminin 5 on the floor of a junctional blister. **C**: Lack of collagen XVII in generalized atrophic benign EB skin. **D**: Reduced collagen VII staining in blister roof in DEB.

Some EB subtypes are very rare, being represented in only single families, and some subtypes have not yet been investigated with molecular markers. Careful clinical, genetic, and biochemical characterization of as many cases as possible will show whether all of these subtypes stand out as distinct defects or whether they represent the same disorders with variable phenotypic expression.

The classification is further complicated by the fact that studies on JEB have indicated that mutations in different genes can lead to a similar clinical presentation [63,64] and that different defects in the same gene can lead to very different clinical phenotypes [65]. An additional problem in the classification of EB is the combination of dominant and recessive gene mutations. It has come as a surprise that a significant number of EB patients are compound heterozygotes for two different mutations and that in several DEB families the combination has been shown to consist of a recessive and a dominant mutation [49,66–68]. Discovery of the extensive variability of combinations of mutations has consequences not only for classification but also for genetic counseling of affected families. Detection of one or two mutations in the index patient does not always allow as unambiguous a determination of the diagnosis, inheritance pattern, and prognosis as previously assumed.

Approach to the Patient

For the careful evaluation of an EB patient, a combination of the family history, clinical examination of the index patient and family members, and a skin biopsy for antigen mapping, and eventually for electron microscopy and keratinocyte and fibroblast cultures, as well as blood samples for mutation analysis, are required (Fig. 3). In the postnatal period, the clinical presentation can be unspecific and, as discussed above, it is not possible to differentiate among the subtypes on clinical grounds. Often, the major EB category can easily be determined by antigen mapping of a skin biopsy of a newborn; however, for many patients the exact clinical subtype cannot be determined until the course of the disease has been observed. Therefore, regular follow-up visits are required.

Relative Frequencies of the Different Types

Relative frequencies of the EB subtypes have not been determined, and only fragmentary data exist in the literature [3]. Generally, EBS is the most common EB category and EBS localisata the most common subtype. DEB is clearly more common than JEB. The striking difference in the incidence of JEB Herlitz between two neighboring countries, Sweden (very high) and Finland (very low), argues against recurrent mutations in hotspot regions. Only one gene is likely to have a "hotspot" mutation, namely the *LAMB3* (laminin β3) mutation R635X, which is found in 40% of JEB Herlitz cases [69]. Gedde-Dahl has calculated that because all DEB mutations have been found at a single locus, *COL7A1*, the pooled frequency of recessively acting alleles is 0.0024 in Norway, implying that 0.5% of the population are heterozygous carriers of *COL7A1* mutations [3].

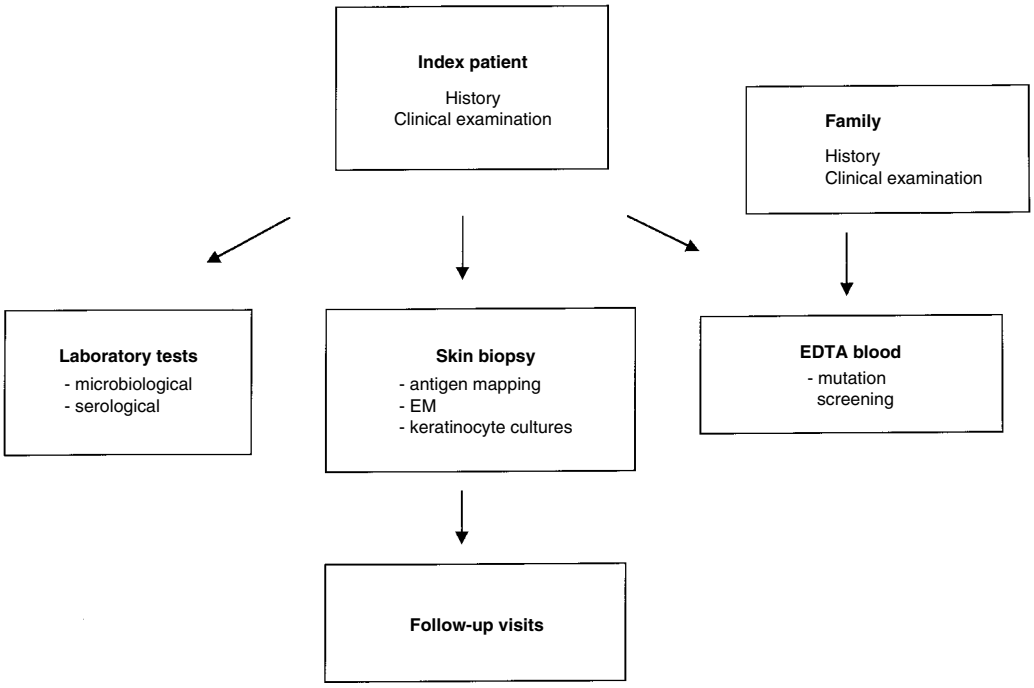

Figure 3. Flow sheet for EB diagnosis.

Prevalence and Epidemiology

It has not been possible to establish reliably the overall prevalence of EB. However, several rather similar estimates of prevalence and incidence have been published from different countries: Gedde-Dahl reports a prevalence in Norway of 42×10^{-6} for EBS (23×10^{-6} if EBS Ogna, which is very common in Norway, is excluded), 2×10^{-6} for JEB, and 10×10^{-6} for DEB [3]. In his study of EB in Finland, Kero [70] reports a prevalence of 27×10^{-6} for all EB subtypes. A similar figure, a morbidity of 25×10^{-6}, was found in Denmark in the 1950s [71]. The incidence of EB in the U.S. and in Europe has been estimated to be about 1:20,000 to 1:50,000, or 20×10^{-6} births [72].

EPIDERMOLYSIS BULLOSA SIMPLEX

Common Aspects

This EB category exhibits trauma-induced skin blistering as a consequence of cytolysis of basal keratinocytes. Keratin intermediate filament scaffolding, which sustains the three-dimensional structure of the cells, is weakened due to defects of the keratins or the linker proteins that attach the intermediate filaments to the hemidesmosomes and collapses as a result of external shearing stress. The blistering is intraepidermal, and healing takes place without scarring. In general, the prognosis is favorable, and blistering activity diminishes with advancing age. Defects in the keratin and plectin genes have been shown to underlie several of the subtypes despite clinical heterogeneity.

Clinical Features

EBS Generalisata (MIM 131900, Koebner Disease, AD-EBS-G)

Blistering occurs from birth, or the first few weeks of life, on the whole integument (Fig. 4) but preferentially involves the skin of the hands and feet, especially the palms and soles [3,4]. The frequency of blistering usually increases at high temperatures or after trivial trauma such as walking. The severity of involvement varies greatly between sibships. Healing usually takes place without scars, although secondary traumatization or infection may lead to scarring and milia formation. Diffuse palmar-plantar hyperkeratosis is common, as is hyperhidrosis of the feet. Nails and teeth are normal, and oral mucous membranes are rarely involved. The primary complication seems to be secondary infection of the blistered skin. The disease may be severe at birth, but although the widespread blistering may be debilitating, the condition is not threatening and patients can have a normal life span. Many experience improvement after reaching maturity.

EBS Localisata (MIM 131800, Weber-Cockayne Disease, AD-EBS-L; MIM 601001, AR-EDS-L)

AD-EBS-L is the most common EBS variant [3,4,70–72]. A few families with identical clinical presentation but with recessive inheritance have been described [73,74]. Blisters are clinically indistinguishable from those in EBS generalisata but are limited primarily to hands and feet. Blistering recurs particularly in the warm seasons. The blisters can be painful and even disabling. Onset is usually by toddler age but can be delayed to the twenties. Nails are uninvolved (Fig. 4), and blistering of the oral mucous membranes is rare. Hyperhidrosis of the feet and palmar-plantar hyperkeratosis are often present. Differentiation between a mild phenotypic variant of EBS generalisata and EBS localisata is arbitrary.

EBS Herpetiformis (MIM 131760, Dowling-Meara Disease, AD-EBS-H)

Generalized blisters and erosions are often present congenitally or develop shortly after birth [3,4]. The symptoms may be severe in the infantile period [75]. Large

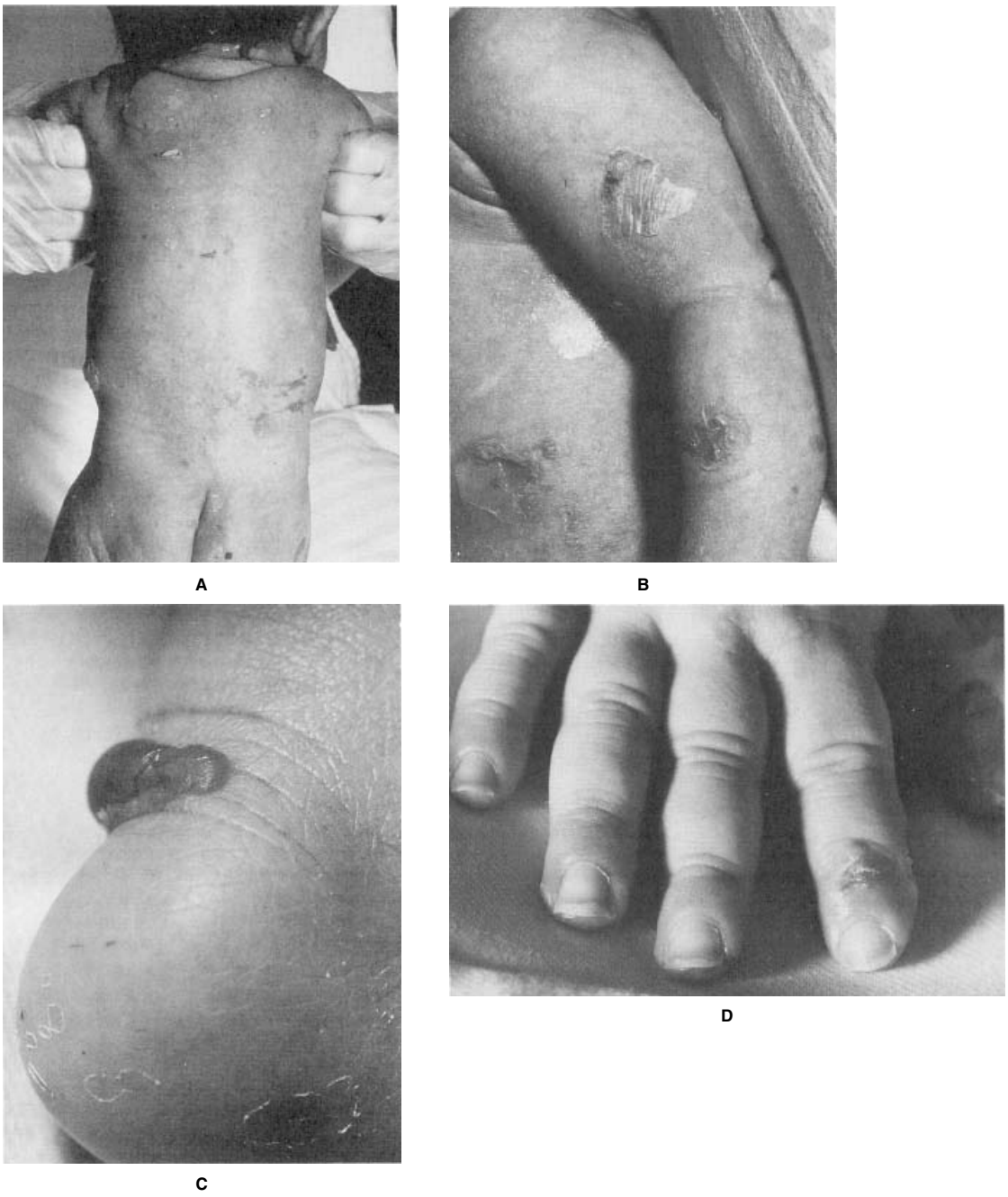

Figure 4. Clinical features of EB simplex. **A**: Generalized blistering in an infant with EBS generalisata. **B**: Superficial flaccid bullae and erosions. **C**: The blisters are occasionally hemorrhagic. Healing takes place without scarring. **D**: Localized blistering of the hand in EBS localisata. The nails are normal.

blisters on the hands and feet, including the palms and soles, and smaller blisters in herpetiform arrangement on the proximal extremities and trunk are typical. Small vesicles can appear on the face. The blisters may spread in a centrifugal manner and heal rapidly, leaving no scars but postlesional hyperpigmentation. Nails may be lost due to subungual blistering but regrow normally. In childhood, hyperkeratotic palms and soles are typical. Oral blistering can occur in early phases. Oddly, the skin clears during high fever, and no typical summertime aggravation is observed. Around school age, the skin improves rapidly, and in adulthood friction blisters rarely occur. At adult age, plantar hyperkeratosis is the main problem. Oral blistering, dental abnormalities, and corneal erosions may accompany this entity.

EBS with Mottled Pigmentation (MIM 131960)

An irregular mottled hyperpigmentation and hypopigmentation (Fig. 9G) of the proximal extremities and trunk accompanying blistering of trauma-exposed sites has been

described in a few European families [3,76,77]. Blisters are present at birth, but the pigment maculae appear during the first years of life. Blistering intensity decreases with age, but the pigmentation tends to persist. The blisters heal without scars, but slight skin atrophy imitating premature skin aging is typical. Most patients have plantar hyperkeratosis and onychodystrophy. Caries and mucosal fragility also occur.

EBS Associated with Muscular Dystrophy (MIM 226670, AR-EBS-MD)

A recessively inherited form of EBS associated with late-onset progressive muscular dystrophy has been described in many families worldwide [5,20,21]. The patients have congenital generalized blistering and healing without scarring but with the development of atrophic skin changes and nail abnormalities. Mild palmo-plantar hyperkeratosis, dental anomalies, and corneal involvement, as well as extensive mucosal erosions and urethral strictures, have been described in some probands. The onset and extent of the muscle symptoms vary greatly among kindreds. Some develop severe progressive muscular dystrophy during early infancy but others not until the third decade of life. In most cases described, the muscular weakness led eventually to the patients being wheelchair-bound.

EBS Ogna (MIM 131950, AD-EBS-O)

This variant has been described only in one large Norwegian kindred with a dominant inheritance pattern [3]. It has its onset in infancy, and patients exhibit a generalized tendency for skin bruising and often onychogryphotic nail changes in addition to seasonal acral blistering. The disease generally follows a mild clinical course.

EBS Herpetiformis with Mottled Pigmentation (AD-EBS-HMP)

This entity has been described in two families [78,79]. Generalized serous and hemorrhagic blisters in herpetiform arrangement were present from birth. The blisters healed rapidly without scarring but left a postbullous pigmentation. The skin rapidly normalized during episodes of high fever, and the blistering showed a marked improvement at school age. A mottled macular pigmentation developed on the neck and trunk in childhood, and the intensity of the staining increased with age. Palmo-plantar hyperkeratosis had an onset at school age and showed increasing severity with age. Subungual blistering in the toes was frequent. Mucous membranes were rarely involved.

EBS Associated with Anodontia and Hair and Nail Disorders (Kallin Syndrome)

A localized EBS associated with anodontia and hair and nail disorders was described in two Swedish families, one clearly demonstrating recessive inheritance [3]. The onset of nonscarring, seasonally aggravated blistering was within the first year of life. Onychogryphosis and transient nonscarring alopecia were observed. Abnormal mineralization led to the underdevelopment of teeth.

EBS Mendes da Costa (MIM 302000)

Trauma-induced blistering with a predilection for the extremities during the first 2–3 years of life is part of a syndrome including alopecia, reticulated hyperpigmentation, and skin atrophy of the face and extremities. Microcephaly is a noncutaneous-associated symptom. This X-linked condition has been described in a Dutch kindred [3].

Other Potential EBS Subtypes

A novel type of EBS with acantholytic blistering within the epidermis was described in one family [80]. The inheritance was dominant, and the blistering was acral and aggravated during summer months. Future research and description of more probands must show whether this represents a distinct EBS subtype or belongs to another disease group.

Genetics

EBS was the first EB category in which causative mutations were identified. First, transgenic mice with a truncated keratin 14 were found to exhibit a blistering skin phenotype on mild mechanical trauma, very similar to human EBS [81]. Parallel independent studies showed linkage of EBS to the type II keratin gene locus on chromosome 12q13, and soon after a number of mutations in the genes for keratin 5 at this locus and keratin 14 on the long arm of chromosome 17 were identified in EBS families [82–85]. Interestingly, mutations in these two genes seem to cause several clinical subtypes, and therefore the EBS subtypes generalisata, localisata, herpetiformis, and EBS with mottled pigmentation are allelic [86–88]. Most keratin mutations in EBS are inherited in a dominant manner. However, rare recessive keratin 14 null mutations have been reported; these seem to result in a somewhat milder phenotype than the dominant mutations [73,74].

Recessively inherited mutations in the gene encoding the hemidesmosomal plaque protein plectin, *PLEC1*, underlie EBS with muscular dystrophy. To date, about a dozen *PLEC1* mutations have been reported [89–94]. Some years ago, a genetic linkage to glutamine-pyruvate transferase (*GPT*; EC 2.6.1.15) at chromosomal locus 8q24.2–q24.3 was demonstrated for EBS Ogna [3]. Because the plectin gene is also located at this same locus, it is possible that dominant plectin mutation are associated with EBS Ogna. Indeed, Koss-Harness and co-workers have evidence for abnormal plectin expression in two EBS Ogna families [95].

Keratins 5 and 14

Keratins belong to the intermediate filament superfamily, which has more than 50 members differentially expressed in nearly all cells of the body (see Chapter 6, this volume). The intermediate filament cytoskeletons seem to be tailored to suit the specific functional needs of each cell type, but despite their functional diversity, the members of the intermediate filament superfamily share a common structure, a dimer composed of two α-helical polypeptide chains oriented in parallel and intertwined in the form of a coiled-coil rod. The highly conserved ends of the intermediate filament rod associate in a head-to-tail fashion and copolymerize to form the supramolecular intermediate filament aggregates. The major keratins expressed in the basal cells of the epidermis are keratins 5 and 14, the former a type II and the latter a type I keratin that, as a pair, form the coiled-coil rod [96]. After the identification of numerous mutations in the genes for keratins 5 and 14 in EBS families, it has become evident that mutations in the highly conserved ends of the rod have more deleterious consequences for filament assembly than mutations in other portions of the rod. Consequently, different mutations in the same gene(s) have been shown to cause a variety of clinical phenotypes [82–88].

Plectin

Abnormalities of plectin, a cytoskeleton-associated protein widely distributed in squamous epithelia and muscle [97], are associated with autosomal recessive EBS with muscular dystrophy [21,22,89–94]. Consistently, plectin has both an intermediate filament and an actin-binding domain. In the skin, it is a hemidesmosomal plaque protein that mediates the attachment of keratin intermediate filaments

to the hemidesmosomes. Consistent with this, experimental evidence exists for ligand interactions between plectin and another component of the hemidesmosomal plaque, β4 integrin [34,35]. Lack or functional deficiency of plectin in keratinocytes leads to defective anchorage of the cytoskeleton to the plasma membrane and to cell fragility as a result of shearing forces in the skin. In muscle cells, plectin bridges intermediate filaments and actin filaments, and its abnormalities cause disorganization of the structural filament networks in the cells and diminished mechanical resistance of the muscle cells, thus leading to muscle wasting in EBS with muscular dystrophy.

Bullous Pemphigoid Antigen-1

Another intracellular hemidesmosome protein with the capacity to bind intermediate filaments, bullous pemphigoid antigen-1, or BPAG1 or BP230, was targeted in mice [98]. In the absence of BP230, the intermediate filaments were severed from the hemidesmosomes, generating intracellular fragility and resulting in skin blistering. Disruption of the locus also resulted in degeneration of the sensory nervous system [98]. This puzzling finding was later explained by the discovery of a neural isoform of bullous pemphigoid antigen-1, BPAG1-n, which was also ablated by the gene-targeting construct [99]. Despite the establishment of the *BPAG1* gene, which encodes bullous pemphigoid antigen-1, as a candidate gene for EBS by these experiments, no mutations in humans have yet been reported.

Physical Properties and Morphology of Keratin Intermediate Filaments and Pathogenesis of the Disorder

In epidermal keratinocytes, the keratin filaments provide a cellular framework that reaches from the nucleus to the specialized cell-cell junctions, the desmosomes, and cell-matrix junctions, the hemidesmosomes. Ultrastructural studies on EBS skin reveal, in addition to intracellular splitting, clumping of the keratin intermediate filament bundles in basal keratinocytes [3]. Genotype-phenotype correlation analysis using filament assembly assays with mutant keratins isolated from patients' cells pointed to a tendency of mutations in the highly conserved ends of the intermediate filament rods to cause severe disruption of the filament network and the more severe phenotypes, whereas mutations in the less conserved domains had less deleterious effects on the filament network and seem to be associated with milder clinical phenotypes [96]. When the network is disrupted as a consequence of dominant missense mutations, insoluble keratin aggregates exacerbate the situation, making the cells sensitive to external mechanical stress and resulting in cell degeneration and skin blistering. Surprisingly, when the network is missing in recessive null mutants, cells become fragile but require greater mechanical stress before rupturing than cells containing mutant keratins [96].

JUNCTIONAL EPIDERMOLYSIS BULLOSA

Common Aspects

Junctional EB (JEB) is a heterogeneous group of disorders with extreme clinical variation and defects in at least six distinct genes underlying the different clinical subtypes [3,5]. In the most severe subtype, JEB Herlitz, the course is usually lethal within two years after birth. In contrast, JEB progressiva only manifests at school age and progresses slowly, and observations indicate that heterozygous carriers may exhibit only minimal associated symptoms, such as enamel hypoplasia, and no skin blistering at all [100,101]. The hemidesmosome-anchoring filament complex is weakened by genetic defects in one of the structural molecules, resulting in epidermal dysadhesion. Healing takes place without scarring; however, a light skin atrophy, nail and dental problems, and alopecia are often associated with this EB category. Because a number of genes are involved, the determination of the candidate gene for each patient necessitates a careful clinical assessment and antigen-mapping analysis of a skin biopsy.

Clinical Features

JEB Herlitz (MIM 226700, JEB Gravis)

This is the most severe subtype of the EB family and has also been called EB letalis because it usually leads to death within the first two years of life [3]. The disorder is characterized by the onset at birth of severe generalized blistering and congenital ulcerations and erosions on the extremities. Blisters on the hands and feet occur soon after birth, but later these locations may be spared. Scalp lesions are common. The blisters heal without scars, but a mild atrophy at the sites of recurrent lesions can be seen. Milia are rarely present. Paronychia-like lesions lead to early loss of the nails. Tooth enamel is defective, but dental growth is normal. At some stage, the healing stops, leaving large excoriations and chronic plaques of granulation tissue, which are characteristically found in the perioral region and at the fingertips. Oral blistering can be moderate to severe, and hoarseness resulting from laryngeal mucosal blistering is a characteristic sign of this disease. Severe blistering of the skin and mucous membranes leads to protein and fluid loss and exposes the organism to secondary infections, thereby resulting in extensive systemic disease, including cardiac and pulmonary problems.

Generalized Atrophic Benign EB (MIM 226650, GABEB, JEB Atrophicans Mitis)

This form has had many names. The first description [102] called it JEB atrophicans mitis, or JEB Disentis, after the village in Switzerland where the affected family resided. Subsequently, the subtype has become commonly known as generalized atrophic benign EB, or GABEB [12]. The name stresses the benign course of the disease and the atrophic changes that develop during the course of the blistering. Generalized blistering begins at birth and continues with at most a moderate improvement to at least middle adult life. The extremities, hands, and feet are predominantly involved. However, the trunk, scalp, and face are usually also affected (Fig. 5). Large serosanguineous blisters heal with local erythema, atrophy, and fragility and sometimes hyperpigmentation. Milia are absent. Nail involvement is common, resulting in dystrophy or loss of nails, and mild palmo-plantar hyperkeratosis may be present. Some degree of alopecia is seen in most patients, and some develop an extensive or total alopecia early in life [65]. Oral blistering is mild and often temporary. Enamel defects may lead to dystrophic dentition and so-called enamel pitting.

JEB Localisata

Blisters indistinguishable from those seen in the generalized JEB are limited to hands, feet, and shins. Onset of the blistering can be from birth to school age. Nail involvement leading to nail dystrophy or loss and oral blistering are sometimes observed. Enamel hypoplasia can be associated with the disease and is sometimes the only symptom in heterozygous carriers [65,100,101].

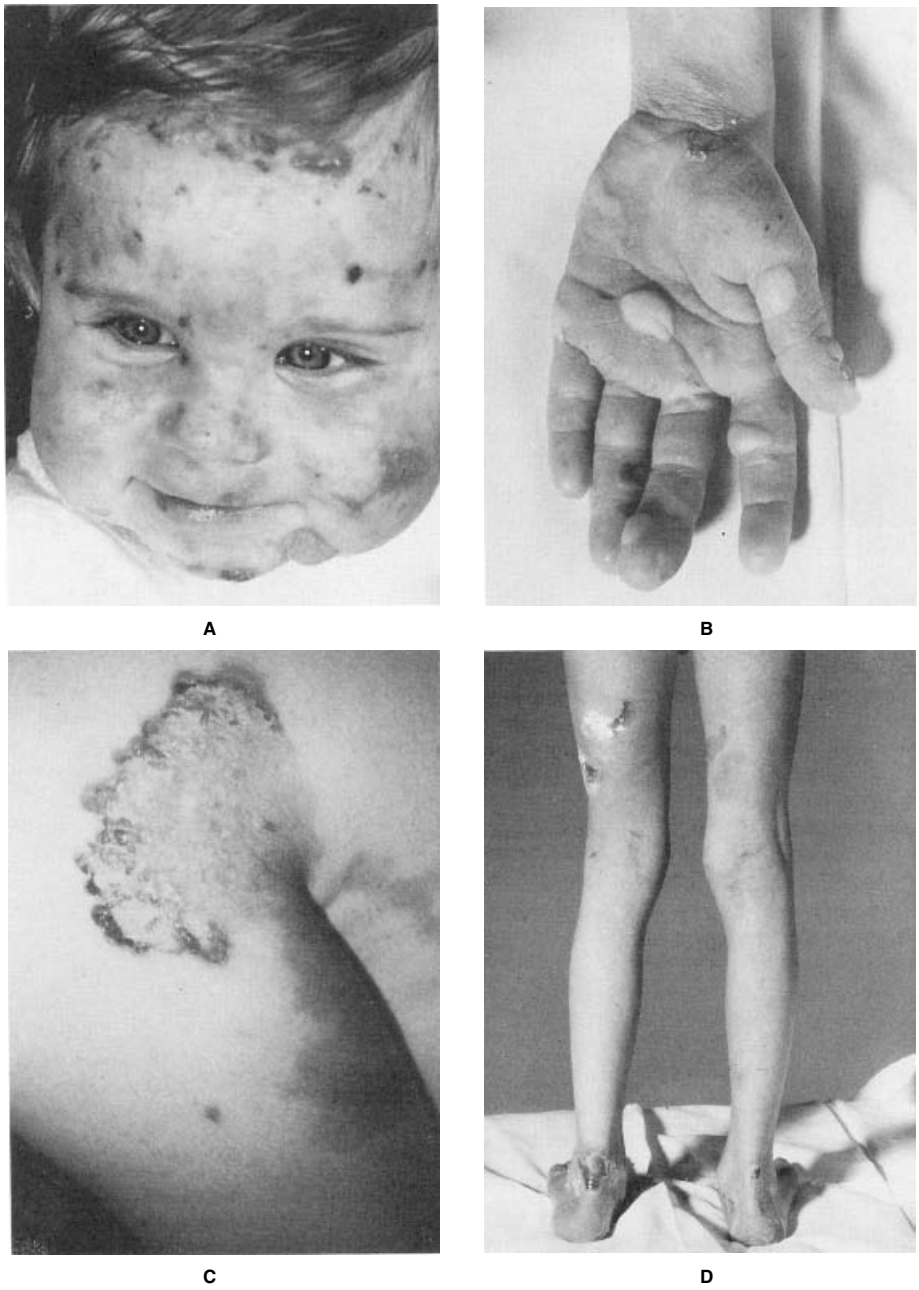

Figure 5. Clinical features of junctional EB. Disseminated blistering in generalized atrophic benign EB (GABEB) typically involves the face, **A**, and palms, **B**. **C**: Polycyclic lesion with blisters, crusts, and postlesional hyperpigmentation in the axillary fold in JEB inversa. **D**: A teenager with generalized atrophic benign EB shows disseminated but not severe blistering.

JEB Inversa

Neonatal blistering may present as pyoderma-like erosions in a generalized distribution. Often, the blistering disappears within a few months only to recur later. Then, bulla formation is concentrated in the inguinal, perigenital, and axillary folds (Fig. 5) and later also in the submammary regions [3]. Blistering of the hands and feet continues. Healing may be slow, leaving erosions, atrophy, and postlesional hyperpigmentation. Nails are sometimes dystrophic, and most patients develop, presumably due to scratching, small, white, atrophic maculae and streaks on the trunk and proximal extremities. Mild to moderate mucosal blistering has been observed. Curiously, the female sex hormones have a positive effect; the course of the disease is better between menarche and menopause, and some patients experience improvement during pregnancy [3].

JEB Progressiva (MIM 226500)

Earlier, this disorder was called EB dystrophica neurotrophica [3]. The discovery of the junctional nature of the blisters and of its progressive course led to the present name.

In this subgroup, dystrophy and loss of nails is usually the first symptom to appear, with late onset between ages 5 and 15 years. Serosanguineous acral blistering, which can also involve palms and soles, follows even after years. Healing leads to gradual mild atrophy with loss of fingerprints. Oral blisters occur, and lingual papillae may disappear similarly to the fingerprints. In the Norwegian families described first, hypoacusis was found as an associated symptom. However, other reports from elsewhere have found individuals with JEB progressiva with normal hearing capacity [103].

JEB Cicatricans

In this subtype, a discrepancy exists between the clinical appearance and the ultrastructural findings. Despite junctional splitting with rudimentary hemidesmosomes visible in the electron microscope, the blisters heal with scarring, and partial syndactylies may occur. Blistering starts at or soon after birth with a generalized distribution but with a predilection for the distal extremities. Blisters heal with scarring and occasionally with milia. The scarring can lead to syndactyly and flexion contractures with apparent shortening of the digits, but a true mitten-like deformity as seen in mutilating recessive dystrophic EB does not develop. Another major feature is the presence of nasal blisters from infancy, which eventually may result in complete closure of the anterior nares. Oral blisters and erosions are moderate and frequently accompanied by dental caries. Nails are completely lost from birth or within the first year of life [104].

JEB with Pyloric Atresia (MIM 226730, AR-JEB-PA)

JEB with pyloric atresia is a rare subtype with skin blistering associated with pyloric atresia at birth. Previously, it was not clear whether JEB-PA represented a distinct subtype or just a coincidence of JEB Herlitz and pyloric atresia. Only after mutations in the genes for α6 and β4 integrin subunits were found in patients with JEB-PA was its identity as a distinct EB form ascertained [3,5,19]. In severe cases, the condition is lethal during the postnatal period and, similarly to JEB Herlitz, affected children die of systemic complications despite surgical correction of the intestinal abnormalities. Mild, nonlethal JEB-PA cases have been recognized. The probands have minimal skin involvement, occasionally noted after surgery to correct the pyloric atresia but sometimes not until the early years of life [20,105]. Acral blistering may be induced by trauma, and dystrophy of some nails may develop. Enamel hypoplasia can be associated with the disease. The prognosis in mild cases is very good after the pyloric atresia is surgically corrected.

Acquired Autoimmune Diseases with Junctional Blistering

The term "acquired JEB" is not used, but certain inflammatory bullous skin disorders could be thought of as acquired forms of JEB. In some autoimmune dermatoses with subepidermal blistering, namely the pemphigoid group, circulating and tissue-bound autoantibodies target hemidesmosomal and anchoring filament proteins. In bullous, cicatricial, and gestational pemphigoid skin disorders, collagen XVII has been identified as a major autoantigen, but reports of autoantibody reactivity with laminin 5 also exist [24,51,52,124,133,134]. In linear IgA dermatosis, IgA autoantibodies, in contrast to mainly IgGs in pemphigoids, target collagen XVII and its soluble ectodomain. Most patients with autoimmune dermatoses are adults, but sometimes children are affected. In such cases, the distinction from a hereditary JEB with late onset may not be trivial, and molecular and immunological diagnostic tests are required to distinguish the two forms.

Genetics

All JEB subtypes are inherited in a recessive manner. Mutations in at least six different genes are involved, including the *LAMA3*, *LAMB3*, and *LAMC2* genes encoding laminin 5 [106–109], the *COL17A1* gene for collagen XVII [110–114], the *ITGA6* gene for integrin α6 [115,116], and the *ITGB4* gene for integrin β4 [19,20,105]. A significant number of patients show compound heterozygosity for two different mutations [5]. Homozygosity or compound heterozygosity for nonsense mutations in these genes is usually associated with severe phenotypes and missense mutations or missense/nonsense combinations with milder clinical presentation. Nonsense mutations in the laminin 5 genes generally lead to JEB Herlitz and missense mutations to a GABEB phenotype [117,118]. Nonsense mutations in the collagen XVII gene are associated with an extensive GABEB subtype and missense mutations with JEB localisata [65,113]. Lethal JEB-PA results from nonsense mutations in the *ITGA6* and *ITGB4* genes, whereas mild JEB-PA phenotypes have been observed in children with missense mutations in *ITGB4* [20,105]. In all these genes, most defects seem to be private mutations and spread over the entire gene, which makes mutational screening tedious, expensive, and time-consuming. Only in the case of the JEB Herlitz subtype does a different situation prevail. *LAMB3* mutations account for 80% of all laminin 5 gene defects in JEB [5]. In 50% of these cases, one of the two common mutations, R42X or R635X, is seen [69]. R635X in combination with missense mutations has been found associated with milder, nonlethal JEB subtypes (Fig. 6). The molecular genetics of JEB may turn out to be more complex than previously anticipated. We have identified a JEB child with digenic mutations in both *COL17A1* and *LAMB3* genes [101] who was mixed heterozygous for *COL17A1* nonsense mutations and carried the *LAMB3* mutation R635X in addition.

Basic Defects

Detachment of basal keratinocytes from the lamina densa and rudimentary or absent hemidesmosomes and anchoring filaments are ultrastructural hallmarks of JEB [119]. Indeed, absence or functional deficiency of the molecular components of the hemidesmosome-anchoring filament complex, laminin 5, α6β4 integrin, and collagen XVII, cause the clinically diverse JEB subtypes [3,5].

Laminin 5

Laminin 5, a heterotrimer of α3, β3, and γ2 chains, is the primary link between the hemidesmosomal α6β4 integrin and the anchoring fibril component, collagen VII. Because laminin 5 is a small laminin isoform with truncated short arms and hence reduced capacity to form self-assemblies, it is cross-linked to the laminin 6 network in the basement membrane, which in turn is connected to the other basement membrane networks [24–26]. Lack of laminin 5 results in extreme cutaneous and mucosal fragility in JEB Herlitz. Homozygous or compound heterozygous null mutations in the *LAMA3*, *LAMB3*, and *LAMC2* genes encoding the α3, β3, and γ2 chains of laminin 5 often lead to nonsense-mediated mRNA decay [120] and lack of synthesis of the corresponding polypeptide chain. Remarkably, absence of any one of the three subunit polypeptides prevents assembly and secretion of the entire heterotrimeric laminin 5 molecule [59]. Further, in the case of *LAMA3* nonsense mutations, laminin 6, with the chain composition α3β1γ1, is also absent from the DEJ. In view of the well-known severe phenotypes caused by lack of laminin 5, the observation of patients between 5 and

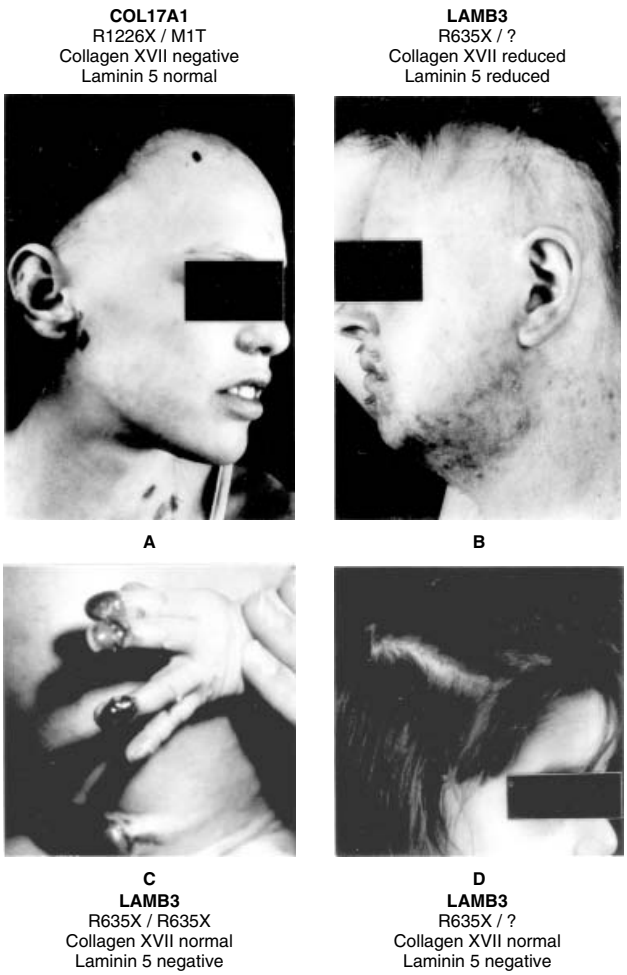

Figure 6. Genotype-phenotype correlations demonstrate unexpected JEB phenotypes. The characteristic phenotype of generalized atrophic benign EB (GABEB) with blistering, slight skin atrophy and temporal alopecia can be caused by mutations in *COL17A1* (**A**) or *LAMB3* (**B**). The common *LAMB3* mutation R635X is associated with 40% of all JEB Herlitz cases (**C**) but has been found in probands with very different, nonlethal phenotypes as well (**B**, **D**). Unanticipated is the clinical presentation of the child in the **lower right** panel; she shows a biological phenotype indicative of JEB Herlitz (i.e., a lack of laminin 5 protein in the skin and heterozygosity for R635X). However, she exhibits only mild acral blistering at the age of 7 years and shows no typical clinical features of either GABEB or JEB Herlitz.

25 years of age who have JEB of mild to moderate severity and who lack laminin 5 in the skin has been very puzzling (Fig. 6). We have shown that two schoolchildren with mild skin blistering practically lack laminin 5 at the DEJ and are heterozygous carriers of the *LAMB3* hotspot mutation R635X (L. Bruckner-Tuderman, unpublished observations). In each case, the second mutation has remained elusive so far. In concert with our observations, a mutation report described compound heterozygosity for *LAMB3* nonsense mutations in an adult with generalized JEB [121]. At present, there is no plausible explanation for the differing phenotypes, and further research on the biology of the laminins and their ligands must clarify this point. The presence of mutant, functionally abnormal laminin 5 at the DEJ has been observed in other nonlethal JEB subtypes, including generalized atrophic benign EB, but essentially nothing is known about the genotype-phenotype correlations in these cases.

α6β4 Integrin

Deficiency of α6β4 integrin is associated with abnormal hemidesmosomes, blistering of the skin, and pyloric atresia [19,20,61,105,115,116]. This JEB subtype often has a lethal outcome due to extensive skin fragility. Because the integrin binds to other hemidesmosome components (i.e., plectin, collagen XVII, and laminin 5), the disruption of ligand interactions is likely to lead to diminished epidermal adhesion. Several components of the anchoring complex have been shown to be expressed in the gastrointestinal epithelia [122,123]. This may explain the association of pyloric atresia with mutations in the genes for α6 and β4 integrin subunits. Nonsense mutations in the *ITGA6* and *ITGB4* genes lead to a complete lack of the integrin in the skin and to severe phenotypes, and missense mutations are found in association with milder phenotypes [105], but very little is known about how the gene defects modify the structure and ligand-binding activities of the integrin.

Collagen XVII

Collagen XVII (see also Chapter 2, this volume) is an unusual transmembrane collagen in type II orientation with an intracellular amino terminus and extracellular carboxyl terminus. It exists in two molecular forms, as a full-length transmembrane protein and as a shed soluble ectodomain [51,52]. The full-length collagen is a homotrimer of three 180 kDa α1(XVII) chains; the globular intracellular domain is disulfide-linked, and the N-glycosylated extracellular domain is triple-helical [52,124]. The soluble form is a triple-helical molecule comprising three 120 kDa polypeptides. The details of shedding are not yet understood, but current data strongly suggest that the soluble ectodomain is generated through furin-mediated proteolytic processing [52]. Thus, collagen XVII is not only an unusual transmembrane collagen but the first collagen with a specifically shed soluble triple-helical ectodomain.

Abnormalities of collagen XVII underlie most cases of GABEB [60,63–65,100,101,110–114]. Many patients are compound heterozygous for two different mutations. Most of the two dozen or so mutations known to date lead to a premature termination codon, nonsense-mediated mRNA decay, and an absence of collagen XVII from the skin (Fig. 7). Only a few missense mutations or missense/nonsense mutation combinations have been described. These allow the synthesis of structurally abnormal collagen XVII and cause milder JEB subtypes, including the JEB localisata subtype with mostly acral blistering, but not with alopecia [65,113]. The genotype-phenotype correlations of collagen XVII abnormalities are only just beginning to emerge. It will be interesting to observe how gene mutations alter the structure-function relationships of this unusual transmembrane collagen and its shedding.

Physical Properties and Morphology of Hemidesmosomes and Anchoring Filaments and Pathogenesis of the Disorder

The ultrastructure of the hemidesmosomes and the anchoring filaments is well-known, but we are only beginning to understand the assembly of these structures. Investigations using the expression of recombinant hemidesmosomal proteins in COS cells have indicated that α6β4 integrin,

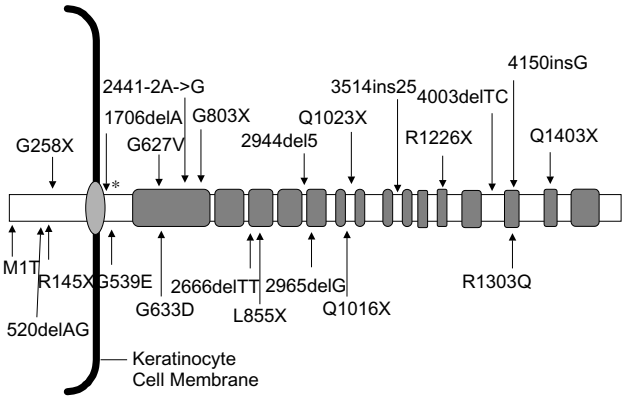

Figure 7. A schematic representation of the collagen XVII molecule and its mutations. Collagen XVII is an unusual transmembrane collagen in type II orientation, with a shorter intracellular amino terminus and a long extracellular carboxyl-terminal domain, which contains 15 collagenous subdomains with Gly–X–Y repeat sequences (gray bars). The oval, light gray symbol indicates the transmembrane domain. Most known mutations in the *COL17A1* gene lead to a premature termination codon and are associated with the generalized atrophic benign EB phenotype. The amino acid substitution mutations G633D and R1303Q have been reported in patients with localized JEB. The asterisk denotes the putative site for shedding of the soluble ectodomain.

collagen XVII, and plectin are essential for the formation of the hemidesmosomal plaque [34]. Ultrastructural studies on JEB skin have shown that the hemidesmosomes can have a rudimentary structure with a hypoplastic inner plaque and outer dense plate or be lacking completely. The most extensive hemidesmosome abnormalities are found in JEB Herlitz and severe JEB with pyloric atresia, suggesting that laminin 5 and the $\alpha 6\beta 4$ integrin are essential for normal hemidesmosome morphology. In JEB Herlitz, the anchoring filaments are also missing, indicating that laminin 5 is an important structural component of the filaments. In milder JEB subtypes, various degrees of lesser ultrastructural abnormalities of the hemidesmosomes are observed. In concert with the assumption that many protein-protein ligand interactions are required for the formation of a functional hemidesmosome-anchoring filament complex, lack of one component sometimes leads to the absence of other components as well, as observed with antigen mapping of JEB skin. For example, in generalized atrophic benign EB, antibodies to both laminin 5 and collagen XVII may show a negative reaction in the immunofluorescence staining of the skin, suggesting that the two proteins represent physiological ligands and that one of them is rendered unstable in the absence of the other.

DYSTROPHIC EPIDERMOLYSIS BULLOSA

Common Aspects

Dystrophic EB (DEB) is characterized by dermal blistering below the basement membrane and subsequent scarring (Fig. 8). Great progress has been made in defining the molecular basis of DEB, and new data have altered some of the concepts about this EB category. In the late 1980s–early 1990s, transmission electron microscopy had revealed abnormalities in anchoring fibrils [3,125–127], and immunohistochemistry of the skin in patients with the most severe DEB forms revealed entirely negative staining for collagen VII epitopes [62,127]. On the basis of these observations, the collagen VII gene was proposed to be at fault in DEB, and as of this writing over 150 mutations have been identified in this gene [5,30]. Traditionally, DEB contained two dominantly and four recessively inherited subtypes, but molecular genetic studies have shown that dominant and recessive mutation combinations and a new subtype also exist. Therefore, DEB may be the first EB category to be reclassified on molecular grounds.

Clinical Features and Complications

Dominant DEB Generalisata (MIM 131750, Pasini Disease, AD-DEB-G)

The blistering is usually seen at birth or in the first week of life and is generalized, involving also the face and ears [3,4]. Later in life, there is a predilection for the extremities, but occasionally blistering of the buttocks and even generalization is seen. Healing occurs with scars (Fig. 9B) and milia formation (Fig. 9E) often leading to large red atrophic areas on the extremities (Fig. 8D). Dystrophic or absent nails are common. Oral blistering is infrequent. Usually around adolescence, but sometimes before, characteristic albulopapuloid, white, scar-like lesions appear spontaneously on the trunk (Fig. 9D). The genesis and significance of these lesions are not yet known. The teeth are not affected.

Dominant DEB Localisata (MIM 131800, Cockayne-Touraine Disease, AD-DEB-L; MIM 131850, Pretibial DEB)

Serosanguineous blistering has its onset in infancy or early childhood, up to the fifth year of life [3,4]. The blisters are strictly localized to the extremities, with a preference for trauma-exposed sites over bony protuberances (Fig. 9A). Healing takes place, occasionally with hypertrophic scars and milia. Joint contractures may follow scarring (Fig. 9F). Some nails may be dystrophic or absent, but others may remain normal. Oral blistering is rare and teeth are normal. In extreme cases, nail changes are the only manifestation. Pretibial DEB cannot clearly be separated from DEB localisata, and since both are caused by mutations in the same gene, these subtypes should be considered the same [128].

Recessive DEB Generalisata Mutilans (MIM 226600, AR-DEB-GM)

This form belongs among the most severe subtypes of the EB family and leads to invalidity, with a poor prognosis owing to extracutaneous complications [3,4]. Severe, generalized blistering is present at birth, extends progressively, and leads to remarkable scarring. Recurrent blistering leads to erosions, which heal slowly with scarring and milia formation. Already in childhood, syndactylies lead to the characteristic mitten-like hands and feet (Fig. 8A,B). Nails become dystrophic and are lost early in life. Scarring causes joint contractures. Mucosal involvement of the oral cavity, pharynx, larynx, and esophagus makes eating difficult. Anal blistering and erosions cause difficulty in defecation and constipation. Impaired nutritional intake and protein loss through chronic erosions lead to anemia and growth retardation. Corneal and conjunctival blistering and erosions are present in some patients.

Recessive DEB Generalisata Nonmutilans (AR-DEB-G)

Generalized blistering is present at birth, with a predilection for the hands and feet. Later, generalized

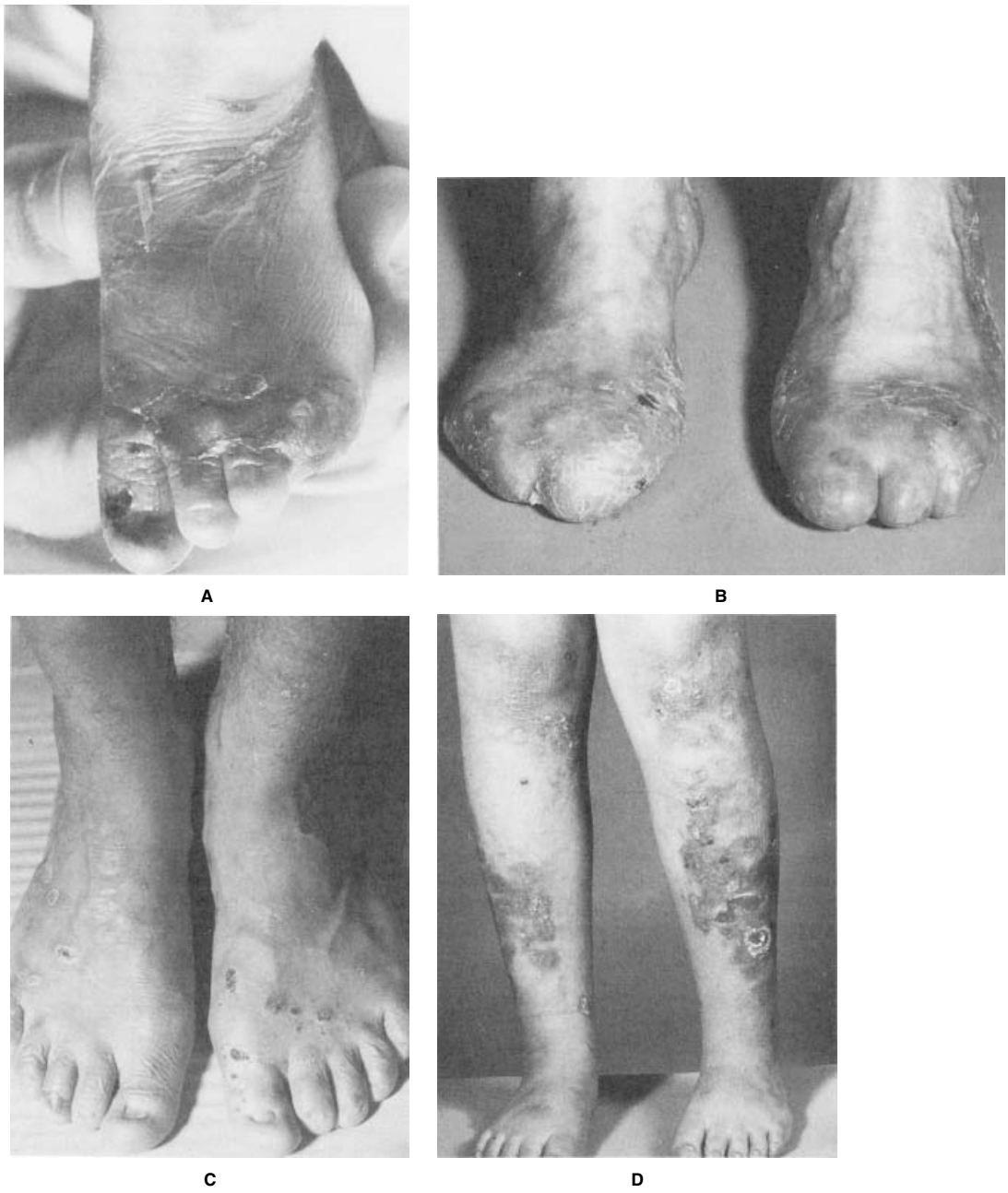

Figure 8. Clinical features of dystrophic EB. **A**: Severe blistering and nail loss in a 4-week-old child with DEB generalisata mutilans. **B**: Mutilation of the feet in a 32-year-old with DEB generalisata mutilans. **C**: Localized blistering, scarring, and nail dystrophy of the feet in recessive DEB localisata. **D**: Blisters, erosions, scarring, and red atrophic areas of the lower extremities in dominant DEB generalisata.

traumatic blisters on the trunk occur as well. Mucosal blistering is extensive, and dystrophy of teeth is evident. Loss of nails is almost always present. Notably, there are no mutilations or syndactylies [3,4].

Recessive DEB Inversa (MIM 226450, AR-DEB-I)

Neonatal blistering and congenital ulcerations are followed by blistering concentrated on the trunk in childhood and later in the axillary, inguinogenital, and perianal folds and belt-like blistering over the sacrum and the neck [3,4]. Some acral involvement continues. The lesions heal with red atrophic scars and only rarely with milia. Early oral blistering leads to scarring and the characteristic shortening of the lingual frenulum. Perianal fissures or blisters may occur. The teeth are normal.

Recessive DEB Localisata (AR-DEB-L)

Acral blistering appears from birth. Atrophic cigarette paper-like scars develop distally from the elbows (Fig. 9B) and the knees [3,4]. Nail and tooth dystrophies are common (Fig. 8C). This subgroup may be clinically indistinguishable from the dominant EBD localisata. A patient with recessive

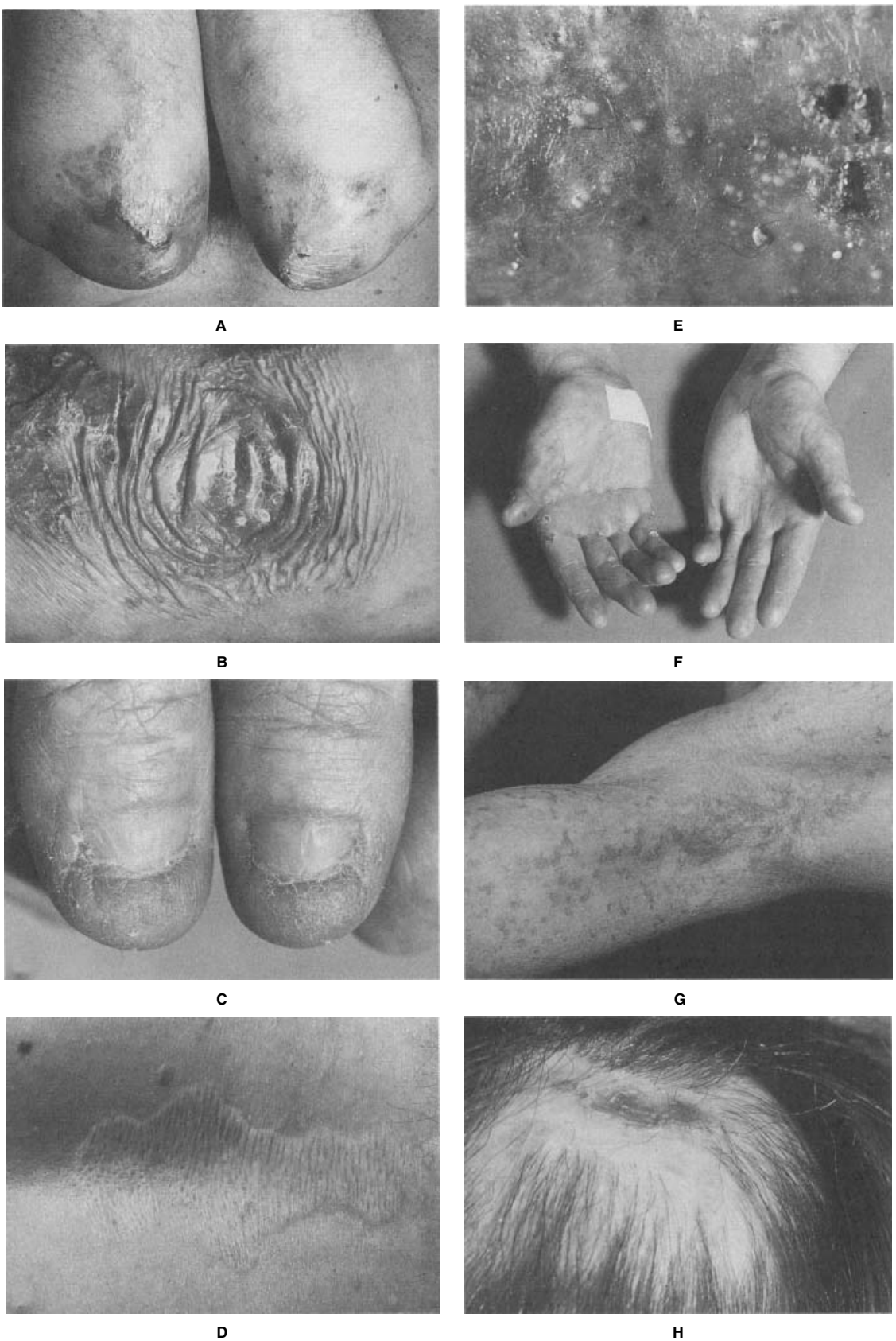

Figure 9. Some characteristic accompanying lesions in EB. **A**: Scarring at trauma-exposed sites. **B**: Atrophic scar. **C**: Nail dystrophy. **D**: Albulopapuloid lesion. **E**: Milia. **F**: Contractures due to scarring. **G**: Irregular pigmentation in EBS with mottled pigmentation. **H**: Alopecia.

pretibial DEB has been described [129], but, as noted above, pretibial DEB cannot clearly be distinguished from DEB localisata, and because both are caused by mutations in the same gene [128], these subtypes should be considered the same.

Transient Bullous Dermolysis of the Newborn (TBDN)

An unusual self-limiting postnatal blistering disease, TBDN is allelic to DEB (Fig. 10). It is characterized by highly fragile skin and blister formation at birth and by rapid amelioration within the first two years of life [3,4,130]. The skin blisters and erosions heal with mild atrophy and scarring, but other secondary signs such as hyperpigmentation, hyperkeratosis, nail dystrophy, and dental anomalies are usually absent. Disappearance of apparent clinical disease during the first years of life is typical, even though mild and variable skin fragility persists in some cases [4,131]. TBDN was previously distinguished from DEB by its benign, limited course and by distinctive pathomorphogenetic changes, such as, retention of collagen VII within the epidermis (Fig. 10), but the discovery of mutations in the collagen VII gene has now established TBDN as a DEB subtype [66,67].

EB Acquisita (EBA)

EBA is an acquired, inflammatory autoimmune blistering disorder that clinically resembles DEB, with blistering at trauma-exposed sites and subsequent scarring and milia formation. Autoantibodies to collagen VII bind to anchoring fibrils and are associated with dermal-epidermal separation below the lamina densa with subsequent scarring of variable degree. The antibodies are believed to perturb functions of the anchoring fibrils and thereby to contribute to blister formation [132–134]. In most cases, the immunodominant epitopes have been shown to reside within the globular NC1 domain of collagen VII [135]. However, a novel EBA subgroup with a milder clinical presentation has been defined in children with tissue-bound and circulating autoantibodies targeting only the triple-helical central domain of collagen VII and not the NC1 domain [136]. The diagnosis of EBA is established by direct immunofluorescence staining of the patient's skin for the demonstration of immunoglobulin deposits at the DEJ and the demonstration of circulating anticollagen VII antibodies in serum. Therapeutic strategies include immunosuppressive regimens with systemic steroids, azathioprine, cyclosporin A, and other agents [132]. Apart from EBA, antibodies to collagen VII have been described in association with bullous systemic lupus erythematosus [137] and some forms of inflammatory bowel disease [138].

Genetics

As far as is known today, all DEB subtypes are caused by mutations in the collagen VII gene, *COL7A1*,

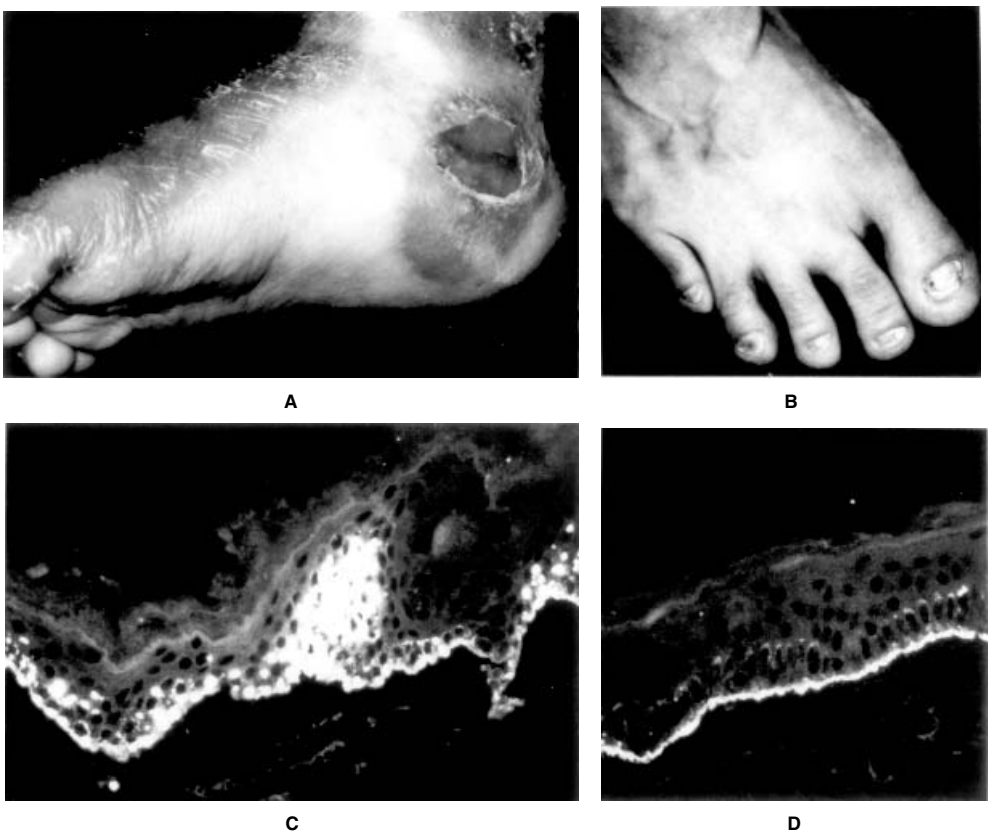

Figure 10. Transient bullous dermolysis of the newborn (TBDN): minimal phenotypes of heterozygous *COL7A1* mutations may be modulated by a second mutation. **A**: A proband with TBDN and massive blistering at birth was compound heterozygous for two different glycine substitution mutations in *COL7A1*, G2251E, and G1519D. **B**: The maternal mutation G2251E caused toenail dystrophy but no skin blistering. The paternal mutation G1519D was silent in the heterozygous state, and the father was clinically unaffected. **C**: In the skin of the newborn, the mutations caused abnormal folding, slow secretion, and intraepidermal accumulation of collagen VII. **D**: After 14 months, in parallel with clinical amelioration, collagen VII was found deposited at the dermal-epidermal junction, with minimal accumulation in the epidermis.

on chromosome 3p21, and are therefore allelic. More than 150 mutations have been reported to date and, remarkably, most patients with recessive DEB are compound heterozygous for two different mutations. Most of the mutations in recessive DEB are nonsense mutations or small insertions or deletions, which lead to frame shift and premature termination codons (PTCs) for translation [5,30]. In general, PTCs are associated with strongly reduced levels of the corresponding mRNA transcripts due to nonsense-mediated mRNA decay [120] and lack of synthesis of the corresponding polypeptide. The PTC mutations are silent in the heterozygous state but when homozygous or combined with another PTC mutation in the other allele, they lead to severe skin fragility, as illustrated by DEB generalisata mutilans.

A large number of missense mutations have also been identified and shown to be associated with an unusually broad spectrum of DEB phenotypes. Particularly interesting are mutations causing the substitution of a glycine residue within the triple-helical domain of collagen VII by another amino acid. In the case of other collagen genes, glycine substitution mutations characteristically cause dominantly inherited disease through dominant negative interference [139] (see also Chapters 8 and 9, this volume). *COL7A1* is, however, an exception among the collagen genes in that not all glycine substitution mutations are dominant, and in several cases remain silent when combined with a normal allele, although they lead to clinical manifestations when combined with another aberration in the other allele [140]. *COL7A1* is also unique among the collagen genes in that a single class of mutations — such as glycine substitutions or deletions — can cause either dominantly or recessively inherited DEB [5].

Most *COL7A1* gene defects are private mutations and spread over the entire gene, with the exception of exon 73. One study implicated this exon as a highly mutable region; over 10% of all *COL7A1* mutations reported to date have been found in exon 73 (Fig. 11). In a series of 53 European DEB families with dominant or recessive inheritance, 28% of the families had a mutation in exon 73 [141].

Basic Defects

Collagen VII

In DEB, the tissue separation occurs at the level of the anchoring fibrils. Abnormalities of collagen VII (see Chapter 2, this volume), the major structural protein of the fibrils, are known to be associated with DEB, and the most recent studies on the consequences of collagen VII mutations in DEB have revealed novel aspects of the biology of this collagen and the anchoring fibrils. Procollagen VII is a homotrimer of three proα1(VII) chains of 2,944 amino acids, each polypeptide consisting of a large 145 kDa central triple-helical collagenous domain flanked by an amino-terminal 145 kDa NC1 and carboxyl-terminal 30 kDa NC2 noncollagenous domain [28,30,142]. The collagenous domain contains repeating Gly–X–Y sequences that fold into a triple helix. The helix is interrupted 19 times by short noncollagenous sequences varying from 1 to 39 amino acid residues, which are thought to provide conformational flexibility to the molecule, a feature that may be of utmost importance for the functions of collagen VII fibrils as anchoring structures of the epidermis. Consistently, the positions of the noncollagenous interruptions within the Gly–X–Y repeat sequences are evolutionarily highly conserved [143]. The longest interruption of 39 amino acid residues lies in the middle of the helical domain. Rotary shadowing electron microscopic studies have shown that the molecule contains at this site a major flexible region [144], a fact that has lent the name "hinge region" to this 39 amino acid stretch. This site is also more sensitive to limited pepsin or trypsin digestion, and the triple helix can be cleaved into the carboxyl-terminal P1 and amino-terminal P2 fragments, each representing approximately one-half of the triple-helical domain [28,30]. During fibrillogenesis, procollagen VII monomers form antiparallel tail-to-tail dimers with a central carboxyl-terminal overlap and with the amino termini pointing outward. The dimers aggregate laterally in a nonstaggered manner into the anchoring fibrils, and before or during this process a C-terminal propeptide is proteolytically removed [45,145]. *In situ*, anchoring fibrils appear also to be transamidated, and both the triple-helical

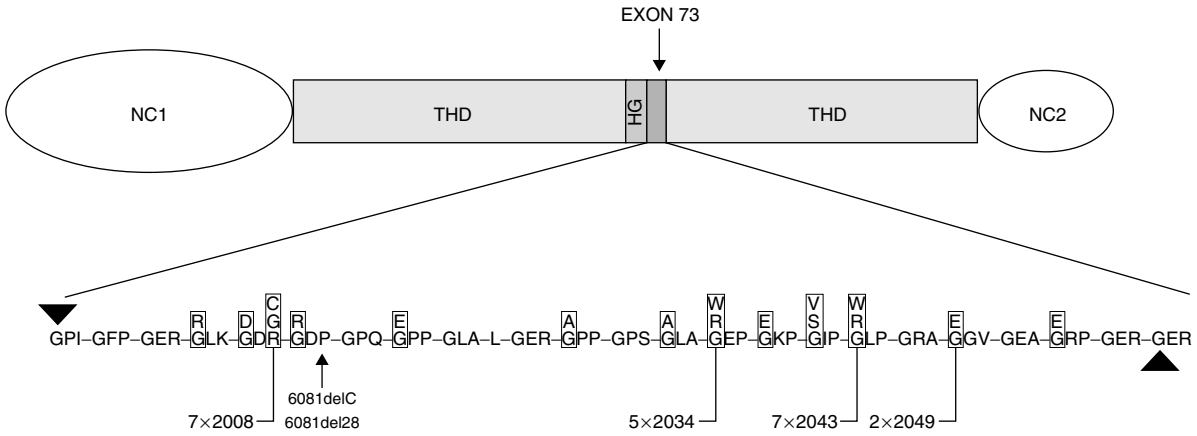

Figure 11. Clustering of *COL7A1* mutations in exon 73. Schematic representation of the collagen VII molecule and the location of the segment encoded by exon 73 (arrowheads). It is part of a 23 Gly–X–Y triplet stretch which is flanked N-terminally by the noncollagenous hinge region (HG) of 39 amino acids; NC1 = noncollagenous domain-1; THD = triple-helical domain; NC2 = noncollagenous domain-2. Amino acid substitutions in positions 2008 and 2043 have been reported in seven families, in position 2034 in five, and in position 2049 in two. In addition to the missense mutations indicated in this figure, two deletion mutations [141] have been reported in exon 73 (arrow).

and the carboxyl-terminal NC2 domains of collagen VII serve as substrates for tissue transglutaminase-2 *in vitro* [146]. These findings suggest that after anchoring fibrils have polymerized, transglutaminase cross-links are formed to stabilize the mature fibrils.

Several lines of evidence suggest that anchoring fibrils contain, in addition to collagen VII, other proteins that may be candidate proteins/genes for DEB [30]. For example, the monoclonal antibodies AF1 and AF2 decorate anchoring fibrils in immunoelectron microscopy but do not react with collagen VII. Another monoclonal antibody, GDA-J/F3, has been used to identify a novel anchoring fibril-associated epitope. The antibody recognizes a 50 kDa noncollagenous protein at the DEJ that by immunoelectron microscopy was localized to the insertion points of the anchoring fibrils into the lamina densa. Its integration into the histoarchitecture of the DEJ is dependent on the presence of collagen VII and the anchoring fibrils because the GDA-J/F3 epitope was missing in several DEB patients with absent or mutated collagen VII [55].

Extensive Molecular Heterogeneity of Collagen VII Mutations

COL7A1 mutations have a broad spectrum of biological consequences and lead to heterogeneous collagen VII abnormalities. From a protein chemical point of view, many of the pathogenetic mechanisms seem predictable; others, however, were completely unanticipated. Predictably, many mutations in severe recessive DEB lead to a premature termination codon (PTC) and a lack of protein synthesis. When the PTC mutations are homozygous or compound heterozygous with another PTC mutation, they cause severe phenotypes, as illustrated by DEB generalisata mutilans. Heterozygous carriers do not express truncated polypeptides but only the normal allelic product. The normal gene product does not appear to be compensatorily up-regulated, because keratinocytes from heterozygous carriers of *COL7A1* nonsense mutations have been shown to synthesize reduced amounts of collagen VII *in vitro* [147]. Therefore, one-half of the amount of collagen VII present in normal skin seems sufficient for adequate dermal-epidermal cohesion.

It has come as a surprise that the triple-helical collagen VII molecule can tolerate a number of structural aberrations without exhibiting any obvious functional defect. For example, glycine substitutions or other mutations causing rather significant changes in the primary structure of the proα1(VII) polypeptide can result in extremely mild clinical phenotypes or no disease phenotype at all. In contrast to other collagens, where glycine substitutions are common and interfere with triple-helical folding causing severe phenotypes [139] (see also Chapters 8 and 9, this volume), only a minority of *COL7A1* mutations cause glycine substitutions [49,66,140,141,148–150]. Furthermore, the mutations cause very variable phenotypes. Some glycine substitutions are associated with a classic dominant DEB

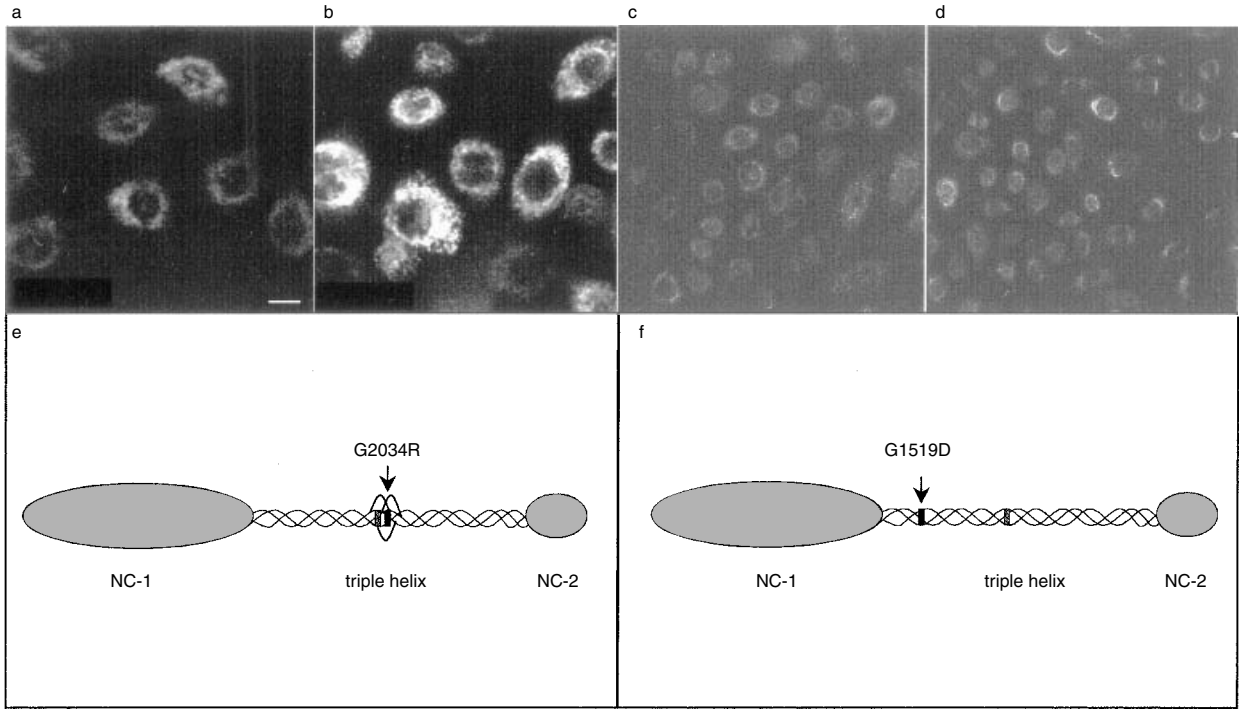

Figure 12. Some but not all glycine substitutions in collagen VII cause dominant negative effects. Immunofluorescence (IF) staining for collagen VII produces a moderate signal in normal control keratinocytes (**a**), whereas keratinocytes with a heterozygous *COL7A1* mutation causing G2034R substitution (**b**) exhibit a clearly stronger IF staining, indicating the intracellular accumulation of procollagen VII. In contrast, IF staining of mutant keratinocytes with the heterozygous glycine substitution G1519D (**c**) show an intracellular IF signal comparable to controls (**d**). The glycine substitution G2034R close to the central noncollagenous hinge region (**e**) interferes more strongly with triple-helical folding than glycine substitution G1519D situated within a long uninterrupted Gly–X–Y sequence close to the amino-terminal end of the triple helix (**f**). Consequently, the extent of the intracellular accumulation of procollagen VII is reflected in the degree of severity of the effects of the mutation. For details, see [140].

phenotype (Fig. 8), such as the mutations G2006D, G2015E, G2034R, and G2043W (Fig. 12) [140,141]. In contrast, the dominant mutations G2251E (Fig. 10) and G2287R merely cause nail dystrophy but no skin blistering, and the mutations G1347R, G1519D, and G2316R result in no disease phenotype at all (Fig. 12) [66,67,140,150].

Another study showed that, surprisingly, mild dominant negative interference resulted from large deletions in collagen VII [151]. Heterozygous intraexonic deletions altered splicing and led to the elimination of ten Gly–X–Y triplets from the center of the triple-helical domain of collagen VII in one DEB patient and to the omission of eight Gly–X–Y triplets from the carboxyl-terminal half of the triple helix in another DEB proband. DEB keratinocytes synthesized equal amounts of normal and shortened collagen VII polypeptides *in vitro* and showed intracellular accumulation of procollagen VII, but some mutant molecules were also secreted. This resulted in a reduced number and abnormal morphology of anchoring fibrils in the skin. These collagen VII abnormalities are analogous to the single-exon deletions in *COL1A2*, *COL3A1*, and *COL11A2* genes reported in, for example, osteogenesis imperfecta type I, Ehlers-Danlos syndrome type IV, and dominant Stickler syndrome, respectively [152–154] (see also Chapters 8, 9, and 23, this volume). However, in comparison with these other collagen disorders, the very mild phenotype and cessation of blistering by adulthood were striking in the case of the DEB patients.

The normal or mild DEB phenotypes associated with heterozygous mutations can, however, be severely modified if combined with another aberration. This is well-illustrated by three unrelated DEB families all of whom had a genomic deletion (8523del14) causing in-frame skipping of exon 115 [45,49]. The deletion alone did not cause a pathologic phenotype, as shown by the unaffected heterozygous carriers (Fig. 13). In the first family, the deletion was combined with a splice site mutation 425A-to-G, which led to abnormal splicing of exon 3 and the generation of several transcripts all of which contained a PTC. Therefore, the proband was hemizygous for the deletion mutation and exhibited a severe phenotype with generalized blistering. Both parents, heterozygous carriers of the mutations, were normal. In the second family, the combination of the same deletion with the glycine substitution mutation G2009R [45] caused a mild phenotype with acral blistering. Again, the mother, who was a carrier of the deletion, was healthy. In a third family, the deletion was combined with a dominant glycine substitution mutation G2043R [49]. This combination resulted in a severe phenotype, whereas the dominant mutation alone caused only mild skin blistering. These examples demonstrate that even significant deletions in collagen VII protein, such as skipping of the 29 amino acids encoded by exon 115, do not necessarily cause a pathologic phenotype. However, combination of the clinically silent deletion with another gene defect can result in drastic modulation of the phenotype (Fig. 13).

Transient bullous dermolysis of the newborn (TBDN) presents another example of modulation of the phenotype by a second mutation. Compound heterozygosity for dominant and recessive glycine substitution mutations in *COL7A1* was found in two unrelated probands [66,67]. In both families, the dominant glycine substitution mutation caused a minimal phenotype of toenail dystrophy but not skin blistering, whereas the recessive glycine substitution mutations remained silent when in the heterozygous state.

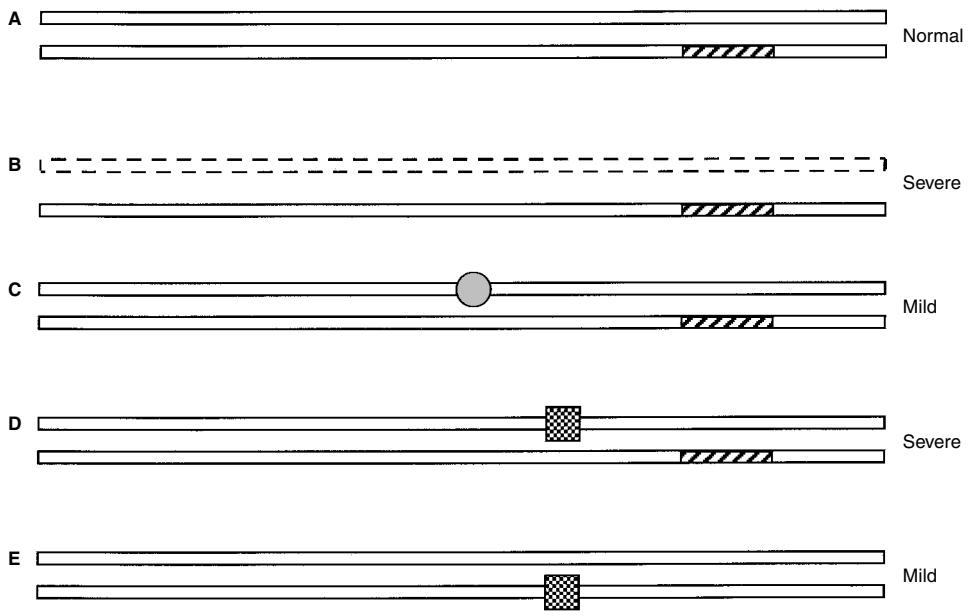

Figure 13. Modulation of the DEB phenotype by a second mutation. The *COL7A1* deletion mutation 8523del14 (striped bar) has been found in several unrelated DEB families from different European countries. Depending on the mutation constellation, the phenotypes differed significantly. (**A**) In combination with a normal allele, *COL7A1* 8523del14 did not cause a pathological phenotype in any of the families. (**B**) In the hemizygous state, when combined with a null allele (interrupted line), a severe phenotype resulted. (**C**) Compound heterozygosity for the deletion and the glycine substitution mutation G2009R (shaded circle) resulted in a mild phenotype. (**D**) In contrast, in combination with the dominant glycine substitution G2043R (shaded square), the deletion led to a severe affection. (**E**) The dominant G2043R mutation alone caused a mild DEB phenotype [49].

Only a combination of the two mutations led to a severe blistering phenotype at birth (Fig. 10). These are the first observations of compound heterozygosity for glycine substitutions in any collagen, and the dramatic intracellular accumulation of the mutant protein in the epidermis may result from "doubly" dominant negative interference in collagen VII folding by two different glycine substitutions. Because the TBDN cells do not synthesize any normal α1(VII) chains, collagen triple helices containing only mutant polypeptides are likely to be retained intracellularly or secreted slowly [66]. Highly active collagen biosynthesis during the postnatal period may be a contributing factor, and a high rate of synthesis of mutant, slowly secreted collagen VII molecules may manifest as intraepidermal accumulation of collagen VII (Fig. 10) and subsequent lack of anchoring fibrils at the DEJ.

Based on the examples above and other similar observations, it seems likely that the specific location of a mutation within a particular submodule of collagen VII, some of them more critical than others for the supramolecular assembly and function of this protein, is important in determining its impact on the overall stability, ligand binding, and other functions. One should keep in mind that the mutant polypeptides fold together with normal polypeptides into collagen triple helices and that the structurally aberrant trimeric molecules are incorporated into fibrils. The effects of the initially rather small structural abnormality are thereby accentuated, or perhaps attenuated, by the supramolecular assembly, and the resulting collagen fibrils are rendered inadequate in different ways. The biological and clinical phenotype is therefore determined by the uniqueness of each particular function and by the extent to which it is lost through the mutation.

Physical Properties and Morphology of Anchoring Fibrils and Pathogenesis of the Disorder

Ultrastructurally, in contrast to normal skin with its well-defined slender and centrosymmetrically cross-banded anchoring fibrils [27], DEB skin displays broad, blunt, whispy fibrils without cross-banding or no fibrils at all [3,125,126]. Complete lack of anchoring fibrils and collagen VII are characteristic signs of the most severe subtype, DEB generalisata mutilans [127]. In milder DEB forms, mutant collagen VII polypeptides are synthesized and incorporated into the fibrils, resulting in variably abnormal morphology and functions of the fibrils. As discussed above, despite the large number of known mutations in *COL7A1* in different DEB subtypes [5,30], correlations between the individual mutations and their suprastructural consequences are only now beginning to emerge. Future studies using mutant collagen VII molecules isolated from DEB skin or keratinocyte cultures, or normal and mutated recombinant collagen VII fragments, must address the effects of a genetic defect on the assembly and fibrillogenesis of collagen VII.

TBDN skin demonstrates very different ultrastructural features at birth. No normal anchoring fibrils can be discerned at the DEJ. Instead, highly dilated rough endoplasmic reticulum in basal and suprabasal keratinocytes of the epidermis is characteristically filled with filamentous material, electron-dense granules and rods [130], which were identified as consisting of collagen VII by immunofluorescence [131]. Curiously, at the age of about 12 months, the morphology normalizes, the intraepidermal accumulation of collagen VII disappears, and anchoring fibrils begin to appear at the DEJ.

DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS OF EPIDERMOLYSIS BULLOSA SUBTYPES

Family History

In families with dominant EB, the pedigree is useful in determining the likelihood of recurrence. However, most EB patients do not have an obvious family history. Nevertheless, a careful history should be taken and all family members be examined for minimal signs of the disease, such as nail dystrophy or enamel hypoplasia, because these minor symptoms may indicate heterozygous mutations. Compound heterozygosity for mutations in a large number of EB patients with different subtypes, and the fact that several families have mixed dominant/recessive mutations [66–68], call for careful (re)consideration of inheritance patterns and the recognition of rare genetic constellations, which seem to be more common in EB than in other genetic disorders.

Clinical Evaluation

At birth, the clinical presentation of EB is unspecific, and it is not possible to differentiate between the main categories and the subtypes on clinical grounds. The secondary signs, such as scarring, milia, hyperkeratosis, alopecia, nail dystrophy, and hyperpigmentation, that are helpful in determination of the EB category only develop within months or years. Therefore, the major category should be defined by antigen mapping as early as possible. The clinical subtype within the major category often becomes obvious when lesions at the predilection sites and the secondary signs have developed. Consequently, regular follow-up visits are required for observation of the course of the disease.

Morphological and Molecular Analysis

A skin biopsy is essential for the diagnosis and classification of EB. The major category can often be determined by antigen mapping of a skin sample from a newborn because a fresh blister shows the typical cleavage plane at the DEJ. The sample should be obtained from clinically uninvolved skin at a distance of 5–10 mm from a blister. Conventional light microscopy is not indicated because in all subtypes blisters form within a narrow zone at the DEJ. The resolution of the light microscope does not suffice for differentiation between intrabasal cell, intralamina lucida, and sublamina densa blistering. Electron-microscopic studies have substantially aided the diagnosis and classification of EB in the past but have been largely replaced by antigen mapping (Fig. 2). However, electron microscopy may still be useful for the study of structural abnormalities of the anchoring complex.

Differential Diagnosis

Table 3 summarizes conditions that may cause difficulty in the differential diagnosis of hereditary EB in the neonate. Genetic disorders, such as bullous ichthyosiform erythroderma, incontinentia pigmenti, congenital poikiloderma with subepidermal blistering, or congenital aplasia cutis, may present with blisters or erosions at birth. However, all of them exhibit characteristic ultrastructural features that can be recognized by electron-microscopic examination [3]. Congenital herpes simplex, staphylococcal scalded skin syndrome, and neonatal candidiasis and impetigo can be differentiated from EB by microbiological means. Neonatal pemphigoid gestationes, pemphigus vulgaris, and, at a later age, other acquired blistering diseases, such as EB acquisita, bullous pemphigoid, and dermatitis herpetiformis, can be diagnosed by direct immunofluorescence on frozen sections to reveal the characteristic immune deposit patterns in the skin. On

rare occasions, suction blisters on the thumb or the radial aspect of the hand in newborns have been observed. These bullae are presumably the self-inflicted results of sucking *in utero* [155].

Mutation Detection Strategies for Epidermolysis Bullosa

As indicated in Table 1, mutations in ten distinct genes have been identified in different EB subtypes thus far. In most cases, the genes were identified as candidate genes and proteins on the basis of ultrastructural and/or immunohistochemical observations. Initial studies identifying mutations in different forms of EB used RT-PCR and direct sequencing of the cDNAs. It soon became evident, however, that many of the mRNA transcripts were relatively large, and the corresponding gene structures were found to be complex, thus rendering mutation search by direct sequencing of the genes time-consuming and expensive. Therefore, mutation detection strategies based on amplification of the exons directly from genomic DNA have been developed [156].

Heteroduplex Scanning Strategy

Mutation screening strategies developed for the identification of mutations in the genes for collagen VII, collagen XVII, laminin 5, α6 and β4 integrins, and plectin [5,93,110,156] have been adopted by many laboratories. For this purpose, primers placed on flanking intronic sequences are used. These primers reliably amplify all coding sequences from the genes in PCR products varying from about 200 to 600 bp in size. The PCR products are then subjected to heteroduplex scanning by conformation-sensitive gel electrophoresis (CSGE) [157], which, in the case of heterozygous sequence variants, displays heteroduplex bands. This is followed by direct automated sequencing of the PCR products displaying heteroduplexes. If the sequence variants are potentially pathogenic mutations, their inheritance will be examined in the family by restriction enzyme digestion or allele-specific oligonucleotide hybridization. Pulkkinen and Uitto [5] have estimated that this methodology is able to detect approximately 80% of all single base-pair substitutions and small insertions or deletions in the genes. However, the strategy may miss large deletions or major rearrangements in the gene. It should also be noted that in the case of recessively inherited homozygous mutations, a proband's PCR products have to be mixed with control PCR products to detect heteroduplexes by CSGE. Alternatively, DNA from the obligate heterozygote parents can be examined.

Mutation screening of JEB and DEB is facilitated by the occurrence of common mutations in two genes. In *LAMB3*, two hotspot mutations, R42X and R635X, have been shown to account for 50% of all mutations associated with JEB Herlitz [69]. Therefore, these two mutations are tested with priority when JEB is suspected. The test is fast because both mutations can be verified by restriction enzyme digestion of DNA amplified by PCR. Evidence suggests that *COL7A1* mutations may cluster in exon 73 [141]. So far, 17 different mutations in this 200 bp exon have been observed, and in our series of 53 DEB families, 28% of families had a mutation in this exon (Fig. 11). Recurrent mutations causing amino acid substitutions in positions 2008, 2034, and 2043 can be verified by restriction enzyme analysis. Therefore, exon 73 is now being screened with priority in our laboratory before initiating the analysis of the 117 other exons of the *COL7A1* gene.

MANAGEMENT OF EPIDERMOLYSIS BULLOSA

No specific cure exists for EB. Although a number of pharmacological regimens have been reported, none has been uniformly successful. The reason for the variable response is not clear, but it may be due to genetic and molecular heterogeneity within the major clinical categories. At present, the management of EB is mainly supportive, and the therapy is symptomatic. A high standard of personal hygiene, careful nursing and daily skin care, protection from trauma, and avoidance of infection are fundamental for optimal management.

Conventional Therapies

General Care

To avoid friction and heat, soft, well-ventilated clothing and footwear should be chosen. Additional reduction of sweating of the feet by administration of aluminum salts or formaldehyde may be beneficial for some patients. Blisters should be burst with a sterile needle or lancet, and erosive skin areas should be soaked with mild cleansing solutions, disinfected, and treated with soft creams or lotions. The dressing materials should not cause friction, and tape should not be applied directly to the skin. Physical therapy is important to prevent joint contractures after scarring. Nutritional advice concerning sufficient calorie, protein, fiber, vitamin, and mineral intake is essential. Patients with laryngeal or esophageal involvement may have to take liquidized food. Good prophylactic dental care and advice are necessary because gingival blisters and erosions may make normal dental hygiene painful or impossible. Detailed instructions about the nursing and care of EB skin can be found in excellent booklets available from DEBRA (Dystrophic Epidermolysis Bullosa Research Association; addresses at the end of this chapter).

Topical Medication

Simple blistering without secondary traumatization or infection usually heals well with everyday skin care and disinfection. Antibiotics are recommended only for secondary infections. Fucidic acid, tetracycline, erythromycin, and clindamycin ointments have proven to be successful regimens. Creams supporting reepithelialization are also useful.

Systemic Therapy

Several therapeutic modalities have been tested in the past, such as phenytoin, retinoids, and tetracyclines [3,4]. Initially, a favorable response to phenytoin was observed in a number of patients with different DEB or JEB subtypes. However, in a multicenter study, no statistically significant effect of phenytoin could be observed [158]. Positive effects of tetracycline and a negative response to retinoids have been reported in individual patients. Systemic corticosteroids have been applied in very severe cases of JEB Herlitz and EBS herpetiformis. Some suppression of blistering activity and inflammation was achieved, but high dosage was required and the side effects were massive. No large studies document the usefulness of these hormones in EB. Systemic antibiotics are indicated when secondary infections occur. Some of these drugs may exhibit an additional beneficial effect on blistering activity, but they should not, for obvious reasons, be used in the absence of infection. Vitamin E has previously been widely used in the therapy of all EB subtypes. Its antioxidant effect was believed to suppress blistering, but the experience of most investigators with this therapy is not encouraging. Dapsone inhibited blistering in an infant with JEB Herlitz, although in other cases the response was not

remarkable. Because nutritional zinc deficiency may occur in some probands, zinc substitution has been used to support wound healing in EB. Oral contraceptives may benefit female patients suffering from JEB inversa whose condition worsens after the menopause.

Surgical Treatment

Surgical degloving of fused hands and feet of patients with AR-DEB generalisata mutilans is routinely and successfully performed in several centers [159,160]. However, repetition of the procedure, often at frequent intervals, may become necessary. Esophageal dilatation, with either a balloon or an instrument, is often required by patients with DEB generalisata mutilans and DEB inversa to release strictures [3,161]. Skin grafting and cultured epidermal autografts have been partially successful in covering denuded areas in DEB [162]. Healing of both the donor and recipient sites has, perhaps surprisingly, been unproblematic. Reepithelialization of chronically denuded areas has been achieved after repeated grafting; however, the autograft contains the same genetic defect as all cells of the patient and thus remains fragile.

Gastrostomy Feeding

Nutrition is a problem in patients who suffer from subtypes of EB involving the oropharynx and esophagus. The use of feeding tube gastrostomy, mostly with button devices, has proven to be a valuable method to complement the oral intake of foods and enhance nutrition in JEB and DEB. Regular overnight feeding via gastrostomy tube resulted in remarkable growth of affected children and the augmentation of blood cell counts and hemoglobin concentrations [163].

Gene Therapy Approaches

For the future treatment of EB, the development of successful gene therapy approaches is required. This could involve the *ex vivo* manipulation of cultured cells in such a manner that a mutation is corrected, followed by grafting of the cells onto eroded areas of skin. Alternatively, direct application of DNA into skin could be used in an attempt to elicit genetic reversal of the underlying mutation [164,165]. Despite the fact that skin, because of its accessibility, seems an optimal target organ for gene therapy, concerns regarding the efficiency of gene therapy approaches have been raised in relation to methods of delivery, targeting of epidermal stem cells, duration of expression of transgenes, and long-term safety. Although the successful application of gene therapy for the treatment of EB may still be several years away, the rapid development of new technologies, such as the use of ribozymes or the application of chimeric RNA/DNA nucleotides for the correction of a mutation by homologous recombination, hold promise for gene therapy for different EB subtypes.

Several *in vitro* studies have already described the correction of deficient gene expression in EB keratinocytes. In one study, epidermal keratinocytes from a JEB Herlitz patient with a homozygous *LAMB3* mutation were immortalized using SV40 virions [166]. The cells were unable to synthesize laminin 5, to assemble hemidesmosomes, or to adhere. Transduction of the β3-null keratinocytes with a retroviral construct expressing a human β3 cDNA rendered the cells able to synthesize and secrete mature heterotrimeric laminin 5. The gene correction fully restored the keratinocyte adhesion machinery, including the capacity for proper hemidesmosomal assembly [167]. In another investigation, collagen XVII-deficient keratinocytes derived from a patient with generalized atrophic benign EB were immortalized and transfected with cDNAs for wild-type collagen XVII. This resulted in the expression of recombinant collagen XVII protein that was correctly polarized at the basal cell surface together with α6β4 integrin [168]. Seitz et al. [169] used a retroviral expression vector for human BP180/collagen XVII to transfect primary JEB keratinocytes and construct skin grafts on immune-deficient mice and showed that the gene-transduced grafts expressed collagen XVII and deposited it at the DEJ *in vivo*. Because cultured keratinocytes are used routinely to make autologous grafts for patients suffering from large skin or mucosal defects, the full phenotypic reversion of primary human epidermal cells defective for a structural protein opens perspectives in the long-term treatment of genodermatoses.

Genetic Counseling of Epidermolysis Bullosa Families

Genetic counseling is an essential part of the management of EB, and all persons involved should be adequately informed. However, the discovery of extensive variability of mutation constellations has consequences for the counseling of affected families. Compound heterozygosity for mutations in a large number of EB patients, and the fact that several families have combined dominant/recessive mutations, should induce (re)consideration of inheritance patterns and the recognition of rare genetic constellations, which seem to be more common in EB than in other genetic disorders. Therefore, the detection of one or two mutations in the index patient does not always allow unambiguous determination of the inheritance pattern or prognostic predictions. For these reasons, the genetic counseling of EB families should be cautious and performed in specialized centers by persons well-acquainted with the genetic and molecular complexity of EB. It is advisable to perform antigen mapping, determine the candidate genes, and identify the mutations in an EB family well before genetic counseling and prenatal diagnosis may be required. Mutation screening of several large candidate genes under time pressure, possibly during a pregnancy at risk for recurrence, is stressful and less likely to succeed.

Prenatal Diagnosis

The identification of specific gene mutations in EB has allowed the development of DNA-based prenatal testing in families at risk of recurrence for severe forms of this disease. Such testing can be performed on chorionic villus samples as early as the tenth week of gestation or on amniocentesis samples at the twelfth week. In fact, prenatal testing has already been applied to families at risk for severe recessive DEB, JEB Herlitz, and JEB with pyloric atresia [5,170–172]. Figure 14 shows verification of the DNA-based prenatal diagnosis of JEB Herlitz using antigen mapping of skin biopsies of the index patient and the aborted affected fetus.

An extension of prenatal testing is the development of preimplantation genetic diagnosis (PGD), a technique that has been successfully applied to a variety of other genetic diseases [173]. PGD is performed in conjunction with *in vitro* fertilization, fertilized embryos being allowed to grow to the eight-cell stage, at which time one cell is removed for mutation analysis. Embryos lacking tested mutation(s) can then be implanted into the uterus to establish pregnancy, which is performed as part of the *in vitro* fertilization procedure. It should be noted that PGD is not legal in all countries at the present time.

When a mutation is not known, prenatal diagnosis of EB using electron-microscopic examination of a fetal

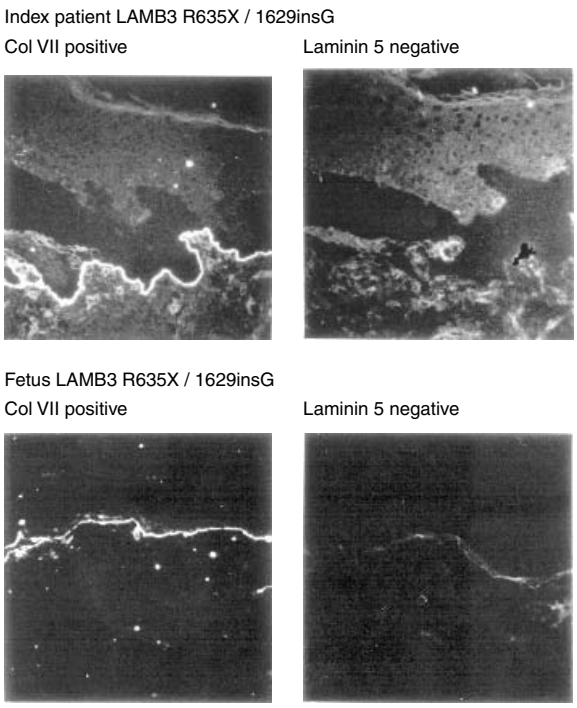

Figure 14. Prenatal diagnosis of JEB Herlitz. The index patient, who died at the age of 7 months, was compound heterozygous for *LAMB3* mutations R635X and 1629insG. Antigen mapping of the skin of this child showed junctional blistering with collagen VII in the blister base (**upper left panel**) and a complete lack of laminin 5 protein (**upper right panel**). Prenatal diagnosis using a chorionic villus sample obtained at the 11th week of the next pregnancy showed that the fetus carried both mutations. After termination of the pregnancy, the diagnosis was verified by antigen mapping of the fetal skin. This showed normal staining with collagen VII antibodies (**lower left panel**) but a negative reaction with antibodies to laminin 5 (**lower right panel**). The faint line visible in the lower right panel represents nonspecific staining of the horny layer.

skin biopsy serves to determine the presence of dermal-epidermal separation and to recognize specific ultrastructural defects. Prenatal diagnosis performed on a skin biopsy obtained by fetoscopy is not a screening method, and certain prerequisites for the tedious and not completely unrisky procedure are necessary [174,175]. The underlying defect must be morphologically demonstrable to allow unequivocal diagnosis, and the fetal skin must be sufficiently developed to allow morphological evaluation of the affected structures. For the DEJ this is the case between the sixteenth and twentieth weeks of pregnancy [176]. Prenatal evaluation is based on recognition of the characteristic ultrastructural abnormalities in fetal skin. Most experience has been obtained with the generalized recessive forms of JEB and DEB because these subtypes are severe enough to warrant the possible termination of pregnancy. Briefly, JEB exhibits detachment of the epidermis through the lamina lucida and defective hemidesmosomes. DEB shows blistering in the uppermost papillary dermis and an absence of anchoring fibrils. Clumping of tonofilaments in EBS herpetiformis and a paucity of anchoring fibrils in dominant DEB makes prenatal diagnosis of these subtypes conceivable as well. Expertise in taking the biopsy, preserving and processing the material, and establishing cell cultures, and great experience in evaluating normal and defective fetal skin, are imperative.

ANIMAL MODELS

Naturally Occurring Defects

Many rare heritable connective tissue disorders occur in domestic or laboratory animals. Such disorders can be used to provide a model system for studies of both normal and abnormal tissues. Although the eponyms of human diseases are not strictly applicable to the analogous diseases in animals, the causes and effects appear to be directly comparable. Affected animals offer opportunities for experimental analysis that are not possible in humans, such as experimental mating for genetic analysis or the provision of large amounts of tissue for biochemical investigations or for organ and cell cultures.

Clinical reports describe hereditary blistering disorders in dogs, bulls, foals, calves, and sheep (Table 4) [177,178], but not all of these have been characterized by electron microscopy or with molecular probes. An autosomal dominant EBS with basal cell cytolysis was found in Simmental bulls, a severe JEB with lamina lucida blistering in a toy poodle, and DEB with sub-basement membrane blistering in New Zealand sheep and Brangus calves. The latter animals presented with oral blistering and sloughing

TABLE 4. Animal Models of Heritable Blistering Disorders of the Skin

Species	Targeted Protein	Gene	Phenotype	Human Disease	Ref.
Dog	Laminin 5	*LAMA3* *LAMB3* *LAMC2*	Skin blistering	JEB Herlitz, JEB localisata, generalized atrophic benign EB	177
Sheep	Collagen VII	*COL7A1*	Severe blistering, exungulation	DEB generalisata mutilans	178
Mouse	Collagen VII	*COL7A1*	Severe blistering	DEB generalisata mutilans	184
Mouse	Keratin 14	*KRT14*	Severe blistering, tonofilament clumping	EBS generalisata	81
Mouse	BP230	*BPAG1*	Skin blistering, neural abnormalities	?	98
Mouse	Plectin	*PLEC1*	Skin blistering, muscular abnormalities	EBS with muscular dystrophy	179
Mouse	Laminin 5	*LAMB3*	Severe blistering, perinatally lethal	JEB Herlitz	180
Mouse	α6 integrin	*ITGA6*	Severe blistering, perinatally lethal	JEB with pyloric atresia	181
Mouse	β4 integrin	*ITGB4*	Severe blistering, perinatally lethal	JEB with pyloric atresia	182
Mouse	α3 integrin	*ITGA3*	Mild skin blistering	?	183

off of the hooves postnatally. Inbred Swiss white alpine sheep carried a severe form of this disorder [178] and developed blisters in the mouth, oral mucosa, tongue, esophagus, and skin areas with sparse hair within two days of birth. Exungulation occurred early, and oral erosions led to difficulty in feeding. Electron-microscopical examination revealed sublamina densa splitting and no identifiable anchoring fibrils. Antigen mapping demonstrated a complete absence of collagen VII in clinically uninvolved tissues of the DEB sheep, whereas in normal sheep skin a bright linear fluorescence at the DEJ was seen. Immunoblotting of dermal and epidermal extracts of EB sheep showed a lack of collagen VII, a phenotype similar to the DEB generalisata mutilans in humans [178].

Transgenic and Knockout Mice

More recently, gene ablation or manipulation in mice has delivered additional information about some DEJ proteins. The functional significance of a molecule can be assessed by elimination of the protein or by the introduction of mutations that cause dominant negative interference in genetically manipulated mice. In addition, in contrast to human mutations, animal models allow investigation of the consequences of gene mutations during embryonic development and assessment of the early consequences of lethal mutations. To date, several such knockout or transgenic mice exist for DEJ components and their cellular receptors, including keratin 14, plectin, BP230, α3, α6, and β4 integrins, the β3 chain of laminin 5, and collagen VII (Table 4).

The first indication of the involvement of keratins in the etiopathogenesis of EBS was derived from transgenic mice. An EBS-like disease was induced when a truncated keratin 14 gene was introduced into the germ line of the animals. Intraepidermal blistering and tonofilament clumping were the key features of the phenotype [81]. Thus, it was shown for the first time that keratin filaments form an essential supportive network in keratinocytes and that abnormalities of basal cell keratins can lead to EBS.

Ablation of the plectin gene in mice resulted in skin blistering due to degeneration of keratinocytes and death 2–3 days after birth [179]. Hemidesmosomes were significantly reduced in number, and their mechanical stability was altered. The skin phenotype of these mice was similar to that of patients with EBS with muscular dystrophy [89–94]. In addition, plectin (−/−) mice revealed abnormalities reminiscent of minicore myopathies in skeletal muscle and disintegration of intercalated disks in heart, suggesting a general role of plectin in the reinforcement of mechanically stressed cells.

BP230, or bullous pemphigoid antigen-1, is located in the inner plaque of the hemidesmosome and hence is likely to bind intermediate filaments. This was confirmed by generating mice with ablation of the *BPAG1* gene encoding BP230. Null mice exhibited a phenotype with a lack of the hemidesmosomal inner plaque, lack of intermediate filament binding, and cytolysis of basal cells. Surprisingly, the phenotype included neuromuscular abnormalities [98]. Molecular analysis of the mice disclosed two isoforms of BP230, an epidermis-specific isoform in the inner plaque of the hemidesmosome, which associates with the keratin intermediate filaments and a neuron-specific isoform, dystonin, that binds actin [99].

Similarly to human probands with JEB Herlitz and JEB with pyloric atresia, knockout mice for the β3 chain of laminin [180] and the α6 and β4 integrins exhibited severe blistering of the skin and other epithelia and postnatal demise [181,182]. In the integrin knockout mice, hemidesmosomes were absent from mutant tissue, but the basement membrane appeared normal. This suggests that these integrins are important for the assembly of hemidesmosomes, but not of the basement membrane proper. These mice provide valuable models for the molecular analysis of these disorders and are useful for developing novel therapeutic strategies.

In contrast, integrin α3-deficient mice revealed regions of disorganized basement membrane in the skin [183]. In neonatal skin, matrix disorganization was frequently accompanied by blistering at the DEJ. Laminin 5 and other matrix proteins remained associated with both the dermal and epidermal sides of blisters, suggesting rupture of the basement membrane itself rather than detachment of the epidermis from the basement membrane. The findings support a role for α3β1 integrin in maintenance of the integrity of the DEJ and establish the gene for α3 integrin as a novel candidate gene for EB.

A collagen VII deficient mouse has also been reported. Similarly to patients with DEB generalisata mutilans and to sheep with severe recessive DEB, the mice exhibited extensive sublamina densa blistering and an absence of anchoring fibrils. The mucocutaneous lesions led to the postnatal demise of the mice [184].

CONCLUDING REMARKS

The surprising heterogeneity of EB mutation constellations and their biological consequences has complicated the unambiguous diagnosis, classification, and prognostic predictions of the disease. Even in situations assumed to be unproblematic, variations have been found, for example, in pedigrees with combined recessive and dominant mutations. Analysis of known genetic aberrations shows that homozygosity is relatively rare and that most patients with recessive EB are compound heterozygotes for recessive, or recessive and dominant, gene defects. For genetic counseling, these findings have a major impact. The identification of one mutation does not suffice for diagnosis and genetic counseling; rather, it is necessary to know whether a second mutation exists and whether it causes a phenotype or acts as a modulator.

Novel genes causing additional EB subtypes are likely to be identified in the future, thereby extending the genotypic and phenotypic spectrum of the disease. A number of candidates have already been identified, such as BP230, α3 integrin, and some of the enzymes involved in the post-translational processing of the macromolecules of the DEJ.

Careful biochemical assessment of as many involved molecules as possible and their mutated counterparts will shed additional light on the molecular consequences of the mutations and help design novel gene therapy approaches for the treatment of EB. The EB subtypes caused by null alleles seem to be optimal diseases to be treated with such therapies, because no interference from endogenous mutant molecules need be expected. The skin is easily accessible for morphologic and biochemical control studies, and keratinocyte culture and transplantation techniques have been well-developed for the treatment of burns. Gene transfer into human keratinocytes *in vitro* has been successfully performed in many laboratories, and procedures for the enrichment of epidermal stem cell populations, stable transfection of the cells, and expression of correctly folded proteins are rapidly advancing. The future development of

successful therapies will also depend on progress in our understanding of suprastructure formation by structural DEJ macromolecules. This not only concerns the mechanism of aggregate formation but also the structural characteristics unique to each molecule. Further, functional redundancies of molecular components within supramolecular aggregates will have to be defined in greater detail than presently possible. This endeavor will not only be assisted by the elucidation of further genetic defects and their consequences in animal or human diseases, as well as the generation of transgenic animals as models for human diseases, but also by the analysis of aggregate formation by isolated matrix macromolecules or their mixtures *in vitro*. This combined information will not only help to understand and treat heritable skin diseases but also many common acquired disorders.

RECENT DEVELOPMENTS

Revised Classification of Epidermolysis Bullosa

At the second international consensus meeting on diagnosis and classification of epidermolysis bullosa, collective clinical, epidemiologic and laboratory data relating to inherited EB were reviewed and a revised classification suggested, better to reflect current knowledge [185]. Future clarification of the molecular abnormalities underlying EB could eventually lead to a completely different classification, but it is presently premature to base any system solely on molecular data.

In generating the new classification system, every attempt was made to simplify and clarify the group of disorders defined as EB, special attention being paid to the creation of a classification that could readily be used by practicing physicians, medical geneticists, and researchers. Several reported EB subtypes were eliminated because they were not considered to represent distinct entities following critical review of current molecular and clinical data, and some subtype names were simplified or renamed to increase consistency across the three major categories. Despite attempts to include the molecular basis of EB subtypes in the classification, a number of eponyms were retained for two particular reasons. First, some eponyms, such as Weber-Cockayne or Hallopeau-Siemens, evoke immediate visual images of highly specific clinical phenotypes, suggesting their continued utility in effective communication between clinicians and scientists all over the world. Second, the precipitous elimination of all eponyms would be counterproductive at this time, because it would impair any linkage with the existing medical literature on EB and result in confusion and, possibly, the generation of a series of alternative classification systems.

The new classification retains the three major EB categories, EBS, JEB and DEB (Table 5). The EBS group comprises EBS Weber-Cockayne (in this chapter, EBS localisata), EBS Koebner (EBS generalisata), EBS Dowling-Meara (EBS herpetiformis) and EBS with muscular dystrophy. The first three subtypes represent allelic variants of keratin 5 and keratin 14 gene mutations, the fourth is caused by mutations in the plectin gene. The new JEB category comprises only three subtypes, namely JEB Herlitz, caused by laminin 5 null mutations, JEB non-Herlitz (which includes JEB localisata and GABEB), caused by missense mutations in the genes for laminin 5 or by nonsense or missense mutations in the gene for type XVII collagen, and JEB with pyloric atresia, caused by mutations in the genes for α6β4 integrin. The new DEB group comprises three subtypes, dominant DEB (in this chapter, autosomal dominant DEB localisata and generalisata), recessive DEB Hallopeau-Siemens (DEB generalisata mutilans) and recessive DEB non-Hallopeau-Siemens (which includes autosomal recessive DEB localisata, autosomal recessive DEB inversa, and DEB generalisata non-mutilans, as described in this chapter). Each of these latter result from defects in the collagen VII gene.

Based on available molecular data relating to the gene defects underlying EB and on epidemiological studies [4], Pfendner et al. [186] have calculated the carrier frequency of EB in the US population as, for all JEB forms, 1/333 (expressed as a fraction); for JEB-Herlitz, 1/781; and for recessive DEB, 1/345. The estimates for dominant EBS and DEB were very low.

Unexpected and Minimal Epidermolysis Bullosa Phenotypes

Clarification of the genetic basis of EB in many more families has shown that minimal phenotypes and unusual symptom combinations may be more common than previously anticipated. It seems that the multitude of genetic and biological constellations in EB provide for a very broad spectrum of clinical manifestations, and that physicians and scientists dealing with EB need to pay particular attention to unexpected symptoms and their combinations in order not to overlook important indices. For example, a *COL17A1* mutation was found in a young child presenting with an EBS-like clinical picture and intraepidermal tissue separation [187]. *COL17A1* gene mutations are known to

TABLE 5. Revised Classification of Epidermolysis Bullosa*

Major EB type	Major EB Subtype	Abbreviation	Protein Involved
EB Simplex (EBS)	EBS Weber Cockayne	EBS-WC	Keratin 5, 14
	EBS Koebner	EBS-K	Keratin 5, 14
	EBS Dowling-Meara	EBS-DM	Keratin 5, 14
	EBS with muscular dystrophy	EBS-MD	Plectin
Junctional EB (JEB)	JEB Herlitz	JEB-H	Laminin 5
	JEB non-Herlitz	JEB-nH	Laminin 5; type XVII collagen
	JEB with pyloric atresia	JEB-PA	Integrin α6β 4
Dystrophic EB (DEB)	Dominant DEB	DDEB	Type VII collagen
	Recessive DEB Hallopeau-Siemens	RDEB-HS	Type VII collagen
	Recessive DEB non-Hallopeau-Siemens	RDEB-nHS	Type VII collagen

*Adapted from [185]

cause JEB, but in this case a large genomic deletion had led to extensive truncation of the intracellular domain of collagen XVII and impaired its interactions with other hemidesmosomal proteins and keratin filaments, leading to an EBS-like presentation. In another interesting case, pyloric atresia in a newborn with skin blistering was associated with dystrophic EB, instead of JEB-PA [188]. Yet another patient with severe JEB-PA experienced spontaneous, remarkable amelioration of skin blistering and subsequently had only very mild symptoms. The "transient severity" was shown to result from the modulation of abnormal splicing of integrin β4 pre-mRNA carrying a branch point mutation [189]. As regards DEB, several cases of *COL7A1* mutations leading to nail dystrophy, but not to skin blistering, have now been published [66,67, 190]. Dysphagia and esophageal stenosis without skin blistering have been shown to represent an extracutaneous form of DEB in a patient with mutations in the collagen VII gene (P. Zimmer, H. Schumann, S. Mecklenbeck, L. Bruckner-Tuderman, unpublished results). These observations extend the phenotypic spectrum of mutations in the genes for DEJ proteins and demonstrate that the clinical consequences of a given mutation depend on its biological effects at the molecular level.

Animal Models and New Candidate Genes

The generation of transgenic mice with ablation of genes encoding proteins of the DEJ has further expanded our knowledge of the molecular mechanisms of skin blistering. Targeted disruption of the *LAMA3* gene prevented the synthesis of both laminin 5 and laminin 6 molecules (both of which contain the laminin α3 chain), resulting in abnormal hemidesmosomes, lack of survival of mutant epithelial cells, severe junctional blistering, and perinathal lethality of the animals [191]. This mouse model also demonstrated that defects in the epithelial basement membrane caused abnormalities of ameloblast differentiation in developing teeth, thus laying a biological basis for understanding the enamel defects observed in JEB patients.

Knockout mice lacking perlecan, a major basement membrane proteoglycan, exhibited abnormalities in many tissues, including basement membranes [192]. Interestingly, the formation of basement membranes was not altered, but they deteriorated in regions under increased mechanical stress, such as myocardium or the skin, resulting in lethal cardiac abnormalities and skin blistering. Based on the severe phenotype in the mouse, perlecan null mutations cannot be expected in EB. Rather, it seems feasible that missense mutations in the perlecan gene may underlie some JEB or DEB subtypes.

Conditional ablation of β1 integrin in mouse skin resulted in severe defects in epidermal proliferation, hemidesmosome structure, basement membrane formation, and skin blistering [193,194]. Perhaps unexpectedly, the phenotype included massive dermal fibrosis and abnormal hair follicle invagination. Cell biological and biochemical analyses indicated that β1 integrin is important not only for hemidesmosome stability, epithelial cell proliferation and hair follicle morphogenesis, but also for the normal processing of basement membrane molecules and the maintenance of epithelial basement membranes [193]. The β1 integrin gene is therefore also a candidate gene for JEB.

In contrast to the above abnormalities, no pathologic phenotype was observed in mice with targeted ablation of the nidogen 1 gene [195]. This unexpected finding led to the assumption that nidogen 2 compensates functionally for the lack of nidogen 1 in the tissues and, therefore, more information on the functions of these ubiquitous basement membrane components can be expected from future double knockout mice lacking both nidogens 1 and 2.

A recent description of inherited DEB in inbred dogs may provide a naturally occurring animal model for somatic gene therapy [196]. The dogs exhibit relatively mild trauma-induced blistering in skin areas with sparse hair, such as the snout, the ears and mucous membranes, and can therefore survive with the disease. The genetic basis of the condition is a homozygous glycine substitution in the *COL7A1* gene [197]. The mild clinical symptoms of these dogs make them a suitable target for somatic gene therapy trials of the transplantation of genetically corrected keratinocyte sheets.

Advances in the Development of Gene Therapy for Epidermolysis Bullosa

Promising gene therapy approaches for EB are now being reported. The current strategy for gene therapy for severe recessive JEB and DEB is gene augmentation via an *ex vivo* approach [198–200]. This is based on the genetic modification of EB keratinocytes *in vitro*, culture of the cells into keratinocyte sheets, and subsequent transplantation of the keratinocyte sheets onto a patient's skin. The well developed techniques of keratinocyte transplantation for burns treatment and the easy access to the transplant offer valuable advantages. Efficient restoration of expression of the laminin 5 β3 chain in keratinocytes derived from several JEB patients was achieved using cDNA in a retroviral vector [201]. Importantly, the genetically corrected cells were fully functional in a skin transplant model in immune deficient mice; no blistering in the transplants was observed. Similarly, successful gene correction of *ITGB4* in keratinocytes from a patient with lethal JEB-PA showed that the corrected keratinocytes were indistinguishable from normal cells in terms of integrin α6β4 expression, the localization of hemidesmosomal components, and hemidesmosome structure and density, suggesting full genetic and functional correction of β4-null keratinocytes *in vitro* [202].

Successful gene therapy for DEB will depend on the restoration of collagen VII synthesis in DEB-Hallopeau-Siemens keratinocytes. Since collagen VII cDNA has a size of about 9.2 kb, it has been considered too large to fit into retroviral vectors. Therefore, a PAC (P1-artificial chromosome) vector containing the entire human *COL7A1* gene was tested [203]. The advantage of gene vectors is that, in addition to a gene, they accommodate the natural promoter and the necessary regulatory elements within the introns, thereby contributing to sustained expression of the transgene. Transfer of the *COL7A1*-PAC vector into an epidermal cell line, using a receptor-mediated, integrin-targeting, liposome-enhanced transfection system, increased collagen VII expression in the cells despite low transfection efficiency [203]. When the same vector was microinjected into immortalized collagen VII-deficient DEB keratinocytes, active synthesis of normal collagen VII was restored. Significantly, the collagen was fully triple helical and efficiently secreted from the cells, suggesting that it was functional (S. Mecklenbeck, S. Compton, R. Cervini, J. Meija, A. Hovnanian, L. Bruckner-Tuderman, Y. Barandon, unpublished results). In another approach, a cosmid clone containing the entire human *COL7A1* gene was used to generate transgenic mice [204]. The cosmid was capable of directing sustained and tissue-specific expression of human

collagen VII in the skin of fetal and adult transgenic mice, and human collagen VII was synthesized by cell cultures derived from the skin of 19-month-old transgenic mice [204], suggesting that the transgene expression was permanent.

Attempts to correct the DEB phenotype of keratinocytes using collagen VII cDNA have now been reported. Chen et al. delivered a truncated collagen VII cDNA into DEB keratinocytes and achieved synthesis of a minicollagen VII *in vitro* [205]. In comparison with parent DEB keratinocytes, the transfected DEB keratinocytes demonstrated enhanced cell-substratum adhesion, increased proliferative potential, and reduced cell motility, features that reversed the DEB phenotype toward normal. Whether the use of the minicollagen VII cDNA will prove a realistic strategy to correct the cellular manifestations of gene defects in DEB remains unclear, since many *COL7A1* deletions have been shown to be associated with pathologic DEB phenotypes. In spite of the large size of the collagen VII cDNA, Meneguzzi and coworkers have succeeded in inserting full-length canine collagen VII cDNA into a retroviral vector. Transduction with the vector induced collagen VII synthesis and secretion in DEB keratinocytes [197]. The advantages of retroviral vectors are high transfection efficiency and stable transgene expression after insertion of the retroviral vector into the genome. The above developments hold promise for the first gene therapy trials for JEB and DEB. At least in the cases of laminin 5 and collagen VII, the transgene products seem to be normal in terms of structure and folding, and can therefore be expected to be functional. After the above gene therapy approaches have been successfully tested in animal models, human treatment regimens using small keratinocyte transplants can be designed. These require careful long-term planning, since, in addition to the generation of genetically corrected skin transplants, extensive governmental approval procedures, the education of highly specialized medical personnel, and acceptance by EB patients will be necessary.

PATIENT SUPPORT GROUPS WORLDWIDE

The Dystrophic Epidermolysis Bullosa Research Associations of Europe and America have informative Web sites: http://www.debra.org.uk/ (DEBRA Europe) and http://www.debra.org/ (DebRA of America).

The contact addresses of the patient support groups in different countries are listed below.

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Chapter 16

Prolidase Deficiency

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SUMMARY

- **Prolidase deficiency is a rare, autosomal recessively inherited disorder in which deficient activity of prolidase, which normally cleaves imidodipeptides containing a C-terminal prolyl or hydroxyprolyl residue, results in a massive urinary excretion of X–Pro and X–Hyp dipeptides derived from generalized protein breakdown, hyperimidodipeptiduria.**
- **The principal clinical feature of the disorder is chronic intractable ulceration of the skin, particularly that of the lower limbs. The phenotype is nevertheless somewhat variable and typically includes a degree of mental retardation and a susceptibility to recurrent respiratory infections.**
- **The onset of clinical symptoms, which may not initially be dermatological, has been reported as occurring as early as shortly after birth or as late as 22 years of age. Although a few prolidase-deficient individuals have been asymptomatic at the time of reporting, it does seem that, given time, the great majority of such individuals do go on to develop symptoms of greater or lesser severity.**
- **The prolidase gene, *PEPD*, is located on chromosome 19 at 19q12-q13.2, and several different mutations have been identified at this site in different prolidase-deficient individuals.**
- **An angiopathic pathogenesis has been suggested for the disorder, small vessels of the dermis frequently showing thickened walls surrounded by multiple basal laminae, and multiple microthromboses in bilateral cerebral white matter suggesting an abnormality of the cerebral vessels. One cause of the vascular anomalies might be superoxide production by neutrophils under the influence of X–Pro imidodipeptides.**
- **Prolidase deficiency may be suspected with a high degree of probability on the basis of hyperimidodipeptiduria and confirmed by measurement of prolidase activity in erythrocytes or leukocytes. Rapid urinary screening may be performed using paper or thin-layer chromatography or by high-voltage electrophoresis. The chromatography of untreated urine in an amino acid analyzer reveals the presence of several abnormal ninhydrin-positive peaks, and quantitative analysis after hydrolysis reveals a striking increase of Pro, Hyp, and Gly.**
- **Various therapeutic measures involving the topical application of glycine and proline, or the systemic or oral administration of various combinations of manganese and factors either stimulating collagen biosynthesis or inhibiting collagenase activity, have provided only occasional and transient relief. Similarly, repeated erythrocyte transfusions, either alone or in combination with $MnSO_4$ and ascorbic acid, have generally been found ineffective in the long term. There is thus little available other than the conservative management of ulcers that can be offered to patients at the present time.**

INTRODUCTION

Prolidase deficiency is a rare, autosomal recessively inherited disorder in which deficient activity of prolidase, which normally cleaves imidodipeptides containing a C-terminal prolyl or hydroxyprolyl residue, results in a massive urinary excretion of X–Pro and X–Hyp dipeptides, hyperimidodipeptiduria. The nature of the imidodipeptides excreted has led on occasion to the disorder erroneously being described as representing a specific defect in collagen metabolism; however, protein degradation in general is affected. The condition is associated with a somewhat variable clinical phenotype, which typically includes a degree of mental retardation and recurrent respiratory infections but of which the main feature is chronic, intractable ulceration of the skin, particularly that of the lower limbs. The pathogenetic mechanisms leading to the skin changes are poorly understood, and even less so are those resulting in mental retardation. To date, attempted therapy has met with little other than transient success.

CLINICAL FEATURES

Since the appearance of this chapter in the first edition of this volume [1], relatively few additional cases have been reported, and still only around just under 60 cases of confirmed prolidase deficiency have been described in the literature [2–4; 5–7; 8; 9–12; 13,14; 15–18; 19; 20,21; 22,23; 24–26; 27; 28; 29; 30,31; 32; 33; 34; 35; 36,37; 38; 39; 40; 41; 42; 43; 44–46; 47; 48; 49,50; 51; 52; 53; 54] (references relating to particular patients have been grouped

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where appropriate, and as far as identities of patients in different publications have been possible to ascertain, and each is either a first report of a particular patient or carries a description of the patient's phenotype).

The clinical features of the disorder show some degree of variability, but certain features do appear to be fairly characteristic. Particular among these are chronic dermatitis with recurrent leg ulceration, a susceptibility to repeated upper respiratory tract infections, a somewhat characteristic facies, a mild to moderate degree of mental retardation, and hepatosplenomegaly.

Dermatological Manifestations

The dominant clinical problem associated with the disorder relates to its dermatological manifestations, and in the majority of cases chronic, repeated slow-healing ulcerations, mainly of the legs and feet (Fig. 1), are a feature. Ulceration of the upper limbs [14] and face [55] has also been reported, and in one case, that of a 17-year-old girl, although active ulcers were confined mainly to the legs, there was scarring from healed ulcers over most of her body [34]. The development of ulcers is often preceded by dermatological manifestations that may occur over the whole body and have been described variously as erythematous papular eruptions [11,40], telangiectasia with pruritus and photosensitivity [14], impetigo-like eruptions [18], pruritic eczematous lesions [19], and necrotic papules [21,35,41,42].

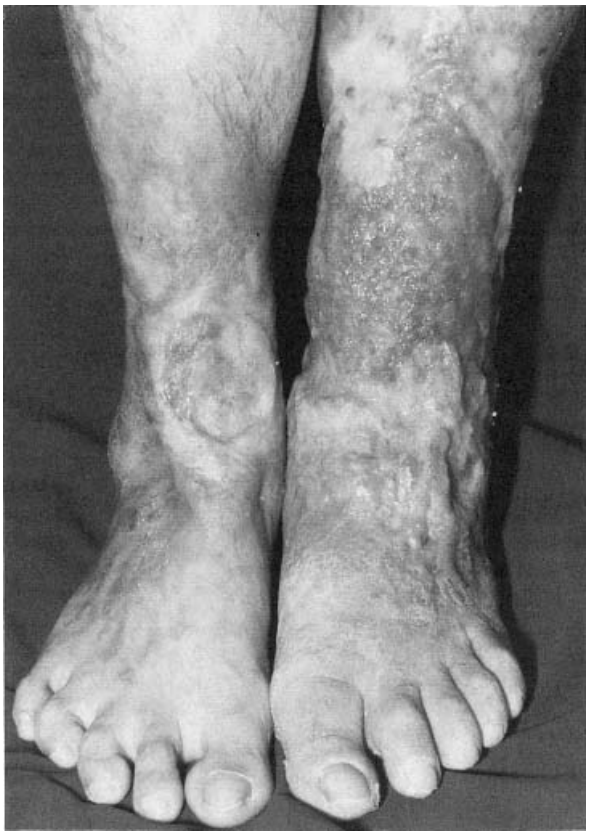

Figure 1. Recalcitrant leg ulcers, surrounded by scars and thickened skin, in a 32-year-old female. (Photograph courtesy of Dr. A. Fujisawa. (Copyright not held by AMA, as discussed with Luna Han, Wiley).)

In the three cases reported by Ogata et al. [19], scar contractures had caused talipes equinus, also observed in one of the sisters described by Voigtländer et al. [35], and in one of the patients of Leoni et al. [33], the same condition was caused by a shortened Achilles tendon. In one case, a lesion on the right foot was so deep that the tendon was exposed and broken by the patient; after healing with scar formation, the foot was grossly deformed [11]. In a further case, the extent of ulceration was so severe that the amputation of both legs below the knee was required, but the patient still suffered from ulceration of the thighs and developed secondary infections of the stumps [36,37,56]. The risk of developing squamous cell carcinoma has been reported to be higher at the sites of venous leg ulcers [57], and one middle-aged prolidase-deficient patient with long-standing ulceration of the lower limbs developed a large, vegetating tumor on the anterior side of the lower right leg that required amputation of the leg above the knee [51].

In many cases, dermatological manifestations are restricted to the lower limbs, but telangiectasia has been reported in other areas including the face in some cases [11,18,19,22,23,35,39,41,43,44], and atrophic scar-like lesions on the face have been noted [11,19,21]. Hyperkeratosis over the elbows and knees has been mentioned in two cases [19,41], and ichthyosis was noted in one [38]. In contrast, several reports mention the thinness of the skin, particularly over the abdomen, with readily visible veins [11,18,19,40,51]. One patient was described as having a "curiously lax" skin with a spongy texture [24,26].

In a small number of patients not presenting with ulcers, perhaps because the clinical course of the disorder had not yet reached that stage, a chronic dermatitis was apparent, predominantly affecting the gluteal areas but also involving the arms, legs, and back and the soles of the feet and the palms of the hands [5,7,8,32].

Facial Features

Although one patient was specifically reported as having no unusual facial features [7], it does seem that most patients present with an "unusual," "peculiar," "characteristic," or "dysmorphic" facies [5,8,11,14,15,18–23,27,33,34,39,40,42–44,47]. Although not common to all patients, features described have included a low hairline and facial hirsutism, a saddle nose, ocular hypertelorism, ptosis, micrognathia, a high-arched palate, mandibular protrusion, and exophthalmos. Three patients have been described as having a "bird-like" facial appearance [24–26,44,46].

Mental Retardation

In several cases, moderate to severe mental retardation has been reported [5,7,11,14,15,19,23,27,31,34,40,42,44,46,49,52], while in another, intelligence was reported to be borderline [20,21]. In other cases, intelligence may be normal [25,44,46]. Epileptic seizures had been recorded in the history of one patient [42].

Other Features

Splenomegaly or hepatosplenomegaly is regularly described [5,7,8,14,18,27,31,33,34,40–42,47,49,50,52], and a tendency toward recurrent otitis media, sinusitis, pneumonia, and gastroenteritis and diarrhea is a frequent feature of the condition [5–8,14,19,27,32,35,42,45–47,49,50,52], although in several instances it has specifically been mentioned that there seemed to be no tendency toward recurrent infection [11,23,25]. On the basis of findings in one patient who suffered from recurrent erosive cystitis apparently unconnected with infection, it has been

suggested that urinary tract inflammation also be considered a consequence of the disease [26]. A degree of obesity has been noted in several patients [5,22,24,40,42]. Blood chemistry appears generally to be unremarkable except for an elevated γ-globulinemia in some cases [5,8,11,14,19,23,24,31,33,42,47,49,50] and occasional anemia [5,7,14,19,22,27,33,37,38,40–43,50]. One patient suffered greatly from lymphedema, which was suggested to have resulted from progressive damage to the lymphatic vessels [14]. In two cases, an association with systemic lupus erythematosus was noted, although it is not clear whether the association was fortuitous or direct [47,50]. Immune function was investigated in detail in one of these latter patients [49] who, in addition to hyper-γ-globulinemia, displayed low levels of complement C3 and C4 and a reduced CH50. In addition, neutrophil chemotaxis was abnormal.

Hypotonia [8,32,52] or muscular incoordination [5,27] has occurred in several patients, and there may also be signs of a generalized disturbance in connective tissue. Several cases of joint hyperextensibility have been reported [7,8,24,32,41], and one patient, displaying pronounced ligamentous laxity in the knees, was described as having a waddling gait, with external rotation of the femora [8]. Congenital dislocation of the hip was noted in the first year of life in two patients [11,43], and hip dislocation has been observed in two adult patients [19]. In one case, a hip dysplasia was operated on at 17 years of age [31].

Skeletal anomalies, which include a mild, diffuse demineralization of the long bones [5,40,41], scoliosis [33], and a retarded bone age [27], have been described. One patient is reported as having suffered frequent fractures of the forearm following only modest trauma from an early age [40]. However, in another case, bones appeared normal on x-ray examination [11], and a further patient was described as having no skeletal abnormalities [25]. Dental dysplasia and dysplasia of the dental enamel have also been described [41,42]. Trichiasis and associated corneal lesions were mentioned in the case of one patient [31].

Clinical Onset

The onset of clinical symptoms, which may not initially be dermatological, has been reported as occurring as early as shortly after birth or as late as 22 years of age; this may represent a genuine heterogeneity of the disorder or failure to recognize it, or it may simply be an artifact of reporting. Four prolidase-deficient individuals, the siblings of patients showing the full range of clinical manifestations, have been asymptomatic at the time of reporting. The first, a six-year-old girl [10], by the age of 15–18 years did go on to develop skin lesions [58–60]. The second, the 26-year-old brother of a severely affected female, was entirely asymptomatic [15], but his present condition and whereabouts are unknown (Dr. F. Gejyo, personal communication, 10 June 1991). The third such patient, asymptomatic at the time of study, was an eight-year-old boy [44], who did shortly after go on to develop small lesions on the feet (Dr. G. Cetta, personal communication, 11 June 1991, and [46]), while the fourth, the sibling of a severely affected child who died at an early age, was only four months old at the time of reporting, and it is not possible to predict the future clinical course [52]. Nevertheless, it does seem that, given time, the great majority of prolidase-deficient individuals do go on to develop symptoms of greater or lesser severity. One man, the brother of a female with confirmed prolidase deficiency, was found to be prolidase-deficient himself when he developed leg ulcerations for one month following a leg injury [25]. Trauma incurred in a road accident also seemed to be the stimulus for the appearance of ulcers on the ankles of a young female patient (Dr. G. Cetta, personal communication, 11 June 1991, and [46]).

Five cases of prolidase deficiency have been detected either directly or secondarily through the demonstration of glycylprolinuria in neonatal screening programs, and prolidase deficiency has been confirmed in four of them [28,29]. At the time of reporting of the three siblings described by Naughten et al. [28], the 19-month-old proband had mild hypotonia, and the two brothers, aged three and four years, had mild developmental delay but were otherwise asymptomatic. Until the age of seven years, the proband suffered chronic respiratory infections with severe neutropenia, which later resolved. At the ages of 13 and 14 years, the two brothers showed mild muscular hypotonia, and both had been hyperactive, with poor concentration and short attention spans, although the proband herself was not thus afflicted. All three sibs had a reduced IQ, to which language difficulties may have contributed, but none of the three had, at that time, gone on to develop the typical dermatological manifestations of the disorder (Dr. M. G. Ampola, personal communication, 13 June 1991; a more recent attempt to follow up the status of the three patients has been unsuccessful). The confirmed prolidase-deficient patient reported by Lemieux et al. [29] had no skin lesions at the age of nine years but did show a degree of impaired development, which may, however, have been secondary to a disturbed family life (Dr. C. R. Scriver, personal communication, 22 February 1991).

Three other patients apparently suffering from prolidase deficiency as determined by the urinary excretion of C-terminal proline-containing dipeptides, but in whom direct confirmation by enzyme assay is lacking, have also been reported. One of these, a five-year-old girl, the first cousin of a severely affected patient who died at eight years of age of septicemia, shortly after her first birthday developed clinical symptoms, including recurrent otitis media, chronic diarrhea, repeated respiratory infections, and abscesses of her face and neck, and later showed a facial rash with a butterfly distribution. She also was diagnosed with systemic lupus erythematosus on the basis of the results of immunological investigations [49,50]. Clinical details of a further such patient, a 22-year-old male, appear to be restricted to the information that he exhibited dermatological purpura [61], but the third patient [62,63], a mildly retarded 47-year-old male, is generally regarded as the first reported case of prolidase deficiency; his clinical symptoms were, however, not entirely typical of the disorder. Skin lesions were absent, although the skin of the lower extremities was described as "atrophic and broken down about the toes," and instead there appeared to be considerable skeletal involvement; there was generalized osteoporosis of the distal tibiae and fibulae and bones of the foot, with eversion of the talotibial joints; hyperostosis was noted in the frontal regions of the skull, but an iliac crest biopsy showed no bony abnormality, and neither was there any radiographic or histological evidence of osteomalacia or hyperparathyroidism. There was no reported increased susceptibility to infections, although the patient did die at about 50 years of age from influenza [63].

Metabolic Phenotype

The hallmark of prolidase deficiency is imidodipeptiduria, an excessive urinary excretion of X–Pro and

X–Hyp dipeptides. In a number of cases of both confirmed [5,7–9,13–15,20,21,23,33,50,64–67] and probable [61–63] prolidase deficiency in which the urinary dipeptides have been analyzed, it has been shown that the C-terminal of the two residues is usually proline, while that in the X position can be one of a number of different amino acids. Those that have been identified include Ala, Ser, Thr, Val, Phe, Ile, Leu, Pro, Asp/Asn, Glu/Gln, Lys, and Tyr, but in all cases the predominant dipeptide has been Gly–Pro, which generally accounts for 20–30% of the amount of all those excreted. Under normal circumstances, the excretion of Gly–Pro is negligible [66–71]. Because the concentration of imidodipeptides in the serum of patients with prolidase deficiency is so much lower than it is in urine, peptides other than Gly–Pro are not readily detectable [23,62]. Nevertheless, if methods are sufficiently sensitive, a variety of X–Pro imidodipeptides can be quantified [72]. Amino acid analysis of cerebrospinal fluid from the previously described case of probable prolidase deficiency was normal, and no peptides were detectable [62].

Despite the apparent relative paucity of hydroxyproline-containing urinary dipeptides, the urinary excretion of peptide-bound hydroxyproline has also been increased some six- to ten-fold in cases of both confirmed [2,5,7,14,20,21,33,64,67] and probable [62,63] prolidase deficiency, although in one patient no urinary hydroxyproline whatsoever was detected [34], a finding that is not readily understandable. The increased urinary excretion of hydroxyproline has been interpreted by some authors as indicative of an increased rate of body collagen turnover [2,5,14,62], but it would seem more reasonable to suppose that it is merely a reflection of the nonsusceptibility of hydroxyproline in peptide-bound form to degradation by hydroxyproline oxidase [63,73] as normally occurs [74]. That a proportion of the imidodipeptiduria is generated by the normal turnover of body protein is demonstrated by the excessive excretion of peptide-bound hydroxyproline by patients while on a gelatin-free diet [6,33,62,63], but gelatin-loading studies have demonstrated that in the absence of intestinal mucosal prolidase activity, dietary protein does contribute considerably, some 40–50% of the bound hydroxyproline ingested being able to cross the intestinal wall in peptide linkage to be excreted within 24 hours [6].

In one or two cases, the urinary excretion of tripeptides such as Gly–Pro–Pro [2,64] and Asx–Pro–Pro and Tyr–Pro–Pro [5] has been noted. Sheffield et al. [14] also note that a number of tripeptides were identified in the urine of their patient, but no details were given. Urinary hydroxyproline-containing tripeptides have been reported in normal individuals in very small amounts [75,76], but others have questioned their existence [77]. It is not clear by what means such tripeptides should escape normal degradation to dipeptides. It is similarly unclear why Pro–Gly should have been found in the urine of one patient [13,14] despite adequate activity of prolinase, this being the enzyme that cleaves dipeptides with an N-terminal Pro or Hyp residue.

INHERITANCE AND PREVALENCE

Prolidase deficiency is inherited in an autosomal recessive manner. In a number of pedigrees, parental consanguinity is evident [9,10,14,15,17,19,22,39,41,49,50,52], and the determination of prolidase in erythrocytes, leukocytes, and plasma of parents and other family members has demonstrated intermediate levels of activity consistent with heterozygosity for a recessive trait [3,5–7,10,15,17,19–21,27,29,32,34,40,47,52,67,78, 79], with the occasional exception [26]. Although originally assigned MIM no. 26413, indicative of its status as an autosomal recessive disorder, prolidase deficiency is currently included under MIM 170100 "Peptidase D; PEPD (prolidase; imidodipeptidase; prolidase deficiency, included)" [80], which reflects rather the codominant inheritance of benign variant alleles of the dimeric enzyme [81].

Although cases of prolidase deficiency have been reported from several ethnic groups, with a wide geographical distribution, the small number of cases reported in the literature makes it clear that it is an extremely rare disorder, although there may well be an element of underreporting as a result of physician unfamiliarity with the condition. Phang and Scriver [74] estimate an incidence of 1–2 per 1 million births, but Myara et al. [82] state that as much as 2% of the Japanese population is heterozygous for the disorder. Such a figure would result in there being over 10,000 cases in Japan and is clearly too high an estimate.

DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

Diagnosis

The diagnosis of prolidase deficiency may be suspected on clinical grounds in pediatric or adolescent patients presenting with the symptoms described, suspicion which may be strengthened by the results of urinary amino acid analysis. Very rapid screening may be performed using paper or thin-layer chromatography or high-voltage paper electrophoresis [82]. High-voltage electrophoresis of urinary amino acids in one case showed a continuous band from Gly to Glu [25], while in another, a large spot that stained apple-green with ninhydrin was observed migrating between Gly and Ala, with a "continuous ninhydrin-positive cascade reaching the glutamine region" [13,14]. Thin-layer chromatography of urine [5,7,8,19] also shows the existence of abnormal spots, the most prominent of which migrates as authentic Gly–Pro. These abnormal ninhydrin-positive spots are not apparent if the urine is first hydrolyzed and, conversely, are still present upon the electrophoresis of urine from which free amino acids have been removed. Imidodipeptiduria may also be simply demonstrated by ion-exchange chromatography with an amino acid analyzer. The chromatography of untreated urine reveals the presence of several abnormal ninhydrin-positive peaks, which are not apparent if the urine is first hydrolyzed, and quantitative analysis reveals an increase in the content of many amino acids after hydrolysis, in particular Pro and Hyp, which are strikingly elevated [5,7,8,13–15,19–21,47,62–64]. Imidodipeptiduria has also been ascertained in a newborn screening program in which newborn urine samples impregnated on filter paper are eluted and analyzed by thin-layer chromatography [29]. The use of capillary zone electrophoresis promises to provide a rapid, simple, and reliable means of detecting imidodipeptides in only small amounts of urine [83], and a complete resolution of imidodipeptides has been achieved using micellar electrokinetic chromatography [84].

As noted above, the excretion of Gly–Pro is negligible under normal circumstances, but enzymatic confirmation of prolidase deficiency is strictly necessary, because the urinary excretion of Gly–Pro has been reported in cases of increased bone turnover, such as in patients with generalized osteopathy and greatly elevated levels of serum alkaline phosphatase [68,85], rickets [69,70], and patients

with osteomalacia and hyperparathyroidism resulting from a parathyroid adenoma [86] (and B. Steinmann, personal observation). Further, a peptide containing proline and hydroxyproline was detected in the urine of three patients with ataxia telangiectasia [87].

Prolidase activity is conveniently determined in erythrocytes or leukocytes. Whole cells should not be used because substrate transport through the plasma membrane is rate-limiting [65]. Cultured skin fibroblasts may also be used, but caution is required because there are large variations in prolidase specific activity in sonicates or homogenates related to cell number [88–90]. Prenatal diagnosis has been accomplished successfully using amniotic fluid cells [52]. A simple procedure suitable for use in mass screening programs has been described whereby activity is extracted from dried blood spots on filter paper [91], and this has been used in prospective ascertainment [28].

For diagnostic purposes, prolidase activity is best measured using Gly–Pro as substrate, and optimal reaction conditions have been described by Myara et al. [92], who have also briefly reviewed means of determining the release of glycine and proline from the substrate [82]. Clinically, a widely used means of determining proline release [10,20,30,32,34,35,46,78,79,91,93] has been the colorimetric procedure of Chinard [94]. Other amino acids, to some extent, interfere with this reaction, and the modification by Troll and Lindsley [95], which removes the basic amino acids lysine, hydroxylysine, and ornithine, has been used also [5]. Others have determined glycine, proline, and Gly–Pro using an amino acid analyzer [8,11,15,23,25,27,28,33,40,45]. A method of measuring dipeptidase activities based on the decrease in absorption at 220–222 nm as peptide bonds are hydrolyzed has been described [96,97] and used for the determination of prolidase activity in leukocytes [5]. A rapid high-performance liquid chromatography (HPLC) procedure has been described for determining the content of unhydrolyzed Gly–Pro in the reaction mixtures [98], and two methods that measure the simultaneous disappearance of substrate and appearance of product have been reported, one based on the difference between the ^{1}H nuclear magnetic resonance spectra of Gly–Pro and free Gly and free Pro [99] and the other on the isotachophoretic separation of Gly–Pro and free Gly [100].

Differential Diagnosis

A differential diagnosis must include familial leg ulcers of juvenile onset [101], an apparently autosomal dominantly inherited condition of unknown cause with expression only in males. In three patients with prolidase deficiency (two confirmed and one probable) in two families, clinical and immunological findings have led to a diagnosis of systemic lupus erythematosus (SLE) [47,50] and the suggestion that prolidase deficiency is likely to be a risk factor for the development of SLE and therefore that patients with SLE for whom there is a family history or childhood presentation of it specifically be investigated for prolidase deficiency because standard immunological or hematological investigations do not identify the biochemical abnormalities characteristic of the disorder [50].

BIOCHEMISTRY AND MOLECULAR GENETICS

Prolidase (imidodipeptidase; peptidase D) (EC 3.4.13.9) functions in the latter stages of catabolism of both endogenous and dietary protein to cleave imidodipeptides in which the imino residue is C-terminal. It is generally considered to be a cytosolic enzyme, but its possible existence at other locations (e.g., bound to the plasma membrane, either internally or externally), has not been excluded [97]. It has a wide tissue distribution, having been found in such diverse situations as intestinal mucosa, kidney, liver, brain, heart, thymus, uterus, and prostate, as well as in erythrocytes, leukocytes, dermal fibroblasts, and plasma [82,102–104]. Dipeptides containing N-terminal imino acid residues are cleaved by a different enzyme, prolinase (iminopeptidase) (EC 3.4.13.8), but there are no reported cases of its deficiency in man or other animals, and it is not considered here.

Prolidase has been purified to apparent homogeneity from several nonhuman sources [103] and in man from liver [105], kidney [106], erythrocytes [65,105,107], and fibroblasts [90] by chromatographic procedures. The native enzyme has an M_r of 97,000–116,000 by gel filtration, and a subunit M_r of 55,000–58,000 by SDS-polyacrylamide gel electrophoresis, consistent with the native enzyme being a dimer. A considerably more rapid and simplified immunoaffinity purification procedure using an immobilized monoclonal antibody to human liver prolidase results in a similar subunit M_r of 56,000 upon SDS-polyacrylamide gel electrophoresis [108]. Analysis of cDNA coding for the entire protein, and protein sequence analysis [109–111], demonstrate that the mature enzyme is composed of 492 residues and has an M_r of 54,305 [110]. Secondary structure predictions indicate the presence of α-helices, β-sheets, and potential β-turns in the protein, with hydrophobic and hydrophilic regions distributed evenly throughout [110]. Comparison of amino acid sequences further suggests that prolidase is related to *Escherichia coli* methionine aminopeptidase (EC 3.4.11.18), the tertiary structure of which comprises a so-called "pita bread" fold with two bivalent metal ion-binding regions within the centrally located active site [112,113].

The optimum pH of the human enzyme is 7.6–7.8 [65]. A relatively early study suggested that there is a specific requirement for Mn^{2+}, which was thought to be bound by a thiol residue [114]. Others also noted the likelihood of a thiol group being involved in enzyme-substrate interaction [115], but the lack of any inhibition of activity by the cysteine-specific reagent 2,2′-dipyridyl disulfide would argue against this [116]. Proposed models of the active site have been suggested and involve a binding site for Mn^{2+}, essential for activity, which interacts with the peptide bond carbonyl group of the dipeptide substrate [117,118] and with the carboxylate group of the prolyl residue in *trans* imidodipeptides in such a manner that the dipeptide functions as a bidentate ligand toward the Mn^{2+} [118,119]. Using the evidence of phenylglyoxal and carbodiimide inactivation, with protection conferred by proline derivatives, Mock and Zhuang [116] deduced the presence of arginine and carboxylate (aspartic/glutamic acid) residues at the active site of prolidase. From a comparison of the amino acid sequences of prolidase and human skin collagenase, they further suggested an evolutionary relationship between the two [116]. Mock and Liu [120] have proposed a catalytic cleavage mechanism for prolidase involving chelative activation of the substrate by one of the pair of active-site metal ions, with nucleophilic addition to the susceptible carbonyl group by the second metal hydroxide. Hechtman et al. [121] and Richter et al. [107], in studies of human erythrocyte prolidase, have presented evidence to suggest that only about 6–30% of the total enzyme within the cells is present in the active holoenzyme form,

possibly corresponding to the low levels of Mn^{2+}, the rest being in an apoenzyme form that can only become active on stimulation by Mn^{2+}. The presence of Mn^{2+} also affects the thermal stability of the enzyme because when crude human erythrocyte lysate is heated at 50°C for 1 hour in the absence of added $MnCl_2$, activity is almost completely lost, whereas, following similar treatment in the presence of $MnCl_2$, full activity is retained [107].

In contrast to the generally held view that Mn^{2+} is necessary for prolidase activity, Mock and colleagues question whether it is in fact an obligatory active site component, pointing out that divalent transition metal ions with similar chemical properties are functionally interchangeable in other metalloproteinases [120,122]. Hechtman [103], however, describes a dramatic loss of activity of prolidase in which Mn^{2+} had been replaced by Zn^{2+} and provides evidence that optimal activity is very clearly achieved with Mn^{2+} over a range of other divalent metal ions. Furthermore, Zn^{2+}-prolidase was thermolabile in comparison with the Mn^{2+}-enzyme, which was thermostable, and although there was little difference in K_m values for different imidodipeptide substrates, V_{max} values were greatly decreased with the Zn^{2+}-enzyme [103]. Nevertheless, Mock and Liu [120] state that they are unable to obtain any benefit from the Mn^{2+} activation procedure widely used during purification of the enzyme.

The prolidase (peptidase D) gene, *PEPD*, is located on chromosome 19 at 19q12-q13.2 [103] and consists of 15 exons ranging from 45 to 528 bases in length and 14 introns ranging in size from 1 to 50 kb, which make up some 98% of the gene, the whole comprising over 130 kb of genomic DNA [123,124].

Several investigators have reported the existence of two forms of prolidase, I and II, separable by ion-exchange chromatography [46,103,104,125–131]. Prolidase I corresponds to the major form described above, while prolidase II is a labile enzyme that would not have survived the preliminary heat inactivation steps of Richter et al. [107] and Boright et al. [90] or the preliminary procedures of Endo et al. [105]. Only prolidase I activity is present in human plasma [131]. The two forms of the enzyme differ in their response to Mn^{2+}, their thermostability, their response to inhibition by *p*-hydroxymercuribenzoate, and their substrate specificity [125,126,128,130,131], in particular, prolidase I having greater activity toward Ala–Pro, Phe–Pro, and especially Gly–Pro than prolidase II [125,126,128–130]. Support for the existence of two forms of prolidase is provided by the failure of antibodies to highly purified prolidase to inhibit all the activity in extracts of cultured skin fibroblasts [59,90]. M_rs of 151,000–185,000 and 95,000 for the native form and subunit, respectively, of prolidase II, as compared with corresponding M_rs of 105,000–112,000 and 56,000 for prolidase I, have been reported [128–130], but it is highly unlikely that prolidase II is a precursor or differently post-translationally modified form of prolidase I encoded either partially or completely by the locus for prolidase on chromosome 19 because there is no immunological cross-reactivity between the two forms [59,90,108]. In prolidase deficiency, the activity of prolidase against Gly–Pro in various cells and tissues, including erythrocytes, leukocytes, plasma, serum, fibroblasts, and cultured lymphoid cells, is reduced to levels ranging from undetectable to less than 7–10% of control values [5–8,10,11,14,15,19–21,23,25,27,29,30,32,33–35,44, 45,47,50,59,65,93,127,130,132]. The level of activity against certain other X–Pro substrates, such as Met–Pro, Phe–Pro, Ala–Pro, Val–Pro, and Leu–Pro, however, may not be completely eliminated and may be reduced by only a moderate extent [32,59,78,79,90,132]. The residual activity may represent the labile prolidase II enzyme [46,59,90,128,130].

Several mutations have been identified in the *PEPD* gene in prolidase-deficient individuals. Those reported at the time of writing are summarized in Table 1. Immunochemical studies of cultured fibroblasts, lymphoblastoid cells, and erythrocytes, using monospecific antiserum or monoclonal antibodies, have revealed that these mutations give rise to a degree of molecular heterogeneity in the polypeptide phenotype in prolidase-deficient patients [59,90,105,109,135] in that both patients who are CRM^- (cross-reacting material negative) and patients who are CRM^+ (cross-reacting material positive) have been identified. Northern blot analysis using a prolidase cDNA probe has shown an absence of prolidase mRNA in CRM^- cells [59].

The first mutation to be identified, in two unrelated CRM^+ patients, resulted in an Asp > Asn amino acid substitution at residue 276, which would predict a change in the secondary structure of the prolidase subunit from α-helix to β-sheet [133]. Bazan et al. [113] note that this residue corresponds to Asp97 in *E. coli* methionine aminopeptidase, a cobalt ligand in the active site of the enzyme. Confirmation that the mutation within the prolidase gene and not some other defect was the cause of the prolidase deficiency was provided by transfection experiments in which plasmids containing either a normal or the mutant prolidase cDNA were incorporated in NIH 3T3 mouse fibroblasts.

In both cases, the synthesis of immunologically cross-reacting material was observed, but active human enzyme was only detected in cells transfected with normal prolidase cDNA [133]. Restriction fragment analysis indicated that the two patients were homozygous for the G826A substitution,

TABLE 1. Mutations in the Prolidase Gene, *PEPD*, in Prolidase Deficiency

Mutation	Exon/Intron	Effect	Notes	Reference
IVS4-1G>C	Intron 4	del exon 5		129
IVS6-2A>G	Intron 6	del exon 7		129
G551A*	Exon 8	Arg184Gln		136
C551T	Exon 8	Arg184Ter		137
G826A	Exon 12	Asp276Asn	In two unrelated patients.	133
G833A*	Exon 12	Gly278Asp		136
G1342A	Exon 14	Gly448Arg	In two unrelated patients.	129
del 774 bp	Exon 14	del exon 14	In two sisters.	123,134
del 3 bp	Exon 15	del Glu452/Glu453		129

*Both of these mutations were detected in a single asymptomatic patient.

which resulted in the elimination of a *Taq*I cleavage site from genomic DNA [133]. In a number of other patients, who were CRM^-, the *Taq*I restriction fragment pattern of exon 12 was similar to that of controls, indicating the probability of different mutations being responsible [133]. Complementation analysis by Boright et al. [90] did, however, indicate that all of the CRM^- and CRM^+ mutations that they studied mapped to a single locus.

In two further prolidase-deficient sisters, the offspring of consanguineous parents from whom they inherited the mutation, Tanoue et al. [123,134] detected a genomic deletion of 774 bp including exon 14, which resulted in the production of mRNA lacking 192 bp of sequence corresponding to exon 14. Transfection and expression analysis using mutant prolidase cDNA resulted in the presence of an inactive mutant protein of M_r 49,000 [134,138]. At the deletion breakpoints there were 7 bp of short repeats, and the mechanism of "slipped mispairing," whereby the repeat sequences misrepair during DNA replication, leading to the formation of a single-stranded loop that is then excised, was suggested as being the cause of the deletion [134].

Ledoux et al. [135,136] subsequently identified six novel alleles from prolidase-deficient patients, listed in Table 1, and Kikuchi et al. [137] have reported a further novel mutation in a Japanese patient. This last mutation, generating a stop codon at amino acid position 184, would be expected to result in the production of a truncated polypeptide, but any mutant protein may have been very unstable because none was detectable [59]. Kikuchi et al. [137] note that the mutation, occurring at nucleotide position 551, involves the CpG dinucleotide, a known hotspot for point mutations. Interestingly, although the patient of Kikuchi et al. [137] was severely affected from birth, the case reported by Ledoux et al. [136], who had a missense mutation at the same position, had residual prolidase activity and was asymptomatic. Position 184 is located in a nonconserved region of the protein, distal to the active site [116], and it would seem that the nonsense mutation (stop) affects the stability of the catalytically inactive enzyme, whereas the missense mutation at the same position does not affect stability and allows the production of a partially active enzyme.

Endo et al. [59] attempted to relate the polypeptide and mRNA phenotypes they observed to clinical phenotype but without success. The only possible correlation observed was that mental retardation occurred only in CRM^- patients, in whom the biochemical defect might be regarded as most severe. However, it did not occur in all CRM^- patients studied. The variable nature of the clinical symptoms observed in prolidase deficiency, especially in affected siblings, has long been puzzling, but the finding of the same gene deletion in two sisters, one of whom developed skin lesions at the age of 19 months but the other of whom did not do so until 18 years of age, makes it clear that factors unrelated to the prolidase gene product influence the course of the disease [134].

In one of the cases reported by Ledoux et al. [136], two novel alleles were detected in a single prolidase-deficient individual, still asymptomatic at the age of 11 years, one carrying a G551A transition in exon 8, resulting in the Arg184Gln substitution mentioned above, and the other carrying a G833A transition in exon 12, resulting in a Gly278Asp substitution. Direct sequencing demonstrated the proband to be a genetic compound for the two mutant alleles on homologous chromosomes, one being paternal in origin and the other maternal, and expression of the mutant alleles in a COS-1 expression system demonstrated that the Arg184Gln mutation was associated with 7.4% residual activity, while the Gly278Asp substitution resulted in inactive enzyme, although in both cases a normal amount of immunoreactive protein of the expected size was detected by western analysis [136]. The expression of mutant alleles previously identified, G1342A (Gly448Arg) and ΔGlu452/453 [135], in the same system resulted in undetectable enzyme activity in each case [136]. Although the former mutation allowed the detection of stable immunoreactive material, the latter did not, being associated with a mutant protein that was synthesized but rapidly degraded [136]. The Gly278Asp and Gly448Arg substitutions both introduce charged side chains in close proximity to the negatively charged active site metal-binding residues Asp276 and Glu452, and Ledoux et al. [136] speculate that the function of these metal-binding residues might be affected by steric and/or electronic hindrance as a result of these mutations, with consequent loss of enzyme activity. Although the ΔGlu452 mutation removes one of the metal-coordinating residues, Ledoux et al. [136] suggest that its destabilizing effect is more likely due to a reversal of polarity in the distribution of residues within the β-motif proximal to the mutation.

Several authors have speculated on the role of the labile prolidase II enzyme in relation to the severity of the clinical phenotype. Butterworth and Priestman [125] first observed a virtual absence of prolidase I in prolidase deficiency and observed that the properties of prolidase II were altered, implying a structural relationship between the two forms. The simplest explanation was that they shared a common subunit that was altered in the disease, but, as noted above, immunochemical studies have shown the two forms not to be immunologically cross-reactive, and Endo et al. [59] could find no correlation between the severity of the clinical phenotype and the residual level of enzyme activity representing prolidase II, concluding that it seemed to be largely irrelevant as a modifier of the clinical phenotype.

The finding of an increased manganese content of erythrocytes from one patient, together with deficient activity of prolidase and arginase, both Mn^{2+}-dependent enzymes, raised the question of there being an entirely different cause of prolidase deficiency in some patients, namely a defect in cellular manganese handling that renders manganese unavailable for Mn^{2+}-dependent enzymes [32], in a manner analogous to the disturbance in copper handling that reduces the availability of copper to Cu-dependent enzymes in Menkes disease and the occipital horn syndrome (see Chapter 14, this volume). However, this particular patient was one of those in whom an Asp276Asn substitution was later detected [133].

PATHOLOGY

The patient initially reported by Isemura et al. [15] died at 25 years of age from cerebral hematoma following an unsuccessful skin-grafting operation and was examined at autopsy by Sekiya et al. [18]. Abdominal and breast skin was thinner than normal, and in some parts the epidermis consisted of only two or three layers of squamous cells. The lamina densa of the epidermal basement membrane displayed irregular disruption, and the basement membrane of the small vessels of the dermis was lamellated and showed interruptions. The basement membranes of the renal tubules

and glomerular capillaries were thickened, irregularly in the first site and with splitting in the second. Both the tubular basement membrane and the vascular basement membranes of the interstitium were highly lamellated. Thickened dermal vessel basement membranes were also observed in five patients by Pasquali Ronchetti and her colleagues [44,139].

In the patients reported by Leoni et al. [33] and Ogata et al. [19], the epidermis appeared to be normal. However, in the two patients described in the former report, dermal fibroblasts and myofibroblasts showed a poorly developed rough endoplasmic reticulum and Golgi apparatus. Vesicles containing heterogeneous electron-dense material were noted, and lysosomes appeared to be abnormally abundant. The dermal fibroblasts observed in skin biopsies of unaffected skin by Quaglino et al. [139] were also characterized by lysosomal inclusions, but the rough endoplasmic reticulum was well-developed and sometimes abnormally dilated. However, in both studies [33,139], the dermis contained numerous macrophages with convoluted plasma membranes and containing numerous lysosomes and vesicles filled with heterogeneous material. In the two patients described by Leoni et al. [33], the small vessels of the dermis were thick-walled because of the presence of aggregates of microfilaments associated with endothelial and smooth muscle cell basement membranes. Histological examination of the spleen of the probable, but atypical, case of prolidase deficiency reported by Goodman et al. [62] revealed moderately widened cords of Billroth, swollen sinusoidal lining cells, and thickened arteriole walls.

Ultrastructural studies of clinically unaffected skin from the thigh have shown both normal [33] and reduced [139] contents of dermal collagen and elastin, but there does seem to be a tendency toward a smaller than normal mean collagen fibril diameter, with a greater size distribution and disturbed packing into fiber bundles, and an abnormal shape and unusually high degree of branching of elastin fibers, indicative of disturbed fibrogenesis [33,44,139]. These changes appear less marked in clinically unaffected forearm skin [44]. Disturbances of collagen and elastin fibers have also been reported in the probable case of Goodman et al. [62], and in both apparently normal skin [4] and skin around leg ulcers [19] in confirmed patients, although Arata et al. [11] and Sekiya et al. [18] mention that no morphological abnormalities of collagen or elastin fibers were apparent in the cases they studied.

The examination of early ulcerations on the legs indicated that they resulted from occlusion by amyloid of the medium-sized vessels of the dermal-hypodermal junction [4]. The overlying epidermis was necrotic, and an infiltrate of neutrophils destroyed the surrounding connective tissue, leading to deep ulceration. The ulcer floor showed dense granulation tissue infiltrated with neutrophils and plasma cells, possibly indicative of a repair attempt, with an increase in the number of fibroblasts as scarring developed. An accumulation of amyloid fibrils around the capillaries in the dermis around leg ulcers was also noted by Ogata et al. [19], and generalized amyloidosis was noted in the patient autopsied by Sekiya et al. [18].

Amyloid was not at all a feature, however, in mild, initial cutaneous lesions in a 19-year-old prolidase-deficient girl who had been asymptomatic at the time of first reporting at seven years of age [11] but had since developed crops of annular erythematous macules with marginal scales on her thighs and buttocks, and atrophies blanches or small ulcers on her lower legs [12]. Blood vessels in the middle to deep dermis of an annular erythema revealed what was described as mild homogenization of their walls, with perivascular edema. Examination of an atrophie blanche without a preceding ulcer demonstrated marked dilatation of capillaries with thickened, hyalinized walls that showed evidence of collagen "homogenization" and had a narrowed or occluded lumen. Multilayered basal laminae were found around small vessels, and capillary endothelial cells had some lysosome-like structures, many mitochondria, and a highly developed endoplasmic reticulum.

PROGNOSIS AND MANAGEMENT

Prognosis

Because the clinical symptoms of prolidase deficiency do not necessarily manifest themselves until late childhood, or even late adolescence or early adulthood, it is not easy to be precise about the prognosis in any particular patient. Only one patient described in the literature, the brother of the autopsy case [18] described above, appears to have remained entirely asymptomatic by the age of 26 years [15], and his present status is unknown. A further late-onset case did not show skin lesions and ulcers until 30 years of age, although he had suffered frequent infections with chronic bronchitis since the age of 10 years [54]. Consequently, it is reasonable to expect that any patient who is prospectively ascertained through routine neonatal urinary screening, for example, will become clinically affected, most probably before the age of 10 years or so. Developmental delay would be apparent before this age.

Although in the majority of instances prolidase deficiency might be seen as severely discomfiting rather than life-threatening, as experience with the disorder grows it does seem that there is a tendency for it to be associated with a reduced life expectancy, at least seven cases out of the approximately 60 reported having suffered premature death. The patient reported by Lapière et al. [2,3], as noted by Endo et al. [59], died at 36 years of age; this was following rehospitalization with very large ulcers up to the thighs, which were deeply infected with *Pseudomonas aeruginosa*. She presented with septic shock and died of an intravascular coagulation syndrome (Dr. Ch. M. Lapière, personal communication, 13 June 1991).

The female patient first reported by Isemura et al. [15] died at 25 years of age [18] and is the patient whose autopsy findings are described above. An unpublished case died at 15 years of age of terminal liver failure and cardiac failure [38], while a further patient died of sepsis in her late twenties [56]. One patient died at 16 years of age of adult respiratory distress syndrome following treatment for cellulitis of the leg [47], and one eight-year-old boy died despite intensive care, plasmapheresis, and high-dose steroids after having been admitted to hospital suffering abdominal pain and subsequently developing septicemia severe enough to require respiratory support [50]. A further severely affected child died at age four years [52], and, in addition, the probable case of prolidase deficiency reported by Goodman et al. [62] died of influenza at around the age of 50 years [63].

Management

Powell et al. [5] suggested that the massive loss of proline and other bound amino acids might result in the decreased availability of the respective amino acids for reuse in protein synthesis, and Jackson et al. [8] proposed that the clinical symptoms of prolidase deficiency resulted from a

"collagen deficit" arising specifically from the excessive urinary loss of proline that would otherwise be available for reuse in new collagen biosynthesis. On this basis, oral proline therapy might reasonably be expected to alleviate symptoms, but results have been disappointing. Although a diet supplemented with 10 g/day of proline, twice the estimated daily loss of 5 g, elevated the serum proline level from the lower limit of normal to the upper limit in one patient [14], no definite benefit was apparent other than the subjective assessment that the patient felt better than before and attended school more regularly. The treatment of a female patient by oral therapy with 4 g of proline/day for a month did not improve her clinical condition [15], and neither did longer-term oral therapy of a further case with 6 g/day of proline in conjunction with wet proline dressings [19] alleviate clinical symptoms.

Although the topical application of an ointment containing 5% glycine and 5% proline was claimed to result in a considerable improvement in the ulcers of three patients [39,140,141], in other cases the same result has not been achieved [47]. It should be noted that proline is not an essential amino acid and that each cell of the body is capable of synthesizing it in sufficient amount.

Sheffield et al. [14] suggested that the therapeutic potential of the manganese cofactor of prolidase might be tested, as might that of drugs that stimulate collagen synthesis or inhibit collagenase, and several workers have tried various combinations of compounds with rather mixed results. Based on the erroneous belief that the condition in their patient represented a genetically determined deficiency of prolyl hydroxylase, Lapière and Nusgens [2] investigated the use of high doses of ascorbic acid, a cofactor of this enzyme, both intravenously and orally, but were unable to demonstrate any improvement or any modification of the urinary excretion of hydroxyproline. The intramuscular injection of ferrous ion, a cofactor of collagen prolyl and lysyl hydroxylases, was similarly unsuccessful. In contrast, De Rijcke et al. [42] reported that a daily oral dose of 4 g of ascorbic acid resulted in healing of their patient's ulcers and a normalization of the previously rough, dry hair. They further reported that when, after two years, the patient discontinued this therapy for psychological reasons, the ulcers reappeared.

Using a combination of oral ascorbate, Mn^{2+}, diphenylhydantoin, and N-acetyl-hydroxyproline, Charpentier and her colleagues [20,21] were able to reduce the urinary excretion of Gly–Pro by their patient by about one-half, although, despite the fact that the treatment prevented the occurrence of further inflammatory rashes, there was no improvement in the patient's leg ulcerations. The effects observed apparently were not related to any restoration of prolidase activity [93]. The antibacterial agent dapsone, administered at a dosage of 75 mg/day in one patient, resulted in accelerated epithelialization of ulcers, but healing was no more rapid [19].

The treatment of one patient, a four-year-old girl, with $MnCl_2$, proline, and ascorbic acid over a nine-month period was, however, accompanied by an apparently dramatic clinical improvement [27]. The skin was almost normalized, growth was normal, bone age increased rapidly, muscular coordination became normal, and IQ was substantially increased. These improvements were accompanied by no great alteration in the urinary excretion of dipeptides during the treatment period. Similar treatment with hydroxyproline instead of proline also resulted in a slight improvement in the condition of the two patients of Voigtländer et al., with an absence of new ulcer formation [35]. The treatment of a further patient with ascorbic acid, diphenylhydantoin, and N-acetyl-hydroxyproline is also reported to have brought about a considerable improvement of skin ulcers to the extent that he was able to resume walking [40].

Repeated erythrocyte transfusions, either alone [3,17,65] or in combination with $MnSO_4$ and ascorbic acid, have generally been found to be ineffective in either altering the pattern of imidodipeptiduria or alleviating clinical symptoms. Hechtman et al. [121] have suggested that such failure is a consequence of prolidase occurring to a large extent in an inactive apoenzyme form in human erythrocytes and propose the use of erythrocytes in which prolidase has previously been activated by preincubation of the cells with Mn^{2+}. In their studies, hemolysates of normal erythrocytes that had been incubated overnight in the presence of 1 mM $MnCl_2$ showed a more than 30-fold increase in prolidase activity, and the intact cells themselves showed a 10–20-fold increase in the rate at which they were able to degrade Gly–Pro. No information is available on whether the procedure has been used successfully in the treatment of prolidase-deficient patients, but given the fact that erythrocytes treated in such a manner retained high levels of enzymatic activity for at least two weeks, the procedure clearly has significant potential. Berardesca et al. [45] reported a dramatic improvement in one 15-year-old Italian patient whose ulcers had worsened to the extent that he was no longer able to walk but in whom, after four transfusions of concentrated erythrocytes at bimonthly intervals, the ulcers showed an improvement such that he was able to resume walking. However, there was no alteration in the level of excretion of urinary imidodipeptides. In this case, the transfused erythrocytes had not previously been activated.

More recent reports suggest the beneficial effects of corticosteroid therapy in managing prolidase deficiency. The patient with probable prolidase deficiency described by Shrinath et al. was apparently making good progress with oral prednisolone, which had prevented further new infections, although her facial rash persisted [50]. Yasuda et al. [55], in contrast, found that oral prednisolone alone was ineffective against skin ulcers in one of their patients but did find that corticosteroid pulse therapy performed using intravenous drip infusion of methylprednisolone (1,000 mg/day) for three days, followed each time by oral prednisolone (30 mg/day), repeated three times over two years resulted in almost complete regression of preulcerative lesions and ulcers 1–2 months after each pulse course. Finally, the topical application of an ointment containing growth hormone to one 13-year-old prolidase-deficient boy in whom growth hormone deficiency had been diagnosed resulted in a complete but transitory healing of the boy's foot ulcers, which was attributed principally to the growth-promoting effects of growth hormone on dermal connective tissue [142].

Although patients with prolidase deficiency are clear candidates for gene replacement therapy [133], on the whole at the present time there is little in the way of treatment confirmed to be effective that can be recommended other than the conservative management of ulcers combined with measures to prevent infection. As an example of this, Ogata et al. [19] used hydrotherapy of ulcers followed by the application of wet dressings containing antibiotics effective against *Pseudomonas aeruginosa*. Split-thickness skin grafting has been found to be unsuccessful [18,19].

As far as genetic counseling is concerned, the risk of a couple of which one member has prolidase deficiency having

an affected infant is almost zero, but the risk of a couple each of whom is heterozygous for the disorder having an affected infant is the same as for any other recessively inherited disorder (i.e., there is a 1 in 4 probability). The activity of prolidase in chorionic villus biopsies appears not yet to have been investigated, but its known expression by amniotic fluid cells [27,89] has permitted the prenatal diagnosis of an affected infant, the sibling of a seriously affected child who died at the age of four years. The diagnosis was later confirmed using the baby's leukocytes [52]. Excessive levels of imidodipeptides might also be apparent in amniotic fluid but, as cautioned earlier, their presence alone would not necessarily be confirmatory of prolidase deficiency.

PATHOGENESIS

The pathogenesis of the clinical manifestations apparent in this disorder is not at all well-understood. An elevated "dermal aldehyde" content, suggested as resulting from an inability of the aldehyde groups to participate in collagen intermolecular cross-links, led Goodman et al. [62] to describe the condition in their patient, a probable case of prolidase deficiency [63], as resembling lathyrism (this being the condition resulting from the inhibition of lysyl oxidase, the enzyme responsible for initiating the cross-linking of both collagen and elastin; see Chapter 9, this volume). In fact, the condition as described bears no resemblance to lathyrism, either biochemically or clinically.

Connective Tissue

The elevated urinary proline:hydroxyproline ratio in their patient, only later confirmed as being prolidase-deficient, initially led Lapière and his colleagues to suspect deficient activity of prolyl hydroxylase (the enzyme responsible for the post-translational modification of specific prolyl residues within the collagen α chains) [2,64,143]. However, the finding of a normal extent of prolyl hydroxylation in collagen produced by cultured fibroblasts from patients with imidodipeptiduria and prolidase deficiency disposed of this possibility [73,93], and Isemura et al. [15] found a normal amino acid content of dermis in their patient, with normal extents of prolyl and lysyl hydroxylation and of hydroxylysyl glycosylation. Nevertheless, the above-mentioned reports [2,62,64,143] may have served to cause an undue amount of attention to be focused on collagen to the extent that in one instance it was felt justifiable to describe prolidase deficiency as an "inborn error of collagen metabolism" [22].

Actual studies of collagen in prolidase deficiency, however, are few. Minimal increases in the ratio of collagen type III to collagen type I have been reported in both dermis [17] and fibroblasts [93], although another study on dermal fibroblasts found an equally minimal decrease [144]. The ratio of the reduced collagen cross-links dihydroxylysinonorleucine:hydroxylysinonorleucine was reported to be elevated in the dermis of one patient [17], but the significance of this is not certain. It is not clear from which region the skin was obtained, and the increased dihydroxylysinonorleucine content may reflect the normal pattern seen in scar or granulation tissue [145]. The most striking observation in studies with cultured fibroblasts from prolidase-deficient patients has been an elevation in the extent of intracellular degradation of newly synthesized collagen [93,144]; however, no such elevation was found in another study [146]. Isemura et al. [15] reported a slightly decreased ratio of the collagen hydroxylysyl glycosides Glc–Gal–Hyl:Gal–Hyl in the urine of their patient, but Leoni et al. [33] in their two patients found a normal content of hydroxylysyl glycosides in urine and a normal diglycoside:monoglycoside ratio, which would indicate a quantitatively normal level of catabolism of collagen. The elevated lysyl aldehyde content of dermal collagen from the patient of Goodman et al. [62] is consistent with reports of disturbed fibrillogenesis and may be a reflection of disturbed alignment of tropocollagen molecules for some reason such that normal cross-linking cannot occur, although it must be said that if alignment and fibrillogenesis were seriously disturbed it would be unlikely that lysyl oxidase could function. In conclusion, one must therefore say that biochemical evidence of any change in collagen metabolism is minimal and that if any such changes do occur they are likely to be secondary to the basic metabolic disturbance.

Because of the frequent occurrence of Gly–Pro sequences in collagen, the occurrence of Gly–Pro as the predominant dipeptide in the urine in prolidase deficiency has also led to possibly greater emphasis than justified being placed on collagen as the principal or even the sole source of the urinary dipeptides [2,5,8,11,13,20,21,62,64,144]. However, several authors have pointed out that the greatly elevated urinary Pro:Hyp ratio, which may be as high as 5:1 or more, indicates that the urinary dipeptides are from sources other than solely the breakdown of body collagen [7,14,15,22,27,73,82,107,121]. It is clear that exogenous protein does contribute to the imidodipeptiduria [6], and Hechtman et al. [121] expressed the view that the principal source of urinary dipeptides is likely to be the absorption of peptides derived from dietary protein.

As mentioned earlier, it has been suggested that the connective tissue alterations might result from a proline "deficit" [8]. Because the C1q component of complement also contains collagen-like sequences, it has also been suggested that there is somehow an immunological deficit related to this fact [8]; however, C1q levels have been found to be normal [21,25], as have levels of plasma acetylcholinesterase [21], another protein with collagen-like amino acid sequences. However, as mentioned above, there are frequently elevated levels of IgG and IgA in plasma from patients. There is a possibility that the frequently observed susceptibility to repeated respiratory infections might result from a failure of IgG function brought about in some way by an elevated concentration of plasma dipeptides. However, Gly–Pro is the principal imidodipeptide to be detectable in the plasma of patients [23,62,72], and although several Gly–X peptides have been examined for their effect on the binding of human IgG to bacterial Fc receptors, none was found [147]. Gly–Pro and X–Pro dipeptides, however, were not examined in this regard.

Piérard et al. [4], in a histological study of the process of dermal ulceration in a prolidase-deficient patient, described developing ulcers as initially appearing as isolated papules resulting from the occlusion of medium-sized vessels of the dermal-hypodermal junction by amyloid. A dense infiltration of neutrophils was above the main vascular alteration, surrounding a core of connective tissue that was eliminated by the neutrophils to form a deep ulceration. The precursors of the fibrous amyloid deposits associated with chronic inflammatory disorders are the serum amyloid A proteins. Mitchell et al. [148] demonstrated the production by rabbit synovial fibroblasts treated with inflammatory mediators of serum amyloid A3 that had the effect of inducing collagenase synthesis by these cells. It is therefore conceivable that such

a mechanism of tissue damage might be operative in the present condition.

Arata et al. [60] examined the cutaneous lesions of one case, a 19-year-old girl who had remained asymptomatic until 15–18 years of age, by light and electron microscopy. Small vessels of the dermis frequently showed thickened walls surrounded by multiple basal laminae, although there were no amyloid deposits, and an angiopathic pathogenesis was suggested. Severe edema of the scrotum and lower limbs, or of just the lower limbs, was specifically noted to be a problem in two patients [14,26] and suggested as resulting from progressive damage to the lymphatic vessels [14]. In the case of both blood and lymphatic vessels, however, the mechanism by which vascular damage might initially be induced is unknown, although there are several indications that collagen-derived peptides are involved in inflammation, and the X–Pro imidodipeptides, some of which will be collagen-derived, may have similar effects.

The small peptide degradation products of bacterial collagenase digestion of acid-soluble calf skin collagen have been found to have a number of bradykinin-like properties. For example, when injected intradermally in rats, they result in an increased permeability of skin capillaries [149] and this may be related to the edema sometimes observed in prolidase deficiency (see above). The same degradation products, when injected into the brain ventricle of rats, depress locomotor activity [149] and have also been shown to cause the release of rat mast cell histamine [150]. Further, dipeptides containing proline and hydroxyproline also induce the release of histamine from isolated mast cells and collagenase from polymorphonuclear leukocytes [151]. In addition to these findings, the intratracheal instillation of collagen peptides resulting from the action of bacterial collagenase on collagen results in an influx of neutrophils into the lungs of rats [152].

High-molecular-weight ($M_r \sim 200$ kDa) fragments derived from mild alkali treatment of insoluble collagen have been reported as being both chemotactic for polymorphonuclear leukocytes and stimulators of neutrophil respiratory bursts with concomitant release of superoxide radicals, lysis, and release of hydrolytic enzymes [153]. The large size of the fragments indicates that the stimulant is either collagen or very large fragments of it. It may be the case in prolidase deficiency that collagen, especially if previously damaged by inflammatory cell enzyme release, might act as a mediator of further inflammation. The tripeptide Pro–Gly–Pro generated by alkali degradation of whole cornea also functions as a chemoattractant toward polymorphonuclear leukocytes [154], and it may be that the imidodipeptides generated in prolidase deficiency have a similar effect.

There is some evidence that the imidodipeptides that accumulate in prolidase deficiency do themselves exert a deleterious effect. Watanabe et al. [155] demonstrated that Pro–Pro, Gly–Pro, and Ala–Pro primed N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced superoxide generation by polymorphonuclear leukocytes from normal, healthy individuals, and Yasuda et al. [55] subsequently examined the process using neutrophils from two prolidase-deficient sisters. The extent of superoxide generation by these neutrophils correlated with the level of several serum imidodipeptides, which, in turn, were highest at periods of active ulcer formation. Pretreatment with Pro–Pro of neutrophils isolated from blood collected at times when serum imidodipeptide levels were comparatively low resulted in a significant enhancement of superoxide generation following fMLP challenge but had no such effect on neutrophils isolated from blood collected at times when serum imidodipeptide levels were elevated, suggesting that they were already fully primed under such circumstances. Yasuda et al. [55] suggested that superoxide production by neutrophils was the direct cause of vascular thickening and inflammatory skin lesions. They further noted that the extent of neutrophil superoxide production was greater in the more severely affected of the two sisters and suggested that clinical severity may depend on the response of neutrophils to the imidodipeptides.

There may also be some involvement of neuropeptides in the dermal ulceration. In the fluid from spontaneous blisters in inflamed skin in a number of disorders, there are significant amounts of substance P, vasoactive intestinal peptide, calcitonin gene-related peptide, somatostatin, and neuropeptide Y [156], and each of these when injected intracutaneously induces flaring, in some cases accompanied by whealing and itching [156–159]. This appears to result from the ability of these substances to elicit histamine release, as demonstrated with rat peritoneal mast cells [157,159], and, in addition, calcitonin gene-related peptide is a potent vasodilator [158]. Substance P is quite likely a mediator of the local inflammatory response [160], is chemotactic for neutrophils and stimulates lysosomal enzyme release from them [161], and enhances the phagocytic activity of both neutrophils and macrophages [162].

Mental Retardation

The pathogenesis of mental retardation is intriguing and has not yet been approached by clinical investigations. However, it is worthy of note that prolidase is normally present in brain at levels 10–100 times higher than would be required for the measured rate of protein turnover in this tissue [117]. Most neuroactive peptides (e.g., thyrotropin releasing hormone, MIF, β-MSH, substance P, β-lipotropin, oxytocin, vasopressin, angiotensin) contain proline in their structure and are thus resistant to the action of conventional amino and carboxyl peptidases. Thus, in addition to its role in protein turnover, prolidase is likely to terminate the activity of proline-containing neuropeptides [163]. One might speculate therefore that deficiency of prolidase might cause significant pathophysiological changes. As mentioned previously, there may be some correlation between the presence of mental retardation and the extent of enzyme deficiency in that of eight patients studied by Endo et al. [59], mental retardation was only apparent in three of the six who exhibited a CRM^- phenotype.

To the best of our knowledge, the examination of cerebrospinal fluid has been performed only in one patient, that with probable prolidase deficiency reported by Goodman et al. in 1968 [62], in which case the amino acid analysis was normal and no peptides were detectable. A broader study to investigate whether imidodipeptides were present in excessive amounts in cerebrospinal fluid would be of interest and might indicate whether one or more functioned as a neurotransmitter or neuromodulator. Furthermore, brain tissue obtained at autopsy from prolidase-deficient patients has not been examined with respect to the extent of residual enzyme activity or the content of free imidodipeptides. However, magnetic resonance imaging of the brain of one of the two siblings initially reported by Arata et al. [11], superoxide production by whose neutrophils was examined by Yasuda et al. [55], demonstrated multiple microthromboses in bilateral cerebral white matter [12]. This finding suggested an abnormality of the cerebral vessels,

consistent with the angiopathic pathogenesis proposed by Arata et al. [12], to which imidodipeptide-induced superoxide production may have contributed [55], and may explain the occurrence of mental retardation in prolidase deficiency [12].

ANIMAL MODEL

No animal model of prolidase deficiency, either naturally occurring or experimentally generated, has yet been described. However, opportunities do present themselves for the creation of an experimental model in which the pathogenesis of the disorder might be investigated by virtue of the fact that the antihypertensive captopril is an inhibitor of prolidase in extracts of various rat and human tissues [164,165], although this effect is weak as far as porcine kidney prolidase is concerned [119] and minimal with respect to erythrocyte prolidase [164]. There is a striking similarity between captopril, N-(3-mercapto-2-methyl-1-oxopropyl-L-proline), and X–Pro dipeptides, and the oral administration of captopril to the rat resulted in the increased excretion of peptide-bound hydroxyproline, suggesting that *in vivo* inhibition of tissue prolidase had occurred [164], although it should be noted that this finding does not necessarily equate with imidodipeptiduria, and, significantly, there was no increase in the urinary excretion of Gly–Pro, normally the most abundant urinary imidodipeptide in prolidase deficiency. The clinical use of captopril has several side effects, including dermatological manifestations similar to those seen in prolidase deficiency [166,167]. Other mechanisms for the development of these cutaneous lesions have been suggested (summarized in [164]), but suspicion must fall on the inhibition of tissue prolidase.

Other inhibitors of prolidase that might be useful in developing an experimental animal model of prolidase deficiency are the naturally occurring glycolytic intermediate, phosphoenolpyruvate, and analogs of it, an effective inhibitor of pig kidney prolidase [97,168], and N-benzyloxycarbonyl-L-pipecolic acid and N-benzyloxycarbonyl-L-proline [119].

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Chapter 17

α_1-Antitrypsin Deficiency

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SUMMARY

- **α_1-antitrypsin (α_1-antiproteinase, α1AT), the most abundant plasma proteinase inhibitor, is a serine protease inhibitor important in the control of tissue proteolysis, particularly in the lungs.**
- **An inherited deficiency of α1AT is associated with an increased risk of chronic obstructive lung disease, liver disease, and tissue destruction in inflammatory conditions such as rheumatoid arthritis.**
- **The locus encoding α1AT (*PI, SERPINA1*) is located at 14q32.1 in a cluster of serine proteases that may also be involved in tissue destruction and/or inflammation.**
- **There are numerous genetic changes in the 392 amino acid residues of α1AT, leading to more than 70 genetic variants, with at least 15 resulting in a reduced plasma concentration.**
- **The most common deficiency variant or PI type is PI*Z. Individuals of PI type ZZ occur as about one in 3,000 to 7,000 in Caucasian populations. The plasma concentration of α1AT in PI ZZ individuals is about 15–20% of normal.**
- **There are other rare variants leading to a reduced concentration of α1AT, some resulting in no detectable α1AT (null variants).**
- **PI Z results in deficiency because of its tendency to polymerize in the liver, which then results in the formation of typical inclusions.**
- **The greatest risk for patients with α1AT deficiency is chronic obstructive pulmonary disease. Smoking has a profound effect on the age of onset and prognosis.**
- **About 3% of all PI ZZ individuals develop severe progressive liver disease in childhood, although about 14% develop signs of liver abnormality that resolve during the early years of life. There is an increased risk of liver disease in adult life.**
- **Avoidance of smoking is important preventative therapy. Replacement therapy, using purified α1AT, is available. The benefit of such therapy is not well-established because of confounding with other factors that may influence the rate of lung degradation. Gene therapy is also under investigation. Antioxidants may aid in tissue preservation; their role needs to be explored. Lung or liver transplantation offers potential therapy for end-stage organ destruction.**

INTRODUCTION

α_1-Antitrypsin (α_1-antiproteinase, α1AT) is a major plasma proteinase inhibitor that plays an important role in the control of connective tissue proteolysis. An inherited deficiency of α1AT is associated predominantly with an increased risk of abnormally rapid degradation of lung elastin, leading to emphysema, and with destruction of the liver in both children and adults. Susceptibility to an increase in joint damage in rheumatoid arthritis and to tissue destruction in inflammatory conditions has also been suggested.

Tissue integrity is dependent on an appropriate balance between proteolytic enzymes, which can facilitate tissue remodeling when required, and inhibitors of these proteinases. The array of proteinases and their inhibitors is extensive. A number of classes of proteinases are involved in forming and maintaining extracellular matrix, including thiol proteinases, carboxyl proteinases, a large group of metalloproteinases, and an even larger group of serine proteinases. Control of proteinases is largely maintained by a variety of proteinase inhibitors. An imbalance between any one of these proteinases and its inhibitors could lead to abnormal tissue destruction (see Chapter 7, this volume).

The Serpins

α1AT, the most abundant plasma proteinase inhibitor on a molar basis, is a serine proteinase inhibitor. Its plasma concentration is similar to that of the much larger proteinase inhibitor α_2-macroglobulin, which inhibits a variety of proteinases by an entrapment mechanism. Of the large number of proteinases and inhibitors found in human tissues and plasma, α1AT, which has been particularly well-studied, can be regarded as part of a model system. Deficiencies of other plasma and tissue proteinase inhibitors may prove to be equally destructive when more is known about their function and deficiency states. The class of serine proteinase inhibitors, referred to as "serpins," typically have a reactive center formed by an X–Ser that acts as the substrate for the target proteinase [1]. The serpins are a superfamily of proteins found in viruses, plants, and animals [2] (see also Chapter 7, this volume). Several of them, such as α_1-antichymotrypsin,

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antithrombin III, C1 inhibitor, and α_2-antiplasmin, are major inhibitors of the inflammatory cascade.

Historical Background

α1AT, first isolated by Schultze et al. [3], was later named α_1-antitrypsin because most of the trypsin inhibitory activity in plasma was associated with the α_1-globulin fraction and particularly with the protein they had initially isolated [3]. The name α_1-antitrypsin has been commonly used since that time and is maintained in this chapter for historical reasons. However, it is now well-recognized that trypsin inhibition is not the major function of this protein, and α_1-proteinase inhibitor has been suggested as an alternate designation.

α1AT deficiency was first noted by C.-B. Laurell, in a number of patients with early-onset emphysema, as an abnormal protein pattern on electrophoresis [4]. Subsequent studies confirmed the presence of early-onset emphysema in individuals homozygous for the deficiency gene [5]. At about the same time, Fagerhol and Briand in Norway [6] observed variable protein bands on acid starch gel electrophoresis, which were shown to be α1AT [7]. A number of variants were observed on starch gels and were named Pi (now PI) for proteinase inhibitor [6] in recognition of the fact that α1AT was an inhibitor of other proteinases in addition to trypsin. The involvement of α1AT deficiency in liver disease in children was recognized by Sharp and colleagues in 1969 [8].

STRUCTURE AND FUNCTION OF α_1-ANTITRYPSIN

Protein Structure

α1AT is a 52 kDa glycoprotein consisting of a single polypeptide chain of 392 amino acid residues and three carbohydrate side chains. The protein is homologous in amino acid sequence to several other plasma proteinase inhibitors: α_1-antichymotrypsin (42% homology), antithrombin III (28%), and C1 inhibitor (27%) [9]. The relatively small size of α1AT permits its diffusion through interstitial body fluids.

The structure of human α1AT, known from x-ray crystallography [10], shows a well-defined secondary conformation, including three β sheets and eight α helices. An inactive, relaxed form of α1AT, proteolytically cleaved at the active site, has been crystallized. α1AT contains only one cysteine residue and therefore does not form an intramolecular disulfide bridge. However, the cysteine residue can form a disulfide bond with other proteins and with thiol compounds. There is microheterogeneity among the α1AT molecules, depending on the type of carbohydrate side chain (biantennary or triantennary). This results in a typical microheterogeneity on isoelectric focusing in which the various protein types have pIs of 4.42–4.67, with the two major bands being at 4.48 and 4.55 [11].

The reactive site of α1AT involves Met and Ser residues at positions 358 and 359, respectively. These two residues are widely separated in the relaxed molecule, which has been crystallized. In the native inhibitor, these residues are adjacent on an exposed loop of approximately 16 residues [1]. This exposed loop then becomes the site for forming a tightly bound one-to-one complex with a target proteinase, particularly neutrophil elastase. This is a common mechanism for serine proteinase inhibitors [12]. The methionine residue, important for functional activity [13], is apparently also crucial for specificity because substitution of Met 358 by Arg in the genetic variant called PI Pittsburgh causes loss of inhibition of elastase. Arginine is present at the identical position in antithrombin III, and this mutant α1AT protein is active as an inhibitor of thrombin [14].

The exposed position of the reactive methionine residue readily allows its oxidation to methionine sulfoxide, which will no longer accommodate the physical complexing between elastase and α1AT [14]. This inactivation of methionine by oxidation could be harmful in individuals already deficient in α1AT but has been proposed to be advantageous in situations of tissue inflammation, where local tissue breakdown would be enhanced [1].

Location and Organization of the Gene

The gene encoding α1AT (commonly known as *PI* for proteinase inhibitor, now officially *SERPINA1*) is 10.2 kb in length and contains four introns [15]. Control of expression and cell specificity requires the region 700 base pairs 5′ upstream of the transcription start site [16], and the specific proteins that bind to this region have been identified [17]. Although the most abundant expression of α1AT occurs in the liver, the gene is also expressed in monocytes [18]. An additional transcription start site 5′ to the hepatocyte initiation site controls expression in macrophages and monocytes [19]. Studies of α1AT in transgenic mice have demonstrated its expression in thymus, chondrocytes, lymphoid tissue of the small intestine, gastric and small intestinal crypt epithelial cells, renal distal tubule brush border, and the lining of pulmonary alveoli [20,21]. The macrophage-specific promoter, and consequently macrophage expression, are reported to be absent in the mouse [22].

The *PI* gene is located in the distal region of chromosome 14 at 14q32.1 and lies within a cluster of homologous genes with some sequence and structural similarities. Members of this cluster, which are involved in blood coagulation, inflammation, and blood pressure control, include corticosteroid-binding globulin (*CBG*), α_1-antichymotrypsin (*AACT*), blood coagulation factor C1 inhibitor (*PCI*), and kallistatin (*PI4*), all of which map within a region of about 300 kb [23–25] (Fig. 1). A *PI*-related gene for which the functional status is not known, designated *PIL* (for PI-like), lies adjacent to *PI* [26]. The close physical proximity of these related genes could have clinical significance. The disease associations ascribed to variants of α1AT could be due to effects of proteins encoded by neighboring genes.

Function of α_1-Antitrypsin

α1AT shows a broad substrate specificity for serum proteinases. The inhibition of pancreatic trypsin, which was the first substrate identified [27], is not considered to be physiologically important. Other serine proteinases inhibited include pancreatic and neutrophil elastase, neutrophil cathepsin G, pancreatic chymotrypsin, collagenase from skin and synovium, acrosin, kallikrein, urokinase, and renin [12]. Proteases within the clotting and fibrinolytic systems (plasmin, thrombin, factor XI, and Hageman factor cofactor) are also inhibited. However, some of these inhibitory activities may be *in vitro* phenomena only. The activity of physiological importance lies in the inhibition of neutrophil elastase. α1AT provides approximately 90% of the antielastase activity in plasma. It gains access to the lower respiratory tract more readily than does α_2-macroglobulin, the other major inhibitor of elastase, although the latter is produced by lung fibroblasts [28]. Inhibition of the clotting cascade does not seem to be of major physiological significance, probably because of other more effective inhibitors.

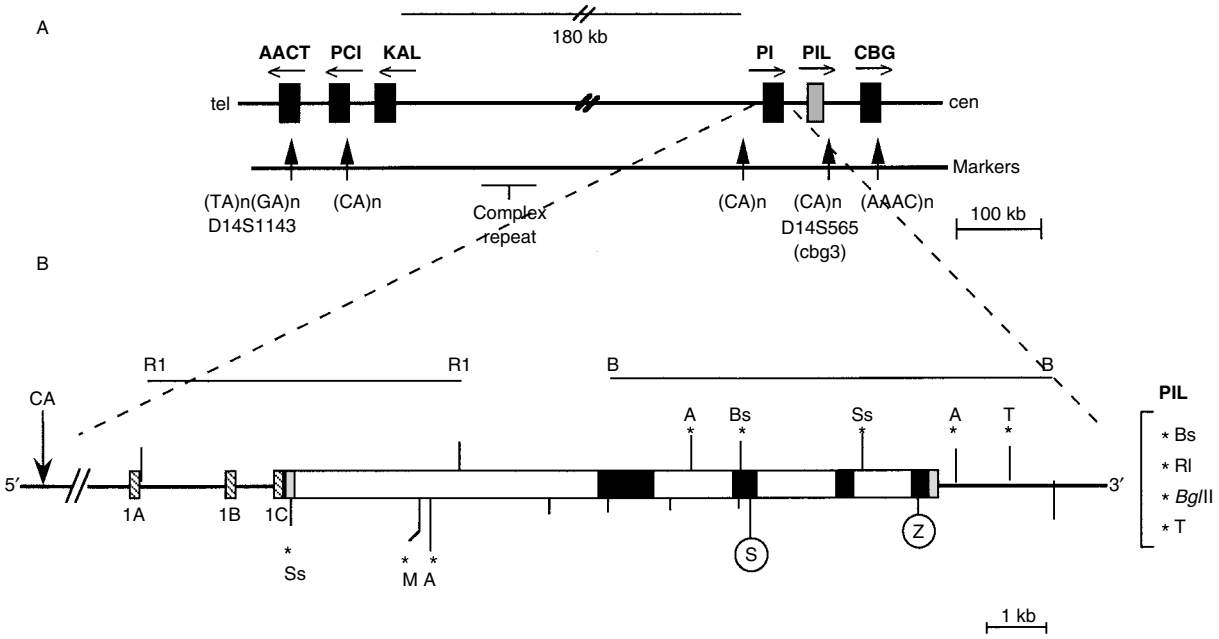

Figure 1. A. Cluster of PI and other loci in the serpin superfamily cluster on chromosome 14, as determined by pulsed field gel electrophoresis [23–25]. AACT: α_1-antichymotrypsin (*SERPINA3*); PCI: protein C inhibitor (*SERPINA5*); KAL: kallistatin (*SERPINA4*); PI: α_1-antitrypsin (*SERPINA1*); PIL: PI-1 like (*SERPINA2*); CBG: corticosteroid-binding globulin (*SERPINA6*). New names, in parentheses, have been assigned by the HUGO Nomenclature Committee. **B**. α1AT gene and flanking regions. Coding regions are solid, introns open, untranslated dotted rectangles. Cross-hatched regions are exons of macrophage DNA. Asterisks indicate sites of polymorphisms (SNPs) identified with the following restriction enzymes (those in square brackets at the right indicate polymorphisms in the PIL locus): A — *Ava*II; B — *Bam*H1; Bs — *Bst*II; T — *Taq*I; RI — *Eco*RI; Ss — *Sst* sites [46]; S and Z circled — sites of mutations in PI*S and PI*Z, respectively. Positions of CA repeats are indicated by arrows in A and B.

GENETIC VARIATION

Many variants of α1AT (PI, or proteinase inhibitor, types; MIM 107400) have been identified by electrophoretic methods. These variants were initially identified by the acid starch gel electrophoresis method developed by Fagerhol [29]. Variants were named according to their relative mobilities in the acid starch gel system: F (fast), M (medium), S (slow), and Z (the most cathodal). Several variants, including the Z deficiency variant, were also recognized by agarose electrophoresis [30]. In most populations, about 90% of the alleles are of the M type. Over 70 alleles, most of them rare, have been described [31–33]; 40 are listed under MIM 107400 [34]. These variants are now identified by their position in polyacrylamide isoelectric focusing (PIEF) gels. Their names have been designated by letters of the alphabet, from the most anodal (presently B) to the most cathodal (Z) in position on the PIEF gel, in addition to a place name designation [32]. PI types are designated MZ, MS, and so forth; alleles are designated *PI*M*, *PI*null*, and so on. PIEF [35] is the most commonly used method for identification of genetic variants, and many modifications have been developed. Names can be replaced by specific sequence designations as sequences of the variants become available.

Normal Variants

Most of the PI variants are associated with a normal concentration of α1AT. The normal mean concentration is 1.32 g/L [36]. PI MM individuals usually have plasma concentrations of 80–120% of this mean. There are several common subtypes of the normal M variant: M1, M2, M3, and others. Typically, the M1 subvariant is most common, at a frequency of about 0.70, then M2 at about 0.15, and M3 at about 0.12. Frequencies are similar in all of the major racial groups [37]. Although there are isolated reports of possible disease association with various M subtypes, these may represent sporadic findings because all of the normal subtypes seem to have a normal concentration and inhibitor activity [38]. Normal variants with a somewhat reduced concentration of α1AT are PI I and PI S, both of which are associated with about 60% of the normal concentration of α1AT [39,40]. These two variants can be considered normal because their presence in the heterozygous state, or even in combination with Z, does not appear to result in a significantly increased risk of disease.

The rare PI P variant, which has also been called Plowell [41,42], is associated with a plasma concentration of α1AT of about 30% of normal [43]. This variant is on the borderline for classification as a deficiency variant because it appears to lead to increased risk of emphysema when combined with the Z allele [42]. There are a number of variants that show a position similar to PI P on IEF that are not deficiency alleles [32,42].

All of these variants are inherited in a codominant manner in that the products of each allele can be visualized on PIEF. This is in contrast to the deficiency state, which is inherited in a recessive manner.

Deficiency Variants

The deficiency alleles can be considered as those associated with less than about 30% of the normal concentration of

α1AT, a concentration at which disease risk is increased. By far the most common deficiency allele, usually representing about 95% of all deficiency alleles, is *PI*Z*. Individuals of PI type ZZ generally have only about 18% of the normal concentration of α1AT. Interestingly, this variant is found in northern European populations and not in blacks or Asians, except through racial admixture [37]. A feature of the Z allele is that it is associated with the formation of liver inclusions due to storage of the abnormal α1AT in dilated endoplasmic reticulum [44].

A number of other rare deficiency alleles make up the remaining 5% of all deficiency alleles in white populations. The mean plasma concentration of α1AT associated with these other rare variants ranges from 0 to about 15% of normal [45]. In addition to the Z variant, there are at least eight rare deficiency variants that produce some measurable product and at least 12 variants for which no product can be detected in plasma. The null (Q0) alleles, by definition, are associated with no detectable α1AT in plasma and are therefore at one end of the spectrum of deficiency alleles. The frequency of all of the null alleles combined was estimated to be 1.4×10^{-4} in a white North American population [45], and the individual Q0 alleles are even more rare. The rare deficiency mutations and their characteristics have been summarized [45,46]. A number of these mutations have been identified by DNA sequencing and indicate various causes for the plasma deficiency. Mmalton [47] and Mnichinan [48] variants produce about 15% of the normal concentration of plasma α1AT, similar to that occurring with the *Z* allele. Both of these have a deletion of the phenylalanine residue at position 52, which results in accumulation of α1AT in inclusions as for the Z gene product. Most of the null variants have base changes that result in a premature stop codon, resulting in intracellular degradation of the abnormal product as observed with one of the more common null alleles, Q0bellingham [49]. Study of the specific mutations involved provides information on factors required for both protein secretion and stability.

The recognition of null alleles has practical significance because null homozygotes seem to have an increased risk of emphysema even in comparison with PI ZZ homozygotes [50]. These affected individuals may be the most likely to benefit from replacement therapy.

DNA Polymorphisms

In addition to the protein variants, both normal and deficiency, found by electrophoretic methods, further variation has been identified in the DNA sequence within and surrounding the α1AT gene. A number of DNA restriction fragment length polymorphisms (RFLPs) have been used to show that the *PI*Z* allele arose about 5,000 years ago, after the divergence of the major racial groups, and has spread extensively through northern European populations [51]. RFLPs have also been used to identify the evolutionary pathway of normal variants [46,52]. Because of extensive linkage disequilibrium throughout the region, the combination of markers, or haplotype, is usually specific for each of the rare deficiency variants and has been used to guide their identification [45]. A polymorphism at a *Taq*I restriction site 3′ of the α1AT gene is suggested to lie in an enhancer region, resulting in an impaired interleukin (IL)-6 acute phase response, thus predisposing to lung disease [53]. All of these RFLPs belong to a subset of SNPs (single-nucleotide polymorphisms) that can be converted for detection using the polymerase chain reaction (PCR).

Highly polymorphic dinucleotide (CA) repeats have been identified close to the PI α_1-antichymotrypsin (AACT) and C1 inhibitor (PCI) loci [24]. Other variants in the serine proteinase cluster are included in marker and SNP databases.

DIAGNOSTIC METHODS

Plasma Concentration of α_1-Antitrypsin

The plasma concentration of α1AT can be measured by immunological or functional methods. Detailed protocols and appropriate references are available [54]. Immunological assays are usually preferred in clinical laboratories, where automated nephelometry is used for the measurement of a variety of proteins. The results of immunological assays can vary considerably between laboratories because of the use of different standards. Many of the commercially available standards have an abnormally high normal range (1.5–3.0 g/L). For this reason, laboratories may express normal values as a percentage of a normal pool, which is established as 100%. A standard from the American Pathological Association can be purchased commercially. This standard has been calibrated against a highly purified preparation of α1AT, which indicates that the normal mean is 1.32 g/L [36].

Functional assays can also be used and usually correlate well with immunological results. However, the inhibitor activity in plasma also includes that due to α_2-macroglobulin. Results can be misleading, particularly in testing children, in whom the α_2-macroglobulin plasma concentration is elevated. As a result, these assays tend to be used only for special investigations. The functional assays evaluate the inhibition of trypsin or elastase using synthetic substrates.

Most of the quantitative variation between individuals is accounted for by PI type [55]. Normal ranges of PI types commonly found in the population and for selected deficiency types, measured immunologically, are shown in Table 1, expressed both as a percentage of a normal pool and as g/L [40,56]. Expressed on a molar basis, the normal mean is 25μM. The measurement of plasma α1AT is the appropriate first test for the diagnosis of deficiency. Any values below 50% of the normal mean require PI typing for verification of genetic status. A low level of α1AT can also occur for nongenetic reasons such as in respiratory distress syndrome in newborns, conditions of severe protein loss, terminal liver failure, and during the course of cystic fibrosis. The plasma concentration of α1AT associated with PI type ZZ is about 18% of normal (range 9–27%) [45,57]. However, in infants and children with liver disease, the concentration of α1AT is frequently elevated to as much as 40% of normal [58].

TABLE 1. Plasma Concentrations of α_1-Antitrypsin Associated with Various PI Types

	α1AT Concentration		Increased Disease Risk	
PI Type	Mean ± SD (% normal)	Mean (mg/dL)	Lung	Liver
MM	100 ± 23	1.32	no	no
MS	86 ± 20	1.12	no	no
MZ	65 ± 15	0.84	no	no
SZ	45 ± 11	0.58	slight	no
ZZ	18 ± 5	0.23	yes	yes
Q0Q0(null)	<1	<0.01	yes	?

Values are from [40] and [56].

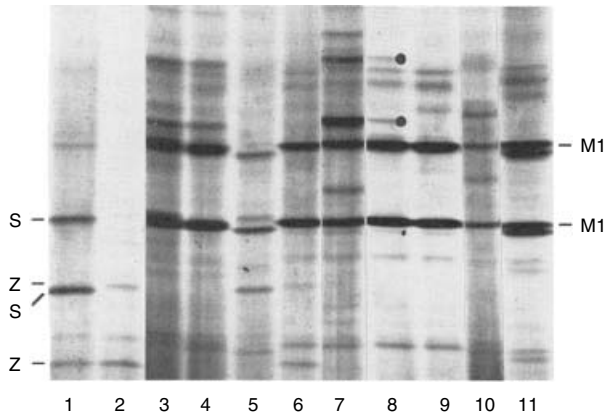

Figure 2. PI types determined by isoelectric focusing, in polyacrylamide gel, pH 4.2–4.9. Lane 1—SZ; 2—ZZ; 3—M1M3 (aged sample, not reduced); 4—M1M3—sample as for 3 but cysteine reduced; 5—M2S; 6—M1Z; 7—IM1; 8—M1 (on penicillamine treatment); 9—M1; 10—FM1; 11—M1M2. The two major bands are indicated for M1, S, and Z. Dots indicate extra bands sometimes observed in patients treated with penicillamine. The anode is at the top.

Measurement of plasma α1AT is not always reliable for the identification of heterozygotes. α1AT is an acute-phase protein that can show a marked increase (up to fourfold) in a wide range of inflammatory conditions, cancer, and liver disease. Pregnancy and estrogen therapy also produce modest increases.

Determination of PI Type

Isoelectric focusing (PIEF) provides confirmation of genetic α1AT deficiency (Fig. 2). Different carbohydrate side chains result in a multibanded pattern. This method identifies a large number of variants; however, most are not clinically significant. The concentration of α1AT below which samples are tested by PIEF will depend on the particular purpose of the test. In general, all samples with a concentration of less than 50% of normal should be further tested by PIEF. This is likely to detect both adults and children of PI type ZZ. If one wishes to detect every PI MZ heterozygote, then all samples must be PI typed. α1AT, as an acute-phase reactant, can easily be elevated into the normal range in PI MZ individuals. In rheumatoid arthritis, the concentration of α1AT in PI MZ individuals was found to be $148 \pm 46\%$ of normal, with some values threefold the normal mean [59]. Agarose electrophoresis, IEF in agarose, or PIEF are all suitable for confirmation of a deficiency state, with the best resolution obtained with an ultrathin PIEF gel and a narrow pH range (pH 4.2 to 4.9) [35]. Commercially available narrow pH gradients, for example 4.2 to 4.9, are typically used with ultrathin PIEF gels to obtain excellent resolution [60]. Similar resolution is also obtained with immobilized pH gradients [61]. Immunofixation following PIEF is appropriate to identify rare variants of α1AT [35].

Occasionally, variants detected by isoelectric focusing are not due to genetic variation. These include a band cathodal to PI Z, which is sometimes seen in liver disease, absent bands due to bacterial degradation, additional bands in aged samples, which can be removed by reduction with an agent such as cysteine, or binding to other proteins or to thiol compounds. Patients on penicillamine therapy show an additional anodal band, which could be interpreted as one of the anodal variants such as F (see Fig. 2). In addition to this anodal band, a cathodal component has also been seen in such patients [62].

Recombinant DNA Methods

Identification of Variants

DNA methods are used in some laboratories in place of PI typing by isoelectric focusing. The appropriate region of exon 5 in the α1AT gene can be amplified using PCR and the Z mutation tested using labeled oligonucleotide probes [63,64]. Another approach is to use a primer with a nucleotide substitution to create a *Taq*I site in the normal allele but not in the Z allele [65,66]. A similar approach has been developed for detection of the S mutation [66]. A disadvantage of this molecular approach is the potential for misclassification. Using specific tests for PI S and Z, the rare deficiency alleles would all provide a "normal" test result. A deficient individual could be classified as PI type MM, particularly if the concentration in the plasma were not taken into consideration. The isoelectric focusing method of PI typing is therefore preferable because a large number of variants can be identified and a deficient allele will not be misclassified. Furthermore, serum is generally a more simple source for testing than DNA, and the procedure is more economical. Rapid methods for mutation detection may make DNA methods more reliable, although probably more expensive, in the future.

Prenatal Diagnosis by DNA Analysis

The molecular approach is preferred for prenatal diagnosis. PCR requires very small quantities of DNA, so that amniotic cells or chorionic villus samples can be assayed conveniently. As with any prenatal diagnosis involving PCR, caution must be taken to avoid maternal contamination. The family must be studied before prenatal diagnosis to identify whether Z or a rare deficiency allele is present. If the mutant allele is known, appropriate specific identification can then be made.

Liver Inclusions

Intracytoplasmic inclusions were one of the first characteristic features to be described in α1AT deficiency [8]. α1AT inclusions are easily visualized as bright pink globules of various sizes, observed with periodic acid-Schiff (PAS) stain following diastase treatment (PAS-D) [8] (Fig. 3). In infants with α1AT deficiency, inclusions may be fine and granular and difficult to visualize. Large inclusions stain brick red with Masson trichrome stain and dark purple with phosphotungstic acid-hematoxylin (PTAH) stain [67]. Although the presence of typical liver inclusions is not the usual way to establish a diagnosis, such inclusions are sometimes discovered in liver biopsies when α1AT deficiency has not been suspected. For null alleles and some of the other rare deficiency alleles, no inclusions are observed in the liver.

It is important to note that these inclusions are present in individuals who carry only one Z allele (e.g., PI MZ). In individuals heterozygous for α1AT deficiency, numerous and large inclusions may be observed in association with liver disease or other inflammatory conditions. The presence of inclusions in periportal granules in patients with chronic liver disease is almost always due to the presence of Z α1AT or other deficiency types of polymerizing protein such as Mmalton. This is not the case in end-stage liver disease or in nonhepatic cancer, in which tumor necrosis factor can stimulate production of α1AT, leading to inclusion formation [68]. Typical inclusions can be observed in any

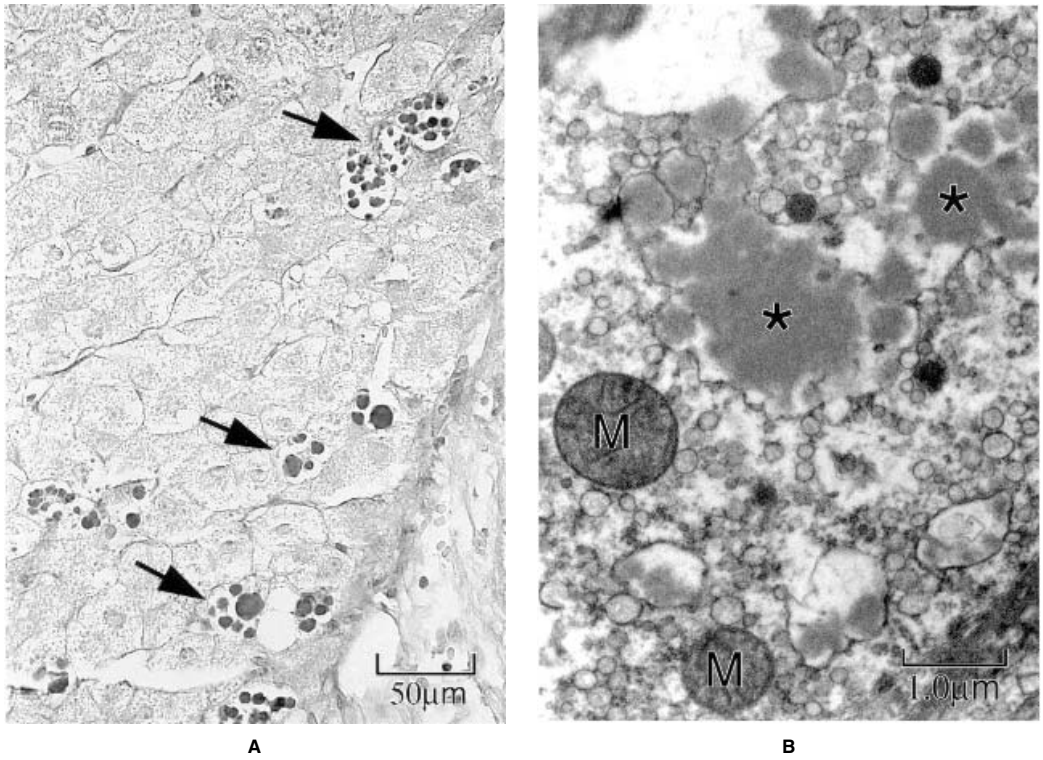

Figure 3. Hepatocyte inclusions seen in α1AT deficiency, PI type ZZ. The mutant Z protein self-aggregates, forming inclusions in the endoplasmic reticulum. **A**. Inclusions (arrows) seen with PAS-diastase stain. **B**. *—inclusion within the dilated cisternae of endoplasmic reticulum, M—mitochondria, as seen by electron microscopy. (Photographs kindly provided by Dr. Laurence D. Jewell, University of Alberta.)

severe systemic disease in which the rate of α1AT synthesis exceeds the capacity of processing enzymes involved in secretion [69,70].

CLINICAL FEATURES AND PROGNOSIS

Obstructive Lung Disease

The major disease risk associated with α1AT deficiency is chronic obstructive pulmonary disease (COPD), specifically emphysema. The incidence of COPD in males is at least twice as high as in females [71], which may be related to smoking exposure. The early observation from Sweden [4] associating α1AT deficiency with early onset of symptoms of COPD has been confirmed in many studies. However, those early studies suggested that 60% of individuals developed COPD before 40 years of age and 90% below 50 years of age. These and subsequent studies have all been plagued by the problem of biased ascertainment because patients are generally ascertained through clinical illness. Although we are now aware that nonsmokers with α1AT deficiency have a much better prognosis, we still do not know the true risks of developing clinically significant disease.

In emphysema associated with α1AT deficiency, the lower portions of the lungs are more severely involved. In patients with well-established emphysema, thoracic x-rays show a symmetric decrease in peripheral vasculature, particularly in the lower lungs [72]. As in emphysema due to other causes, changes in lung mechanics are noted, with reduction in lung volumes and expiratory flow rates. The decreased expiratory flow is due to loss of elastic recoil, which in turn is due to destruction of the elastin fibers. The most sensitive parameters for detecting abnormalities in α1AT-deficient patients with no clinical symptoms are reported to be closing volume, nitrogen washout volume, and lung mechanics, as for other types of emphysema. Evidence suggests that CT scans may be particularly sensitive for detecting changes [73].

The age of onset and severity of disease are highly variable, probably due in part to both genetic and environmental factors. Nonsmokers can maintain normal lung function to at least 30 years of age [50], and the course of the lung destruction is generally long and slow. In a large Swedish study of 246 adult nonsmokers with the deficiency, the age of onset of dyspnea was 53 years in males and 54 years in females, with symptoms rarely occurring before 43 years of age [74].

Effect of Smoking

Smoking is the most important known factor in determining the rate of deterioration of lung function and the ultimate prognosis. In the Swedish study quoted previously [74], the age of onset of dyspnea for male smokers was 40 years and for female smokers 39 years. In the majority, symptoms were rarely observed before 29 years of age. Lung function deteriorates continually as the elastin matrix of alveolar walls is destroyed. In a study of 65 PI ZZ Danish patients (60% of whom were identified because of symptoms), the age at which 50% of FEV_1 (forced expiratory volume in 1 second) predicted for age and height was reached was 17 years greater in those who had never smoked than in smokers [75]. A large proportion of individuals with α1AT deficiency may never come to medical attention because of mildness or absence of symptoms. Data from the α1AT

Registry in the U.S. show a mean decline of 57.5 mL per year in FEV_1 among 208 nonsmokers, 52.0 mL per year in 697 ex-smokers, and 104.8 mL per year in 22 current smokers [76]. Males showed a greater decline than females. Initiation of smoking during adolescence is likely to be particularly detrimental because attainment of maximal lung function is prevented even in normal individuals [77].

Although true survival figures are difficult to obtain, again because of biased ascertainment, the most extensive data are available from the Swedish study, as summarized in Figure 4. At 55 years of age, the survival rates for patients with α1AT deficiency were found to be 83% for nonsmokers and 22% for smokers [74].

A study of 52 patients with α1AT deficiency, about half ascertained through family studies rather than through the presence of COPD, indicates that the risk of developing COPD has been overestimated because of study biases [78]. Of those deficient patients not ascertained through COPD and between 30 and 60 years of age, only one-third, almost all of them smokers, had developed COPD. In addition to the pronounced effect of cigarette smoking on lung function, the possibility was raised that the presence of emphysema in a parent, or the presence of asthma, might also increase the risk of individuals with α1AT deficiency developing COPD.

Risk of Lung Disease in Heterozygotes

Knowledge of the potential risk of developing COPD is important because of the high incidence, about 2–5%, of heterozygotes in most populations. Early studies, which have been reviewed [56], reached varying conclusions. There appear to be no significant differences in lung function between PI MZ and MM nonsmokers [79]. However, PI MZ smokers show a decrease in lung function parameters, which reflects a loss of elastic recoil [79,80]. Over a six-year period, MZ smokers showed a significantly increased mean annual reduction in FEV_1 of 75 mL/year in comparison with 40 mL/year for nonsmoking MZ individuals [81]. This suggests, in comparison with data presented above, that the loss in elastic recoil for an MZ smoker is similar to that of a PI ZZ nonsmoker.

Liver Disease

Childhood Onset

The first report of liver disease in infants and children with α1AT deficiency suggested that a poor prognosis was typical [8]. Based on a follow-up study of 120 PI ZZ children ascertained from the screening of 200,000 Swedish newborns [82], we now have a more accurate concept of the prognosis. Although approximately 17% of PI ZZ children developed clinically recognized liver abnormalities, of which more than half were prolonged jaundice, a follow-up of these infants up to 12 years of age indicated that only 2.4% of the PI ZZ infants developed liver cirrhosis with early death, corresponding to 13.6% of those with clinically obvious liver abnormalities in infancy [82].

In infants, the most common sign of liver abnormality associated with α1AT deficiency is jaundice, with increased conjugated bilirubinemia and raised serum aminotransferase levels in the early days and months of life. α1AT deficiency is a common cause of the "neonatal hepatitis syndrome". In infants presenting with hyperbilirubinemia, histopathological features include intrahepatic cholestasis, varying degrees of hepatocellular injury, and moderate fibrosis with inflammatory cells in portal areas [67]. The reason for the continued liver destruction leading to cirrhosis in a small proportion of children with early hyperbilirubinemia is not known. While the serum level of liver enzymes (alanine aminotransferase and glutamyl transpeptidase) tends to remain somewhat elevated in a large percentage of PI ZZ children, persistent elevation at greater than three times the normal upper limit tends to be associated with a poor prognosis [83,84].

The typical PAS-D positive inclusions may be scarce in young infants and show no correlation with the severity of the liver disease. Confirmation of the deposits as α1AT can be made by immunofluorescence microscopy, which usually shows the presence of stored material in asymptomatic children when very little can be observed by PAS-D staining. Although the presence of these typical inclusion bodies is a diagnostic feature, the diagnosis is made more easily and reliably by PI typing of a serum sample.

The overall risk of an individual of PI type Z developing severe liver disease in childhood, although generally low (about 2%), is higher among sibs of PI ZZ children with liver disease. The frequency of recurrence of severe liver disease in a subsequent sib varies considerably among studies. However, the overall conclusion from all studies indicates a risk dependent on the severity of liver disease in the index patient. When liver abnormalities are mild and resolve, the risk of liver disease in a subsequent PI ZZ sib seems to be about 13%. When liver disease is severe in the index patient, the risk in subsequent PI ZZ sibs may be about 40% [85].

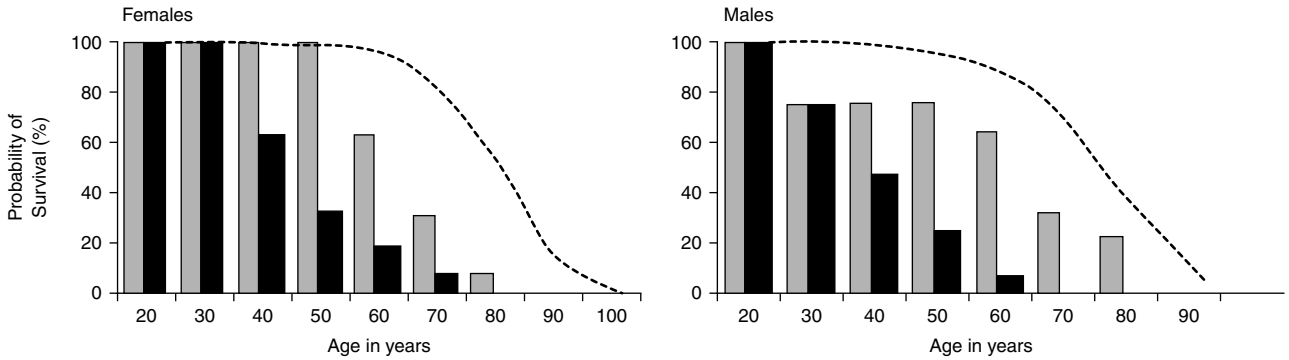

Figure 4. Survival curves for 141 males and 105 females with α1AT deficiency compared with the normal population. Dotted line indicates survival curve (percentage surviving at each age shown in years) for normal males and females in the Swedish population. Stippled bars, nonsmokers; solid bars, smokers, Based on data from [74].

Adult Onset

Soon after the discovery of the association of α1AT deficiency with lung disease, an association with hepatic cirrhosis and fibrosis was documented in Swedish adults [86]. Liver disease in adults does not seem to be associated with a history of neonatal hepatitis. The incidence of liver disease apparently increases with age and is higher in males. Among PI ZZ individuals over 50 years of age, 15–19% develop cirrhosis [74,87]. The risk of liver disease at 20–40 years of age has been reported to be about 2% and at 41–50 years of age about 4% [87]. Destruction of the liver is exceedingly rapid when onset occurs in adults. Risk of the development of hepatocellular carcinoma (hepatoma) has been reported for males [88], but an excess of patients with α1AT deficiency has not been found among hepatoma patients [89].

PI MZ and SZ Heterozygotes

The risk of liver disease in individuals heterozygous for α1AT deficiency seems to be low. There could be a slightly increased risk for PI SZ adults and for PI MZ individuals because two or three times the normal frequency of MZ individuals has been found in patients with liver disease [90]. There is unlikely to be a significantly increased risk of liver disease in PI SZ children beyond that expected by chance. At least some cases reported in the literature have been due to a mistyping of PI ZZ as PI SZ. The strongest support for the lack of association with liver disease comes from the Swedish newborn screening program [83].

Rheumatoid Arthritis

Reduced plasma α1AT probably contributes to the progression rather than the initiation of rheumatoid arthritis. Of 246 Swedish patients with α1AT deficiency, 4.4% had rheumatoid arthritis, and a further 3% had considerable joint pain [74]. While there could be an increased tendency toward rheumatoid arthritis in homozygotes, most studies have focused on heterozygotes.

An increased prevalence of PI MZ among adult patients with rheumatoid arthritis was first observed in 1976, when a prevalence rate of 9% was found in 55 patients studied [91]. When the size of this patient group was increased, 9.3% were Z heterozygotes (PI MZ and SZ) among patients with classical rheumatoid arthritis who had joint erosions radiologically [59]. Among female patients with onset of arthritis before to 42 years of age, 8 of 46 (17.4%) were Z heterozygotes. Possibly the increased prevalence of Z heterozygosity is associated with more severe disease because nine out of ten Z heterozygotes had stage 3 or 4 rheumatoid arthritis [59]. In patients with both rheumatoid arthritis and pulmonary disease, no increased frequency of Z heterozygotes has been observed [92,93]. Such studies could be biased, however; for example, in the latter study, patients were required to be sufficiently ambulatory to be able to volunteer to take part in extensive tests of pulmonary function.

There have now been at least 14 studies carried out on the frequency of PI types associated with rheumatoid arthritis in several countries, including Canada, Sweden, Switzerland, the U.K., and the U.S. An increased prevalence of Z heterozygotes has been observed in some studies [94–96] but not in others [97,98]. Possibly the differences between these studies represent differences in the patient populations. In summary, it seems that no association with Z heterozygotes is found when patients are drawn from a general arthritis clinic population, whereas an increase of three to four times that found in the normal population is found when patients are selected for erosive rheumatoid arthritis. This is demonstrated, for example, in Swedish studies, where the heterozygote frequency was found not to be increased in random patients from a clinic [97], while in a study of 200 patients with erosive rheumatoid arthritis, 6.1% were found to be Z heterozygotes versus 2.1% in controls [95]. We can conclude that Z heterozygosity does not necessarily predispose to initiation of rheumatoid arthritis. However, although the risk to Z heterozygotes is relatively low, at least some of these individuals may undergo particularly extensive joint destruction.

In one study, an increased frequency of the rare F variant α1AT in FM heterozygotes was reported [98]: a prevalence of 2.1% compared with about 0.4% in the control population. This association has not been noted in most other studies, except that by Beckman et al. [95]. The second cysteine residue, resulting from the mutation in the F variant [99], could in some way impair inhibitor activity in the inflamed joint tissue. These cases may have been mistaken as having the unusual IEF pattern observed in patients treated on penicillamine [62], which is frequently administered to patients with rheumatoid arthritis.

In Canadian children with juvenile rheumatoid arthritis, a numerical but nonsignificant increase in Z heterozygotes was found [59]. More severely affected British children showed a significant increase of PI type MZ to 10.4% versus 1.6% in a control population [100].

There is reason to assume that a reduced amount of α1AT might be more conducive to severe degradation of the joint. α1AT is a potent inhibitor of elastase and other proteolytic enzymes released by neutrophils, which would occur particularly at the site of inflamed joints. Therefore, although not initiating the rheumatoid process, a reduced concentration of α1AT, as in MZ heterozygotes, could contribute to increased destruction of the proteoglycans, collagen, and other connective tissue components in the joints. This hypothesis is discussed further in the section on disease mechanisms.

Vascular Disease

Data from several studies suggest that α1AT may play an important role in the protection of blood vessels. Various disorders affecting arterial integrity have been described [101]. In a study of 47 patients with infrarenal abdominal aortic aneurysms, five (11%) were of PI type MZ phenotype, where about 2% would have been expected [102]. A male patient with emphysema and deficiency (PI ZZ) was reported to have a ruptured colic artery aneurysm and multiple other small aneurysms of a middle colic artery [103]. An increase in intracranial aneurysms has also been suggested. In an examination of the medical records of 362 patients with α1AT deficiency, including both PI MZ and homozygous (PI ZZ) deficiency, a ruptured intracranial aneurysm was found in one PI ZZ patient and two Z heterozygotes [104]. An additional PI MZ heterozygote had spontaneous dissection of the cervical internal carotid artery. In a study of a larger series of patients with intracranial aneurysms, 6,696 patients had a post-mortem examination, and six were recorded as having α1AT deficiency (four PI ZZ, one PI MZ, and one not PI typed), although it appears that PI typing was carried out on only a small proportion of the patients [105]. Interestingly, arterial fibromuscular dysplasia was found in two of the six patients, both with the PI ZZ phenotype and hepatic or pulmonary failure. Fibromuscular dysplasia is reported in about 12% of patients with spontaneous cervical artery dissection and

up to 20% of those with ruptured intracranial aneurysms. Two of three women with bilateral internal carotid artery stenosis due to fibromuscular dysplasia were found to be of PI type MZ [106]. Fibromuscular dysplasia involving a cerebral artery was found in a PI ZZ child with decreased antioxidant status [107], and this observation may indicate an underlying tendency to arteriopathy later in life in PI ZZ individuals. The stiffness of the abdominal aorta in 19 men with α1AT deficiency was reduced in comparison with normal controls; however, no difference was found for 17 women in the study [108]. These observations provide additional evidence for an effect on the connective tissue of blood vessels.

Both plasma α1AT and plasma α_1-antichymotrypsin (a cathepsin G inhibitor) are bound to the surface of endothelium [109]. Neutrophils release proteinases, such as elastase and cathepsin G, to the endothelium at the time of an occlusion. Therefore, it might be predicted that if the concentrations of inhibitors were inadequate, tissue damage could result. The arterial weakening and fibromuscular dysplasia may represent a response to damage of the vascular endothelial matrix due to inadequate inhibition of proteinases. A history of smoking may be especially important because of the impaired function of α1AT that occurs as a consequence of oxidation of the critical methionine residue. More studies are needed to document the extent of vascular defects and also the potential preventative role of antioxidants.

Kidney Disease

Membranoproliferative glomerulonephritis (MPGN) has been identified in PI ZZ children who also have liver disease [58,110]. The kidney abnormality may be a consequence of the liver disease. Nephropathy was observed in 79% of 19 patients of PI type ZZ who had chronic liver disease [111]. However, among 246 Swedish PI ZZ patients, only 15% showed signs of renal glomerular damage, manifested by proteinurea or hematuria [74]. Some of these may have been mild and not have advanced to MPGN. In two-thirds of those affected, there was no coexisting liver cirrhosis. The abnormal Z α1AT probably plays a role in the formation of the immune complex in the kidney. Concentration of Z α1AT at the glomerular membrane could lead to its polymerization, with subsequent formation of antibody to the foreign aggregate. A role for Z α1AT is supported by the report of one patient with liver disease whose MPGN was cured following liver transplantation [112]. A PI ZZ adult with IgA glomerulonephritis and hypertension as well as cirrhosis was reported to have sudden deterioration of renal function [113]. Immune complex deposition may play some, but not necessarily the only, role in producing symptoms of kidney damage. α1AT deficiency is apparently not a frequent cause of MPGN. In one study of 53 patients with idiopathic MPGN, none were homozygous or heterozygous for PI Z [110].

Other Diseases with an Inflammatory or Immune Component

There are reports of a number of other inflammatory conditions associated with α1AT deficiency. Panniculitis is characterized by inflammation of the panniculus adiposus, the fat layer under the dermis. Although the risk of patients with α1AT deficiency developing panniculitis must be relatively small, there may be other predisposing factors in certain individuals to cause the disease. A number of cases have now been reported of severe ulcerative panniculitis associated with α1AT deficiency. Two of five patients with panniculitis were found to have α1AT deficiency [114]. In a survey of 96 patients with panniculitis, 15.6% were found to be α1AT-deficient, as defined by serum concentration [115]. Recognition of such patients is important for successful treatment. Administration of purified α1AT has been successful for the treatment of severe cases, and deoxycycline, a tetracycline that inhibits collagenase activity, has also been effective [116,117]. Histoplasmosis has been suggested as one of the triggering agents that might lead to panniculitis in individuals of PI type ZZ [118]. Further studies on this association are required.

Anterior uveitis, an immunologically mediated inflammatory eye disease, has been associated with an increased frequency of Z heterozygotes [119,120]. Two of 16 patients with posterior uveitis were of PI type MZ [121]. Corneal ulceration was observed in a PI ZZ child, who had markedly reduced α1AT in tears [122]. Testing of individuals with severe corneal ulceration might be productive and lead to improved therapy because α1AT is normally expressed in the cornea [122].

Other inflammatory conditions reported in association with α1AT deficiency are systemic necrotizing vasculitis [123] and persistent cutaneous vasculitis [124]. A fatal case of Wegener granulomatosis has been reported [125]. Panarteritis in association with emphysema and glomerulonephritis [126] and Hashimoto thyroiditis [127] has been reported in PI ZZ individuals.

PATHOLOGY AND DISEASE MECHANISMS

The Basic Defect

The Z deficiency allele is associated with typical inclusions in the liver and PAS-D globules within the endoplasmic reticulum, which show positive fluorescence with α1AT antibodies. These inclusions result from a defect in secretion of the abnormal Z α1AT. The protein within the liver inclusions has an abnormal carbohydrate composition, with a mannose-rich core, typical of incompletely processed glycoproteins. However, this incomplete processing is a secondary effect because impaired secretion also occurs when Z mRNA is injected into *Xenopus* oocytes [128]. The portion of Z α1AT that is secreted may have decreased specific elastase inhibitor capacity [129]. Z α1AT has a normal rate of synthesis [130] and a half-life of 5.2 days, identical to that of M α1AT [131].

The mutation in the Z protein is Glu342Lys [132,133]. Examination of the three-dimensional crystallographic structure indicates that this residue is involved in a salt bridge that is important for stabilization of the molecule and occurs at a sharp bend in the major β sheet [10]. A series of site-directed mutagenesis experiments, in which the salt bridge was altered and replaced, suggest that the salt bridge defect has only a modest effect on the stability of Z α1AT, and the lack of secretion seems to be due to a change to a positive charge at amino acid residue 342, resulting in an alteration in tertiary structure [17]. The Glu342 residue of M α1AT is located at a hinge region of the reactive center loop. This loop is mobile and can adopt varying configurations. With mild denaturation, the loop locks into a structural sheet (A sheet) within the protein, forming a thermostable inactive protein [134]. Under the same conditions, when the temperature is elevated to 37 °C, the reactive center loop inserts into the A sheet of a second molecule, a phenomenon that has been called loop-sheet polymerization [135]. Substitution of the hinge residue 342 in Z α1AT makes the molecule a receptor for a second Z molecule and results in dimerization and finally

aggregation of the protein [136]. The same type of self-aggregation, or polymerization, occurs spontaneously with the deficiency variant PI Mmalton, which has a deletion of residue 52, phenylalanine [137]. Like the Z type α1AT, Mmalton forms hepatic inclusions. Because polymerization is increased at higher temperatures, elevated body temperatures associated with inflammation could be predicted to enhance the formation of inclusions.

In addition to polymerization, inclusion formation by the abnormal protein could involve the abnormal binding or the lack of binding of proteins required for secretion. There is evidence that heat shock proteins, produced in cells under stress, could be involved (reviewed in [138]). Heat shock proteins have been shown both to bind to secretory proteins until the assembly of the proteins is complete [139] and to be induced by the presence of abnormal proteins [140]. Further, the synthesis of stress proteins has been shown to be increased in PI ZZ individuals with liver disease [141]. However, it is not clear whether such proteins induce damage or are innocuous bystanders involved only in degradation of the abnormal protein. There is variability among PI ZZ individuals in their tendency to develop liver disease. Some individuals may have an inherently different metabolism, increasing their susceptibility to liver damage. Studies have been carried out on cell fibroblasts from patients with and without liver disease and transfected with a mutant *PI Z* gene. In cell lines from patients with liver disease, protein degradation lags, suggesting an inadequate mechanism for degrading abnormally folded protein [17]. The liver injury probably results from a combination of a slow degradation pathway, inadequate antioxidant protection from the effects of absorbed uninhibited proteinases, subsequent damage to the cell membrane due to oxidant free radicals, further destruction of weakened cells by the presence of inclusions, and possibly autoimmune effects.

Pathogenesis of Lung Disease

Elastin, the major component of the tissue matrix of the lung, can be destroyed by neutrophil elastase. A reasonable explanation for the destruction of elastin tissue in α1AT deficiency has been supported by several types of data. The alveolar destruction leading to emphysema can be explained by an imbalance between proteolytic enzymes, particularly elastase, and their inhibitors. α1AT has been demonstrated to be the major inhibitor of elastase at the epithelial surface by studies of a homozygous null individual who had less than 15% of normal inhibitor capacity against neutrophil elastase in lavage fluid [142]. This suggests that other elastase inhibitors in lung tissue, such as low-molecular-weight proteinase inhibitor or α_2-macroglobulin, make only a minor contribution. α1AT enters lung tissue readily because of its small molecular size and can be recovered in lavage fluid. The plasma deficiency found in patients of PI type ZZ is also reflected in a low concentration in the lung alveoli, as reflected in bronchiolar lavage fluid [143]. A number of experimental studies have been carried out in animals that demonstrate that experimental emphysema can be produced by administration of porcine pancreatic elastase [144] and human neutrophil elastase [145,146]. The extensive studies that support this hypothesis and the role of neutrophils have been reviewed [147,148].

Effect of Smoking on Enhancement of Lung Destruction

The destructive effects of smoking on lung tissue are even more enhanced in individuals with α1AT deficiency. One of the major mechanisms of increased destruction seems to be the effect of oxidation. Methionine residue 358 of α1AT, when oxidized, is unable to complex with elastase. A direct effect of smoke can occur through the oxidizing agents found in the gas phase of tobacco smoke (reviewed in [147]). No consistently reproducible effect has been shown in studies examining the elastase inhibitor capacity in the serum of smokers and nonsmokers, which is not surprising in view of the generous supply of antioxidants in plasma. However, inactivation of α1AT has been noted in lavage fluid after smoking, and oxidized α1AT with a reduced inhibitor activity has been recovered from the lungs of smokers [149]. In addition to oxidation of α1AT, smoke triggers the release of elastase and also causes the release from neutrophils of myeloperoxidase, which inactivates α1AT [150]. Any excess elastase produces complexes with α1AT and cleaves the α1AT to an inactive form. A local imbalance between elastase and α1AT inhibitor will therefore lead to enhanced tissue destruction.

Pathogenesis of Liver Disease

One hypothesis for the liver disease associated with α1AT deficiency is that the α1AT accumulated in the endoplasmic reticulum of liver cells directly causes liver injury. Mouse models have provided equivocal results. In one study, transgenic hepatitis-free mouse lines containing multiple copies of human M or Z α1AT genes per mouse were established [151]. There were no differences between M and Z mice at five copies of gene per mouse genome. In a Z line with 12 copies, abundant material accumulated in the liver, resulting in areas of liver necrosis. However, this excessive overexpression of the Z allele does not represent the human situation in early neonatal life. In another study in which one, ten, and more than ten copies of the Z gene were present, inflammation and necrosis occurred to the same degree in both M and Z mice, possibly relating to the presence of hepatitis virus in the colony [152]. There was an increase in inflammation and necrosis in mice with ten or more copies of the Z gene, although the difference was not significant. In a more recent study in which large numbers of transgenic M and Z mice were produced, accumulation of Z protein was achieved in the liver but no evidence of cirrhosis or fibrosis was seen in any animal. This study suggested that the mouse model was most useful for investigating the liver manifestations occurring in ZZ adults but that other factors must be involved in neonatal liver disease [153].

Further arguing against this hypothesis is the observation that infants with α1AT deficiency and liver symptoms have very little accumulated α1AT in their hepatocytes. On the other hand, adults with α1AT deficiency frequently have numerous and large inclusions and yet may be free of liver disease. The intracellular accumulation of α1AT has been shown to increase hepatic lysosomal enzyme activity [154]. This could cause the elevation of liver enzymes frequently observed in apparently clinically well children and adults with the deficiency. The extensive accumulation, equivalent to that seen in the transgenic mice, could play some role in adult liver disease. However, given the very slight amount of α1AT stored in the livers of newborns and infants, the accumulation may not be the only cause of early liver destruction.

Another hypothesis for the cause of liver injury is a lack of circulating α1AT, particularly as a cause of liver disease in young infants. The typical PAS-D positive inclusions

are often difficult to identify in percutaneous liver biopsy specimens from infants [67]. The amount and size of liver inclusions show no clear correlation with the severity of liver disease; severe liver disease in the early months of life can be associated with only very sparse liver inclusions. An apparent lack of liver disease in null homozygotes cannot be used as evidence against a lack of α1AT as the causative factor for liver disease. Null homozygotes are present at only 1/1,000 the frequency of PI ZZ individuals. Therefore, if only 2% of PI ZZ individuals were affected with severe and fatal liver disease in childhood, the dozen or so null homozygotes described to date would not, by chance, be expected to have liver disease in infancy. α1AT is expressed in human jejunal epithelium [155]. Secretion from this source could be important in combating exogenous proteinases in the gut, as proposed below in discussion of the role of breast-feeding.

The choice between these two hypotheses is important for therapy. If the liver inclusions cause damage, then infusion of additional α1AT would not be a rational therapy. However, if the damage occurs because of lack of α1AT, there is potential for the use of α1AT as therapy for liver disease, at least during the early sensitive weeks. At the present time, such therapy is not warranted, pending the availability of further information on the basic disease mechanism.

Pathogenesis of Disorders with an Autoimmune or Inflammatory Component

In rheumatoid arthritis, the Z heterozygote may have inadequate α1AT present in the joint fluid to prevent leukocyte elastase, cathepsin G, and collagenase from attacking the structural proteins of joint cartilage [95]. In addition to its role as an antiproteinase, α1AT may also reduce inflammation through the inhibition of hydrogen peroxide oxidation [156], and the deficiency state could therefore ineffectively control inflammation. However, it is not clear whether this effect occurs *in vivo*, where α1AT may be inactivated by exposure to hydroxyl radicals generated from hydrogen peroxide [157].

Several of the abnormalities in inflammatory response also have an autoimmune component. It is not clear whether a deficiency of α1AT has an effect on the immune response or whether disease results entirely from an inability to handle inflammation adequately. An inappropriate immunological response could be triggered by a lack of proteinase inhibitor to exert its effect on cell-bound proteinases. Proteinases such as trypsin, neutrophil elastase, and cathepsin G can substitute for helper T-cells in B-cell mitogen assays [158]. Increased T-helper cell activity and B-cell inactivation could trigger exaggerated cell-mediated immunity. Alternatively, the wide variety of inflammatory abnormalities may result entirely from inadequate handling of the proteinases released during the inflammatory response in various tissues. Individuals who develop such disorders may have some additional predisposing factor. However, recognition of PI deficiency in individuals with any of these severe inflammatory disorders is important because of the potential for treatment with purified α1AT.

Another possible reason for these disease associations could be the effect of impairment in function of adjacent genes in the serine proteinase inhibitor cluster. Particularly noteworthy is the close association with corticosteroid-binding globulin, which is involved in control of the immune response.

THERAPY

Preventative Therapy

Avoidance of Smoking

An effective way to prevent the lung destruction of α1AT deficiency is to avoid smoking and exposure to other agents from the environment that can damage the alveolar connective tissue. Given the high incidence of α1AT deficiency in the population, a large percentage of affected individuals may never come to medical attention. For some individuals, the lung destruction will be sufficiently slow that a relatively normal life span can be achieved. This complicates the assessment of any therapy because it will not necessarily be possible to determine whether a particular rate of decline is altered by that therapy. The dramatic effect of smoking in accelerating the rate of lung destruction has been described previously in this chapter.

If the establishment of smoking behavior is to be avoided, patients must be recognized before the age when smoking usually begins. Population (newborn) screening has been shown to be feasible using dried blood samples on filter paper [159]. The wisdom of population newborn screening is a topic of lively debate [160]. The goal of such a screening program would be to counsel parents to encourage their affected children to avoid smoking completely. For the best effect, parents of children with α1AT deficiency should also be advised to avoid smoking. In Sweden, however, studies showed that about one-third of parents will continue their smoking [83]. Negative attitudes in parents regarding their child's illness were shown to last for at least five to seven years [161], stressing the necessity for extensive education to accompany the screening. Screening during the teen years is an alternative, although usually less practical. Although parents were unlikely to be persuaded to give up smoking, the Swedish screening program did have an impact on smoking by affected children [162]. Of 50 children followed, 18–20 years after their initial diagnosis in the newborn screening program, only 12% were previous or current smokers compared with 36% of 48 control children. Five of the six affected smoking children had one or both parents who smoked.

Antioxidant Therapy

Because excessive free radicals from any source can lead to tissue damage, antioxidants might be expected to modify or even prevent tissue damage. There is evidence that an excess of pro-oxidants can damage both liver and lung [163–165]. Tissue exposure to oxidants will be dependent on genetic variation in metabolic pathways as well as antioxidant intake. Optimal antioxidant activity should be maintained in lung and liver tissue to help prevent tissue damage. Vitamin E status might be important in determining individual susceptibility to liver or lung destruction. Vitamin E, administered in doses adequate to cover daily requirements, may help to protect tissues and should be considered as a possibility for preventative therapy. Because this is an inexpensive approach to therapy, data need to be collected and evaluated in appropriate trials.

Other Therapies

Because the cause of liver disease in a small percentage of PI ZZ individuals is unknown, preventative measures are uncertain. Given the hepatotoxicity of alcohol, avoidance or limitation of intake would seem to be prudent for α1AT-deficient individuals. For liver disease of infancy, breast-feeding during the early months of life may offer

some protection from liver disease. A significant difference was found between breast-fed and nonbreast-fed infants in developing clinical signs of liver disease in the early months of life, although breast-feeding did not necessarily prevent the development of cirrhosis [166,167]. The rationale for the effectiveness of breast-feeding is that proteinase inhibitors in human milk help limit proteinase uptake and reduce their transport to the liver via the portal circulation [168]. Human milk has high concentrations of α1AT, particularly in the first week of lactation, and a breast-fed infant can have an intake in the range of 100–400 mg/24 hours [169]. Although there are inadequate data to prove the effectiveness of breast-feeding, this is a subject requiring further study.

Replacement of α_1-Antitrypsin

Replacement by Infusion

The destruction of lung tissue in patients of PI ZZ results from the uninhibited activity of proteases in the lung. Effective therapy must prevent this tissue destruction, because the adult human lung has a limited capacity for self-repair. The goal of augmentation therapy is to increase the α1AT plasma concentration sufficiently to delay the accelerated rate of lung deterioration found in α1AT-deficient patients. Because PI MZ and even SZ heterozygotes, with 60% and 35%, respectively, of the normal amount of α1AT are unlikely to be at increased risk of lung disease, maintenance of a partial level (about 35% of normal) through augmentation therapy should prevent excessive lung destruction. The protective plasma level is calculated to be about 0.52 g/L according to the internationally established standards [170,171].

Augmentation of serum levels by the infusion of purified human α1AT is the only currently approved therapy for α1AT deficiency. In initial studies, administration of 60 mg/kg weekly was shown to increase α1AT and antineutrophil elastase levels in lung and serum to appropriate protective levels [172]. Clear evidence for significant benefit from this therapy has not yet been established. A randomized clinical trial, predicted to require 500 patients and controls for a three-year period to show a 40% decrease in the decline of FEV_1, was not considered to be feasible [173]. A more sensitive way to measure decline in lung function, possibly by CT scan [73], could make random trials more feasible. As an alternative, patient registries have been established in the U.S. and Europe to monitor α1AT-deficient patients receiving augmentation therapy by infusion. In a study of 927 patients from the U.S. registry, among whom 10% of those treated were current smokers and 389 received continuous therapy, survival over a period of at least six months was assessed. Significant improvement was shown only for patients with an FEV_1 of 35–49% and not for those with lower or higher FEV_1 values. However, it is noteworthy that the change in relative risk was slight in comparison with the effects of education, which presumably reflects socioeconomic status. Initial FEV_1 was a high predictor of survival. Those on augmentation therapy showed a decreased relative risk for mortality overall. A significant difference in the decline of FEV_1 was shown only in those with an initial FEV_1 of 35–79% predicted. However, these results cannot easily be interpreted because there is a strong possibility that differences were due to other factors such as socioeconomic status. Another comparison is available from Europe, although the controls and patients are from different populations. A German group of patients received therapy, and a Danish group who received no therapy were used as controls. Again, those in the mid-range of initial FEV_1 showed a slowed rate of decline in lung function (83–93 mL/year with no treatment, 64–66 mL/year with treatment). No effects of therapy were observed in those with higher or lower FEV_1 values. There is a suggestion that augmentation therapy may be useful in some individuals; however, there are many questions still unanswered. Of particular importance is the possible effect of socioeconomic status, perhaps reflecting improved nutrition. All figures in these studies had large standard deviations. Because of the high degree of variability in decline of lung function, baseline data on the rate of decline of lung function in any particular patient will be critical in deciding when and if therapy will be warranted.

No data are available to indicate whether the protective level must be maintained at all times. Patients with α1AT deficiency have been shown to have free elastase activity, as measured by lung lavage, only during periods of respiratory infection [174]. Augmentation therefore may be effective when administered only during times of infection [175]. The patients in these studies have no documentation of their nutritional and antioxidant status, which may well be important factors in maintaining a slow decline in lung function.

Because an unknown proportion of nonsmoking individuals of PI type ZZ maintain relatively normal lung function for many decades, there will be some individuals who have a relatively normal life span even without therapy. For this reason, it would seem appropriate to monitor the rate of decline of lung function, if possible over a one- or two-year period, to assess the rate of progression of lung destruction. This may help in rationally deciding who will benefit most from the therapy. Replacement therapy is costly, so consideration needs to be given to the initial slow rate of decline in some patients who may not require continuous therapy for effective results. Assessment of therapy only in times of respiratory stress also needs evaluation.

Replacement by Aerosol

More direct targeting of α1AT specifically to the lungs, the only major organ to be significantly damaged by elastase degradation, could avoid the large requirement for purified α1AT. The challenge for effective aerosol therapy is to deliver appropriate-sized droplets into the lung alveoli. Aerosolized α1AT has been shown in sheep to pass through the lower respiratory tract epithelium, gaining access to the alveolar walls, and in individuals with PI type ZZ to be absorbed into the serum [176]. Administration of 100 mg twice daily appears to be safe and effective in raising the lung epithelial lining fluid levels of α1AT to protective levels [176]. The half-life of α1AT in the lungs after a single dose of 200 mg is 69.2 hours and that of its antineutrophil elastase activity is 53.2 hours [177]. This approach requires only about 10% of the purified α1AT used for intravenous administration and could provide an economically feasible approach, in combination with antioxidant administration, for the treatment of α1AT deficiency.

Use Of Recombinant α_1-Antitrypsin

Normal human α1AT, like many proteins, can be produced in high yields in bacteria and yeast. An interesting modification to α1AT replaces methionine 358 at the active site with valine to produce a molecule that is an active inhibitor but one which resists oxidation, providing a potential advantage in replacement therapy [178]. A major problem in the use of synthetic forms of α1AT

is their extremely short half-life due to the absence of carbohydrate side chains, which provide stability for the normal protein [178].

Another biotechnological approach to the production of α1AT is the use of transgenic animals. α1AT is expressed at a high level in the milk of transgenic mice, and the use of transgenic sheep or cattle could help supply large amounts of α1AT for therapeutic purposes [179].

α1AT deficiency provides a useful model for the testing of gene therapy approaches; however, it seems unlikely that this will be the preferred approach for treatment of this condition. Three potential vector systems, plasmid, retrovirus, and replication-deficient adenovirus, have been evaluated. The gene therapy approach is to remove cells from a patient and to reintroduce them after genetic modification. This approach is difficult where the product must be targeted to the lungs. Plasmids, relatively inefficient in gene transfer, are sometimes linked to a ligand to target to a particular cell type. Retroviral vectors are of concern because of the possibility of incorporation into a gene involved in tumorogenesis, leading to malignancy. Most of the retroviral DNA does not get targeted into chromosomes. Adenoviral vectors bypass the problem of slow replication of endothelial cells because the virus has avidity for epithelia of the respiratory tract. Transfer of genes into the liver has been tested as another approach. Genes transferred into hepatocytes could create a source of circulating α1AT to prevent both liver and lung damage if levels remained sufficiently high. Hepatocytes from an α1AT-deficient individual would be transfected with the α1AT gene and then returned into the peripheral circulation. Using this approach, expression of α1AT has been maintained in rats for at least four months when accompanied by partial hepatectomy [180,181]. This approach generally results in low levels of expression, which may not be adequate to protect from the development of emphysema. It is not known whether it would help avoid liver disease, particularly if such disease is due to the storage of inclusions. The simpler approach of α1AT infusion or delivery by aerosol and/or antioxidant therapy may be more feasible and cost-effective than gene therapy approaches.

Other Therapeutic Approaches

Another approach to therapy is to alter the intracellular fate of Z α1AT in the liver either by preventing degradation or increasing secretion. At the cellular level, both the protein translation inhibitor cycloheximide and the specific inhibitor of proteasome function, lactacystin, are able to prevent intracellular degradation of Z α1AT, partially restoring Z intracellular transport [182]. This observation has been made only in an experimental cell system. The use of chemical chaperones to increase secretion of mutant Z α1AT is another interesting possible route for future therapy. Chemical chaperones that reverse cellular mislocalization or misfolding have been shown in a cell-culture system and in mice to increase the secretion of α1AT, in the mouse studies producing 20–50% of the levels present in PI M mice [183].

Transplantation

There is no specific treatment for the severe liver disease that occurs in a small proportion of individuals of PI type ZZ. Fortunately, only about 3% of such individuals will experience progressive liver disease during childhood; however, a higher proportion of affected adults over the age of 50 develop liver disease. Supportive management, as for other types of liver disease, involves optimal nutrition, the prevention of fat-soluble vitamin deficiency, and diuretic treatment for ascites as required. Liver replacement provides the only definite treatment for children with end-stage liver disease associated with α1AT deficiency, a situation that will occur in only about 3% of all α1AT-deficient individuals. All of 21 PI ZZ children who received liver transplants were alive at a median follow-up of 40 months [184].

Lung transplantation has now become a feasible treatment for selected patients with end-stage lung disease. Double lung transplantation is not required as was initially believed. The survival rate may be somewhat better for individuals with single lung rather than bilateral lung transplantation [185]. Survival rates following single lung transplantation after one year have been reported as 77–90% [185,186]. Morbidity, particularly due to immunosuppression, results in a 50% five-year survival. Transplantation is a possible option when the FEV_1 falls below 30% [187]. However, a careful analysis of risk and benefit will be required. Ideally, with good nutrition, maintenance of adequate tissue antioxidants, and possibly therapy during respiratory infections, extreme measures such as transplantation can be avoided. Nevertheless, the most effective treatment of all, particularly for the lungs, remains early detection and the avoidance of smoking.

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Chapter 18

Heritable Forms of Rickets and Osteomalacia

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SUMMARY

- **Rickets and osteomalacia are generic terms for the clinical consequences of impaired mineralization of skeletal matrix in the pediatric and adult populations, respectively. Deficiency of vitamin D, due to inadequate cutaneous biosynthesis or depletion secondary to hepatobiliary or gastrointestinal disease, causes malabsorption of dietary calcium (Ca^{++}) and explains most cases.**
- **Heritable rickets/osteomalacia usually reflects defective bioactivation of vitamin D, peripheral resistance to the hormone 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), or, more commonly, hypophosphatemia due to renal phosphate (Pi) wasting. Subsequently, low extracellular levels of Ca^{++} and/or Pi compromise hydroxyapatite crystal deposition into osteoid.**
- **X-linked hypophosphatemia (XLH), the most common heritable rickets/osteomalacia, is a sex-linked dominant disorder. Short stature and bowing of the lower limbs without muscle weakness are the principal pediatric complications. Adults are troubled by osteoarthritis, pseudofractures, enthesopathy, and dental abscesses. XLH is caused by mutations in *PHEX* (phosphate regulating gene with homologies to endopeptidases on the X chromosome). An uncharacterized phosphaturic factor, "phosphatonin," seems to escape *PHEX* inactivation causing the selective renal Pi wasting. Treatment with $1,25(OH)_2D_3$ and Pi supplementation is helpful, but nephrocalcinosis and hyperparathyroidism are potential complications of treatment.**
- **Other heritable causes of "phosphate diabetes," leading to "hypophosphatemic bone disease," include autosomal dominant hypophosphatemic rickets, hereditary hypophosphatemic rickets with hypercalciuria, McCune-Albright syndrome, primary or secondary Fanconi syndrome, and X-linked hypercalciuric nephrolithiasis. This last condition is caused by deactivating mutations in the *CLCN5* gene that encodes a channel protein, ClC-5, expressed predominantly in the kidney. Depending on the specific disorder, Pi, thiazides (when there is hypercalciuria), or $1,25(OH)_2D_3$ treatment may be helpful.**
- **Vitamin D-dependent rickets, types I and II, are caused by impaired bioactivation of vitamin D due to deactivating mutations in the gene that encodes a component of the vitamin D, 1α-hydroxylase in the kidney and $1,25(OH)_2D$ resistance resulting from defects in the gene encoding the hormone's receptor (VDR), respectively. Consequently, clinical and biochemical disturbances resemble vitamin D deficiency. Treatment consists of physiologic or large doses of $1,25(OH)_2D_3$ (often with Ca^{++} supplementation), respectively.**
- **Hypophosphatasia features low serum alkaline phosphatase (ALP) activity. Premature loss of deciduous teeth is a clinical hallmark. The extreme range of disease severity reflects more than 100 different deactivating mutations in the gene that encodes the tissue nonspecific isoenzyme of ALP (TNSALP). Three phosphocompounds accumulate endogenously and therefore seem to be natural substrates for TNSALP: phosphoethanolamine (PEA), inorganic pyrophosphate (PPi), and pyridoxal 5′-phosphate (PLP). PPi inhibits hydroxyapatite crystal growth and may be a key pathogenic factor. Severe cases reflect autosomal recessive inheritance, whereas dominant-negative mutations cause relatively mild disease in successive generations. There is no established medical treatment.**

INTRODUCTION

Rickets is a generic term for the clinical consequences of impaired mineralization of matrix throughout a growing skeleton. Infants, children, and adolescents can be affected. Osteomalacia refers to the problems engendered by the same disturbance in adults (i.e., after growth plates fuse). Neither designation denotes a specific disease [1].

Often, the cause of rickets or osteomalacia is an acquired condition that diminishes endogenous stores of vitamin D. Rarely, vitamin D bioactivation or target tissue effects are impaired. Consequently, there is the cascade of gastrointestinal malabsorption of calcium, hypocalcemia, secondary hyperparathyroidism, hyperphosphaturia, and hypophosphatemia. Occasionally, however, the pathogenetic defect is kidney tubule dysfunction, leading to urinary wasting of phosphate and hypophosphatemia, sometimes associated

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with reduced bioactivation of vitamin D. Sometimes, abnormalities of chondrocytes and osteoblasts, faulty bone matrix, or other perturbations block ionized calcium [Ca^{++}] and inorganic phosphate [Pi] entry as hydroxyapatite crystals into the skeleton [1].

The number of disorders that result in rickets and osteomalacia is considerable; most are sporadic diseases but some are heritable and comprise the subject of this chapter (Table 1) [1,2].

In rickets, all three processes of skeletal formation described below are adversely affected, whereas osteomalacia disturbs only bone remodeling [3].

Growth. For long bones to lengthen, chondrocytes in columns within growth plates (physes) proliferate, hypertrophy, and then degenerate, preparing the matrix they secrete to mineralize (endochondral bone formation).

Modeling. Correct shaping of growing bones requires simultaneous deposition and removal of osseous tissue at the outer and inner surfaces of cortical bone (subperiosteum and endosteum, respectively).

Remodeling. Trabecular (spongy) and cortical (compact) bone are resorbed and then reformed in countless microscopic areas periodically throughout life to fulfill the metabolic, structural, and repair requirements of the skeleton.

Rickets features short stature (physeal disturbances) and skeletal distortions (modeling defects) [3]. The abnormal bone remodeling (''turnover'') can appear radiographically as generalized osteopenia or, occasionally, as osteosclerosis (coarse trabecular bone) and contributes importantly to associated aberrations in mineral homeostasis. Osteomalacia will usually not deform the skeleton, unless there are fractures, because in adulthood growth plates are closed and bone modeling has essentially ceased.

Vitamin D and Mineral Metabolism

Vitamin D

Much is now known about the control of mineral homeostasis [4], including the biosynthesis, bioactivation, and physiological actions of vitamin D [5,6]. Despite the availability of supplements or the fortification of foods with vitamin D in several countries (e.g., 400 IU per quart — 0.946 liters — of milk or infant formula in the United States), antirachitic activity in most healthy people is derived primarily from cutaneous synthesis in which 7–dehydrocholesterol is converted by ultraviolet (UV) light of 290–310 nm wavelength to cholecalciferol (vitamin D_3) [7]. Ergocalciferol (vitamin D_2) is the product of UV irradiation of ergosterol extracted from plants or animals and is used as an additive or as a pharmaceutical [5,6].

Both vitamins D_2 and D_3 are prohormones that can be stored in muscle and fat or transported by vitamin D–binding protein to the liver and subsequently to the kidney for bioactivation [5,6]. First, with little regulation, vitamin D is hydroxylated in hepatocyte mitochondria by the enzyme $P450_{c25}$ to form the 25-hydroxyvitamin D derivative, 25(OH)D, called calcidiol. Then, with precise control, somehow conditioned by Ca^{++}, Pi, and parathyroid hormone (PTH) levels, 25(OH)D is further hydroxylated

TABLE 1. Heritable Causes of Rickets and Osteomalacia

	MIM No.
I. Disorders of Vitamin D Bioactivation	
A. Vitamin D dependency, type I (1α-hydroxylase deficiency)	264700
B. Vitamin D dependency, type II (hereditary Vitamin D–resistant rickets)	277420
II. Renal Tubular Acidosis, Distal (Classic, Type I)	
A. Primary (specific etiology not determined)	179800 (AD), 602722 (AR)
B. Galactosemia	230400
C. Hereditary fructose intolerance with nephrocalcinosis	229600
D. Fabry disease	301500
III. Phosphate Depletion from Impaired Renal Tubular Reabsorption (See also Table 5)	
A. X-linked hypophosphatemia	307800
B. Autosomal dominant hypophosphatemic rickets	193100
C. Syndrome of lipoatrophic diabetes, vitamin D–resistant rickets, persistent Müllerian ducts	mentioned in 277440
D. X-linked hypercalciuric nephrolithiasis	310468
E. Hypophosphatemic rickets with hypercalciuria	241530
F. McCune-Albright syndrome	174800
IV. General Renal Tubular Disorders (Fanconi Syndrome)	
A. Primary idiopathic	134600,227700, 227800
B. Cystinosis	219800
C. Fanconi-Bickel syndrome	227810
D. Lowe syndrome	309000
E. Wilson disease	277900
F. Tyrosinemia	276700
V. Primary Mineralization Defect	
A. Hypophosphatasia	146300,171760, 241500,241510
VI. Miscellaneous	
A. Axial osteomalacia	109130
B. Osteopetrosis (osteopetrorickets)	259700
C. Neurofibromatosis	162200

in renal proximal convoluted tubule cell mitochondria by $P450_{c1\alpha}$ — more commonly known as 25(OH)D, 1α-hydroxylase or simply 1α-hydroxylase [8,9]. The product is the fully active 1,25-dihydroxyvitamin D derivative, $1,25(OH)_2D$, called calcitriol. Vitamins D_2 and D_3 seem equally susceptible to these two hydroxylation steps, and their bioactivated metabolites are essentially equipotent in influencing mineral homeostasis [5,6]. $1,25(OH)_2D$ increases intestinal absorption of Ca^{++}, kidney reclamation of Ca^{++}, and bone resorption [5,6]. Rightfully, vitamin D is now regarded as a steroid hormone, not as a nutrient [4–6].

In target tissues, $1,25(OH)_2D$ has genomic and nongenomic actions [10]. It binds to the vitamin D receptor (VDR), a member of the nuclear hormone receptor superfamily [8,9], which possesses both hormone-binding and DNA-binding domains. After also combining with a retinoid X receptor, this VDR complex activates the transcription of several genes in enterocytes, kidney, and bone ultimately to augment extracellular concentrations of Ca^{++} and Pi [10].

Minerals

Extracellular concentrations of Ca^{++} and Pi are conditioned by gastrointestinal absorption, fluxes involving the skeleton and small bowel lumen, and urinary excretion [4]. Circulating levels of Ca^{++} are more tightly controlled than are levels of Pi [4]. Increments in blood Ca^{++} concentrations occur from the direct actions of PTH on the skeleton and the kidneys to accelerate bone turnover and to reclaim filtered Ca^{++}, respectively, and from the indirect action of PTH on the gut mediated by enhanced $1,25(OH)_2D$ production [5,6]. Blood Pi levels are regulated primarily by the kidney [11]. Although PTH causes phosphaturia, control of Pi homeostasis at the renal level is incompletely understood, and studies of heritable rickets and osteomalacia discussed subsequently reveal that an additional important hormonal factor(s) awaits discovery [11].

Diagnosis of Rickets and Osteomalacia

The diagnostic approach to the patient with rickets or osteomalacia is founded on an especially careful medical history and physical examination [12].

Medical History

Depending on the patient's age, disturbances in vitamin D and mineral homeostasis can cause a considerable variety of symptoms and skeletal problems. With deficiency (or impaired bioactivation) of vitamin D, the derangements can be metabolic or anatomic (Table 2) and are likely to be especially severe if extracellular Ca^{++} levels are low (Table 3).

Physical Examination

Rickets affects especially the most rapidly growing bones [13,14]. Because the neonatal calvarium enlarges particularly quickly, congenital disease sometimes causes craniotabes, with flattening posteriorly. A rachitic rosary (resulting from widened costochondral junctions) may appear during infancy. Metaphyseal flaring expands wrists and ankles. Harrison's groove is due to horizontal ridging along the inferior thorax at costal insertions of the diaphragm. Weight-bearing typically bows the lower limbs (*genu varum*). Onset of rickets later in childhood or adolescence can cause knock-knee deformity (*genu valgum*). Occasionally, the curves of the lower extremities are in the same direction ("windswept legs") [13]. Nevertheless, limb deformity may not manifest unless the patient stands. Bone pain and tenderness on palpation can reflect fracture, but they are also a prominent feature of osteomalacia due to vitamin D

TABLE 2. Vitamin D–Deficiency Rickets: Age-Dependent Signs and Symptoms

I.	Metabolic
	Hypocalcemia (see also Table 3)
II.	Neuromuscular
	Hypotonia
	Waddling gait
	Pot belly with lumbar lordosis
	Listlessness
	Proximal myopathy
III.	Dental
	Delayed eruption
	Enamel defects
	Caries
IV.	Skeletal
	Cranial sutures widened
	Craniotabes and skull asymmetry
	Rachitic rosary
	Harrison groove
	Rib deformity → respiratory compromise
	Bone tenderness
	Dystocia
	Flared wrists and ankles
	Fracture
	Frontal bossing
	Kyphosis
	Lax ligaments
	Limb deformity
	Low back pain
	Short stature
	Sternal indentation or protrusion
	"String-of-pearls" deformity in hands
V.	Other Features
	Failure to thrive
	Pneumonia

TABLE 3. Hypocalcemia: Signs and Symptoms

I.	Nervous System
	Increased irritability with latent or overt tetany
	Seizures
	Mental status change
	Basal ganglia calcification
	Retardation
II.	Cardiovascular
	Prolonged electrocardiographic ST interval with arrhythmia
	Hypotension
	Cardiomyopathy with congestive heart failure
III.	Other
	Dysplastic teeth
	Integument changes (hyperkeratotic skin, brittle nails, hair loss)
	Intestinal malabsorption
	Joint contractures
	Lenticular cataracts
	Papilledema
	Rickets/osteomalacia
	Vertebral ligament calcification

deficiency. Additional problems include a "metabolic myopathy" with reduced muscle tone and strength, leading to a waddling gait, but no (or nonspecific) changes on electromyography [13]. Proximal muscle weakness is suspected because of difficulty negotiating stairs, combing hair, and arising from a chair. Gower's sign detects this manifestation when patients must push with their hands on their thighs to stand up. Myopathy is an especially prominent feature when there are disturbances in vitamin D homeostasis.

Hypocalcemia typically results from biochemical derangements that diminish vitamin D action [5,6,14]. In infancy, floppiness, hypotonia, and failure to thrive are common. Young children are often listless and irritable [13]. Symptoms may be elicited during the medical history, and signs of overt or latent tetany appear during the physical examination (Table 3) [14].

Skull size and shape can be distorted by disturbances involving the cranial sutures. Premature fusion of the sagittal suture often causes dolichocephaly in X-linked hypophosphatemia but usually amounts only to a cosmetic concern. In hypophosphatasia, however, functional or true fusion of sutures can lead to scaphocephaly, sometimes with raised intracranial pressure [15].

There can also be lax ligaments, pectus excavatum from diaphragmatic and intercostal muscle traction, delayed eruption of permanent teeth, and enamel defects [13,14]. Alopecia is a distinctive feature in hereditary vitamin-D–dependent rickets type II [8,9]. However, it is also a manifestation of rickets from malnutrition [13].

Radiologic Studies

A posteroanterior radiograph of a wrist and an anteroposterior radiograph of a knee best document the severity of physeal and metaphyseal distortion in rickets and are used for diagnosis and to judge response to therapy [16]. Typically, physes are uniformly widened with rickets of recent onset. However, weight-bearing can asymmetrically change growth plates in the knees, sometimes masking the diagnosis (Fig. 1). Metaphyses are generally splayed, ragged, and concave, with epiphyses appearing as though they are held within cups. In osteomalacia, intervertebral disks may compress softened end plates, causing biconcave ("cod fish") vertebrae [16]. Pseudofractures (Looser's zones), often found in the femora, scapulae, and pubic rami, are a radiographic hallmark of osteomalacia (Fig. 2) [16,17].

Radiographs can provide clues concerning the etiology and pathogenesis of rickets [12,16]. Disturbances in vitamin D homeostasis, which typically engender secondary hyperparathyroidism, often lead to osteopenia and sometimes subperiosteal bone resorption. Conversely, X-linked hypophosphatemia features normal or sometimes increased skeletal radioopacity without changes of PTH excess. In hypophosphatasia, peculiar "tongues" of radiolucency project from physes into metaphyses (Figs. 3 A,B) [12,15]. Nevertheless, not all disorders that cause growth-plate distortions and extremity bowing are forms of rickets [18].

Determining how rapidly rickets responds to pharmacologic therapy may be diagnostically helpful. In primary vitamin D deficiency, radiographic improvement occurs just several weeks after a single large oral dose of ergocalciferol [13,19]. Other forms of rickets, especially those due to renal Pi wasting, often take several months to improve substantially with current medical treatments [1].

Bone scintigraphy is effective for uncovering abnormalities in the skeleton in rickets or osteomalacia but does not reveal the etiology or pathogenesis. Enhanced radioisotope uptake occurs when there is an excess of unmineralized matrix (osteoidosis); hence, rickets and osteomalacia can produce a "superscan." In osteomalacia, bone scanning is important for detecting fractures and pseudofractures. This study, however, is usually not necessary in children.

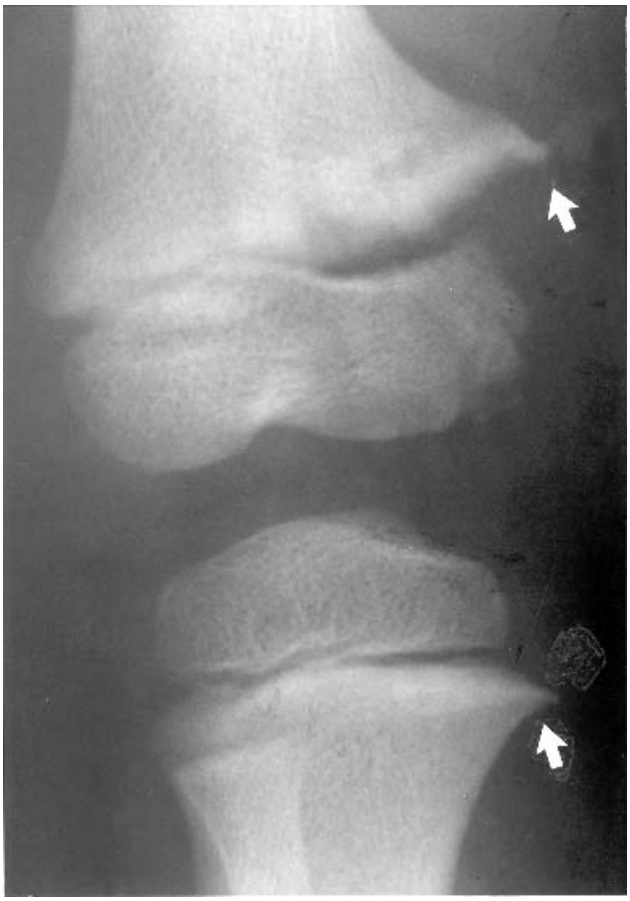

Figure 1. X-linked hypophosphatemia. Physeal widening in the knee of this 4-year-old boy with XLH is asymmetrical due to longstanding bowing deformity of his lower limbs. There is associated characteristic "beaking" of the medial femoral and tibial metaphyses (arrows).

Bone densitometry can be imprecise or misleading in rickets or osteomalacia for several reasons. Dual energy x-ray absorptiometry (DEXA) provides an areal measurement of bone mineral density (gm/cm^2) that is artifactually lowered by small body size [20]. Skeletal deformities can also compromise interpretation. Furthermore, osteoid accumulation may be recorded by various methods. Nevertheless, these techniques can be helpful to monitor responses to medical therapy, especially for individual patients with osteomalacia.

Biochemical Investigation

In acquired or heritable rickets or osteomalacia from disturbed vitamin D homeostasis, hypocalcemia can be severe [13]. Secondary hyperparathyroidism causes a mild hyperchloremic metabolic acidosis reflecting bicarbonaturia. Significant acidosis, however, suggests Fanconi syndrome or renal tubular acidosis [1,13,14]. Additionally,

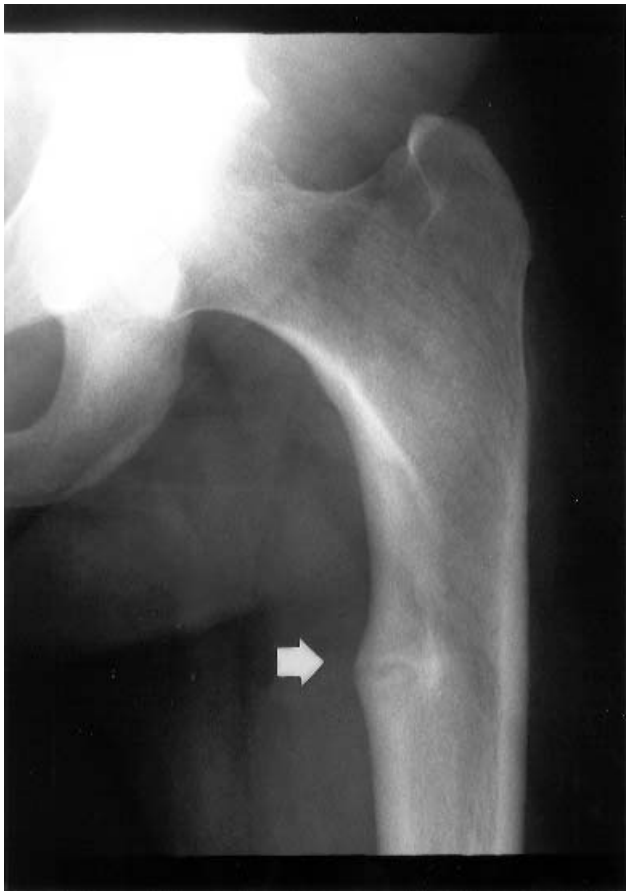

Figure 2. Pseudofracture. This 21-year-old woman with osteomalacia from XLH has *coxa vara* and a characteristic pseudofracture (Looser's zone) in the medial cortex of her left proximal femur (arrow).

nearly all forms of rickets or osteomalacia also manifest hypophosphatemia that can be due to renal Pi wasting from kidney tubule dysfunction or to excessive PTH levels.

Although quantification of circulating vitamin D_2 and vitamin D_3 levels directly evaluates vitamin D repletion, assaying these prohormones is not provided for commercially [5,6]. Instead, measuring serum 25(OH)D concentration is possible and an excellent surrogate. Assays for serum $1,25(OH)_2D$ levels are useful for diagnosing forms of vitamin D–dependent rickets and are also available on a service basis.

Serum alkaline phosphatase (ALP) activity (bone isoform) is elevated in nearly all patients with rickets or osteomalacia. A striking exception is hypophosphatasia, which features hypophosphatasemia [15]. Levels of other biochemical markers of skeletal turnover can be disturbed, depending on the precise pathogenesis of the rickets or osteomalacia [21], but need not be measured routinely.

Histopathologic Findings

Although the medical history and physical examination findings, results of routine biochemical studies, and radiographic abnormalities together usually suffice to diagnose and initiate appropriate treatment for rickets or osteomalacia, histopathologic investigation showing defective mineralization of skeletal matrix is the *sine qua non* for these disturbances [3]. A transiliac specimen obtained with a 5 mm internal diameter trephine is ideal for assessment. Both cortical and trabecular bone are sampled. Two 3-day courses (separated by a 2-week interval) of oxytetracycline or demeclocycline hydrochloride (20 mg per kg body weight daily in divided oral doses) are given to characterize *in vivo* fluorescence labeling of osseous tissue at the calcification front. The final dose is swallowed several days before biopsy.

In rickets or osteomalacia, properly stained, nondecalcified sections of skeletal tissue will reveal increased quantities of osteoid covering cortical and trabecular bone surfaces, and fluorescence microscopy will fail to show two discrete tetracycline "labels" produced by active mineralization at the osteoid-mineralized bone interfaces. Instead, absent or smeared fluorescence is noted [3]. Evidence of secondary hyperparathyroidism includes excessive numbers of osteoclasts and peritrabecular fibrosis.

Guidelines for Pharmacologic Therapy

For heritable forms of rickets or osteomalacia (Table 1), the primary pathogenic disturbance cannot be fixed. Depending on the precise diagnosis, however, it is often possible to compensate for the gene defect or to circumvent it [1].

Successful medical therapy for heritable rickets or osteomalacia must recognize the specific problem leading to aberrant mineral homeostasis [1]. Most forms of these disorders can be treated. However, an accurate diagnosis and appropriate follow-up are essential for a favorable clinical outcome while avoiding intoxication with vitamin D or mineral supplements [22]. Pharmacologic doses of vitamin D (or an active metabolite), sometimes with mineral supplementation, often effect considerable improvement but rarely achieve complete yet perfectly safe control.

As of this writing, seven sterols with vitamin D activity are prescribed worldwide [5,6,23]: vitamins D_2 and D_3, $25(OH)D_3$, $1,25(OH)_2D_3$, and three synthetic analogs of vitamin D called dihydrotachysterol (DHT) and alfacalcidiol [$1\alpha(OH)D_2$ and $1\alpha(OH)D_3$]. Vitamin D_3 and alfacalcidiol are not available by prescription in the U.S. These seven drugs differ importantly in potency, biological half-life, and cost (Table 4) [5,6,23].

Medical therapy for heritable rickets and osteomalacia can be rewarding for patient and physician. Given sufficient time before growth-plate closure, defects in the three processes of skeletal development can often be substantially corrected [3]. Relief from bone pain, reversal of deformity, correction of short stature, reduction in the risk of developing osteoarthritis, and fracture prevention are major goals of treatment [1].

Physical examination provides critical information [12]. Linear growth rate is a key parameter to monitor in infants, children, and especially adolescents. Height is best determined with a wall-mounted stadiometer. Rapid increases in body size during the pubertal "growth spurt" can significantly augment dose requirements for medical therapy. Inordinate weight gain in girls sometimes accelerates puberty, causing an early growth spurt, which, if unrecognized, can exacerbate limb deformity and compromise final height. Measurements of arm span and upper and lower body segment lengths help to quantify skeletal distortion. With heritable rickets, treatment will be necessary throughout growth, and some time can pass before control of the

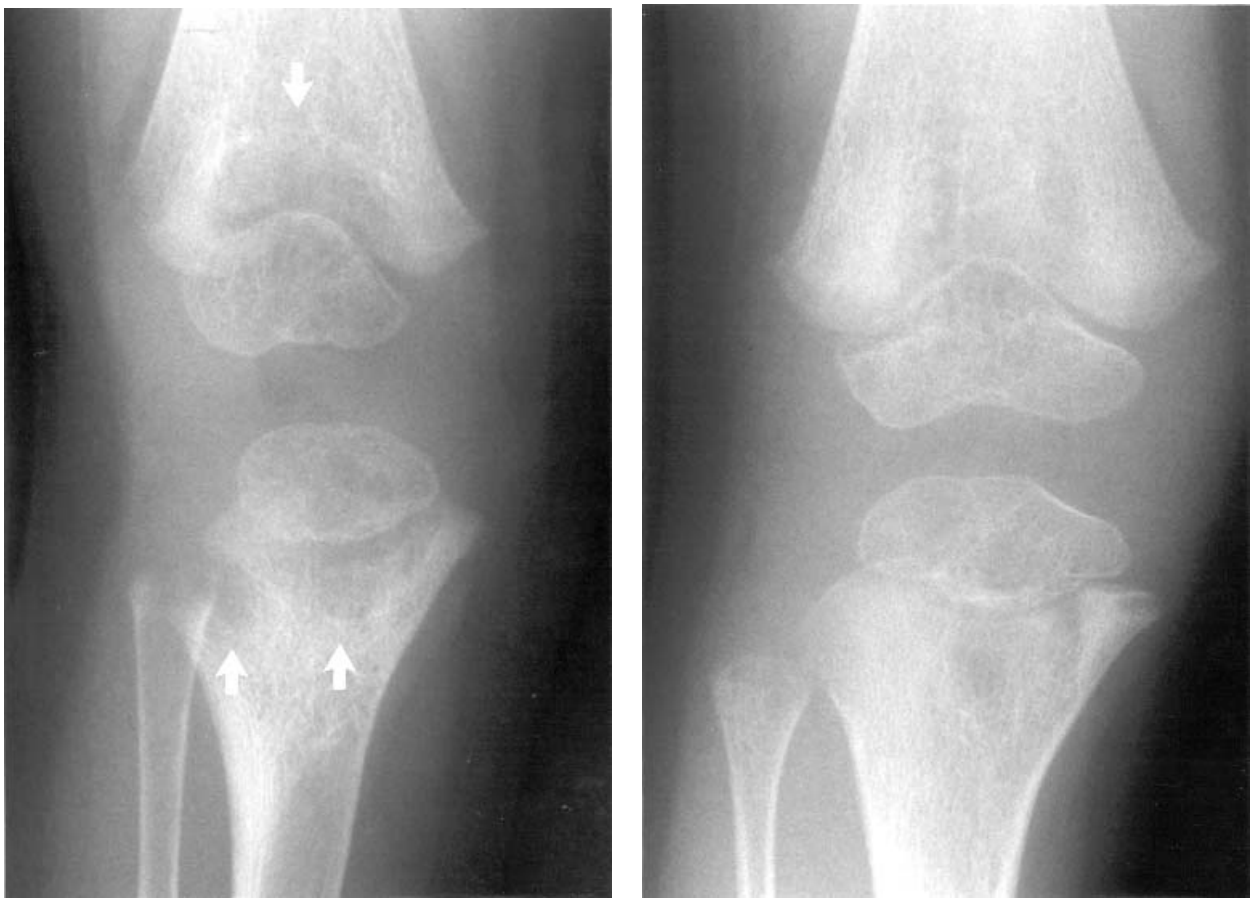

Figure 3. Hypophosphatasia. A) Pathognomonic ''tongues'' of radiolucency (arrows) project from physes into metaphyses in the knee of this 2-year-old boy with the infantile form of hypophosphatasia. This finding is especially noticeable in the proximal tibia. **B**) By age $3\frac{1}{2}$ years, there has been considerable spontaneous radiographic improvement.

TABLE 4. Preparations of Vitamin D and Active Metabolites*

Preparation	Ergocalciferol	Dihydrotachysterol	Calcifediol	Calcitriol
Abbreviation	D_2	DHT	$25(OH)D_3$	$1,25(OH)_2D_3$
Trade name	Calciferol	Hytacherol, Intensol	Calderol	Rocaltrol, Calcijex
Dosage form	a. Capsules: 1.25 mg	a. Tablets: 0.125,0.200, and 0.400 mg	Capsules: 20 and 50 μg	a. Capsules: 0.25 and 0.50 μg
	b. Liquid: 200 μg/mL	b. Liquid: 0.2 mg/mL		b. Liquid: 1 μg/mL
	c. Intramuscular injection: 12.5 mg/mL in sesame oil			c. Injection: 1 and 2 μg/mL
Physiologic daily dose	2.5–10 μg	25–100 μg	1–5 μg	0.25–0.5 μg
Time to reach maximum biologic effect	4–10 weeks (oral)	2–4 weeks	4–20 weeks	$\frac{1}{2}$–1 week
Persistence of biologic effect after cessation	6–30 weeks (oral)	2–8 weeks	4–12 weeks	$\frac{1}{2}$–1 week

*Available in the U.S.

skeletal disease is achieved. Occasionally, it is a family member who sees the patient only every few months who best appreciates gradual changes in deformity. Accordingly, clinical photography, videotaping, and even gait analysis can be helpful [12].

Radiographic assessment of skeletal abnormalities, such as physeal widening and metaphyseal irregularity, is essential for monitoring rickets. In osteomalacia, there are fewer x-ray features to follow, but pseudofractures can heal with treatment. Other findings are less helpful;

osteopenia or coarsening of trabecular bone is found only in some patients [16]. Nevertheless, sequential bone density measurements should, with potential technical limitations, be worthwhile in osteomalacia.

The most important biochemical parameters for diagnosis and follow-up of rickets and osteomalacia are serum Ca^{++} and Pi concentrations, ALP activity, and PTH levels. Depending on the etiology and pathogenesis of the specific disorder, knowing serum 25(OH)D and, more rarely, $1,25(OH)_2D$ concentrations can be helpful [1]. Ca^{++} excretion in 24-hour urine collections (expressed per creatinine content) should also be assayed to guide therapy and to help monitor against vitamin D or mineral intoxication[1] [1].

The diseases that give rise to heritable rickets and osteomalacia are numerous and diverse (Table 1). Successful treatment will require an accurate diagnosis and thorough understanding of a disorder's pathogenesis [1]. Although administration of vitamin D_2 can cure vitamin D deficiency, heritable forms of rickets or osteomalacia respond best to regimens that bypass or compensate for the intrinsic metabolic defect. However, a range of effective doses must be anticipated, even among patients with a specific diagnosis. The optimal approach will likely include one of the vitamin D metabolites, perhaps with mineral supplementation. Significant adverse consequences (failed treatment, hypercalcemia, tertiary hyperparathyroidism, or renal damage) can result from an incorrect diagnosis or excessive therapy.

Treatment with an active metabolite of vitamin D has advantages and disadvantages. Importantly, these drugs can circumvent defects in the bioactivation of vitamin D [1,5,6]. Additionally, they are more potent than vitamins D_2 or D_3 and have faster onset of action (Table 4) [23]. Fortunately, toxicity can be corrected easily. However, they also have shorter biological half-lives, and more frequent dosing is necessary. Furthermore, cessation of therapy will quickly decompensate mineral homeostasis. Finally, these pharmaceuticals are expensive.

Many Ca^{++} and Pi supplements are available [23]. $CaCO_3$ given orally is least costly, but Ca^{++} citrate is best absorbed. Calcium gluconate is especially high-priced. Tablets rather than liquid preparations for Pi supplementation are more convenient, better tasting, well-absorbed, and seem less prone to cause diarrhea. Those containing high amounts of Na^+ should be avoided.

Follow-up clinic visits are essential for all types of heritable rickets and osteomalacia, but the interval should reflect the diagnosis as well as the patient's age and compliance with, and response to, therapy. Because these disorders represent deficiency of mineral content within the skeletal matrix, decreases in medication dosages are frequently necessary when healing becomes complete (i.e., acute and maintenance therapies often differ). Satiation of "hungry bones" can abruptly increase urinary Ca^{++} excretion because the skeleton no longer acts as a mineral sump [1]. Fortunately, hypercalciuria generally precedes hypercalcemia unless there is renal failure or sustained elevation in circulating PTH levels (acting to reclaim Ca^{++} from the glomerular filtrate). Correction of abnormal biochemical findings should herald hypercalciuria. Lower doses of medication may then be needed. Thus, 24-hour urine collections, not random specimens, assayed for Ca^{++} and creatinine are especially important for monitoring patients. In fact, when there is hypocalciuria but no sustained hyperparathyroidism, rising urinary Ca^{++} levels will indicate effective therapy.

Consultation and follow-up with the pediatric orthopedic surgeon is often essential for optimally managing heritable rickets. Straight lower limbs with alignment of growth plates parallel to the ground helps to prevent osteoarthritis. Leg bracing, physeal stapling (epiphysiodesis), or osteotomy may be beneficial.

Pharmacologic regimens for specific types of heritable rickets or osteomalacia are outlined in the following sections.

Heritable disorders that cause rickets or osteomalacia are listed in Table 1. Some reflect disturbances in the bioactivation or action of vitamin D; several are characterized by renal Pi wasting. As discussed below, most of these conditions have proven to be inborn errors of metabolism due to enzyme deficiencies. However, for a few of the diseases that feature renal Pi wasting, the genetic basis awaits discovery.

HYPOPHOSPHATEMIC BONE DISEASES

Most acquired and some heritable forms of rickets or osteomalacia reflect aberrations in vitamin D homeostasis leading to reduced levels of Ca^{++} in extracellular fluid [1,14]. Nevertheless, it is important to appreciate that these disorders also diminish extracellular Pi concentrations due to dietary malabsorption as well as phosphaturia from secondary hyperparathyroidism. Consequently, hypocalcemia and hypophosphatemia act in concert to decrease the extracellular $Ca^{++} \times$ Pi product, thereby impairing mineralization of newly synthesized osteoid [3].

The importance of Pi for skeletal formation is exemplified best by rickets or osteomalacia due to renal wasting of Pi ("phosphate diabetes"). Here, hypophosphatemia, without associated hypocalcemia, causes osteopathy [11,24]. "Hypophosphatemic bone disease" is a useful generic term that emphasizes the diagnostic and pathophysiologic significance of this biochemical disturbance. Many of the disorders that cause hypophosphatemic bone disease are heritable (Table 1). Although pharmacologic treatment for these types of rickets or osteomalacia has certain themes, it is important to recognize that optimal regimens differ among the various entities.

X-Linked Hypophosphatemia

X-linked hypophosphatemia (XLH) (MIM 307800) is the most common heritable form of rickets or osteomalacia [2]. Its prevalence in North America is approximately 1 : 20, 000 live births [11]. All races seem to be affected [25].

XLH was first described in 1937 after the epidemic of vitamin D–deficiency rickets, a socioeconomic scourge centered in smoggy northern industrialized cities, became treatable [26]. Discovery of vitamin D in 1919 and successful therapy and preventive measures for "nutritional" rickets was a triumph of medical science at the beginning of the last century [5,6]. Nevertheless, some patients with rickets were puzzling because they were not cured even by massive doses of vitamin D_2 [26]. XLH became the prototypic example of "vitamin D–resistant rickets" (VDRR), an acronym that survived until the disorders discussed below became sufficiently delineated and understood.

In 1958, "VDRR" was recognized to manifest sex-linked dominant inheritance [27]. Females and males are affected in a characteristic 2 : 1 ratio, although early on it was believed that the disease is more mild in females [11,24].

[1] If incomplete healing of rickets or osteomalacia is suspected, nondecalcified iliac crest histomorphometry is required [1,3].

In 1972, hypophosphatemia from selective renal Pi wasting was emphasized as a key pathogenetic factor requiring treatment [28]. In fact, XLH seems to be the best name for this disorder because of the lifelong persistence of this kidney defect [29]. In 1980, an inappropriately normal circulating level of $1,25(OH)_2D$ despite hypophosphatemia was documented but is still an enigmatic biochemical feature of XLH [30]. In 1995, an international consortium of researchers identified a gene now called *PHEX* (phosphate regulating gene with homologies to endopeptidases on the X-chromosome) that was defective in XLH [31]. More than 100 different mutations in *PHEX* have been reported worldwide [24,25].

Clinical Presentation

XLH causes bowing of the lower limbs and short stature in toddlers as they begin to bear weight (Figs. 4 A,B). Adult height Z-scores will typically be minus 2 to 3 standard deviations unless there is effective treatment [29]. Affected children can seem clumsy but are otherwise well. When skeletal disease sometimes presents later during childhood, knock-knee deformity may occur. There is no muscle weakness (in contrast to many forms of rickets). The skull often becomes dolichocephalic due to early closure of the sagittal suture, but this feature seems to be only of cosmetic concern. Deafness is not a problem in pediatric patients [32]. The chest and upper extremities are not distorted. Fractures are uncommon in children but occasionally occur later in life. Doppler and two-dimensional echocardiography of the heart is normal [33].

Adults with XLH suffer three principal complications [29,34,35]. Arthralgias, primarily involving the lower limbs and especially the knees, are usually caused by osteoarthritis. The degree of extremity deformity predicts the likelihood and severity of joint deterioration [29]. Bone pain, especially in the thighs and elicited by palpation, is often explained by pseudofractures (Fig. 2). Dental abscesses develop because brittle "shell teeth" have formed early in life due to defective mineralization of dentin [29]. Calcification of tendons, ligaments, and joint capsules, called enthesopathy, is common, but it is unclear how often enthesopathy causes symptoms [29,34]. Perhaps sensorineural hearing loss and occasionally spinal stenosis occur with increased prevalence in adults [11,24]. Obstetrical histories, however, seem benign [29].

Radiologic Findings

Radiographs of the lower limbs show physeal widening in the knees that is pronounced medially when there is bowing deformity (Fig. 1). Osteopenia and evidence of secondary hyperparathyroidism are typically absent unless dietary Ca^{++}intake is poor. In fact, the skeleton sometimes appears dense even before treatment (in contrast to other forms of rickets associated with increased circulating PTH levels). In adults, axial skeletal mass is usually normal, although sometimes the bones look sclerotic [35].

Laboratory Findings

The biochemical hallmark of XLH is hypophosphatemia [11,24]. Low blood Pi levels are documented if important age-related changes in the reference range for serum Pi are appreciated [36]. Healthy infants and children have considerably higher serum Pi concentrations (and ALP activity) than adults [36]. Because serum Pi levels may increase or decrease depending on what is eaten, fasting blood specimens are necessary for diagnosis [1]. Additionally, a considerable variety of disorders and perturbations can cause chronically low blood Pi levels and must be excluded for a correct diagnosis (Table 5). In XLH, quantification of renal Pi reclamation by calculating the tubular reabsorption of phosphorus (TRP) or, better yet, assessing the transport maximum for phosphorus corrected for glomerular filtration rate (TmP/GFR)[2] shows that hypophosphatemia is due to "phosphate diabetes" [36].

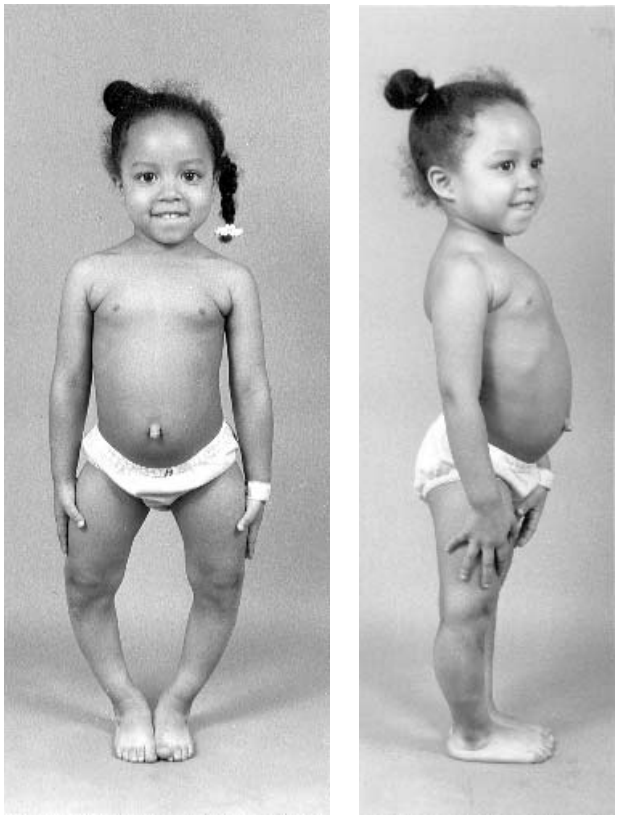

Figure 4. X-linked hypophosphatemia. A) Severe bowing deforms the lower limbs of this newly diagnosed, 4-year-old girl with a three-generation family history of XLH. **B**) Despite her hyperlordotic posture and "pot-bellied" appearance, she has no muscle weakness. Mild frontal bossing is characteristic of XLH.

Serum Ca^{++} levels are often low-normal, but not distinctly reduced, unless there are additional perturbations of mineral homeostasis [29,37,38]. Circulating $1,25(OH)_2D$ concentrations in XLH are, as a rule, normal or low-normal despite the hypophosphatemia that typically increases renal 25(OH)D,1α-hydroxylase activity in healthy individuals [11,24,30]. Unless patients receive Pi supplements insufficiently matched by doses of $1,25(OH)_2D_3$ (see below), PTH levels are also generally unremarkable [29,37,38]. Without pharmacologic treatment, serum ALP is invariably increased in children [37,38] but not always in adults [29]. Occasionally, trace glucosuria is detected; however, other reflections of renal proximal tubular function (e.g., serum K^+, HCO_3^-,

[2] $\frac{TmP}{GFR} = S_p - \left[\frac{U_p \times S_{Cr}}{U_{Cr}}\right]$ where S_p, S_{cr}, U_p, U_{Cr} are concentrations of phosphate and creatinine in serum and urine [36].

TABLE 5. Causes of Chronic Hypophosphatemia

I.	Decreased Intestinal Absorption of Pi
	Antacid excess
	Heritable forms of rickets and osteomalacia
	Malabsorption
	Starvation, debility from alcohol abuse
	Vitamin D deficiency
II.	Increased Urinary Loss of Pi
	Abnormalities of vitamin D metabolism
	Vitamin D deficiency
	Vitamin D–dependent rickets (types I and II)
	Alcohol abuse
	Drugs: calcitonin, diuretics, glucocorticoids, bicarbonate, ifosfamide
	Extracellular fluid volume expansion
	Heritable forms of rickets and osteomalacia
	Hyperparathyroidism
	Metabolic or respiratory acidosis
	Oncogenic (tumor-induced) rickets and osteomalacia
	Poorly controlled diabetes mellitus
	Proximal renal tubular defects (Fanconi syndrome)
	Pseudo-(tumor-induced) rickets
	Renal transplantation
	Respiratory alkalosis
	Severe burns
	X-linked hypophosphatemia

and uric acid levels) are normal in XLH (i.e., renal Fanconi syndrome — see later — is absent).

Histopathologic Findings

Histologic examination of the skeleton shows rickets or osteomalacia in untreated patients with XLH [28–30]. It is of interest that in appropriately stained, nondecalcified bone sections there will also be pathognomonic "halos" of osteoid surrounding individual osteocytes [29]. This peculiarity seems to be diagnostic of XLH, reflecting a mineralization defect that persists when osteoblasts become osteocytes. In fact, halos remain despite successful $1,25(OH)_2D_3$ and Pi therapy [39–43]. When circulating PTH levels are elevated, there will also be changes of hyperparathyroidism including abundant osteoclasts and peritrabecular fibrosis.

Pathogenesis and Genetics

The pathogenesis of XLH is incompletely understood [11,24]. Malabsorption of dietary Ca^{++} is a manifestation of the vitamin D resistance that requires further investigation. A murine model (*Hyp*) for XLH [44] has revealed a defect in the transport of Pi from the glomerular filtrate across kidney proximal tubule cells where PTH and somehow dietary Pi levels control urinary Pi reclamation [45]. Here, Pi transfer through the brush border membrane is the rate-limiting step. In *Hyp* mice, there is decreased activity of a high-affinity, low-capacity, Na^+-dependent, Pi cotransport system [11,24]. The transporter (NPT-2) gene in humans has been cloned from chromosome 5q35 and therefore is not a candidate for XLH [24]. Nevertheless, there is ~50% reduction in NPT-2 mRNA and protein in *Hyp* mice [11]. However, *Hyp* mice also manifest a blunted response to activators of $1,25(OH)_2D$ synthesis in kidney mitochondria [11]. In these animals, Pi deprivation and supplementation inappropriately accelerate and suppress, respectively, $1,25(OH)_2D$ catabolism [46]. Nevertheless, the precise intracellular disturbances in the kidney that diminish Pi transport and alter vitamin D bioactivation in XLH are not known.

Of great interest to the field of metabolic bone disease are observations from parabiotic and renal transplantation studies using the *Hyp* mouse that implicate a phosphaturic factor in XLH [47,48]. The nature of this factor, provisionally called "phosphatonin," is incompletely understood [49,50]. It seems to be a heat-stable protein of ~57,000 kDa with PTH-like immunoreactivity [49,50]. Additionally, however, tissue culture studies of *Hyp* and XLH bone explants suggest that osteoblast function is also intrinsically impaired [39,51,52]. Indeed, osteoblasts express PHEX and may be a source of phosphatonin [53,54].

XLH maps to chromosome Xp22.3-21.3 [25]. The gene that is defective is now called *PHEX* [2,11,24]. More than 100 different mutations affecting the coding sequence of *PHEX* have been identified in patients worldwide [24]. These defects are expected effectively to eliminate PHEX protein production. However, such mutations have been found in only ~50% of patients [25]. Defects within the noncoding regions of *PHEX* or perhaps in an additional nearby gene(s) could be the explanation for XLH in other affected individuals. In fact, preliminary analysis indicates that these essentially null mutations in *PHEX* are associated with the most severe clinical and biochemical findings among the XLH patient population [55]. Nevertheless, the putative substrate for *PHEX*, "phosphatonin," remains elusive [53,54]. Possibly, PHEX acts enzymatically at osteoblast surfaces to inactivate phosphatonin or to activate a Pi-conserving hormone [11,24].

In XLH, no gene-dosage effect emerges from comparison of prepubertal heterozygous girls versus prepubertal hemizygous boys [38]. Nevertheless, pseudofractures and enthesopathy are more prevalent and severe in men than in women [34]. Accordingly, sex (sex steroids and/or physical labor) seems to influence the long-term outcome of XLH [38].

Renal Pi wasting is a major pathogenetic abnormality in XLH [27,28]. TmP/GFR correlates positively with height Z-score and negatively with the degree of bowing deformity in untreated children and adults, respectively, with XLH [29,38]. Accordingly, this fundamental disturbance is addressed by medical therapy. Resistance to vitamin D and aberrations in renal $1,25(OH)_2D$ biosynthesis are also compensated (see below).

Treatment

A bioactive form of vitamin D together with Pi supplementation is currently the preferred medical treatment for XLH [37]. High doses of vitamin D_2 (e.g., 100,000 IU daily) improve, but will not heal, XLH rickets [26,28]. Furthermore, this therapeutic approach can prove toxic [56]. Large doses of vitamin D_2 are converted to 25(OH)D. Although the affinity of the VDR for 25(OH)D is two to three orders of magnitude lower than for $1,25(OH)_2D$ [10], excessive vitamin D_2 therapy sometimes produces sufficiently great concentrations of 25(OH)D to cause prolonged hypercalcemia, hypercalciuria, nephrocalcinosis, and renal failure [56]. The period of intoxication also reflects the long biological half-life of vitamin D itself (Table 4). In fact, vitamin D_2–induced hypercalcemia can persist for months, necessitating dietary Ca^{++} restriction and glucocorticoid treatment. Additionally, "high dose" vitamin D_2 therapy requires cessation months before osteotomy to avoid hypercalciuria or hypercalcemia if there is postoperative immobilization.

When the renal Pi wasting of XLH was first addressed therapeutically, improved clinical, biochemical, and radiographic responses became apparent [28,37]. Transient augmentation of circulating Pi levels is achieved using frequent oral Pi dosing. The amount depends on body size. About 1–2 grams of phosphorus (as Pi) are given each day for an older child or adolescent [37].

However, it is clear that combined use of $1,25(OH)_2D_3$ and Pi supplementation is important and the best regimen for XLH [37]. $1,25(OH)_2D_3$ enhances both Ca^{++} and Pi uptake from the gut. Improved dietary Ca^{++} absorption is necessary to prevent secondary or tertiary hyperparathyroidism from hypocalcemia provoked by the effect of Pi in binding Ca^{++} in the circulation or in the gut lumen. Growth hormone and $24,25(OH)_2D_3$ have been tested individually for XLH with uncertain outcome using relatively small groups of patients [57,58].

In fact, treatment of XLH requires a combined medical and orthopedic approach. Ideally, this is provided by centers experienced with XLH. $1,25(OH)_2D_3$ and Pi therapy can correct deficits in growth and restore skeletal modeling and remodeling in pediatric patients [37]. The goals of treatment are essentially twofold and clear-cut. Elimination of lower-limb deformities by the time growth plates fuse is necessary to prevent osteoarthritis later in life. Additionally, both boys and girls can achieve normal height. However, two potential complications of $1,25(OH)_2D_3$ and Pi dosing are secondary or tertiary hyperparathyroidism (compromising the clinical outcome and perhaps necessitating parathyroidectomy) and renal damage [55]. Unfortunately, the margin for error in treating XLH patients with these drugs is not great. Accordingly, medical management seems best from physicians experienced with this disorder.

Therapy should begin with affected toddlers to help avoid lower-extremity deformities that could require surgery. However, dosing and biochemical monitoring will understandably be difficult at first. Control, but not complete correction, of the rickets is a reasonable early objective. $1,25(OH)_2D_3$ is available in 0.25 and 0.50 μg capsules and in a solution of 1 μg/mL (Rocaltrol®; Hoffman-LaRoche, Inc., Nutley, New Jersey) (Table 4). Approximately 40 ng of $1,25(OH)_2D_3$ (i.e., 0.040 μg) per kg of body weight daily (divided doses are ideal) should be achieved safely over 2–3 months. Pi supplementation must be introduced at the same time as $1,25(OH)_2D_3$, with gradually increasing doses of both medications and follow-up of their biochemical effects. Pi supplementation is given three to four times daily. Tablets of Na^+/K^+ phosphate (e.g., K-Phos Neutral®; Beach Pharmaceuticals, Tampa, Florida) are convenient and generally well-tolerated, and the low Na^+ content does not cause hypertension [37,59]. Occasionally, Pi leads at first to diarrhea, but patients often tolerate the drug better after perhaps one week of treatment. If they do not, supplementation can be adjusted to acceptable levels.

In some ways, $1,25(OH)_2D_3$ and Pi produce opposite effects on Ca^{++} homeostasis, and this must be dealt with as dosages of $1,25(OH)_2D_3$ or Pi are regulated [37]. If either $1,25(OH)_2D_3$ or Pi treatment has been inadvertently stopped temporarily, both supplements should be withheld. Sudden decreases in Pi dosing should be avoided because blood and urinary Ca^{++} levels may rapidly rise [60]. Accordingly, patients should be cautioned not to go without one of the medications.

Careful biochemical surveillance is essential for XLH patient treatment because $1,25(OH)_2D_3$ and Pi supplements potently increase Ca^{++} and Pi absorption from the gut. Ca^{++}, P, and creatinine should be assessed using 24-hour urine collections (not random specimens) [1,37]. Initially, monitoring should be monthly; testing can then occur perhaps every three months when dosing becomes established. Urinary Ca^{++} to creatinine ratios of about 150–180 mg/g (424–509 mmoles/mole) demonstrate adequate gut effects of $1,25(OH)_2D_3$ and should reflect Ca^{++} absorption that will control circulating PTH levels. Unless serum PTH is nonsuppressably elevated, hypercalciuria will herald excessive $1,25(OH)_2D_3$ dosing. If PTH levels remain increased, hypercalcemia can occur before hypercalciuria. Partial parathyroidectomy can become necessary when tertiary hyperparathyroidism causes hypercalcemia and/or difficulty in controlling the skeletal disease. Fortunately, $1,25(OH)_2D_3$ has a short biological half-life, permitting rapid correction of dosing (Table 4). Reducing dietary Ca^{++} intake also works rapidly to correct endogenous Ca^{++} levels. For Pi supplementation, urine levels of 3–3.5 gP/g creatinine (8.6–10.0 moles P per mole creatinine) are efficacious yet seem reasonable to help prevent nephrocalcinosis. Renal ultrasonography, creatinine clearance, and the serum PTH level should be monitored at least yearly to screen for complications of treatment (Fig. 5). Nephrocalcinosis in XLH represents Ca^{++}-Pi deposits [61,62]. Subradiographic nephrocalcinosis, however, seems not to compromise renal function [56].

Dosage increases will be necessary as the child grows. Hypercalciuria (Ca^{++} >4 mg per kg of body weight or >220 mg per g creatinine or >628 moles/mole) can occur abruptly when skeletal mineralization is restored or when growth plates fuse. Approximately one-half the dose of $1,25(OH)_2D_3$ and Pi should then provide maintenance therapy. If hypocalciuria persists during $1,25(OH)_2D_3$ and Pi treatment, increased milk consumption or Ca^{++} supplementation may be helpful.

Orthopedic evaluation should occur at least annually during childhood and biannually during the adolescent growth spurt because bracing or epiphysiodesis can help straighten lower-limb deformities. For severe distortions, osteotomies are performed ideally after growth plates have fused to minimize the likelihood of postoperative curvatures.

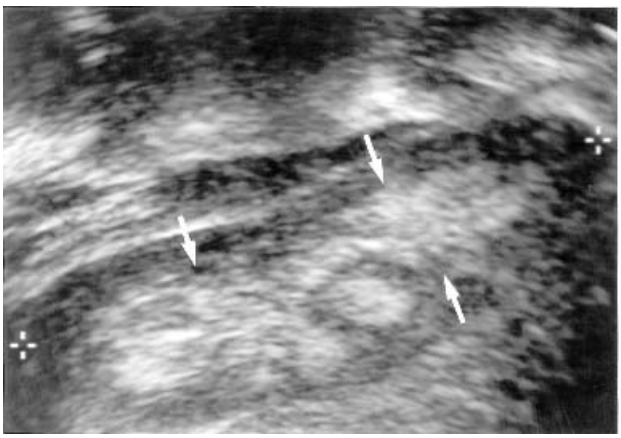

Figure 5. X-linked hypophosphatemia. Medullary nephrocalcinosis detected by renal sonography appears as patches of white material (arrows) in this 6-year-old girl with XLH receiving $1,25(OH)_2D_3$ and Pi supplementation (the symbols ⊹ help measure kidney length).

Unless patients are weight-bearing within two days of surgery (or a fracture, etc.), both $1,25(OH)_2D_3$ and Pi therapy should be discontinued temporarily to avoid immobilization-induced hypercalciuria and hypercalcemia [63].

Pubertal closure of physes does not signify that XLH is cured [29]. Renal Pi wasting persists throughout life [29]. Accordingly, affected adults should be monitored perhaps yearly. Some of these individuals seem to benefit from $1,25(OH)_2D_3$ and Pi therapy to prevent fractures or worsening deformity or to heal pseudofractures [29,64]. However, the efficacy and outcome of medical treatment for men and women with XLH is not well-understood [64]. In our experience (unpublished), five untreated adult patients tolerated knee or hip replacement well and benefited significantly. The impact of XLH during old age has not been studied, but life expectancy is probably not compromised.

Genetic Counseling/Prenatal Diagnosis

Approximately 20% of XLH patients represent new mutations [11,25]. In keeping with X-linked dominant inheritance, affected males then transmit the disorder to all of their daughters but none of their sons. Affected females pass on XLH to half of both their sons and their daughters. Prenatal diagnosis has not been reported for this treatable disease.

X-Linked Hypercalciuric Nephrolithiasis

X-linked hypercalciuric nephrolithiasis (XLHN) (MIM 310468) maps to chromosome Xp11.22 and is caused by deactivating mutations in the *CLCN5* gene involved in Cl^- transport [65,66]. It is a sex-linked recessive condition that affects males, who manifest proximal kidney tubule failure and progressive glomerular disease leading to renal insufficiency. Renal Pi wasting sometimes causes rickets [67–69]. In response to hypophosphatemia, serum $1,25(OH)_2D$ levels are elevated and associated with hypercalciuria, nephrocalcinosis, and nephrolithiasis. β_2-microglobulinuria reflects an early renal disturbance [66,68]. The severity of the hypophosphatemic rickets seems to vary considerably worldwide, and several additional names have been given to this disorder, including Dent's disease and X-linked recessive hypophosphatemic rickets [66].

Treatment of XLHN rickets is not well-established, but Pi supplementation (with caution not to induce hyperparathyroidism or to exacerbate nephrocalcinosis) has had some success [66]. Thiazide diuretics have also been used to diminish the hypercalciuria [66]. If renal failure occurs, Pi diabetes ceases and defective mineralization of skeletal matrix corrects as circulating Pi levels rise.

Autosomal Dominant Hypophosphatemic Rickets

Autosomal dominant hypophosphatemic rickets (ADHR) (MIM 193100) is an extremely rare disorder that also features isolated renal Pi wasting. It has been fully characterized in one large kindred [70], but patients with the rare disorder called "hypophosphatemic bone disease" (HBD) may also represent cases of ADHR [24]. Affected individuals manifest relatively mild rickets that can appear either during early childhood or adolescence [70]. In some patients, renal Pi wasting corrects after puberty. Serum $1,25(OH)_2D$ levels are inappropriately normal [24]. The disorder has been mapped to chromosome 12p13, but the defective gene has not been identified [71]. Medical treatment for ADHR involves the regimen used for XLH, but lower doses of $1,25(OH)_2D_3$ and Pi seem effective [24,70].

Hereditary Hypophosphatemic Rickets with Hypercalciuria

Hereditary hypophosphatemic rickets with hypercalciuria (HHRH) (MIM 241530) has been characterized in one large Bedouin kindred [72,73]. The mode of inheritance seems to be autosomal recessive [2]. Renal Pi wasting causes hypophosphatemic rickets, but circulating $1,25(OH)_2D$ levels are elevated, presumably as a physiologic response to the low Pi concentration in blood. Subsequently, patients develop hypercalciuria and kidney stones. Medical treatment of the rickets could include cautious introduction and close follow-up of Pi supplementation and the use of thiazide diuretics [24].

Fanconi Syndrome

Fanconi syndrome features proximal renal tubule dysfunction leading to low serum levels of Pi, K^+, HCO_3^-, and uric acid, as well as to aminoaciduria [74]. There are several heritable causes, including cystinosis, tyrosinemia, and Lowe syndrome (Table 1). Therapy with $1,25(OH)_2D_3$ and Pi supplementation (see XLH) seems helpful, but urinary Ca^{++} levels must be monitored especially carefully because there can be hypercalciuria.

McCune-Albright Syndrome

McCune-Albright syndrome (MAS) (MIM 174800) is a sporadic condition caused by somatic cell mosaicism involving rare gain-of-function mutations of the *GNAS1* gene (chromosome 20q13). *GNAS1* encodes the α subunit of the G protein (Gs_α) that stimulates adenyl cyclase [75]. MAS is characterized clinically by polyostotic fibrous dysplasia, café-au-lait spots, and endocrine gland overactivity, most frequently causing precocious puberty, thyrotoxicosis, or acromegaly [75].

Although the precise pathogenesis is uncertain, acquired hypophosphatemic rickets due to renal Pi wasting is not uncommon in MAS, especially when there is extensive skeletal disease (Fig. 6). We find the onset of the rickets to be at about 8 years of age [76]. The Pi wasting could reflect excessive cAMP formation in kidney tubule cells, but we speculate instead the elaboration of a phosphaturic factor, perhaps phosphatonin, from the increasing mass of fibrodysplastic tissue [76]. In fact, hypophosphatemic rickets can also complicate neurofibromatosis, ossifying and nonossifying fibroma, fibrous dysplasia that occurs with nevus unius lateris, and mixed-mesenchymal tumors (oncogenic rickets), all associated with fibrous tissue accumulation [1].

Treatment with $1,25(OH)_2D_3$ and Pi can help to minimize the added skeletal burden of rachitic disease in MAS. However, responses can be difficult to evaluate because of premature closure of growth plates when there is precocious puberty. Furthermore, the remarkable appearance of the underlying fibrous dysplasia itself can divert attention from rickets (Fig. 6). In fact, even bone histopathology may not be helpful because the defect in mineralization can be obscured if fibrous dysplasia is inadvertently sampled.

VITAMIN D–DEPENDENT RICKETS

Vitamin D–dependent rickets (VDDR) types I and II (VDDR I and VDDR II) are rare autosomal recessive disorders that mimic vitamin D deficiency [5,6,77]. However, they manifest no aberration in cutaneous synthesis of vitamin D_3 or hepatobiliary or gastrointestinal disease that can disturb vitamin D homeostasis. Despite low circulating Ca^{++}

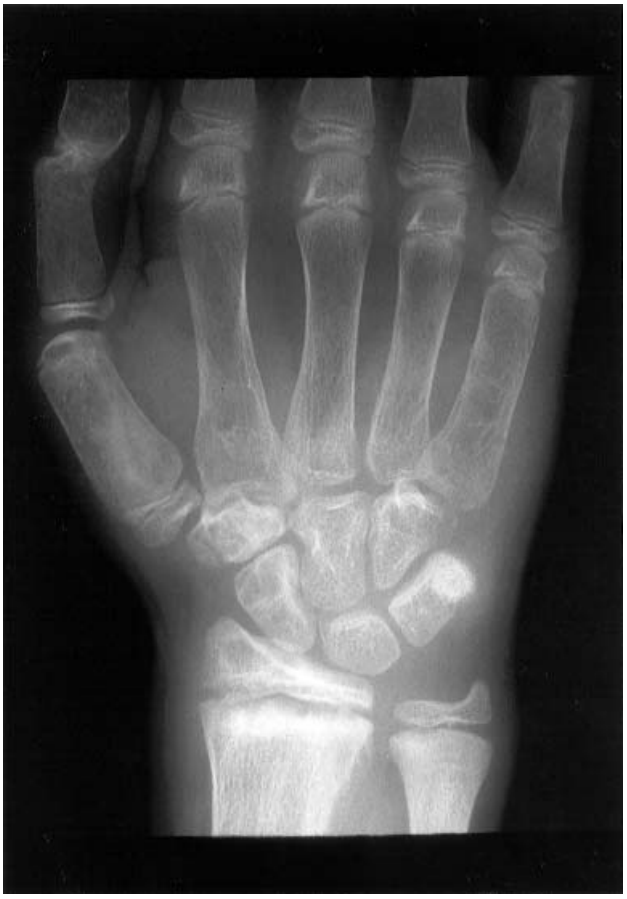

Figure 6. McCune-Albright syndrome. The right wrist of this 9-year-old girl with MAS shows widening of the physes and irregularity of the metaphyses (especially in the distal radius) indicative of rickets. She has gradually developed hypophosphatemia with serum Pi = 2.3 mg/dl (normal 4.3–5.7). Cortical thinning and a "ground glass" appearance, characteristic of fibrous dysplasia, are present in her metacarpals.

levels, patients are replete with vitamin D, as shown by normal serum levels of 25(OH)D. In fact, there is only one report in the medical literature suggesting that a heritable defect in hepatic 25-hydroxylase occurs in humans [78].

VDDR I and II feature, respectively, diminished biosynthesis of, and target tissue resistance to, $1,25(OH)_2D$. Because there is either a block in renal conversion of 25(OH)D to $1,25(OH)_2D$ (VDDR I) or peripheral resistance to $1,25(OH)_2D$ itself (VDDR II), serum levels of $1,25(OH)_2D$ that are low and high, respectively, readily distinguish these disorders [5,6,77]. Nevertheless, both types of VDDR alter mineral homeostasis in a similar way. Dietary Ca^{++} is malabsorbed, leading to hypocalcemia, secondary hyperparathyroidism, renal Pi wasting, and hypophosphatemia. Decreased extracellular fluid levels of Ca^{++} and Pi together impair skeletal mineralization. Because the pathogenesis of VDDR I involves defective production of $1,25(OH)_2D$ by the kidney, physiologic doses of $1,25(OH)_2D_3$ easily control the disorder [79]. However, in VDDR II, even enormous doses of $1,25(OH)_2D_3$ may prove ineffective [5,6], and Ca^{++} supplementation becomes crucial [80,81]. As discussed below, both VDDR I and II are now understood at the gene level [77].

Vitamin D–Dependent Rickets, Type I

In 1961, Prader and colleagues [82] reported two children with "pseudovitamin D deficiency" who had clinical, radiographic, and biochemical features of nutritional rickets yet responded only to large doses of vitamin D. The term "vitamin D dependency" was used later for this disorder (i.e., VDDR) [77]. Subsequently, with recognition of a different entity featuring tissue resistance to $1,25(OH)_2D$, these conditions became known as VDDR I and II, respectively [5,6,77]. Early reports of affected siblings in inbred kindreds indicated that VDDR I is an autosomal recessive condition (MIM 264700) [2]. Serum levels of $1,25(OH)_2D$ are low or undetectable despite normal levels of 25(OH)D and otherwise intact renal function. Because VDDR I is now understood at the molecular level, it is also called 1α-hydroxylase deficiency [2,77,79].

Clinical Presentation

Patients appear healthy at birth. However, features consistent with nutritional rickets are usually noticed before 2 years of age and often within the first 6 months of life. There is growth retardation and poor gross motor development. Muscle weakness, irritability, pneumonia, seizures, and failure to thrive are prominent findings [77,79,82].

Radiologic and Histopathologic Features

Radiographic changes are in keeping with nutritional rickets. In addition to growth-plate abnormalities and rachitic deformities, osteopenia and other features of secondary hyperparathyroidism are present [77,79,82]. Histological examination of nondecalcified bone specimens shows defective matrix mineralization and changes of hyperparathyroidism, including osteoclastosis and peritrabecular fibrosis [79].

Laboratory Findings

Malabsorption of dietary Ca^{++} leads to hypocalcemia, secondary hyperparathyroidism, renal Pi wasting, and hypophosphatemia. Serum ALP activity is elevated. Serum concentrations of 25(OH)D are normal in VDDR I (elevated if pharmacologic doses of vitamins D_2 or D_3 or $25(OH)D_3$ are given), but serum levels of $1,25(OH)_2D$ are subnormal or remain only partially corrected by vitamin D or 25(OH)D therapy [79]. Because high doses of vitamin D or $25(OH)D_3$ produce therapeutic responses in VDDR I, it seems that 25(OH)D (or some metabolite) at sufficient levels can activate the VDR (vitamin D receptor). Theoretically, enhanced local biosynthesis of $1,25(OH)_2D$ could also occur with pharmacologic doses of the prohormone.

Pathogenesis and Genetics

VDDR I is especially prevalent in French Canadians in whom a genetic founder effect seems manifest [83,84]. In 1990, linkage studies of five such families mapped VDDR I to chromosome 12q14 [79]. Although this is the chromosomal region of the VDR gene, the molecular defect in VDDR I involves the renal mitochondrial cytochrome $P450_{cl\alpha}$ enzyme that causes rate-limiting, hormonally regulated hydroxylation of 25(OH)D to $1,25(OH)_2D$ (i.e., the 25(OH)D,lα-hydroxylase) [81]. Actually, the 1α-hydroxylase enzyme has several components: cytochrome P-450D10t, ferredoxin, and ferredoxin reductase [79]. The complete structure of the $P450_{cl\alpha}$ gene has been reported, and several mutations have been found in this single-copy gene in VDDR I patients [83–85]. French Canadians are commonly homozygous for a 958ΔG mutation. Other ethnic groups harbor a variety of $P450_{cl\alpha}$ gene changes [84]. Although

quantitative and/or qualitative defects (e.g., decreased substrate affinity) are possible for the 25(OH)D,1α-hydroxylase enzyme, to date only absent activity has been documented [83,84].

Treatment

Clinical remission has followed high-dose therapy with 1–3 mg of vitamin D_2 or with 0.2–0.9 mg of $25(OH)D_3$ given orally each day. However, physiologic doses of $1,25(OH)_2D_3$, using 0.25–1.0 μg daily, bypass the lα-hydroxylase defect and provide effective treatment [79]. Because there is no problem with hepatic conversion of vitamin D_2 to $25(OH)D_2$, vitamin D_2 rather than $25(OH)D_3$ is an effective yet cheaper therapy. Nevertheless, $25(OH)D_3$ or $1,25(OH)_2D_3$ administration, although expensive, has advantages. The physiologic half-lives of these sterols are much shorter than for vitamin D_2. Excessive dosing will therefore respond more rapidly to temporary cessation of therapy. Most patients, however, can be managed with vitamin D_2, but periodic follow-up is essential.

Vitamin D–Dependent Rickets, Type II

This disorder was characterized in 1978 when a patient with features of "pseudovitamin D deficiency" was found to have high serum levels of $1,25(OH)_2D$ [86,87]. Thus, "hereditary resistance to 1,25-dihydroxyvitamin D" or VDDR II refers to this condition [5,6]. The presence and absence of alopecia now distinguish VDDR II types A and B, respectively (MIM 277420; 277440) [2].

Clinical Presentation

Most VDDR II patients have been from the Mediterranean region [5,6,77]. Autosomal recessive inheritance is well-established, and parental consanguinity has been reported in approximately 50% of cases [5,6,77]. Affected neonates appear normal but then develop features resembling VDDR I. Additionally, ~50% of affected newborns have hypotrichosis or alopecia, or develop this manifestation during the first few months of life. Oligodontia, epidermal cysts, and cutaneous milia are further findings [5,6]. Ectodermal abnormalities tend to occur with especially severe disease. Rickets is noted before 2 years of age, although several sporadic cases have developed skeletal problems as late as their teenage years or middle age. In general, the earlier the disease presentation, the more severe the clinical and biochemical features [5,6]. Obligate heterozygotes do not have clinical manifestations.

Radiologic and Histopathologic Features

Radiographic findings in VDDR II resemble those of nutritional rickets (i.e., growth-plate changes, rachitic deformities, osteopenia, and evidence of secondary hyperparathyroidism). Bone specimens have shown normal or increased numbers of osteoclasts but with sparse areas of bone resorption. In a patient with total alopecia, hair follicles were present [88,89].

Laboratory Findings

Hypocalcemia in VDDR II causes secondary hyperparathyroidism, hypophosphatemia, and elevated serum ALP activity. Additionally, circulating $1,25(OH)_2D$ levels are increased, sometimes as much as tenfold [5,6]. This abnormality reflects peripheral resistance to $1,25(OH)_2D$, causing malabsorption of dietary Ca^{++} and the combined effect of the three activators of renal 25(OH)D,1α-hydroxylase activity (i.e., hypocalcemia, increased serum PTH, and hypophosphatemia). Furthermore, some patients seem to have diminished feedback inhibition by $1,25(OH)_2D$ on the kidney 1α-hydroxylase itself [90].

Pathogenesis

In VDDR II, the nature of the resistance to $1,25(OH)_2D$ and aberrations in the VDR effector system have largely been elucidated [5,6,77]. Molecular studies show that a variety of VDR or post-VDR abnormalities block the target tissue actions of $1,25(OH)_2D$ [91–97]. There can be absence of the VDR, diminished $1,25(OH)_2D$ binding capacity or binding affinity by the VDR, or failure of the $1,25(OH)_2D$-VDR complex to localize to the nucleus and interact with DNA [5,6,77]. Hair follicles normally contain the VDR, but the pathogenesis of alopecia is not understood [5,6]. Patients lacking VDR expression are the most difficult to treat [5,6]. Interestingly, a VDR-positive, mild variant of this condition has been reported in Colombia [98]. A mouse model has been developed by targeted ablation of the *VDR* gene [99].

Treatment

If untreated, most patients with VDDR II die in early childhood [5,6]. However, good control of the disorder is possible with medical therapy, especially in those individuals without alopecia [77,89]. Patients with VDDR IIB (normal hair) can respond fully to pharmacologic doses of bioactive vitamin D metabolites [88,89]. However, only some patients with VDDR type IIA (total alopecia) do so. Remarkably, older patients with VDDR IIA may no longer need $1,25(OH)_2D_3$ therapy, or they may require only low doses later in life [5,6]. Depending on its severity, VDDR II may require management with calciferols that enhance endogenous production of $1,25(OH)_2D$, the administration of high doses of both calciferols and Ca^{++} to compensate for the target tissue resistance to $1,25(OH)_2D$, or high doses of Ca^{++} alone (given orally or intravenously) to circumvent the target cell $1,25(OH)_2D$ resistance [80,81,100–102]. Whereas most patients respond to very high oral doses of $1,25(OH)_2D_3$ (10–40 μg daily), some can experience clinical, radiographic, and biochemical corrections with high doses of vitamin D_2 or $25(OH)D_3$ [5,6]. There can be unexplained periods of disease fluctuation.

OSTEOBLAST DISORDERS

Hypophosphatasia

In 1948, the term "hypophosphatasia" was coined by J. C. Rathbun [103] to distinguish a lethal form of rickets featuring deficient activity of alkaline phosphatase (ALP) in serum and tissues (MIM 146300, 241500, 241510). We now know that hypophosphatasia is an inborn error of metabolism characterized biochemically by low levels of activity of the tissue-nonspecific (liver/bone/kidney) isoenzyme of ALP (TNSALP) [15,104]. At least 100 different mutations in the TNSALP gene have been identified in patients worldwide [104]. Hence, hypophosphatasia is an instructive disorder. It validates the theory first promulgated in 1923 by the discoverer of ALP, Robert Robison, that ALP conditions mineralization of skeletal matrix [105]. TNSALP is ubiquitous and especially rich in liver and kidney as well as in cartilage and bone, yet hypophosphatasia seems to affect directly only the skeleton and dentition [106].

Clinical Features

Approximately 300 cases of hypophosphatasia have been described [15]. It is apparent that the severity of this disorder is remarkably variable, spanning intrauterine death from profound skeletal hypomineralization to merely premature loss of teeth [15]. Five clinical types are reported,

depending primarily on the patient's age when skeletal disease is documented. Perinatal, infantile, childhood, and adult hypophosphatasia cause skeletal and dental disease [15]. Children and adults who manifest premature tooth loss without bone defects have odontohypophosphatasia. Although artificial and somewhat imprecise, this clinical classification, reviewed below, provides a sense of the patient's prognosis and serves as the foundation for a potentially more helpful molecular nosology [104].

Perinatal Hypophosphatasia

Perinatal hypophosphatasia is diagnosed at birth and is almost invariably lethal [15]. Polyhydramnios and stillbirth are common. Profound skeletal hypomineralization with caput membranaceum and short and deformed limbs is obvious. Occasionally, bony spurs protrude from the shafts of major long bones [107,108]. Severe osteogenesis imperfecta (see Chapter 8, this volume) or cleidocranial dysplasia (see Chapter 23, Part V, this volume) may be suspected but can be distinguished by radiographic studies [17]. Failure to gain weight, irritability with a high-pitched cry, unexplained fever, seizures, anemia, periodic apnea with bradycardia, and intracranial hemorrhage can occur. Respiratory compromise from pulmonary hypoplasia and chest deformity proves fatal.

Infantile Hypophosphatasia

Infantile hypophosphatasia presents before 6 months of age with poor feeding, failure to thrive, widened fontanelles, hypotonia, and rickets [15]. Hypercalcemia and hypercalciuria may explain bouts of vomiting and nephrocalcinosis. Sometimes there is significant renal impairment. Rachitic deformity of the chest and rib fractures predispose to recurrent pneumonia. Seizures and spells of apnea may occur. Despite the impression from palpation or radiographs that fontanelles are wide open, this is a reflection of skull hypomineralization. In fact, functional craniosynostosis is common. Infantile hypophosphatasia often features progressive clinical and radiographic deterioration, and approximately 50% of patients die within the first year of life. However, the prognosis seems better if there is survival past infancy [109].

Childhood Hypophosphatasia

Childhood hypophosphatasia is particularly variable [15]. Premature loss of deciduous teeth (< age 5 years is abnormal) from dysplasia or aplasia of cementum may be the only clinical manifestation. Cementum anchors the tooth root to the periodontal ligament. Therefore, teeth are shed without the usual root resorption. Incisors are usually lost first, and some young children are rendered nearly edentulous. Enlarged pulp chambers and root canals also result in "shell teeth" that are predisposed to infection in later life. Skeletal deformity can include scaphocephaly with frontal bossing, a rachitic rosary, bowed legs or knock-knees, short stature, and metaphyseal widening causing wrist, knee, or ankle enlargement. When radiographs disclose physeal and metaphyseal irregularity, delayed walking and a characteristic but unexplained waddling gait are common. Features of a static myopathy are often present. Childhood hypophosphatasia may improve when growth plates fuse after puberty, but recurrence of symptoms is likely during middle age [15,110]. It has been noted that in some families patients manifest limb bowing *in utero* that can be mistaken for perinatal hypophosphatasia or a life-threatening bone dysplasia [107,108].

Adult Hypophosphatasia

Adult hypophosphatasia generally presents during middle age. Approximately 50% of patients, however, recall rickets and/or premature loss of teeth during childhood. Often, they are troubled by recurrent, poorly healing, metatarsal stress fractures. Painful femoral pseudofractures may then be found proximally in the lateral cortices [17]. Chondrocalcinosis is common, but Ca^{++} pyrophosphate dihydrate crystal deposition less frequently causes arthritis or pseudogout. Premature shedding of teeth can occur [15].

Odontohypophosphatasia

Odontohypophosphatasia is diagnosed when there are dental manifestations but no radiologic or even histopathologic evidence of skeletal disease.

Radiologic Findings

Radiographs are helpful for diagnosing hypophosphatasia—especially in pediatric patients. Perinatal hypophosphatasia causes pathognomonic changes. The skeleton can be so hypomineralized that only the skull base is apparent. In other affected fetuses or newborns, individual vertebrae appear to be "missing" and bony spurs protrude from the shafts of major long bones. Alternatively, severe rachitic changes are seen. Calvarial bones can be mineralized only centrally, giving the illusion that cranial sutures are widely patent. Fractures are not uncommon. In infants, abrupt transition from well-mineralized diaphyses to hypomineralized metaphyses suggests sudden metabolic deterioration. Relentless skeletal demineralization, worsening rachitic disease, and progressive bony deformity predict a lethal outcome. Bone scintigraphy showing little tracer uptake in widely separated cranial "sutures" suggests their functional closure [111]. Patients who survive infancy can have true premature pan-suture fusion, causing a "beaten-copper" radiographic appearance and raised intracranial pressure (Fig. 7). In children, characteristic "tongues" of radiolucency extend from the physes into the metaphyses of major long bones (Figs. 3 A,B). Adult hypophosphatasia can cause recurrent, poorly healing, metatarsal stress fractures as well as osteopenia, chondrocalcinosis with changes of pyrophosphate arthropathy, calcific periarthritis, and proximal femoral pseudofractures that occur laterally (rather than medially as in other forms of osteomalacia) [17].

Laboratory Findings

Subnormal serum ALP activity for age and sex (hypophosph*atasemia*) is the biochemical hallmark of hypophosphatasia. In fact, the ALP level generally reflects the disease severity [15].

Patients with odontohypophosphatasia have only mild hypophosphatasemia. Nevertheless, this finding is especially impressive because rickets or osteomalacia typically cause *hyper*phosphatasemia [1]. Several other disorders, some with skeletal manifestations, lower blood ALP levels [15], but these conditions should be readily differentiated from hypophosphatasia, and such patients do not accumulate TNSALP substrates endogenously (see below).

Serum levels of Ca^{++} and Pi are not diminished. Indeed, hypercalciuria and hypercalcemia often complicate the infantile form. The pathogenesis seems to involve a "dyssynergy" between gut absorption of Ca^{++} and defective skeletal mineralization; however, skeletal demineralization may also be a factor. Circulating levels of PTH, 25(OH)D, and $1,25(OH)_2D$ are normal unless there is hypercalcemia or renal compromise [112]. Serum Pi concentrations are above

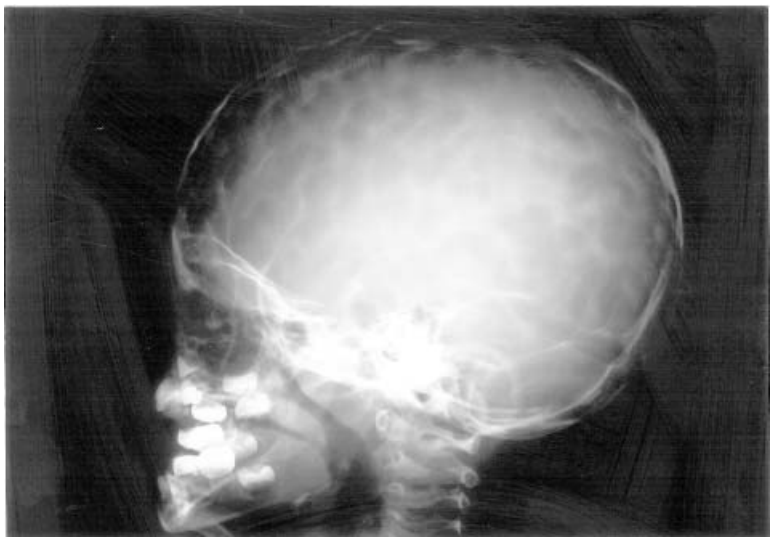

Figure 7. Hypophosphatasia. Lateral view of the skull of this 4-year-old girl with infantile hypophosphatasia shows a ''beaten copper'' appearance characteristic of pan-suture closure in the cranium.

control mean levels, and mild hyperphosphatemia is noted in approximately one-half of affected children and adults. The pathogenesis reflects enhanced renal reclamation of Pi, sometimes associated with suppressed PTH levels [113].

Three phosphocompounds, putative natural substrates for TNSALP, accumulate endogenously in hypophosphatasia: phosphoethanolamine (PEA), inorganic pyrophosphate (PPi), and pyridoxal 5′-phosphate [PLP] (Fig. 8) [15,106,114]. Preliminary evidence suggests that there may be several more [115]. Commercial laboratories will assay urinary PEA and plasma PLP. Although accumulation of PPi seems to be the key pathogenetic factor in hypophosphatasia (see below), quantification of PPi remains a research technique. Increased plasma PLP concentration is a sensitive and specific marker for hypophosphatasia [114]. However, subjects must not be taking vitamin B_6 when tested. Mild phosphoethanolaminuria can occur in several additional metabolic bone diseases, including postmenopausal or idiopathic osteoporosis [116].

Histopathologic Findings

Defective skeletal mineralization characterizes all clinical forms of hypophosphatasia except odontohypophosphatasia [15]. Unless assessment of the ALP activity in bone is undertaken, the histologic findings are those of other types of rickets or osteomalacia without secondary hyperparathyroidism [117].

Pathogenesis

ALP [orthophosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] is found in nearly all organisms as a glycosylated, plasma membrane–bound ectoenzyme [15,106]. The discovery that three phosphocompounds — PEA, PPi, and PLP — accumulate endogenously in hypophosphatasia revealed how TNSALP probably functions [15,106]. Accumulation of PLP, the principal cofactor of vitamin B_6, indicates that TNSALP acts primarily on cell surfaces [118,119]. Except for seizures in severely affected infants, patients with hypophosphatasia do not have symptoms or signs of vitamin B_6 deficiency or toxicity despite markedly increased plasma PLP levels [114]. In 1965, the discovery that urinary levels of PPi are elevated in hypophosphatasia disclosed a possible pathogenesis for the skeletal disease [120]. PPi is a potent inhibitor of biomineralization [4,120]. In patients, matrix vesicles are devoid of ALP activity but do contain hydroxyapatite crystals [121]. However, only a few isolated crystals are observed outside these extracellular structures. Hence, PPi could be blocking hydroxyapatite crystal formation in the extracellular matrix of bone [121].

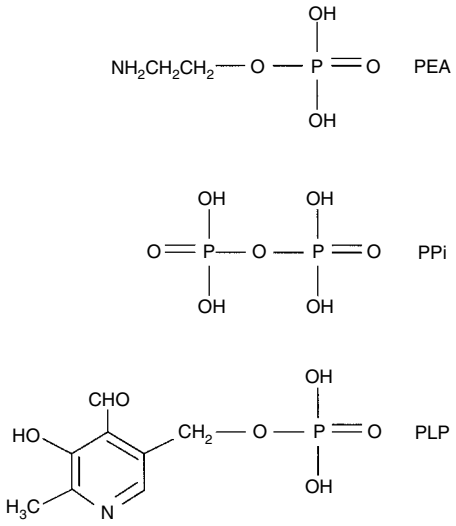

Figure 8. Natural substrates for TNSALP (tissue nonspecific alkaline phosphatase isoenzyme). Three phosphocompounds — phosphoethanolamine (PEA), inorganic pyrophosphate (PPi), and pyridoxal 5′-phosphate (PLP) — accumulate endogenously in hypophosphatasia, suggesting that they are natural substrates for TNSALP. Note: bisphosphonates are analogs of PPi in which the central oxygen atom is replaced by a carbon atom, rendering them resistant to ALP cleavage (See Chapter 1, Part III, this volume).

Genetics

Hypophosphatasia occurs worldwide. It is most common in Mennonites and Hutterites in Canada, among whom the incidence of severe disease is approximately 1/2,500 live births [122]. The incidence among the general population in Toronto is estimated to be 1/100,000 [123]. In our experience, hypophosphatasia seems to be especially rare in blacks.

Perinatal and infantile forms are transmitted as autosomal recessive traits. Obligate carriers can have diminished or low-normal levels of serum ALP activity and sometimes demonstrate elevations in plasma PLP levels, especially after a vitamin B_6 challenge [122].

The inheritance pattern(s) of the childhood, adult, and odonto forms of hypophosphatasia may be more complex [15,104]. Clearly, some cases reflect autosomal recessive inheritance. In others, however, there has been generation-to-generation transmission of biochemical features of the disorder, occasionally with mild clinical expression. Accordingly, autosomal dominant inheritance has also been reported [107,108,124,125].

The gene for TNSALP exists as a single copy in the haploid genome on the tip of the short arm of chromosome 1 (lp36.1-lp34) [126]. In 1988, a missense mutation was identified in a severely affected homozygous infant from an inbred Canadian kindred [126]. Studies of individuals with hypophosphatasia have disclosed more than 100 different mutations in the TNSALP gene [104]. Perinatal and infantile hypophosphatasia patients are homozygotes or compound heterozygotes. Molecular studies confirm that the childhood and adult forms of hypophosphatasia can indeed be the "same" disease [110]. A mouse model that recapitulates the infantile form of hypophosphatasia has been developed by TNSALP gene knockout [127].

Treatment

Conventional treatments for rickets or osteomalacia are probably best avoided in hypophosphatasia because patients are usually vitamin D replete and serum levels of Ca^{++} and Pi are not reduced [15]. Indeed, such therapies could exacerbate or provoke hypercalcemia and hypercalciuria. Hypercalcemia in infantile hypophosphatasia generally responds to reduction in dietary Ca^{++} intake but may require glucocorticoid or calcitonin therapy. Enzyme replacement by intravenous infusion of various forms of ALP has generally been disappointing [15,108,128,129]. One infant who seemed destined to die from infantile hypophosphatasia showed clinical and radiographic improvement after bone marrow and stromal cell transplantation [109].

Supportive therapy is important for hypophosphatasia. Fractures do mend, but delayed healing after casting or osteotomy has been observed. Placement of an intramedullary rod, rather than load-sparing devices such as plates, seems preferable for immediate or prophylactic treatment of femoral fractures or pseudofractures [17]. Expert dental care is especially important for children because their nutrition and speech development can be impaired by premature tooth loss. Dentures may be necessary. Craniotomy can prevent brain damage in cases with functional or true craniosynostosis. Naproxen reportedly helps pain and weakness of the lower limbs in affected children [130].

Genetic Counseling/Prenatal Diagnosis

Severely affected fetuses (perinatal form) have been detected *in utero* by ultrasonography and by assay of ALP activity in chorionic villi or amniotic fluid cells [131]. TNSALP gene mutation analysis holds promise for improved prenatal diagnosis, including for the infantile form [104].

Axial Osteomalacia

Axial osteomalacia (MIM 109130) is characterized radiographically by a coarse trabecular pattern in the axial but not the appendicular skeleton [132]. Fewer than 20 case reports have been published. Most have reflected sporadic disease, but dominant transmission is possible [133].

Clinical Presentation

Patients with axial osteomalacia generally have been middle-aged or elderly men; a few affected middle-aged women have been described. Radiographic manifestations would probably be detectable earlier in life [133]. The majority of cases have presented with vague, dull, chronic axial bone pain (often in the cervical region) that prompted x-ray studies. Family histories are usually negative for skeletal disease.

Radiologic Features

Disturbances are essentially confined to the spine and pelvis, where trabeculae are coarsened with a pattern resembling that found in other types of osteomalacia [134]. However, Looser's zones have not been reported. The cervical spine and ribs seem to contain the most severely affected bones. Two patients also had features of ankylosing spondylitis [135]. Radiographic survey of the appendicular skeleton is unremarkable.

Laboratory Studies

In four patients, serum Pi levels tended to be low [135]. In others, osteomalacia occurred despite normal serum levels of Ca^{++}, Pi, 25(OH)D, and $1,25(OH)_2D$. Serum ALP (bone isoenzyme) may be increased.

Histopathologic Findings

Iliac crest specimens have distinct corticomedullary junctions, but the cortices can be especially wide and porous. Trabeculae are of variable thickness, and total bone volume may be increased. Collagen has a normal lamellar pattern on polarized-light microscopy. There is increased width and extent of osteoid seams throughout. Tetracycline labeling confirms the defective skeletal mineralization, producing fluorescent "labels" that are single, irregular, and wide [133]. Osteoblasts are flat and apparently inactive "lining" cells, with reduced Golgi zones and rough endoplasmic reticulum, and increased amounts of cytoplasmic glycogen, but they do stain intensely for ALP activity. Changes of secondary hyperparathyroidism are absent [133].

Etiology and Pathogenesis

Axial osteomalacia is possibly due to an osteoblast defect [118]. Electron microscopy of iliac crest from one patient revealed osteoblasts that appeared inactive but had been able to release matrix vesicles within the abundant osteoid [116].

Treatment

Effective medical therapy has not been reported for axial osteomalacia. The natural history, however, seems relatively benign. Long-term follow-up of one patient showed that symptoms and radiographic findings did not change [136]. Methyltestosterone and stilbestrol have been tested unsuccessfully [136]. Vitamin D_2 (as much as 20,000 U/day for 3 years) was similarly without benefit [136]. Slight improvement in skeletal histology, but not in symptoms, was reported for calcium and vitamin D_2 therapy in a study of four cases [135].

RECENT DEVELOPMENTS

Major recent advances in relation to heritable rickets and osteomalacia include further understanding of the molecular bases of these conditions and additional insight into the phosphaturic factor(s) that cause some forms of hypophosphatemic bone disease [137].

Autosomal Dominant Hypophosphatemic Rickets

Autosomal dominant hypophosphatemic rickets (ADHR) is due to missense mutations in the gene encoding fibroblast growth factor 23 (FGF23) [138]. Proteolytic cleavage of FGF23 is prevented, resulting in its enhanced biological activity [138]. Oncogenic osteomalacia tumors have been found to contain high levels of FGF23 mRNA. Hence, this secreted protein is a candidate "phosphatonin" [139,140]. The potential role of FGF23 in acquired and inherited hypophosphatemic bone disease was reviewed in 2001 [141].

McCune-Albright Syndrome

Evidence has been published that the hypophosphatemic rickets which sometimes complicates McCune-Albright syndrome (MAS) is caused by a humoral factor [142]. Medium conditioned by lesional bone cells in culture diminished inorganic phosphate (Pi) transport in several test systems, but did not alter Pi transport in the kidney. The authors concluded "it is unlikely that the mechanism of hypophosphatemia in MAS is related to a renal phosphate leak... it is possible that the intestine is the target site..." [142]. Apparently they were unaware of our preliminary report in 1996 [76] documenting that significant Pi wasting is acquired in MAS, perhaps due to a circulating factor emanating from the expanding mass of fibrodysplastic tissue.

X-Linked Hypophosphatemia

In X-linked hypophosphatemia (XLH), phenotype-genotype correlation is difficult because of variability in pharmacologic regimens, compliance with medical therapy, and orthopedic interventions [143]. Nevertheless, as discussed in our preliminary report in 1999, *PHEX* mutations which compromise the structure of the encoded PHEX protein seem to produce the most severe clinical and biochemical disease [55]. *PHEX* mutations can lead to trapping of the encoded protein in the endoplasmic reticulum, precluding terminal glycosylation [144]. A preliminary report indicates that frizzled-related protein (FRP-4) inhibits Pi uptake and is a substrate for PHEX [145].

In children with XLH, dipyridamole treatment was found not to improve renal Pi wasting [146], and acute challenges with growth hormone only slightly altered parameters of Pi homeostasis [147]. However, growth hormone therapy given until physes fused reportedly caused a transient improvement in Pi homeostasis and had a positive effect especially on poor growth [148]. In a preliminary report, the synthetic vitamin D analog ED-71, 2β-(3-hydroxypropyl)-1α, 25-hydroxyvitamin D_3, seemed helpful for the *Hyp* mouse model of XLH [149]. Urinary oxalate and Pi concentrations are increased in XLH during $1,25(OH)_2D_3$ and Pi supplementation treatment but do not reflect supersaturation of these molecules, and the levels seem unable to predict the likelihood of nephrocalcinosis [150].

Vitamin D-Dependent Rickets

A murine model of vitamin D-dependent rickets, type I (VDDR I), has been reported as a consequence of the targeted ablation of the 1α–hydroxylase gene [151]. Reductions in CD4–and CD8–positive peripheral T lymphocytes occur, and female mice are infertile due to uterine hypoplasia and absent corpora lutea [151].

In vitamin D-dependent rickets, type II (VDDR II), specific vitamin D analogs seem promising for partially or completely restoring the responsiveness of the mutated vitamin D receptor (VDR) [152].

Hereditary Hypophosphatemic Rickets With Hypercalciuria

Hereditary hypophosphatemic rickets with hypercalciuria (HHRH) does not involve mutations in the gene encoding the type II sodium/phosphate co-transporter (NPT 2) [153,154].

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Chapter 19

Osteopetrosis

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SUMMARY

- **Osteopetrosis results from diminished osteoclast action. Defective skeletal resorption is revealed by the persistence of primary spongiosa deposited during endochondral bone formation. Consequently, enlargement of marrow spaces and neural foramina is compromised, and bone quality is reduced because skeletal remodeling is uncoupled.**
- **Complications of osteopetrosis can include myelophthisic compromise leading to anemia, infection and bleeding, cranial nerve deficits, recurrent fractures, and osteomyelitis. Severe disease can cause hypocalcemia.**
- **Infantile ("malignant") and adult ("benign") osteopetrosis, inherited as autosomal recessive and autosomal dominant traits, respectively, are best known. However, there are at least eight types of human osteopetrosis spanning a great range of disease severity. Neurodegenerative disease or renal tubular acidosis can complicate additional rarer forms.**
- **Although osteopetrosis is usually heritable, transient *in utero* and perhaps post-infectious forms of unknown etiology occur sporadically.**
- **The genetic basis is established only for a subset of patients with malignant osteopetrosis and for carbonic anhydrase II (CA II) deficiency. Mutations of the *TCIRG1* gene, that encodes a subunit of the vacuolar proton pump, and the *CA2* gene compromise acidification of the osteoclast pericellular milieu thereby impairing bone resorption.**
- **Several naturally occurring animal models as well as increasing numbers of gene knockout mice have osteopetrosis and provide insight concerning osteoclast formation and action.**
- **Malignant osteopetrosis, featuring nonfunctioning osteoclasts, can be cured by bone marrow transplantation. Pharmacologic treatment may involve high-doses of calcitriol with a diet low in calcium, interferon gamma 1b, and/or prednisone therapy. Hyperbaric oxygenation can help heal osteomyelitis.**
- **Prenatal diagnosis will require molecular information. Radiologic procedures are generally not useful.**

INTRODUCTION

In connective tissues, extracellular matrix is comprised of a rich assortment of macromolecules that together provide scaffolding for the body. While achieving its purpose, this "ground substance" also influences the metabolism and the behavior of cells embedded within this complex material that was once regarded as inert [1].

In the skeleton, the composition and physiological role of extracellular matrix is more complicated compared with connective tissue elsewhere because bone contains mineral [2,3]. Hydroxyapatite crystals become arranged in type I collagen fibers and interact with a variety of specialized noncollagenous proteins giving the body both rigid support and protection [2–4]. Furthermore, osseous connective tissue is unique because it participates in several systemic processes [5,6]. Bone is a reservoir for calcium, phosphate, bicarbonate, magnesium, and other ions [2,3], and hormonally regulated, cell-mediated events may recruit the skeleton to either release or store these constituents during health or disease [5,6]. Accordingly, among the connective tissue disorders, those that involve bone can reflect metabolic or endocrine disturbances and demonstrate novel clinical manifestations [6].

Three cell-mediated processes — growth, modeling (shaping), and remodeling (turnover) — are the foundation for the two major physiological functions of the skeleton distinguished above [2,7,8]. Structural integrity is an important reflection of the outcome of growth and modeling of individual bones [2]. Biochemical interactions are dependent on remodeling of osseous tissue [2,7,8]. The skeleton is formed when chondrocytes or osteoblasts synthesize matrix in cartilage or in bone, respectively, which then mineralizes [2,8]. Resorption occurs when multinucleated osteoclasts create an acidic pericellular milieu to leach mineral from these tissues and then immediately degrade the decalcified matrix [8–13]. Skeletal remodeling is the sequence of bone breakdown followed by bone formation occurring lifelong and involving both cortical (compact) and trabecular

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(spongy) bone [2,7,8,13]. At the microscopic level, this process takes place in discrete bone remodeling units [2,7]. In health, turnover is precisely regulated by an intricate system of increasingly understood humoral and mechanical factors acting in concert to preserve the structural function of the skeleton while assuring mineral homeostasis [5,6,8].

A great variety of disorders disturb the skeleton by adversely affecting growth, modeling, or remodeling of bone, or by altering the biochemical environment necessary for matrix mineralization (see also Chapters 8,18,21,23, this volume) [6–8,14,15]. Among the heritable connective tissue diseases, one group of conditions features impaired resorption of skeletal tissue. Defects in osteoclast-mediated degradation of cartilage and bone limit modeling and "uncouple" remodeling, causing pathological increases in skeletal mass [11,12]. Such disturbances result clinically in *osteopetrosis*. Hypothetically, osteopetrosis could be the result of aberrations in the genesis, proliferation, differentiation, or fusion of precursor cells to form multinucleated osteoclasts or reflect inactivity of mature osteoclasts themselves [8,11,12,16–18].

This chapter provides an overview of the various types of osteopetrosis, emphasizing the forms encountered in humans.

HISTORY

Osteopetrosis was first described in a single-paragraph, unillustrated, case report published in 1904 [19] by the German radiologist Heinrich Ernst Albers-Schönberg (1865–1921) [20]. He recounted how diffusely dense bones, including some peculiar circular areas of osteosclerosis, were discovered by röntgenography in a 26-year-old man who broke his femur stepping into a shallow hole [19]. Accordingly, the eponym *Albers-Schönberg disease* increasingly refers to the mild, autosomal dominant form of this disorder (see below) [21].

Soon after Albers-Schönberg's report, the memorable but orthopedically inaccurate term *marble bone disease* captured the solid x-ray appearance of the affected skeleton. An improved alternative, *osteopetrosis*, was coined in 1926 when Karshner sought to emphasize the associated bone fragility [22].

Many conditions are now known to engender high bone mass (Table 1). Despite progress in elucidating the etiology and pathogenesis of a number of these disorders [23], the term osteopetrosis is, unfortunately, still being used generically for any type of increase in skeletal density. However, true forms of marble bone disease are caused by deficiency of osteoclast-mediated resorption of cartilage and bone (as described below) [17,18,24–26]. Hence, the term osteopetrosis reflects a specific pathogenesis and can be used with precision.

Osteopetrosis of any type is rare. Prompted by its remarkable clinical and radiographic manifestations, more than 500 case reports have been published [27]. By the 1960s, two major clinical types had been delineated: an autosomal dominant "benign" (adult) form (MIM 166600) that can be asymptomatic [25] and likely affected Albers-Schönberg's patient [19], and an autosomal recessive "malignant" (infantile) form (MIM 259700) that, unless treated, usually kills during infancy or childhood [28]. Benign osteopetrosis has an estimated prevalence of 1 per 100,000–500,000 in the United States [25]. In Europe, the figure is approximately 1 per 20,000 [29]. Malignant osteopetrosis is even rarer [30], with an incidence of about 1 per 500,000 in North America and perhaps 1 per 200,000 elsewhere [29,30]. Subsequent to these relatively early descriptions and characterizations of marble bone disease, additional phenotypes were elucidated as summarized below [31].

In 1972, osteopetrosis with renal tubular acidosis was described in three separate preliminary reports [32]. Comprehensive accounts of this syndrome were first published in 1980, when cerebral calcification was recognized to be the third principal feature [32,33]. In 1983, this disorder was discovered to be a new inborn error of metabolism characterized by carbonic anhydrase II (CA II) isoenzyme deficiency (MIM 259730) [34,35].

Beginning in 1979, cases of "intermediate" osteopetrosis were reported [36–38]. This form of marble bone disease presents during childhood with some of the symptoms and signs of the malignant type. Although survival to adulthood is likely, lifespan seems uncertain and variable [37,38]. Intermediate osteopetrosis seems to be transmitted as an autosomal recessive trait (MIM 259710) [37,38].

Even rarer types of marble bone disease have been delineated. In 1986, osteopetrosis that proved lethal *in utero* was described in stillborn siblings who were conceived by Moroccan first cousins [39]. In 1987, benign osteopetrosis was reported from Denmark to manifest in two autosomal dominant forms, types I and II [29,30]. In 1988, malignant osteopetrosis complicated by neuronal storage disease was characterized [40]. This disorder seems to be inherited as an autosomal recessive trait. In 1990, it was hypothesized that osteopetrosis in humans could follow retroviral infection [41]. Also, a "transient infantile" type of osteopetrosis, which seems to develop sporadically *in utero*, was first reported in 1991 [42]. In 1995, it was proposed that there is an autosomal dominant, type III ("centrifugal") form of benign osteopetrosis that affects primarily the limbs [43]. In fact, recent gene mapping and locus exclusion studies have demonstrated further genetic heterogeneity for benign osteopetrosis [44] (see below). Finally, osteopetrosis has occurred with other disorders [45,46], but it is uncertain whether such cases represent new diseases, or merely chance associations. Accordingly, the nosology for the human forms of osteopetrosis is becoming complex [23,31]. It seems that at least eight entities have been distinguished on the basis of their clinical/genetic features (Table 2), and other types of marble bone disease will surely be delineated or discovered.

The pathogenesis of osteopetrosis focuses on diminished osteoclast-mediated resorption of cartilage and bone as revealed many years ago by histopathologic studies that showed unresorbed primary spongiosa deposited during endochondral bone formation [7,17,18,26]. This concept was advanced significantly by the observations of Donald Walker published between 1973 and 1975, when he demonstrated that parabiosis or transplantation of bone marrow or spleen cells from normal to osteopetrotic animals cured their disease [47]. The origin of the osteoclast was revealed to be a circulating, marrow-derived cell [11,16,48].

Despite a variety of subsequent important advances in osteopetrosis — improved understanding of osteoclast ontology and function [11,16], the discovery or creation of animal models [8,9,11,12,16–18,26,48,49], and successful treatment of severe disease by bone marrow transplantation [50–57] or by hormonal approaches [23,49] — precisely why skeletal breakdown is compromised in patients remains largely unknown. Only CA II deficiency [35] and malignant osteopetrosis in some patients [58] are understood at the molecular and biochemical levels. Indeed, as noted previously, it seems that not all types of human osteopetrosis have

TABLE 1. Disorders that Cause High Bone Mass[1]

Disorder	MIM No.
Dysplasias and Dysostoses:	
Autosomal dominant osteosclerosis	
Central osteosclerosis with ectodermal dysplasia	
Craniodiaphyseal dysplasia	218300,122860
Craniometaphyseal dysplasia	218400,123000
Dysosteosclerosis	224300
Endosteal hyperostosis	239100,144750
Van Buchem disease	239100
Sclerosteosis (craniotubular hyperostosis)	269500
Frontometaphyseal dysplasia	305620
Infantile cortical hyperostosis (Caffey disease)	114000
Lenz-Majewski hyperostotic dwarfism	151050
Melorheostosis	155950
Metaphyseal dysplasia (Pyle disease)	265900
Mixed-sclerosing bone dystrophy	
Oculodento-osseous dysplasia	164200
Osteodysplasia of Melnick and Needles	
Osteoectasia with hyperphosphatasia (hyperostosis corticalis)	239000
Osteomesopyknosis	166450
Osteopathia striata	166500
Osteopetrosis	166600, 259700, 259720, 259710, 600329, 259730
Osteopoikilosis	166700
Pachydermoperiostosis	167100
Progressive diaphyseal dysplasia (Camurati-Engelmann disease)	131306
Pyknodysostosis	265800
Tubular stenosis (Kenny-Caffey syndrome)	127000,244460
Metabolic Disorders:	
Carbonic anhydrase II deficiency	259730
Fluorosis	
Heavy metal poisoning	
Hepatitis C-associated osteosclerosis	
Hypervitaminosis A,D	
Hyper-, hypo-, and pseudohypoparathyroidism	
Hypophosphatemic osteomalacia	
Milk-alkali syndrome	
Renal osteodystrophy	
X-linked hypophosphatemia	307800
Other:	
Axial osteomalacia	109130
Diffuse idiopathic skeletal hyperostosis (DISH)	106400
Fibrogenesis imperfecta ossium	
High bone mass	601884
Ionizing radiation	
Leukemia	
Lymphoma	
Mastocytosis	
Multiple myeloma	
Myelofibrosis	
Osteomyelitis	
Osteonecrosis	
Paget disease	167250
Polycythemia rubra vera	263300
Sarcoidosis	
Sickle cell disease	141900
Skeletal metastases	.
Tuberous sclerosis	191100

[1]Heritable disorders are designated by MIM nos. [27].

TABLE 2. Forms of Human Osteopetrosis

Type	Inheritance[1]	MIM No.
Benign (adult)	AD	
? Type I[2]		
Type II Albers-Schönberg disease (at least two loci)		166600
? Type III (''centrifugal'' form)[2]		
Malignant (infantile)	AR	259700
Carbonic anhydrase II deficiency	AR	259730
Intermediate	AR	259710
Lethal *in utero*	AR	259720
Malignant with neuronal storage disease	AR	600329
Transient infantile	Sporadic	
Postinfectious	Sporadic	
Other		

[1]AD, autosomal dominant; AR, autosomal recessive.
[2]?, Uncertain validity.

a genetic basis. Sporadic cases are relatively common, and some evidence suggests that a disturbance *in utero* [42] or a viral infection [41] can also cause marble bone disease (see below). The nosology for the human osteopetroses outlined in Table 2 will surely require expansion as additional variations are discovered and more becomes known about their etiology.

CLINICAL PRESENTATION

Malignant Osteopetrosis (MIM 259700)

Malignant osteopetrosis typically presents during infancy [28,52,54,57,59]. This disorder is inherited as an autosomal recessive trait (Table 2) [27]. Nasal stuffiness resulting from underdevelopment of the mastoid and paranasal sinuses is often the earliest symptom. Soon after, because cranial foramina cannot widen fully, palsies of the optic, oculomotor, auditory, or facial nerves can occur [28,59–63]. Failure of the skeleton to model properly (including formation of medullary cavities) together with marrow crowding by increased numbers of excessively large but ineffective osteoclasts and accumulation of fibrous tissue cause a myelophthisic disturbance in blood formation. Patients survive because of extramedullary hematopoiesis, but secondary hypersplenism exacerbates the marrow-based anemia [28,50,54,57,59]. Furthermore, although less well understood, hemolytic disease can occur. Leukopenia contributes to recurrent infections and thrombocytopenia leads to bruising and bleeding. There is failure to thrive. Some children are troubled by hydrocephalus [63], and sleep apnea has been described [64]. Because basal foramina in the skull do not widen properly, symptoms of cerebral ischemia can be the result of arterial stenosis [65]. Other neurological manifestations of malignant osteopetrosis include developmental delay, mental retardation, cerebral atrophy with irritability, dysarthria, and hypocalcemic tetany (see below) [63]. Vision can be impaired also by retinal degeneration, long-standing papilledema, or failure of myelinization of the optic nerves [66]. Deafness may reflect conductive defects secondary to sclerosis of middle ear ossicles or impaired drainage of the eustachian tubes [67]. Eruption and shedding of the deciduous dentition is delayed or prevented because osteoclasts fail to resorb tooth roots, or because teeth become ankylosed within a sclerotic jaw. Consequently, osteomyelitis of the mandible is a frequent complication [68]. Poor blood flow predisposes the skeleton to infection. Despite their dense appearance on radiographic study, bones are fragile and fracture is common [69]. Developmental coxa vara can be another difficult orthopedic problem [70].

Physical examination typically reveals short stature, frontal bossing with a large head, ''searching'' nystagmus, proptosis, strabismus, optic atrophy, papilledema, anosmia, facial nerve palsy, a protuberant abdomen resulting from hepatosplenomegaly, and knock-knee deformity (Fig. 1). The posterior nasopharynx may be small. Obligate mouth breathing reflects obstruction of the upper airway [28,36,59,61,63].

Children who are unsuccessfully treated usually die during the first decade of life from severe anemia, bleeding, pneumonia, or sepsis [28,59,61,71]. Hematological abnormalities with blindness by 3 months of life seem to herald a fatal outcome [71]. However, visual deficits alone do not predict early death [71]. As many as 30% of patients

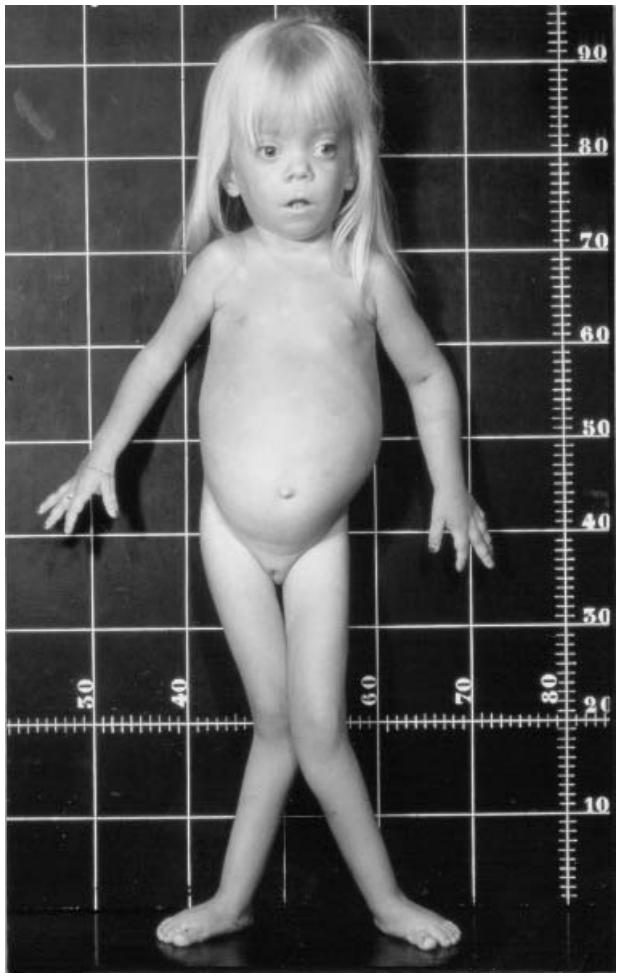

Figure 1. Osteopetrosis (malignant form). This 5-year-old girl has macrocephaly, divergent strabismus, short stature, genu valgum, a protuberant abdomen because of hepatosplenomegaly, and anemia, thrombopenia and leukopenia (Courtesy B. Steinmann, Zurich.)

given a diagnosis of malignant osteopetrosis survive to 6 years of age and, although handicapped, occasionally some live to the second or third decade of life [62,71,72].

Benign Osteopetrosis (MIM 166600)

Benign osteopetrosis features radiographic abnormalities that increase during childhood or adult life [25,30,73]. Rarely, disturbances are documented by x-ray at birth [74]. This condition is inherited as an autosomal dominant trait (Table 2) [27]. Extensive studies beginning in the 1980s of families with dense bones in Denmark suggested to the investigators that two entities were distinguishable by their clinical, radiographic, and biochemical findings [29,30,75–77]. Type II disease seems to be the disorder described by Albers-Schönberg [19]. However, the author remains uncertain whether the especially rare type I disease is truly a form of osteopetrosis. Furthermore, in 1995, a type III (''centrifugal'') form of benign osteopetrosis was proposed (Table 2), although no histopathological or biochemical evidence of impaired bone resorption was presented [43]. Indeed, in 1997, a mixture of phenotypes was reported in a Norwegian family, making subclassification of autosomal dominant osteopetrosis, discussed below, even less secure [78].

Type I disease, identified in several kindreds, features a generalized increase in bone mass, including within the calvarium [30,77]. Symptoms appear with aging and include bone pain, headache, discomfort in the back, and cranial nerve palsies, but patients are unlikely to experience fractures [30,77].

Type II disease is characterized radiographically by sclerosis of the skull base and pelvis and a ''rugger-jersey'' spine [30,73,77]. Affected individuals may be asymptomatic [25], but more often experience clinical manifestations, often including fractures (especially ''chalk-stick'' breaks in the femur) [19,73]. In fact, index cases are typically men, perhaps reflecting their predisposition to trauma compared with women [29,30]. Often, fractures manifest delayed healing or malunion [25,77]. Occasionally, there may be deafness, loss of vision, facial nerve palsy, psychomotor delay, osteomyelitis of the mandible, osteoarthritis, spondylolysis/spondylolisthesis, carpal-tunnel syndrome, or hypogonadotropic hypogonadism [25,73,79–81]. Coxa vara, sometimes with osteoarthritis of the hip, and scoliosis are common [70,73,81]. In a few kindreds, the disorder has been nonpenetrant in some individuals; i.e., they have unremarkable radiographic findings despite obvious skeletal disease in their predecessors and their offspring [25,30]. Bone densitometry may not disclose their status [82]. Conversely, severely affected children have been described in families with otherwise benign disease [83]. In Europe, the term ''benign'' is proving to be a misnomer for this type of osteopetrosis [73].

Intermediate Osteopetrosis (MIM 259710)

Intermediate osteopetrosis can cause short stature and some patients develop mild or, occasionally, moderately severe anemia, ankylosed teeth with osteomyelitis of the jaw, macrocephaly with cranial nerve palsies, and recurrent fractures [36–38]. Hepatosplenomegaly is reportedly rare [84], but can develop with myelophthisic anemia (personal observation). This disorder is inherited as an autosomal recessive trait (Table 2) [27]. The long-term outcome is poorly understood.

Osteopetrosis with Neuronal Storage Disease

Osteopetrosis with neuronal storage disease exhibits features of the malignant form but manifests additionally with cerebral and optic atrophy, ventricular dilatation, retinal pigmentary changes, hypotonia or hypertonia, central apnea, and seizures [62,71,85]. The disorder is inherited as an autosomal recessive trait (Table 2). Accumulation of ceroid lipofuscin and storage of both carbohydrates and lipids in neurons has been reported [86]. Bone marrow transplantation [62] and pharmacologic efforts to improve osteoclast function [84] are ineffective. The condition is fatal before 2 years of age [40,62,71,85]. Testing to exclude other neuronal storage disorders should be considered when osteopetrosis features early, severe, neurological abnormalities [62].

Osteopetrosis Lethal *In Utero*

Osteopetrosis lethal *in utero* is probably inherited as an autosomal recessive trait (Table 2). It causes fractures prenatally followed by stillbirth and is the one type of marble bone disease that can be detected early during gestation by ultrasonography because of dense, broken bones and hydrocephaly [39].

Carbonic Anhydrase II Deficiency (MIM 259730)

Carbonic anhydrase II (CA II) isoenzyme deficiency is an inborn error of metabolism caused by a variety of defects in the *CA2* gene [35]. This disorder is inherited as an autosomal recessive trait (Table 2) [27]. Because the etiology is certain, its manifestations, including differences between siblings and ethnic groups [35], can be described with confidence.

CA II deficiency was first reported as a distinctive syndrome in three independent preliminary publications that appeared in 1972 from the United States and Europe [32,87]. The patients had a combination of osteopetrosis and renal tubular acidosis that seemed to be a new autosomal recessive disorder [32]. Cerebral calcification was recognized to be the third principal feature of the syndrome when the first detailed descriptions were published in 1980 [32,33]. Consequently, the memorable but insensitive term ''marble brain disease'' has been used to describe this genetic disorder [33,88]. In 1983, the discovery that three American sisters had each inherited profound deficiency of CA II [34] represented our first understanding of the biochemical basis of human osteopetrosis [31]. Since 1991, when the *CA2* (''candidate'') gene was fully characterized, a variety of structural mutations have been documented in patients. We now know that specific *CA2* gene defects often cluster among ethnic groups [35,89,90].

More than 50 patients with CA II deficiency, primarily children, have been described in the medical literature [35,91,92]. Most cases have been reported from the Mediterranean region and the Middle East [35,59,92,93]. The birth history is usually unremarkable. The diagnosis is made in late infancy or in early childhood because of developmental delay, failure to thrive, fracture, and/or short stature [87,88,91]. Proximal and/or distal kidney acidification defects have been reported [94]. Hypotonia, apathy, and muscle weakness trouble some patients and may be the result of the associated renal tubular acidosis. Periodic hypokalemic paralysis can be an additional complication of the kidney dysfunction. Blindness from compression of the optic nerves and dental malocclusion occur frequently. Mental subnormality is common but not invariable [59]. Although most patients with CA II deficiency do not suffer fractures,

recurrent long bone breaks may be the source of significant morbidity [91–93]. Some patients have hypercalciuria and medullary nephrocalcinosis or recurrent nephrolithiasis [93]. Reports of the long-term outcome of CA II deficiency are lacking. In the American sibship [32], the youngest patient has developed significant sleep apnea (unpublished)—a problem that can also complicate malignant osteopetrosis [64].

Other Osteopetroses

Table 2 shows that the nosology for human osteopetrosis seems more complex than the above descriptions. A rare, transient, infantile form that is detected incidentally in the first few months of life reflects diminished resorption of skeletal tissue, apparently beginning *in utero* [42,95,96]. Severe changes are apparent on radiographic study, and there is anemia and thrombocytopenia, but no visual impairment. The abnormalities correct soon after they are discovered. The cause is unknown [42,95], but exposure to acetazolamide (a CA II inhibitor) should be considered [84]. The benign outcome of this form of osteopetrosis demands accurate diagnosis so that treatment, especially bone marrow transplantation, can be avoided. Perhaps some sporadic or inherited cases of marble bone disease result from viral infection [41]. It is uncertain whether rare occurrences of osteopetrosis with additional conditions such as Hirschsprung disease [45] or poikiloderma [46] reflect new syndromes or are merely chance associations of two disorders.

RADIOLOGIC FEATURES

Radiography

Because the pathogenesis of all true forms of osteopetrosis features a global defect in bone resorption, the growth, modeling, and remodeling of the skeleton are disturbed [97–99]. Typically, on radiographic study, there is generalized osteosclerosis (thickening of trabecular bone) and hyperostosis (widening of cortical bone) [100]. The combination of manifestations distinguish marble bone disease from other disorders that cause high bone mass, including craniometaphyseal dysplasia, pyknodysostosis, and hepatitis C–associated osteosclerosis (Table 1) [23]. However, the severity and the evolution of these abnormalities depend upon the particular type of osteopetrosis. The usual radiographic findings are described below.

Impaired bone modeling in the limbs contributes to the symmetrical increase in skeletal density because tubulation of long bones does not occur properly. Osteoclasts fail to resorb the cartilaginous and osseous tissue deposited by endochondral bone formation at the periphery of growth plates. Hence, the ends of the long bones are "undertubulated," causing a characteristic finding, i.e., metaphyses that are broad with a so-called Erlenmeyer flask or club-like deformity (Fig. 2) [97–100]. An unexplained additional aberration in some patients is a pattern of varying bone density in the pelvis and at the ends of the major long bones, i.e., perhaps the "circles" of osteosclerosis first observed by Albers-Schönberg [19]. This finding may reflect fluctuating disease activity. Rarely, the distal phalanges are inexplicably resorbed, but this irony is more common in the sclerosing bone disorder caused by defects in the cathepsin K gene [27], pyknodysostosis (MIM 265800), that increasingly seems to represent a form of osteopetrosis [23].

Rachitic-like changes occur occasionally in severe osteopetrosis (see below) [101,102]. The limb bones of affected children appear diffusely dense, yet their growth plates and metaphyses are widened and irregular, respectively. Actually, "osteopetrorickets" [102] may be the result of secondary hyperparathyroidism rather than defective skeletal mineralization (analogous to pediatric renal osteodystrophy) [97,99].

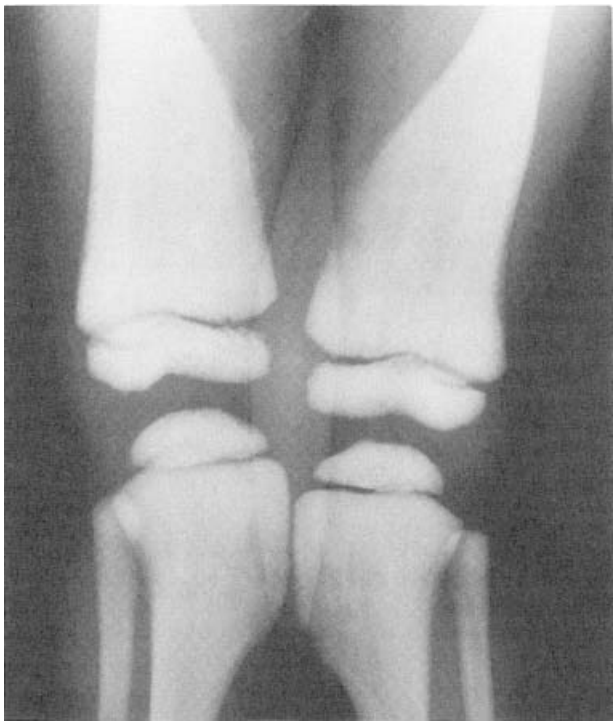

Figure 2. Osteopetrosis (intermediate form). This $4\frac{9}{12}$-year-old boy has characteristic symmetrical osteosclerosis and evidence of defective bone modeling including absence of medullary cavities and Erlenmeyer flask deformities in the distal femora.

In the axial skeleton, typically the cranium is thick and dense, especially at its base. This can cause a mask-like appearance on anteroposterior projection. The mastoid and paranasal sinuses are underdeveloped and underpneumatized. On lateral view, the vertebrae may show a characteristic "bone-within-bone" (endobone) configuration (Fig. 3) from the persistence of cartilage anlagen, or demonstrate endplate hyperostosis causing a "rugger-jersey" or "sandwich" appearance resulting from impaired modeling of the endosteal surfaces of cortices. Endobones also occur typically in the pelvis, hands, and feet [97–99] and are analogous to the "islands" of unresorbed primary spongiosa seen histologically elsewhere (see below).

In so-called autosomal dominant osteopetrosis type I, there is widening and increased density of the cranial vault with diffuse osteosclerosis of vertebrae [30]. In type II disease, there appears to be selective thickening of the skull base with typical endobone formation or a "rugger-jersey" spine [30]. In both conditions, abnormalities may be detected in infancy and progressive osteosclerosis occurs afterwards, but skeletal modeling defects are usually mild or absent [103]. Fractures are seen commonly in type II (Albers-Schönberg) disease [30]. In intermediate osteopetrosis, the radiographic changes can resemble those of the malignant form and may be found in infancy [84]. In CA II deficiency,

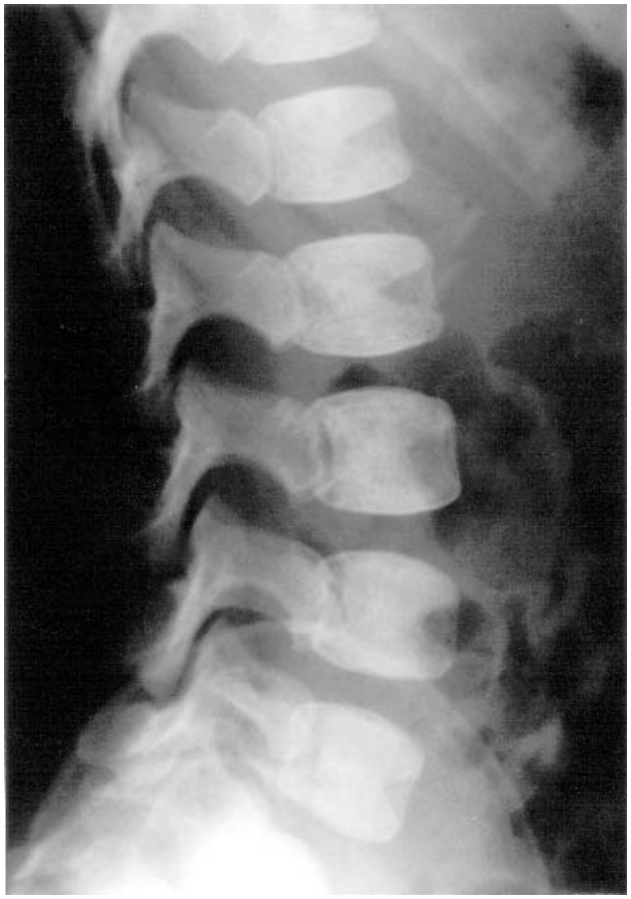

Figure 3. Osteopetrosis (benign form). At 2 years of age, the lumbar spine displays a ''bone-within-bone'' appearance.

Figure 4. Carbonic anhydrase II deficiency. At age 2 years, the patient shown on the extreme left in Figure 7 had diffuse osteosclerosis with absence of medullary cavities and flared metaphyses. Transverse lines (arrowheads) are present, and there are healing fractures (arrows). (Reprinted with permission from Sly et al. [91].)

skeletal radiographs have been abnormal at the time of diagnosis in all cases, although one neonate had only subtle defects [104]. The x-ray findings are generally similar to those in other forms of osteopetrosis (Fig. 4). However, cerebral calcification appears during childhood. Remarkably, the osteosclerosis and the defects in skeletal modeling noted in infancy or childhood can diminish over time in CA II deficiency [33,104]. This observation contrasts with other forms of marble bone disease in which bone density seems to increase with aging [70,97–99].

Bone Densitometry

Little has been published concerning this technology in osteopetrosis, although the various devices should be helpful for evaluating natural history and response to treatment [105]. Investigation of one individual suggests that dual energy x-ray absorptiometry (DEXA) may fail to identify nonpenetrant carriers of Albers-Schönberg disease [82].

Bone Scintigraphy

Bone scintigraphy in osteopetrosis retains its utility to disclose fractures, osteomyelitis, or other focal skeletal defects [106,107]. Recently, bone marrow immunoscintigraphy has proven helpful in assessing medullary space, especially in response to bone marrow transplantation [108].

Magnetic Resonance and Computed Tomographic Imaging

Magnetic resonance imaging (MRI) seems to be useful for monitoring the effects of bone marrow transplantation in osteopetrosis (see below) because it can document increases in medullary space [109]. MRI is crucial in malignant osteopetrosis to search for degeneration of cerebral white matter [62,67].

Computed tomography (CT) is the best technique to evaluate cranial nerve impingement resulting from narrow optic foramina [6,110,111]. In CA II deficiency, CT reveals cerebral calcification beginning at about 5 years of age [112]. Subsequently, the mineral deposits increase and affect cortical gray matter as well as the basal ganglia (Fig. 5). This calcification is similar or identical to the abnormality found in idiopathic hypoparathyroidism or in pseudohypoparathyroidism [112]. CT may also help to reveal cerebral ventricular dilatation.

LABORATORY FINDINGS

In malignant osteopetrosis, some affected infants or children experience hypocalcemia of sufficient severity to

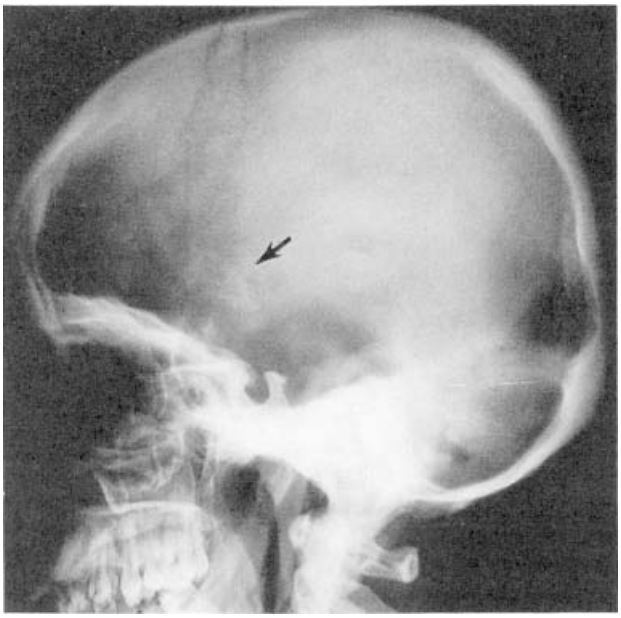

Figure 5. Carbonic anhydrase II deficiency. At age 22 years, basal ganglia calcification (arrow) is apparent in the patient shown on the extreme right in Figure 7.

cause symptoms, including seizures, and to produce rachitic changes of their growth plates [62,101,102,113]. Defective skeletal resorption likely explains the low serum calcium levels that consequently reflect dietary mineral intake [114]. Secondary hyperparathyroidism, with appropriately elevated serum $1,25(OH)_2D$ levels, is common in these cases [113–115]. Conversely, severe hypercalcemia may follow restoration of osteoclast function after successful bone marrow transplantation [116]. Heparan and chondroitin sulfate levels can be increased in patient urine [117].

Benign osteopetrosis typically demonstrates unremarkable biochemical parameters of mineral homeostasis. However, elevated serum levels of parathyroid hormone and tartrate-resistant acid phosphatase (TRAP) have been reported in Albers-Schönberg disease [30].

In 1996, from Shriners Hospitals for Children, St. Louis, Missouri, we reported that the "brain" isoenzyme of creatine kinase (BB-CK) (EC 2.7.3.2) is aberrantly present in the serum of patients with true forms of osteopetrosis, but not in that of patients with other sclerosing bone disorders [118]. Indeed, in some cases of marble bone disease BB-CK accounts for the majority of circulating CK, raising total CK values to supranormal levels [118,119]. This finding does not seem to reflect neurological damage, because it occurs in mild cases of osteopetrosis. Instead, the observation could reflect a pathogenetic biochemical defect common to each of the forms of osteopetrosis because BB-CK is the CK isoenzyme found in osteoclasts [118]. It has been suggested that anaerobic osteopetrotic bone alters osteoclast metabolism causing greater dependence upon high-energy creatine phosphate [119]. Additionally, however, TRAP from osteoclasts [11,12] can increase in serum in various forms of osteopetrosis [25,30]. Perhaps, elevated BB-CK and TRAP levels in patient serum reflect enhanced recruitment of osteoclast precursor cells [17,118].

In CA II deficiency, bone marrow appears undisturbed. When anemia is detected, it is usually mild and nutritional in origin [87,91,92]. Metabolic acidosis, featuring low serum bicarbonate levels and hyperchloremia, with increased urinary pH has been documented as early as the perinatal period [104]. Distal and/or proximal renal tubular acidosis have been reported [94,120]. Glucosuria and aminoaciduria are absent, and there is no further evidence of Fanconi syndrome [91].

HISTOPATHOLOGIC FINDINGS

For patients with severe skeletal manifestations of osteopetrosis, the radiographic features are diagnostic [97–99]. There is generalized osteosclerosis and hyperostosis causing absence of medullary space. In the mild forms of marble bone disease, however, the x-ray changes may engender less confidence. Fortunately, however, failure of osteoclasts to resorb skeletal tissue in osteopetrosis causes a pathognomonic finding that unmasks the disorder's pathogenesis [7,121–123]. Primary spongiosa (the calcified cartilage deposited during endochondral bone formation), which should be removed during skeletal modeling or remodeling, persists in osteopetrosis. Consequently, this material becomes entrapped within osseous tissue, appearing as distinctive "islands" or "bars" of cartilage. Although there seems to be a variety of pathophysiologic defects causing osteopetrosis in humans, all true forms of this disorder share this histopathologic feature. This residuum is found wherever epiphyseal or apophyseal bone formation once occurred (Fig. 6) [121,122]. In other pediatric disorders with high bone mass, there is sufficient osteoclastic activity to remove this primary spongiosa during skeletal turnover so that these cartilage remnants are not observed [23]. In CA II deficiency, cartilage bars have been demonstrated within trabecular bone in iliac crest specimens from four affected individuals representing two families [87]. However, this finding has not been reported in patients with autosomal dominant osteopetrosis types I or III. In osteopetrosis, failure of skeletal remodeling to remove this cartilage, as well as to interconnect osteons, probably contributes to the poor quality of bone tissue and thereby to recurrent fractures.

Osteoclasts are often excessive in the more common forms of osteopetrosis in humans [11,122]. Occasionally, normal counts of these cells are noted [122]. Few osteoclasts are sometimes reported, as in osteopetrosis lethal *in utero* [39], unusual cases of malignant osteopetrosis [66], and type I benign osteopetrosis [124]. Patients with few osteoclasts are of special interest because this finding also occurs in some well-understood animal models of marble bone disease (see below) [11,12]. Malignant osteopetrosis typically shows numerous osteoclasts, appropriately on the surface of trabeculae, with especially great numbers of nuclei [71,113,122,125]. Furthermore, the electron microscope generally, but not always, fails to show a "ruffled border" of the plasma membrane of these polykaryons. Ruffled borders normally form in osteoclasts that are actively resorbing bone [7,9,10]. Additionally, osteoclasts in malignant osteopetrosis usually lack the "clear zone" that normally develops between cytoplasmic organelles and the ruffled border [7,126]. Although unexplained, fibrous tissue proliferates in the medullary space in severely affected patients (Fig. 6) and further crowds hematopoietic cells [122,126]. Possibly, this abnormality reflects, in part, secondary hyperparathyroidism.

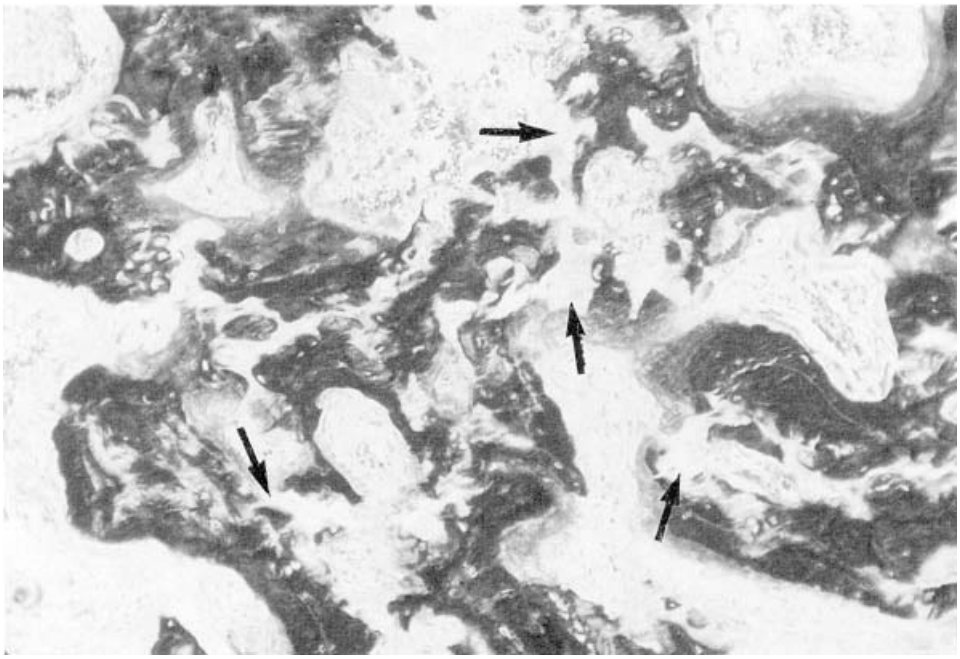

Figure 6. Osteopetrosis (malignant form). This nondecalcified section of bone shows areas of calcified primary spongiosa that stain lightly (arrows) within well-mineralized, darkly staining trabeculae. There is abundant fibrous tissue within the marrow space (Masson trichrome stain, ×160, original magnification).

Albers-Schönberg disease also features increased numbers of osteoclasts that can lack ruffled borders [76]. Unmineralized osteoid can be excessive. Woven (disorganized) bone in trabeculae and in cortices is common [76]. The histopathologic findings in other forms of osteopetrosis are less well characterized.

PATHOGENESIS AND ETIOLOGY

High bone mass can occur by several means [23,100] (Table 1). It may result from osteoclast failure, exuberant bone formation, or long-standing uncoupling of skeletal remodeling favoring net accretion of osseous tissue. Consequently, there can be focal or generalized osteosclerosis and/or hyperostosis [23,100]. Many such disorders are heritable and, like osteopetrosis, offer important clues concerning skeletal homeostasis [127].

As noted previously, osteopetrosis is caused by defective osteoclast-mediated bone resorption [17,18,24–26] (see also Chapter 1, Part III, this volume). Why this failure occurs in humans is incompletely understood. Osteoclasts are members of the mononuclear phagocyte system [11]. Their origin is a pluripotential hematopoietic stem cell that gives rise to a circulating precursor cell of monocyte-macrophage lineage [11,12,18,51]. A compelling illustration is the treatment success based upon the pioneering animal model studies of Walker [47], showing that defective osteoclast-mediated bone resorption can cause osteopetrosis and that the osteoclast progenitor is a marrow-derived cell [51,55]. Children with severe osteopetrosis have benefited dramatically from bone marrow transplantation (see below). They were cured by osteoclasts of donor origin [50,51,54,56]. However, the ontology of the osteoclast is quite complex [11,48]. A variety of growth factors, cytokines, and hormones condition osteoclast precursor cell growth, differentiation, and fusion, as well as regulate mature osteoclast function [11,18,48]. Hypothetically, therefore, many disturbances could cause marble bone disease [49]. Recently, it has become possible to generate osteoclasts using marrow cells or peripheral blood and thereby to reproduce *in vitro* fundamental pathogenetic features of osteopetrosis [128,129].

Two inheritance patterns (autosomal recessive and autosomal dominant) and several clinical phenotypes of osteopetrosis in patients (Table 2) indicate that a number of gene defects significantly impair osteoclast-mediated bone resorption in humans. Nevertheless, as reviewed below, the molecular basis for this disorder is largely unknown. Only a few patients with osteopetrosis have profoundly reduced numbers of osteoclasts [39,62]. Hence, diminished function rather than lack of these cells seems to be the more common pathogenetic disturbance [84].

Carbonic Anhydrase II Deficiency

CA II deficiency was the first form of osteopetrosis in humans for which the biochemical and genetic basis became understood [35]. The enzymopathy was discovered in 1983 [34]. In 1991, when the *CA2* gene located on the long arm of chromosome 8 had been fully characterized, mutations in patients were identified [35,89,90]. The American sisters [32] proved to be compound heterozygotes for both a splice-site mutation and a missense mutation [35]. However, the widespread geographic distribution and variable clinical severity of CA II deficiency predicted further allelic heterogeneity that is now well documented [35]. To date, a variety of *CA2* gene defects have been identified [35,89,90,130]. Accordingly, CA II deficiency is the best understood form of human osteopetrosis [35]. In the Arabian peninsula, consanguinity is a common etiologic risk factor [59,104]. Nevertheless, a range of clinical expression

occurs within sibships, and additional genetic or epigenetic factors require elucidation to fully comprehend this inborn error of metabolism [35].

The pathogenesis of CA II deficiency has been unfolding since the discovery in 1983 of a profound reduction of CA II in erythrocyte lysates of three American sisters (Fig. 7) [34]. Consistent with autosomal recessive inheritance, approximately one-half normal CA II levels were found in the red cells of their parents. Levels of CA I were undisturbed [34]. In 1985, CA II deficiency was documented in all 21 patients available for study, representing 12 unrelated kindreds of diverse ethnic and geographic origin [91]. Although autopsy investigations have not been reported, the molecular pathology of CA II deficiency establishes an important role for CA II in human bone and kidney and perhaps in brain [32–35].

Several studies show that osteoclasts substantially acidify their pericellular space during skeletal resorption [10,131]. When osteoclasts are degrading bone, ruffled membranes form an enclosure over osseous tissue in which the pH may decrease to as low as 5.5 [10,131]. At this high concentration of H^+ created by a vacuolar proton pump, mineral is solubilized. Subsequently, collagenases and other proteases become active as the pH increases, destroying the remaining matrix [132] (see also Chapter 1, Part III, this volume).

CA catalyzes the union of CO_2 and H_2O to form H_2CO_3, which then dissociates to H^+ and HCO_3^-. Accordingly, CA plays an important role in acid/base balance. CA II is the most catalytically active CA isoenzyme with the widest tissue distribution, including erythrocytes, kidney, and brain [35,130,133]. Notably, CA II is the CA of the osteoclast and is found in the cytosol [11,134–136].

In CA II deficiency, osteoclasts cannot convert CO_2 and H_2O to H_2CO_3, thereby blocking subsequent production of H^+ for acidification of the pericellular space. In fact, pharmacologic inhibitors of CA (e.g., acetazolamide) hamper skeletal resorption both *in vivo* and *in vitro* and can increase bone mass [131].

Enhanced bicarbonaturia after taking acetazolamide in CA II deficiency suggests that an additional CA isoenzyme acts importantly in renal physiology [120]. Indeed, normal levels of CA IV are detected in cell membranes shed into patient urine [137]. In CA II deficiency, intact erythrocytes seem to have sufficient CA I activity to fulfill the minimum resting requirements for respiratory gas exchange in the blood [138]. Why cerebral calcification occurs is unclear. Development of several mouse models for CA II deficiency provides tools to enhance our understanding of this inborn error of metabolism [139,140].

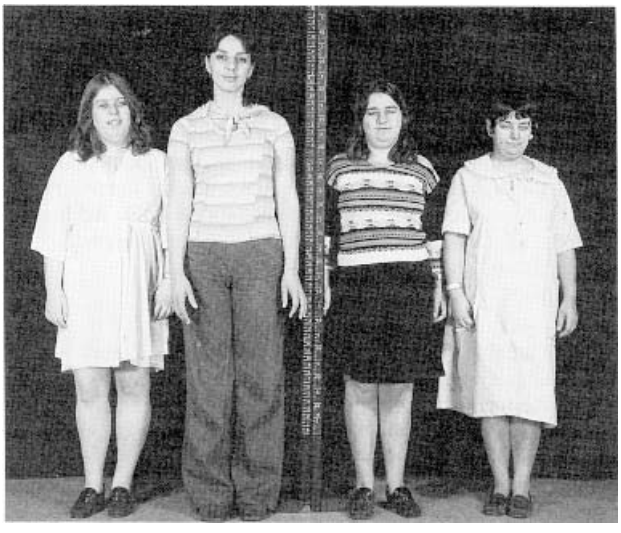

Figure 7. Carbonic anhydrase II deficiency. Three of four sisters (ages 22,24, and 29 years, left to right) are affected and have short stature. (Reprinted with permission from Whyte et al. [32].)

Malignant Osteopetrosis

The molecular basis for severe osteopetrosis remained unknown until 2000. There have been reports of diminished granulocyte and monocyte function [141], yet problems with the immune system itself seem uncommon. In 1997, malignant osteopetrosis was found to map to chromosome 11q13 in two consanguineous Bedouin kindreds [142]. M-CSF (macrophage colony-stimulating factor) production seemed to be normal [143] and in 1998, in follow-up of animal model studies (see below), the protein tyrosine kinase $p60^{c\text{-}Src}$ was found not to be implicated in malignant osteopetrosis [144]. Furthermore, there is evidence that some patients have a primary defect involving osteoblasts rather than osteoclasts [145]. In 1993, lack of expression of the osteoclast-reactive vacuolar proton pump responsible for H^+ secretion by these cells [132] was reported in a patient with craniometaphyseal dysplasia [146]. In 2000, defects in the TCIRG1 subunit of this pump were discovered in a major subset of patients with malignant osteopetrosis [58].

Osteopetrosis with Neuronal Storage Disease

In osteopetrosis with neuronal storage disease, postmortem studies show degenerative changes in brain cells, including widespread axonal dystrophy, with spheroidal bodies, storage of ceroid lipofuscin [40,84,85], and accumulation of carbohydrates and lipids [86]. Accordingly, there may be a defect(s) in lysosomal function [40].

Benign Osteopetrosis

In benign osteopetrosis, resistance to the actions of thyroid hormone and $1,25(OH)_2D$ have been reported in types I and II, respectively [74,76]. In 1997, Albers-Schönberg disease was mapped to an 8.5 cM candidate region on chromosome 1p21 in a Danish kindred, but the M-CSF (CSF-1) locus was excluded [21]. In 1999, linkage exclusion analysis of two affected families from Indiana, in the United States, indicated genetic heterogeneity [44]. Possibly, different disease loci reflect genes encoding a protein complex or a humoral factor and its receptor [44].

Other Forms

In other patients with osteopetrosis, there may be deficient production of interleukin-2 [147], synthesis of an abnormal parathyroid hormone molecule [148], or failure to generate superoxide [149]. Animal models of marble bone disease show skeletal resorption associated with induction of superoxide in cells of granulocyte/monocyte lineage in response to interferon-γ [149], M-CSF [150], and interleukin-2 [151]. Indeed, severe osteopetrosis in patients responds to recombinant human interferon-γ 1b therapy (see below) [152].

Finally, some cases of osteopetrosis may have an infectious etiology [41,153]. In 1988, electron microscopy of osteoclasts from several patients with sporadic, benign osteopetrosis revealed viral inclusions resembling the nucleocapsids of *Paramyxoviridae* [153]. Measles or respiratory

syncytial virus antigens were detected in these cells [153]. In 1990, a reverse transcriptase that resembled the enzyme made by retroviruses was discovered in the supernatant of circulating mononuclear blood cells taken from a patient with benign osteopetrosis [41]. The investigators hypothesized that retroviruses could block osteoclast action and account for some heritable forms of marble bone disease resulting from genomic integration of the virus [41]. Indeed, several animal models of "osteopetrosis" are caused by viral infection; however, they feature exuberant bone formation [84]. Whether viral inclusions observed in patients reflect the cause of their osteopetrosis or are an epiphenomenon remains unclear. Viral inclusions in osteoclasts have been seen in other bone disorders, e.g., pyknodysostosis and Paget bone disease [23]. Nevertheless, the considerable variety and growing number of animal models of osteopetrosis and their genetic heterogeneity suggest that osteopetrosis in humans will reflect abnormalities in a diversity of genes [11,12,18,82,154–159].

ANIMAL MODELS

Rapidly advancing studies using animal models provide potential clues to the etiology and pathogenesis of the human osteopetroses [11,12,18,84,154–159]. Many possible defects that could impair osteoclast action have been identified, ranging from an altered microenvironment containing osteoclast precursor cells (e.g., failure of marrow elements to produce factors that condition stem cell maturation) to skeletal tissue refractory to resorption. There could, hypothetically, be disturbances involving the proliferation or maturation of osteoclast precursor cells, fusion of progenitor cells to form polykaryons, recognition by mature osteoclasts of bone tissue for resorption, response by osteoclasts to hormones, or degradation of skeletal tissue due to lysosomal defects [11,17,18,26].

There are now more than 15 animal models of osteopetrosis involving several mammalian species [11,17,18,26, 154–159]. Mutations have either occurred spontaneously or have been induced, but each type of gene defect causes osteopetrosis that is transmitted as an autosomal recessive trait [17]. All animal models characterized molecularly reflect gene knockouts, except the *opg* mouse featuring overexpression of the osteoprotegerin gene [17].

Among the naturally occurring ("classic") mutations, the *op/op* mouse showing few osteoclasts was found in 1990 to reflect a single base-pair insertion generating a stop codon in the M-CSF gene [160]. Subsequently, in patients, however, normal serum levels of biological and immunological M-CSF activity were detected in all of 6 and 13 individuals tested, respectively [143].

The microphthalmic (*mi/mi*) mouse harbors a gene defect that affects a transcription factor [158]. Patients with an abnormality in this gene, however, have Waardenburg syndrome, type 2, which does not feature bone disease [17].

Malignant osteopetrosis maps to chromosome 11q13 in two consanguineous Bedouin kindreds — a candidate region predicted by the murine osteosclerosis (*oc*) mutation [142]. This observation led to the discovery of the *TCIRG1* defects causing vacuolar proton pump deficiency in some patients with malignant osteopetrosis [58].

Murine osteopetrosis also occurs with inactivation of the genes encoding *c-src* (a tyrosine kinase), *c-fos*, NF-κB1 and NF-κB2 (double knock-out), PU-1 (myeloid- and lymphoid-specific transcription factor), and Acp5 (acid phosphatase) [11,17,18,154,155,161]. However, defects in none of these genes have been detected in patients with marble bone disease [144,161,162].

TREATMENT AND PREVENTION

Because the outcome varies greatly for the various human forms of osteopetrosis, a correct diagnosis is critical before treatment is chosen. Radiologic investigation of the patient's family and evaluation of disease progression may be necessary. However, for malignant osteopetrosis, a "window of opportunity" for bone marrow transplantation (BMT) can close if there is a delay in diagnosis and treatment because medullary space becomes filled by excessive skeletal and fibrous tissue [57,84]. Early transplantation is considered important for such patients [57,84].

In most infants and children with severe osteopetrosis, osteoclasts are present or even numerous. BMT is designed to effect a cure by replacing these dysfunctional cells [57]. However, BMT is a complex, expensive, and potentially hazardous treatment that can be ineffective in some patients with osteopetrosis [57,84]. Currently, BMT seems appropriate only for severely debilitating or life-threatening disease. Therefore, attempting to stimulate osteoclasts has been an additional approach to therapy, especially when excellent donor matches for BMT are unavailable. The major treatment strategies for the osteopetroses in humans are discussed below and have been reviewed elsewhere [49,56].

Dietary/Hormonal Treatment

Since first used in 1965, calcium-deficient diets have reportedly had some success in treating osteopetrosis [163]. Furthermore, reduction in dietary calcium intake has been recommended when children receive interferon therapy (see below) [164]. However, liberalization of the diet or calcium supplementation may be necessary to correct symptomatic hypocalcemia, sometimes causing seizures or rachitic-like changes on x-ray [101,102]. Indeed, administration of a low-calcium diet in severe osteopetrosis has provoked rickets, which was transiently mistaken for a good response to BMT, when bone density decreased near growth plates [165].

Calcitriol [$1,25(OH)_2D$], the most potent metabolite of vitamin D, conditions the formation and function of osteoclasts [114]. Hypothetically, some cases of malignant osteopetrosis could involve either resistance to $1,25(OH)_2D$ or defective 25(OH)D, 1α-hydroxylase activity. High doses of $1,25(OH)_2D_3$ in children with osteopetrosis reportedly cause disease stabilization and, in 25% of cases, marked improvement [84,114,123]. A preliminary report of one adult, seemingly with an intermediate form of osteopetrosis, also described better hematopoiesis and decreased skeletal mass with prolonged $1,25(OH)_2D_3$ therapy [166]. Similarly, intravenous infusions of PTH appeared to be beneficial for one affected infant, perhaps by enhancing renal $1,25(OH)_2D$ biosynthesis [148].

Methylprednisolone therapy reportedly caused remission of malignant osteopetrosis in two neonates [167]. M-CSF has been assessed recently, perhaps resulting in some disease stabilization [61,84].

Bone Marrow Transplantation

Malignant osteopetrosis has been treated since 1977 [168] by transplantation of allogeneic HLA-identical marrow [50–57,169]. Retention of small numbers of donor cells is effective [84]. Successful engraftment has been followed by significant clinical, laboratory, radiologic, and histopathologic improvement in some patients [50–57]. In fact, BMT

offers the possibility of a cure. Some neurological abnormalities may improve, others are halted or prevented, but some can remain [57,62]. Indeed, visual impairment often persists despite successful marrow engraftment. Hence, BMT before 3 months of age seems best [57,71]. Permanent recovery from malignant osteopetrosis is more likely in young patients (<4 years of age) [55,56]. However, severe hypercalcemia occurs in 29% of patients who have engraftment [57,116].

Nevertheless, not all patients with malignant osteopetrosis respond well to allogeneic BMT [57]. The likely pathogenetic heterogeneity underlying malignant osteopetrosis predicts that successful engraftment of normal osteoclasts might not benefit all patients [26,55–57,62,123]. When there is primary retinal degeneration and/or generalized neurodegeneration, BMT will not stop the progressive and ultimately lethal course [62,84]. Patients whose medullary space is severely crowded by osteoclasts and fibrous tissue seem less likely to engraft [56]. Especially large osteoclasts with increased numbers of nuclei and ruffled border membranes also predict a poor response. Accordingly, histopathological study of an iliac crest specimen and electron microscopy of the patient's osteoclasts have been recommended before BMT [55,84]. The outcome of BMT is also dependent on successful HLA matching [56,57,170]. BMT from HLA-nonidentical relatives has a significantly poorer outcome [54,57,84,170]. In this situation, only about 15% of transplanted cells survive; nevertheless, there are some cures. Importantly, 30% of patients with untreated severe osteopetrosis will live. Accordingly, this is generally considered to be an unacceptable rate of failure for unmatched BMT because patient survival is well below 50% [84,170]. Recently, however, transplantation of cord blood progenitor cells, with several advantages over BMT, has been used when there is no HLA-compatible relative [56].

Interferon

Since 1992, there have been reports of treatment successes for severe osteopetrosis using recombinant human interferon-γ [84]. Predisposition to infection among these patients, attributable to diminished superoxide generation in circulating leukocytes, led to these therapeutic trials [84]. Interferon-γ 1b increases leukocyte superoxide production [152]. This treatment is followed by fewer infections, improved anemia and platelet counts, diminished trabecular bone mass with widened medullary spaces, and enlarged cranial nerve foramina [84,152,171]. There is improved survival with reduced morbidity [84,152,172]. Developmental assessment shows a wide range of cognitive and adaptive abilities in these patients [60]. Careful attention to nutritional requirements is necessary [84]. Interferon-γ 1b and calcitriol together may be especially effective [84,171].

Supportive Therapy

Hematologic problems in malignant osteopetrosis result primarily from myelophthisis. However, other factors can exacerbate the low blood cell counts. With extramedullary hematopoiesis, there is hepatosplenomegaly and secondary hypersplenism. Additional causes of hemolysis may also be present. High-dose glucocorticoid therapy helps to stabilize the hematologic situation when there is pancytopenia and hepatosplenomegaly [167]. Indeed, prednisone therapy alone, or together with a low-calcium/high-phosphate diet, can effectively treat some cases of severe osteopetrosis [173].

Hyperbaric oxygenation can be an important adjunctive treatment for osteomyelitis [174,175]. When dental sepsis is refractory to antibiotic and hyperbaric oxygen therapy, tooth extraction may be necessary, although the procedure is difficult [174,175]. Otolaryngological approaches to malignant osteopetrosis were summarized in 1998 [61]. Children with osteopetrosis are more likely to have tracheas that cannot be intubated [176]. Perioperative morbidity and mortality are increased [176].

Orthopedic complications of malignant osteopetrosis and their management were reviewed in 1999 [177]. Although technically arduous, hip replacement and correction of neuromuscular scoliosis have been performed successfully in adults with osteopetrosis [178,179]. Cervical or lumbar spondylolysis in children will generally respond to nonoperative measures [80].

Surgical decompression has benefited some patients suffering from palsies of the optic or auditory nerves [63]. The best response for optic nerve involvement occurs if retinal disease is excluded by ophthalmoscopic examination and electroretinography [65]. Transcranial Doppler ultrasonography seems promising for planning operative intervention for visual loss [180].

In CA II deficiency, whether the systemic acidosis should be corrected is unclear. In some untreated patients, skeletal density can decrease over time and radiographic changes become more normal [32]. Reversing the metabolic acidosis could diminish bone resorption, by blunting liberation of HCO_3^- from the skeleton [32]. When attempted for CA II deficiency, long-term alkali therapy seems disappointing [181,182]. Furthermore, caution is indicated because hypercalciuria, medullary nephrocalcinosis, and nephrolithiasis affect some patients [94]. In 1988, transfusion of CA II–replete erythrocytes for one of the American sisters did not improve her systemic acidosis [183]. This observation supports an acidification defect intrinsic to the kidney [120]. In CA II–deficient mice, BMT leaves the renal tubular acidosis uncorrected; however, retrograde injection of cationic liposomes complexed with a *CA2* gene transiently corrected urinary acidification [139].

PRENATAL DIAGNOSIS

In 1943, a routine x-ray examination reportedly disclosed osteopetrosis at 6 months gestation [184]. The unique sibship with osteopetrosis lethal *in utero* was evaluated by ultrasound at 18 weeks gestation [39]. Malignant osteopetrosis has been detected by ultrasound at 18 weeks gestation [185] and by x-ray [186], and can sometimes now be detected by mutation analysis of the gene coding for the TCIRG1 subunit of the vacuolar proton pump. Generally, however, radiological studies have not been helpful. CA II deficiency has been diagnosed prenatally by molecular testing [92]. Evaluation of CA II deficiency must be made at the DNA level because CA II is not expressed in fetal erythrocytes [92]. Whether cordocentesis for measurement of BB-CK or TRAP levels in serum would be helpful has not been explored.

RECENT DEVELOPMENTS

Benign Osteopetrosis

Autosomal dominant adult (benign) osteopetrosis, type I, has been localized, according to a preliminary report, to chromosome 11q12-13 [187]. This is the same region to which a high bone mass phenotype distinguishing a family from Omaha, Nebraska had been mapped [188].

Autosomal dominant adult (benign) osteopetrosis, type II (Albers-Schönberg disease), previously believed to be genetically heterogeneous, is now understood at the

molecular level. After a report that in one large Danish family the condition mapped to chromosome 1p21 [21], this region was excluded in two American kindreds [44]. The disorder was found not to map to chromosome 1p21 in an additional family [189], indicating that 1p21 is, at best, a minor locus [190]. Instead, a genome-wide search involving a large French family mapped this disorder to chromosome 16p13.3 [191]. In fact, the original Danish family [21] could not be excluded from showing linkage here, suggesting genetic *homo*geneity for Albers-Schönberg disease [191].

Malignant Osteopetrosis

Loss of the ClC-7 chloride channel as a result of mutations in the *CLCN7* gene on chromosome 16p13.3 has been reported in one of 12 patients with autosomal recessive malignant osteopetrosis [192]. Subsequently, mutations in *CLCN7* were discovered in 12 families worldwide with adult osteopetrosis, type II, and in a second patient with autosomal recessive malignant osteopetrosis [193]. This revelation of mutations in a single gene leading to two clinically distinct types of osteopetrosis has added another defect of ion transport in osteoclasts to the pathogenesis of impaired bone resorption in marble bone disease [194].

As noted previously, autosomal recessive infantile osteopetrosis had been found in a small number of patients to reflect mutations in *ATP6i* (*TCIRG1*) encoding the α3 subunit of the vacuolar proton pump [58]. It is now known that approximately 50% of such patients worldwide have deactivating mutations in this gene [195]. Accordingly, malignant osteopetrosis can be caused by *TCIRG1* and *CLCN7* (and perhaps other) gene defects. As also noted above, craniometaphyseal dysplasia was reported in one patient on the basis of monoclonal antibody studies to involve deficiency of the vacuolar proton pump [146]. Recently, however, several patients with this disorder have been discovered to have defects in the *ANKH* gene that conditions the cellular transport of inorganic pyrophosphate [196].

Other Osteopetroses

The existence of an extremely rare condition, "asynchronous asymmetric heterogeneous osteopetrosis," is supported by a report of a second patient [197].

Prenatal Diagnosis

Prenatal diagnosis of malignant osteopetrosis has recently been successful in some families using genetic testing. In several inbred Bedouin kindreds, in which the disorder had been mapped to chromosome 11q12-13 [142], linkage analysis was helpful [198]. Mutation information concerning *TCIRG1* has enabled prenatal diagnosis of malignant osteopetrosis in Costa Rican families [195]

Treatment

Advances in the treatment of osteopetrosis include a case report of apparent cure of the malignant form using prednisone therapy [199]. In 2000, interferon γ-1b received Food and Drug Administration (FDA) approval in the United States for severe osteopetrosis. Interferon-γ enhances osteoclast generation and normalizes superoxide production [200], helping, perhaps, to control infection in malignant osteopetrosis [201]. As predicted [183], bone marrow transplantation corrects osteopetrosis but not renal tubular acidosis in carbonic anhydrase II deficiency [202]. The efficacy of stem cell transplantation for severe osteopetrosis has recently been assessed [203], and the diagnosis, management, and outcome of autosomal recessive osteopetrosis has been briefly reviewed [204].

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Chapter 20

Alkaptonuria

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SUMMARY

- **Alkaptonuria is a rare, hereditary, disease of phenylalanine and tyrosine metabolism in which the intermediary compound, homogentisic acid, cannot be further metabolized. The metabolic defect results in a characteristic triad of findings: homogentisic aciduria, connective tissue ochronosis, and arthritis of the larger joints.**
- **The cause of the disease is a lack of the enzyme homogentisic acid oxidase, normally found primarily in the liver and kidney. It requires oxygen, ferrous iron, and sulfhydryl groups to cleave the aromatic ring of homogentisic acid. No effective treatment is available, but avoiding severe stress to the spine and large joints will reduce the progress of the ochronotic arthritis.**
- **Alkaptonuria is inherited as an autosomal recessive disorder. Although no general methods are available to detect heterozygous carriers of the condition, recent advances in understanding the molecular basis of the genetic defects in alkaptonuria may permit heterozygous detection within families carrying identified homogentisic acid oxidase mutations.**
- **Exactly how this simple metabolic defect causes tissue ochronosis and arthritis is still not clear, and solving these problems will be a challenge for the future. Animal models of this disorder are now available to study the mechanism of the pathophysiology in alkaptonuria, and to assess possible approaches for treatment of the metabolic disorder.**

INTRODUCTION

Alkaptonuria (MIM 203500) is a rare, hereditary, metabolic disease in which the enzyme homogentisic acid oxidase (HGO) is deficient or missing. Homogentisic acid (HGA) produced by the metabolism of phenylalanine and tyrosine cannot be further metabolized in alkaptonuric individuals; it accumulates to some degree in the tissues, and is excreted in the urine. Urine containing HGA gradually turns dark upon standing, as the acid is oxidized to a melanin-like product. The oxidation is much faster under alkaline conditions, and is greatly retarded in acidic urine. Some alkaptonurics do not report noticing dark urine, and many are not diagnosed until they require a surgical procedure which reveals extensive ochronotic pigmentation of their cartilage and other connective tissues. However, most alkaptonuric patients do report that they pass dark urine, or urine that darkens on standing. The medical literature of the 16th and 17th centuries describes individuals who continually passed dark urine, and some of them probably had alkaptonuria [1]. An Egyptian mummy dated approximately 1500 B.C. showed the characteristic x-ray changes in the spine seen in alkaptonuric patients (extensive intervertebral disk calcification and narrowing of the hip and knee joints). Spectroscopic analysis of pigment obtained from the hip region of the mummy closely resembled the reference pigment produced by oxidation of HGA [2]. Although it was not possible positively to identify HGA in the mummy tissues, this may be the earliest case of alkaptonuria on record.

The first alkaptonuric patient diagnosed with certainty was that of Boedeker in 1859 [3]. Boedeker determined that the reducing compound in his patient's urine was not glucose, and noted the rapid darkening of the urine after alkali was added. He used this property of avid oxygen uptake in alkaline solution to name the unknown substance "alcapton" [3]. Two years later, Boedeker spelled it "alkapton" [4], and since then this condition has been called *Alkaptonurie* in the German literature and *alcaptonurie* in the French. Boedeker precipitated "alcapton" from alkaptonuric urine as the lead salt [3,4], but was unable to determine its chemical structure. About 30 years later, Wolkow and Baumann [5] identified it as 2,5-dihydroxyphenylacetic acid and named it homogentisic acid because of its similarity to gentisic acid (2,5-dihydroxybenzoic acid).

After the aromatic structure of HGA had been identified, suggestions were made about the possible metabolic sources of this unusual urinary product. Tyrosine and phenylalanine, as aromatic components of the dietary proteins, were, of course, the primary suspects. In 1891, Wolkow and Baumann demonstrated that feeding extra tyrosine or a diet high in protein greatly increased the amount of HGA excreted by an alkaptonuric patient [5]. Similar clinical investigations by several other groups allowed the construction of a metabolic pathway by which phenylalanine and tyrosine could be metabolized to HGA. It was assumed that compounds in the metabolic sequence should increase the excretion of HGA in an alkaptonuric subject, but that chemicals that were not intermediates would fail to do so. From all of these studies Neubauer suggested a sequential scheme for tyrosine metabolism in 1909 [6], the first such metabolic

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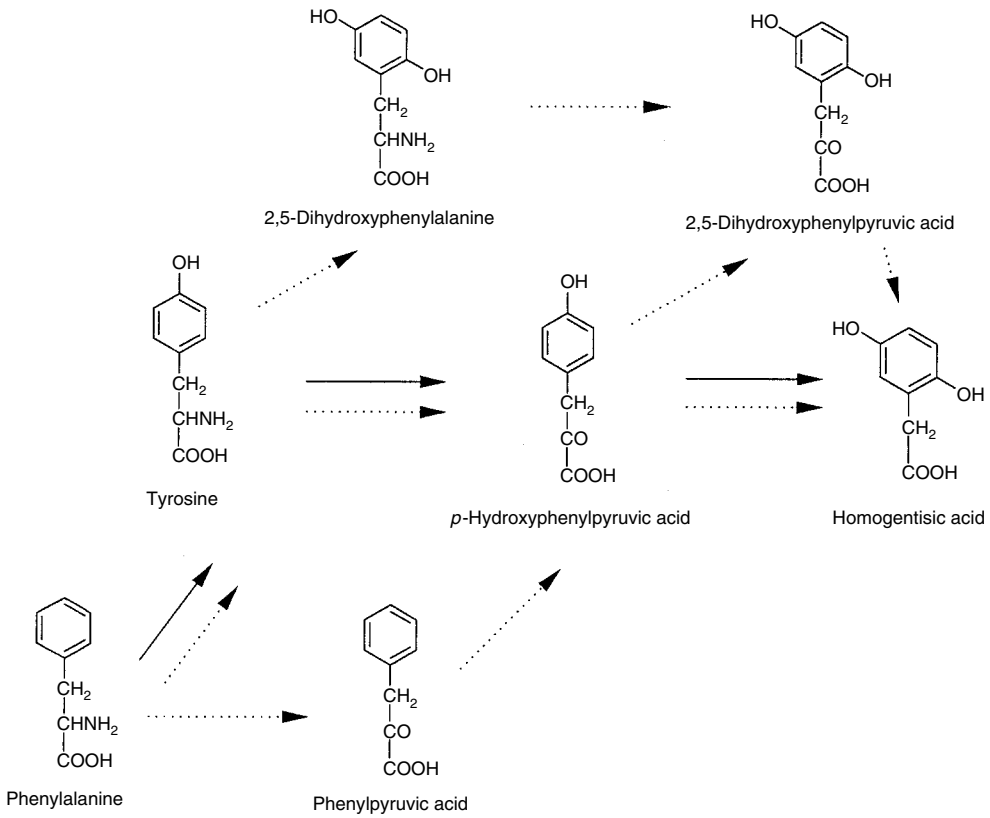


Figure 1. Scheme of phenylalanine and tyrosine metabolism to homogentisic acid based on feeding experiments with alkaptonuric patients. Dotted arrows show the possible pathways considered by Neubauer [7]; solid arrows indicate the pathway as it is believed today.

scheme proposed for any amino acid. He revised it slightly in 1928 from additional data [7] (Fig. 1), and this scheme has remained essentially unchanged over the past 70 years.

Alkaptonuria has served as a valuable model in the historical development and understanding of metabolic diseases. Garrod's brilliant concepts about inborn errors of metabolism were based on his insightful studies on alkaptonuria. In 1908, he discussed alkaptonuria in one of the Croonian Lectures [1], and in the following year he expanded his ideas more completely in his classic book *Inborn Errors of Metabolism*. [8]. He considered alkaptonuria to be an extreme example of the wide variation possible in metabolic capacity, and more like an inherited structural abnormality than an acquired, infectious disease. He predicted that some degree of metabolic variation was present in every person, according to one's hereditary background. Alkaptonuria and the other inborn errors of metabolism represented extreme examples of such variational possibilities [9]. He suspected that these variants ultimately depended on differences in the activity of specific enzymes. In this way, Garrod anticipated by many years the important interpretation of Beadle and Tatum [10] that a single defective gene is correlated with a metabolic deficiency in one enzymatic reaction. In 1909, Garrod even proposed the remarkable conclusion that the enzyme which splits the benzene ring of homogentisic acid was defective in alkaptonuric subjects, and that this enzyme was normally present in human liver [8]. This deduction was supported by all the circumstantial evidence gathered during the subsequent years, and was directly confirmed in 1958 by enzymatic assays on a biopsy sample of alkaptonuric liver [11].

CLINICAL FEATURES

The three major features of alkaptonuria are the presence of HGA in the urine, pigmentation of cartilage and other connective tissues, and nearly always, in later years, arthritis [12]. Alkaptonuria does not appear to reduce the normal life span of affected subjects [13].

Urinary Changes

Although it is generally said that people with alkaptonuria give a history of dark urine or urine which turns dark on standing, it should be noted that many reported alkaptonuric patients never observed any abnormal color of their urine during childhood [14–16], and the diagnosis was made only after they obtained medical intervention for arthritis during their later years [15–20]. For others, it may have been a false positive test for diabetes [15,19], or the finding of unusual, distinctive x-ray changes in the spine [21], or some surgical procedure revealing marked pigmentation of articular cartilage [22] that led to the diagnosis.

Alkaptonurics on a normal diet void a urine which seems to be normal, and it may not darken for several hours if it remains at an acid pH. Thus, in those instances in which freshly voided urine has been reported to darken quickly, there must have been other factors involved. Two of these are: the excretion of an alkaline urine, and the presence of a lower than normal concentration of vitamin C, or possibly

other reducing agents, in the urine. Vitamin C protects HGA against oxidation, and ascorbic acid was suggested as a therapeutic agent to prevent ochronosis because of this property [23]. The abnormal findings in alkaptonuric urine can all be attributed to the presence of HGA. No abnormal amino acid pattern [24], or other tyrosine metabolic products are found [25].

Diagnostic urinary tests for alkaptonuria are based on the detection of HGA and its unusual chemical properties. Its ease of oxidation in the presence of alkali results in the gradual darkening of the urine downward from the surface until the entire sample is dark brown. Alkaptonuric urine also reduces copper in Benedict's sugar reagent, producing an orange precipitate in a muddy-brown solution. Reduction of molybdate is the basis of the Briggs test, which has been used in the past to follow the course of urinary excretion of HGA [26]. Reduction of silver in photographic paper emulsion has also been used as a simple, qualitative test [27], and as the basis of a quantitative method to measure HGA [28]. HGA is not fermented by yeast, and it does not fluoresce under ultraviolet light.

A presumptive diagnosis of alkaptonuria can be made with the above nonspecific tests, but specific tests to identify HGA are also available. This acid has been isolated from alkaptonuric urine after precipitation as the lead salt [25,29] and the product analyzed for its chemical composition and melting point. Paper chromatography of the urine directly, or after ether extraction from acidified urine, is another simple technique to identify HGA [30]. A specific enzymatic method has been developed that permits the quantitative analysis of HGA in urine, blood, and other tissues [31,32]. Rapid analysis of HGA in urine and plasma is also possible by an HPLC method [33]. A stable isotope dilution gas chromatography-mass spectroscopic method can be used to determine the concentration of HGA in normal human plasma; normal levels are reported to be from 2.4 to 12 ng/mL, about 1/1000th of the levels found in alkaptonuric plasma [34].

Ochronosis

In 1866, Virchow first described a peculiar, generalized pigmentation in the connective tissues of a 67-year-old man [35]. The pigment was gray to bluish-black on gross examination, but ochre microscopically, and so he named this condition "ochronosis." The patient's clinical history was not available to Virchow, but there is little doubt that he had described for the first time the generalized connective tissue pigmentation that gradually develops in alkaptonuric individuals. It was nearly 40 years later that Albrecht, in 1902 [36], clearly demonstrated the causative association between ochronosis and alkaptonuria. Not long thereafter, Sir William Osler diagnosed ochronosis clinically for the first time in two alkaptonuric brothers [37]. He recognized the pigmentation of the sclerae, cerumen, and ears to be signs of the same metabolic abnormality that had previously been diagnosed only by the dark pigment in alkaptonuric urine and autopsy connective tissues.

The long delay in clinical recognition of ochronotic pigmentation as a basic feature of alkaptonuria is not surprising. Generally, the earliest external signs of ochronosis that can be detected are a slight pigmentation of the sclerae and the ears. However, these changes are rarely apparent until alkaptonuric patients are about 20 to 30 years old. Pigmentation of the eye, found about midway between the cornea and the outer and inner canthi, is found at the sites of insertion of the rectus muscles (Fig. 2). In addition, a more diffuse pigmentation may involve the conjunctiva and cornea [39]. The typical pigmentary changes in the ear cartilages also can be detected only after longstanding alkaptonuria. The cartilage is slate blue or gray and feels irregular and thickened.

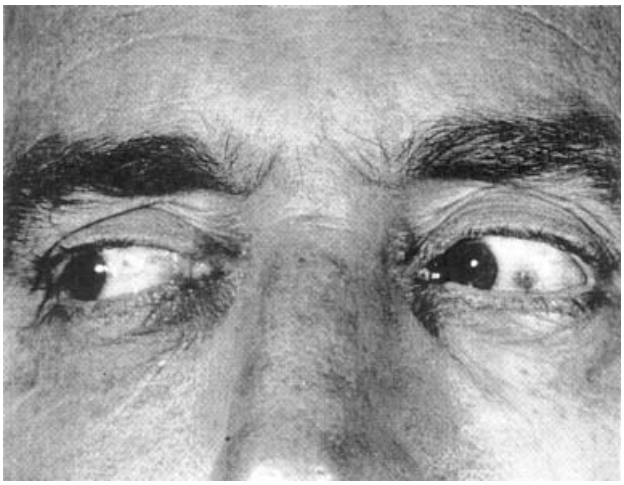


Figure 2. The bilateral deposition of ochronotic pigment in the sclerae, best seen in the left eye. From Bunim et al [38].

It is sometimes reported that a dusky discoloration, corresponding to underlying tendons, can be seen through the skin over the hands. The degree of prominence of this pigmentation is variable, and in many instances is scarcely evident at all. The pigment appears in perspiration; clothing near the axillary regions may be stained, and the skin may have a brownish discoloration in the axillary and genital regions.

In contrast to these minimal external findings, the pigmentation observed in the tissues of an elderly alkaptonuric patient at operation or postmortem is indeed striking [35,40–43]. Cartilage in many areas, such as the costal, laryngeal, and tracheal cartilage, is deeply pigmented. It has been described as being coal black in some areas (Fig. 3). Pigmentation is also present throughout the body in fibrous tissues, such as fibrocartilage, tendons, and ligaments (Fig. 4). Pigmentation is also found in the endocardium, the intima of the larger vessels, in various organs such as kidney and lung, and in the skin epidermal layer. Microscopic examination shows the pigment may be either granular or homogeneous, and is deposited both intercellularly and intracellularly [44]. Electron microscopy of alkaptonuric synovial membrane [45], hip joint tissue [46], and articular cartilage [47] shows the fragments of cartilage to be stained with yellow-brown pigment derived from HGA.

Like melanin, the ochronotic pigment can be bleached by treatment for 24 hours with hydrogen peroxide. The pigment is soluble in alkali, but only slightly soluble in hydrochloric acid. Thus, in many of its chemical characteristics the ochronotic pigment resembles melanin, which arises from the oxidation of 3,4-dihydroxyphenylalanine (dopa). To date, there is no specific stain that distinguishes between the ochronotic pigment of alkaptonuria, and melanin derived from dopa. Although Fitzpatrick and Lerner [48] had reported that Becker's silver stain for melanin was not darkened by ochronotic pigment and that the latter was stained intensely black by polychrome methylene blue,

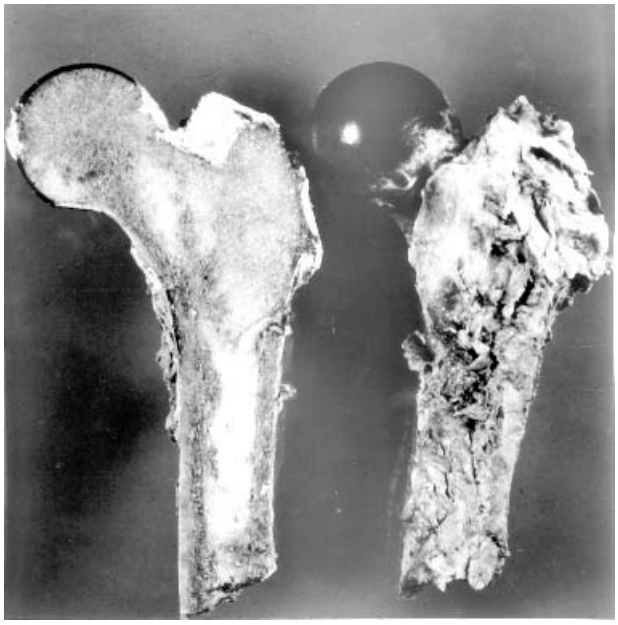


Figure 3. Ochronotic pigmentation of the femur of a 56-year-old alkaptonuric patient. (Courtesy of Dr. H.W. Edmonds of the Washington Hospital Center, Washington, D.C.)

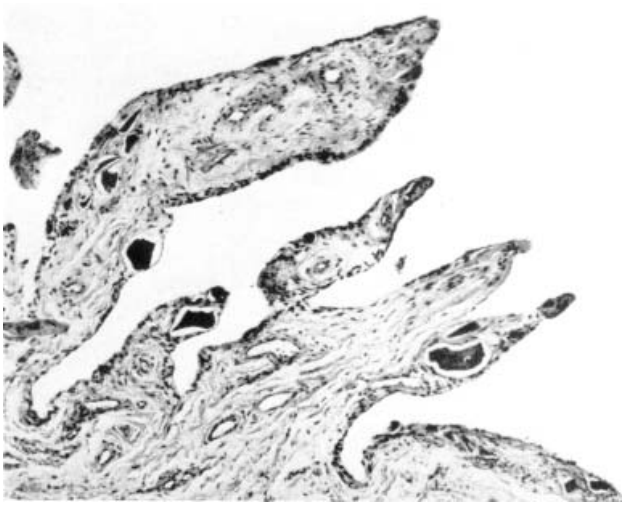


Figure 4. Deposition of ochronotic pigment in synovial tissue from the knee joint of a 40-year-old alkaptonuric male subject. (From O'Brien et al [43].)

Cooper and Moran [42] compared the staining properties of ochronotic pigment and melanin with a number of special stains and concluded that no specific differentiation could be made with any of the stains they tested. Both pigments are best detected by the Nile blue stains of Lillie [49].

The ochronotic pigment is a polymer derived from HGA, but its exact chemical structure has never been determined. It is possible that other constituents in addition to HGA are included in the product, just as melanin pigment contains an appreciable quantity of sulfur [50] as well as polymerized dopa units.

Formation of the pigment in the tissues may be entirely nonenzymatic, as with the darkening of alkaptonuric urine. Pure HGA solutions made alkaline and aerated with air or oxygen form a dark-brown product that has an ultraviolet absorption peak at 250 nm [51]. Unfortunately, the natural pigment deposited in alkaptonuric tissues as ochronosis has not been analyzed in this way, and so the relevance of the model polymer to the *in vivo* process has not been established. Milch and Titus [52] suggested that since polymerization of pure HGA solutions does not proceed at neutral pH and low partial pressure of oxygen (as occurs in cartilage), it is likely that if molecular oxygen is involved *in vivo*, it involves the participation of an enzymatic system present locally. This conclusion assumes not only that the component steps of formation of the ochronotic pigment are the same *in vivo* as in solutions of pure HGA, but that the pigment is formed as well as deposited in the cartilage. It is not possible to say whether enzymes do play a role in the synthesis of the ochronotic pigmentation in alkaptonuria, but on the basis of their substrate specificities, it is reasonable to exclude enzymes like tyrosinase in this process.

Mammalian (human, rabbit, and guinea pig) skin and cartilage contain the enzyme homogentisic acid polyphenol oxidase, which catalyzes the oxidation of HGA to an ochronotic-like pigment [53]. Benzoquinoneacetic acid has been identified as an intermediary metabolite in that oxidation. The enzyme is a copper-protein, but it clearly is distinguished from tyrosinase. For example, tyrosine, dopa, and other catechols are not substrates for this polyphenol oxidase. Earlier studies by La Du and Zannoni [54] demonstrated that *p*-quinones, such as benzoquinoneacetic acid, can form 1,4 addition products with sulfhydryl groups, and Stoner and Blivaiss [55] observed similar derivatives with the amino groups of glycine. Binding and chemical reactions of benzoquinone (or polymers derived from the acid) with the connective tissues may produce important chemical changes that alter tissue constituents and lead to ochronosis and ochronotic arthritis [24]. A possible scheme for the enzymatic oxidation of HGA and the formation of ochronotic pigment in the connective tissues of alkaptonuric patients is given in Figure 5.

Ochronosis Not Due to Homogentisic Acid

Clinically, ochronosis might be confused with the pigmentation resulting from the antimalarial agent atabrine [56], or that secondary to use of carbolic acid dressings for chronic cutaneous ulcers [57]. Both are reversible after medication is discontinued, but both treatments are rarely used today. Long-term use of skin-lightening creams and lotions containing hydroquinone may also cause a chemically induced ochronosis [58,59]. Melanotic tumors may produce a generalized ochronosis, but such patients do not survive long enough for this to be a diagnostic problem. One case of alkaptonuria was unfortunately misdiagnosed as a melanosarcoma of the eye because of the ocular pigmentation, and the eye was removed [60].

Arthritis

"Ochronotic arthritis" is a regular manifestation of longstanding alkaptonuria. From a review of the case reports, it appears that alkaptonuric arthritis occurs earlier and is more severe in males than in females [61], even though the sex incidence of alkaptonuria is approximately equal. According to Hench [62], ochronotic arthritis resembles rheumatoid arthritis clinically but is like osteoarthritis radiographically. The earliest symptoms are likely to be

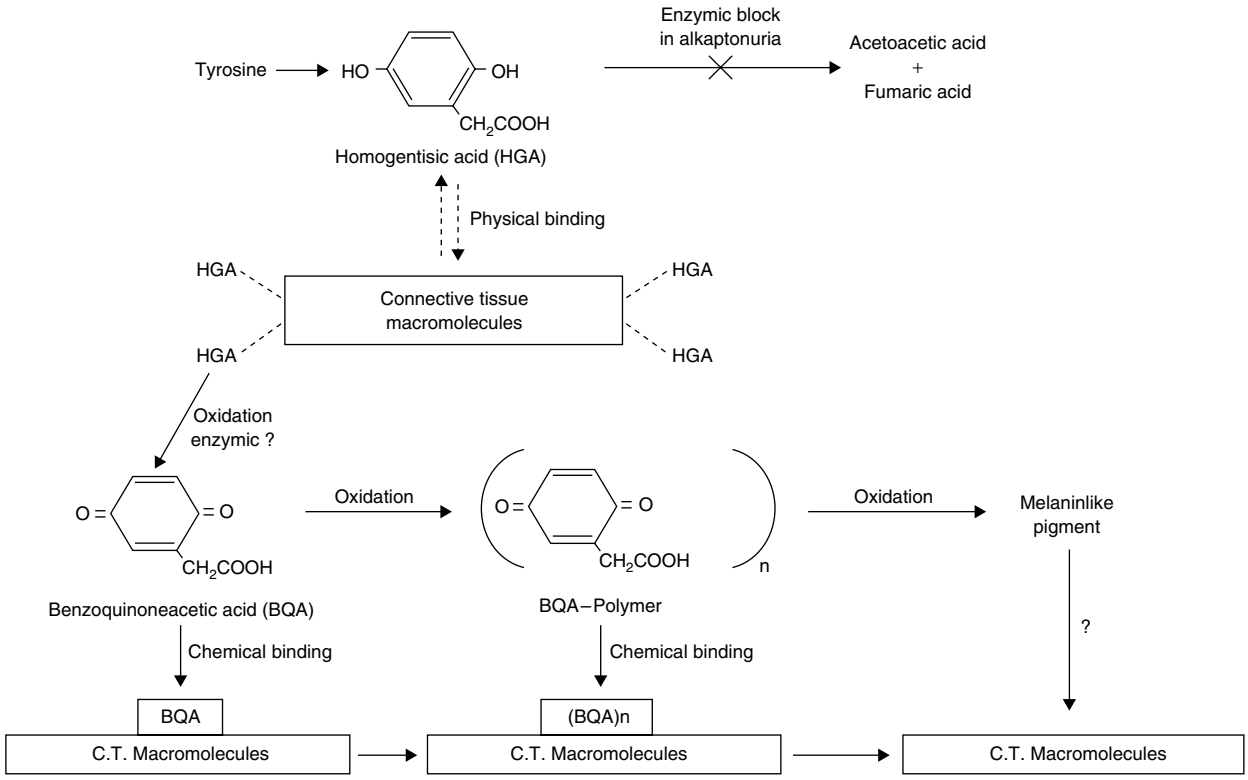


Figure 5. Postulated scheme for the formation of ochronotic pigment in alkaptonuric tissues (From Zannoni et al. [53].)

some limitation of motion of the hip, knee joints, and occasionally, the shoulders. Periods of acute inflammation resembling rheumatoid arthritis are common, followed by rather marked limitation of motion and ankylosis in the lumbosacral region.

X-ray changes of the spine are considered almost pathognomonic for alkaptonuria [21]. The vertebral bodies of the lumbar spine show degeneration of the intervertebral disks and a narrowing of the intervertebral space and dense calcification of the remaining disk material (Fig. 6). There is also a variable degree of fusion of the vertebral bodies. From x-ray changes in the lumbar spine alone, it is often possible to diagnose alkaptonuria. In contrast to rheumatoid spondylitis, alkaptonuria shows little osteophyte formation and minimal calcification of the intervertebral ligaments. The large peripheral joints involved in alkaptonuria differ from those involved in osteoarthritis in that degenerative changes are most commonly found in the shoulder and hip joints, but the sacroiliac region may be completely spared.

Calcification of the ear cartilage is another sign of alkaptonuria that may be seen on x-ray films. Occasionally, free intra-articular bodies are found [63]. In contrast, the smaller joints usually show little or no abnormality.

The common occurrence of arthritis in the population and the long interval before it occurs in alkaptonuric patients probably account for the failure of earlier investigators to connect arthritis with alkaptonuria. The first case of Boedeker [3] was said to have neuralgia of the lower lumbar spine. The early investigators considered alkaptonuria to be a completely benign disorder without symptoms and of clinical importance only because it might be misdiagnosed as diabetes, because of the nonspecific copper-reduction tests on urine used many years ago. Review of the earlier case reports shows that in most instances osteoarthritis was mentioned, and, in fact, arthritis developed in nearly all alkaptonuric patients during their later years. The arthritic complications are often severe and painful, and may lead to a completely bedridden existence in later life.

The relationship between the deposition of ochronotic pigment in the connective tissues and the degenerative changes which occur in some areas of the connective tissues, particularly the cartilage and the intervertebral disks, remains unknown. It has been suggested that the pigment acts as a chemical irritant which accelerates a degenerative process in the cartilage, with pathological changes similar to those seen in osteoarthritis [18,63]. Intra-articular injection of HGA into the knee joints of rabbits produces local lesions of the cartilage and soft tissues resembling those seen in alkaptonuria [64]. Presumably, either the ochronotic pigment or HGA itself inhibits some of the enzyme systems involved in cartilage metabolism. Greiling [65] found that low concentrations of the artificial pigment prepared by treating HGA with alkali inhibited the action of hyaluronidase on chondroitin sulfate, and hyaluronan. However, HGA itself did not inhibit hyaluronidase at equivalent concentrations. Dihlmann et al. [66] extended these studies to show inhibition of several additional enzymes, particularly glutamic dehydrogenase, hexokinase, and malate dehydrogenase, by the oxidized, polymerized pigment derived from HGA.

Inhibition of lysyl hydroxylase [67] (see also Chapter 2, Part I, this volume) could be particularly important, if

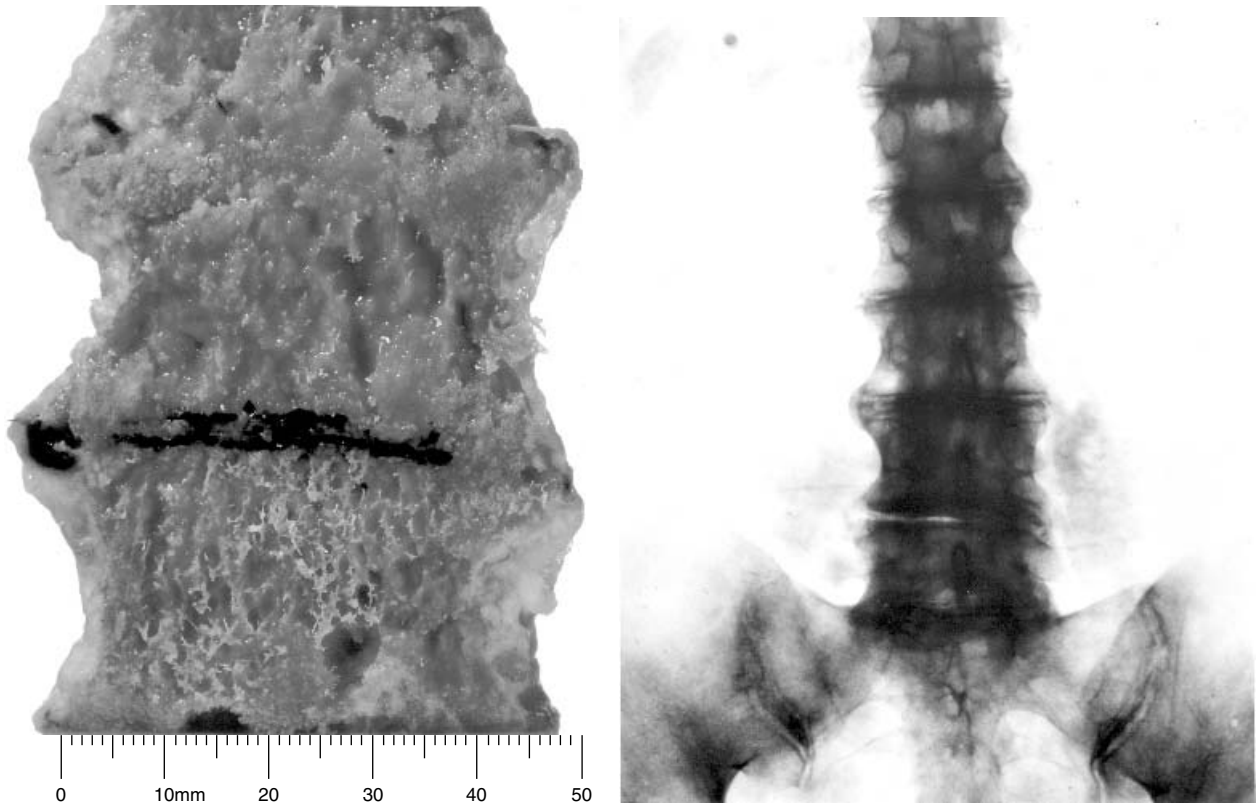


Figure 6. Alkaptonuric spines showing the typical narrowing and calcification of the intervertebral disks (Photograph on the **left**, see also Legend to Figure 9, courtesy of Dr. B. Bode, Department of Pathology, University Hospital, Zurich, Switzerland; radiograph on the **right**, from Bunim et al [38]).

this were to reduce the number of hydroxylysine residues and the nature of the cross-links essential to give tensile strength to collagen fibers. Recent studies [67a] question this hypothesis, based on the finding of normal urinary ratios of lysyl pyridinoline / hydroxylysyl pyridinoline residues in three alkaptonuric patients. Reducing agents, such as ascorbic acid and 1,4-dithiothreitol, competitively protect against inhibition of lysyl hydroxylase by HGA in the chick embryo [67], so the actual inhibitory agent in this model system might be an oxidation product of HGA, such as benzoquinoneacetic acid.

In recent years the techniques of molecular biology have made it possible to discover a number of molecular mutations that affect the structure of collagen [68](see also Chapters 8,9, and 23, Part II, this volume); some of these are responsible for hereditary forms of arthritis [69]. Whether HGA or its metabolites are responsible for inducing a similar defect in the collagen structure by altering the nature of the cross-links and thereby decreasing its resistance to shearing stress in alkaptonuric subjects remains to be established. If the arthritic complications in alkaptonuric subjects could be prevented or greatly reduced, the major clinical burden of this disorder would be eliminated.

Other workers have evaluated the effects of HGA on articular chondrocytes [70]. Cytotoxic effects were noted with rabbit adult articular chondrocytes at HGA concentrations of 5 μg/mL or above; cytotoxic effects were seen with fetal articular chondrocytes at concentrations of 1 μg/mL. Another laboratory [71] has shown that hyaline cartilage incubated with the polymerized products of HGA oxidation develops an increased hardness and decreased elasticity. It is suggested that these structural changes, as well as the mechanical stress to the large joints, lead to cartilage destruction [72].

Other Findings in Alkaptonuria

In addition to the features mentioned above, some other complications seem to occur in alkaptonuric patients with a greater frequency than might be anticipated in the general population. A relationship between ochronosis and cardiovascular disease has not been clearly established, but a review of the case histories of alkaptonuric patients indicates there to be a relatively high incidence of heart disease [19]. In 1910, Beddard [73] tabulated the autopsy findings in eleven patients with ochronosis and found that eight had chronic mitral and aortic valvulitis, one had an aortic aneurysm, and one had an aneurysm of the left ventricle. Other investigators [40,74] have noted generalized arteriosclerosis and calcification in the heart valves and of the annulus of the aortic and mitral valves [41,75]. Myocardial infarction was a common cause of death in this group.

Other complications regularly found in alkaptonuric patients are ruptured intervertebral disks [76], prostatitis, associated with prostatic stones [14,19,77], and renal stones [77]. One patient with alkaptonuria also had polycythemia [78], one had nephrocalcinosis [79], and one had severe renal disease called "ochronotic nephrosis" [42], but these are probably examples of chance association with

the other diseases. Conditions that favor the expression of alkaptonuria, such as consanguineous marriage, would also favor the presence of other recessive but unrelated traits.

SYNTHESIS AND DEGRADATION OF HOMOGENTISIC ACID

In mammals, most of the dietary phenylalanine and tyrosine is oxidized to acetoacetic acid by enzyme systems localized primarily in the liver and kidney. The scheme of this metabolic pathway is shown in Figure 7. As mentioned above, the scheme is based on the earlier studies with alkaptonuric patients and many animal experiments *in vivo*. Studies with isotopically labeled phenylalanine and tyrosine showed the fate of each of the carbon atoms in the aromatic ring and the side chain [80–82], and the distribution of the isotope in the products — CO_2, acetoacetic acid, and fumaric acid. Two of the four carbon atoms of acetoacetic acid were derived from carbon atoms 2 and 3 of the side chain and the other two came from the aromatic ring. Furthermore, the position of the isotope from the ring carbon atoms indicated that the side chain migrated during the oxidation, in agreement with the scheme postulated earlier by Neubauer (see Fig. 1), to account for the 2,5-dihydroxyphenyl intermediary product, HGA, from the 4-hydroxyphenyl precursors. This rearrangement involves a quinol intermediate, so hydroxylation of the ring, and migration, and oxidative decarboxylation of the side chain all take place as a complicated single step [83–86].

The present scheme of tyrosine oxidation is therefore very much like that of Neubauer (Fig. 1) in the steps leading to the formation of HGA, with the notable exception that neither 2,5-dihydroxyphenylalanine nor 2,5-dihydroxyphenylpyruvic acid is now considered to be an intermediate. Both compounds do produce extra HGA when fed to alkaptonuric patients [6,28], but were found to be inactive as substrates when tested with mammalian liver preparations which oxidize tyrosine or *p*-hydroxyphenylpyruvic acid to homogentisic acid [80–82].

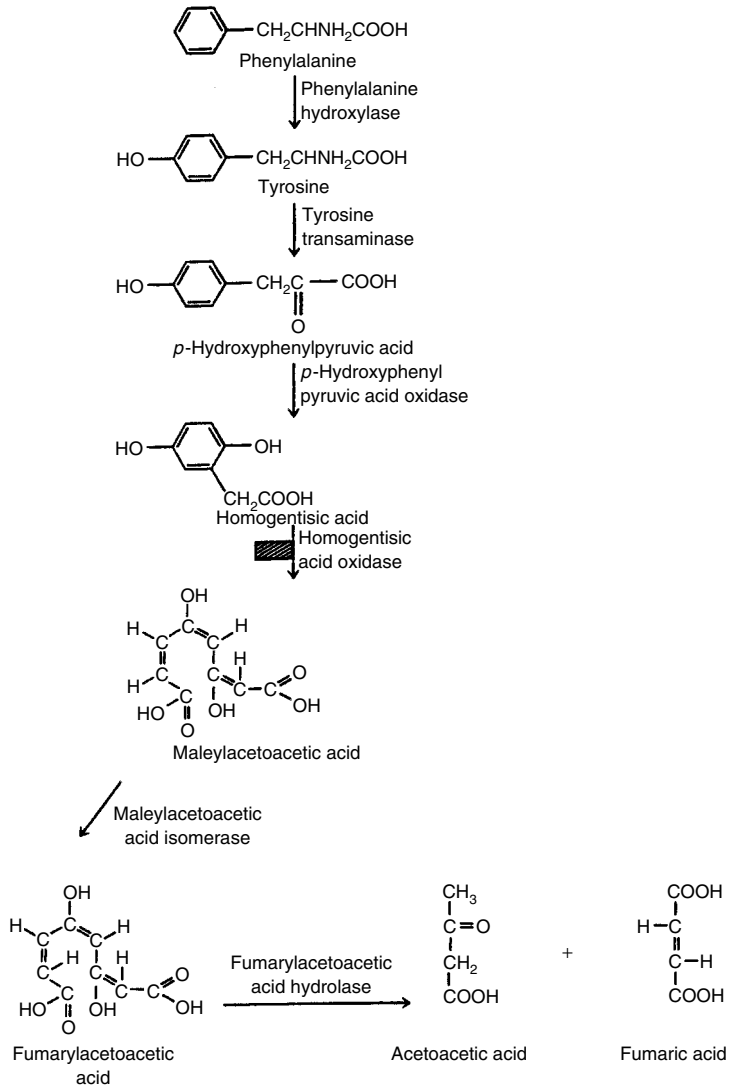


Figure 7. Enzymatic steps in the oxidation of phenylalanine and tyrosine to acetoacetic acid. ▨ = block in alkaptonuria.

TABLE 1. Activity of Tyrosine Oxidation Enzymes in Homogenates from Alkaptonuric and Nonalkaptonuric Human Liver

Enzymes	Enzyme Activity (μmol Substrate Oxidized per hour per gram Liver)	
	Nonalkaptonuric	Alkaptonuric
Tyrosine transaminase	36	32
p-Hydroxyphenylpyruvic acid oxidase	67	46
Homogentisic acid oxidase	268	<0.048
Maleylacetoacetic acid isomerase*	960	780
Fumarylacetoacetic acid hydrolase	288	222

*Units calculated as Δ log density (hour per 0.1 g) wet weight liver [87].
(From La Du et al. [11])

METABOLIC DEFECT IN ALKAPTONURIA

Garrod suggested in 1909 [8] that the metabolic defect in alkaptonuria was the absence of a liver enzyme catalyzing the oxidation of HGA, but other possible explanations were suggested from time to time after that by other workers. However, direct analysis of the enzymes involved in tyrosine metabolism in biopsied normal and alkaptonuric liver in 1958 showed that only HGO was missing in the alkaptonuric liver. All the other enzymes involved in tyrosine metabolism to acetoacetic acid were present at about the same level of activity as in normal liver [11,38] (Table 1). Evidence was also obtained that the lack of activity was not due to the presence of inhibitor or to the lack of any known cofactor [11]. Later, autopsy samples of kidney tissue from two alkaptonuric patients made it possible to show that HGO is also absent from alkaptonuric kidney [88], and presumably from any other tissues where it is usually found.

It is now reasonable to define the defect in alkaptonuria as the failure to synthesize active HGO and to attribute all the findings in alkaptonuria to this specific enzymatic defect. Recent cloning of the gene coding for HGO and the identification of specific mutations affecting the structure of the enzyme protein now provide a molecular basis for classifying various HGO deficiencies in different alkaptonuric patients.

The metabolic abnormality in alkaptonuria is present essentially from birth. Garrod noted in 1901 that staining of the diapers was scarcely evident 38 hours after birth, although after 52 hours they were deeply stained and continued to be thereafter [89]. The reason for the delay in the excretion of HGA in newborn alkaptonuric patients is probably because the enzyme systems involved in tyrosine oxidation are not completely developed at birth and increase in activity during the first few days thereafter [90]. Once established, the defect continues relentlessly throughout life. No therapeutic agent has been found that substantially alters the degree of the defect. The amount of HGA excreted per day is usually from 4 to 8 g; it can be altered by changing the content of phenylalanine and tyrosine in the diet. In starvation there is a marked decrease in HGA excretion [91], as would be expected, although Mittlebach [92] showed that on a diet very low in protein, an alkaptonuric patient continued to excrete some HGA, presumably from the breakdown of endogenous tissue proteins.

HOMOGENTISIC ACID OXIDASE

The enzymatic step missing in alkaptonuric subjects is the further metabolism of HGA by an oxidative cleavage of the benzene ring to yield maleylacetoacetic acid [93], which, in turn, is isomerized enzymatically to fumarylacetoacetic acid [87,94,95] (see Fig. 7). Human maleylacetoacetic acid isomerase has recently been cloned [96] and shown to have an identical amino acid sequence to another enzyme, glutathione S-transferase [97]. The next step is hydrolysis to fumaric and acetoacetic acids by a hydrolase which appears to be the same as that shown to hydrolyze a number of α-γ-diketo acids by Meister and Greenstein [98] and to hydrolyze triacetic acid [99,100]. In 1950, Suda and Takeda [101] solubilized an enzyme from *Pseudomonas* adapted to tyrosine which catalyzed the oxidation of HGA, and named it homogentisicase. They also studied the properties of a similar enzyme from rabbit liver [102]. Homogentisicase, or, as it is now generally called, homogentisic acid oxidase (HGO)(EC 1.13.11.5), has been purified and characterized by several laboratories [93,103–105]. It is an oxygenase, and both atoms of oxygen come from atmospheric oxygen in the cleavage of the benzene ring, based on experiments with ^{18}O. The enzyme contains essential sulfhydryl groups and requires ferrous iron [102,106]. It resembles other oxygenases involved in ring cleavage reactions, such as pyrocatechase [107,108], hydroxyanthranilate oxidase [109], and protocatechuic acid oxidase [110]. No other cofactors have been clearly implicated in this reaction, and ascorbic acid simply maintains iron in its reduced form. HGO activity in isolated rat hepatocytes depends on the oxygen tension [111]. The enzyme is inhibited by various quinones [104], sulfhydryl-binding agents [106], and metal-chelating agents such as α,α′-dipyridyl and *o*-phenanthroline [112] which react with ferrous iron.

HGO activity has been measured manometrically, because the oxidation requires the uptake of two atoms of oxygen, and spectrophotometrically [113], by following the absorption of the product maleylacetoacetic acid at 330 nm (provided that product is stable and not further metabolized under the assay conditions). HGO is localized in the soluble fraction of liver and kidney [114], as are all other mammalian enzymes involved in the conversion of tyrosine to acetoacetic acid [115,116]. The level of HGO activity in the rat is highest in liver, and appreciably lower in kidney, and there is no significant activity in any other tissues so far examined [105,106], such as blood, salivary glands, germinal epithelium, and muscle. A similar tissue distribution pattern was observed in rats, rabbits, guinea pigs, and pigeons [105]. In humans, too, HGO is highest in liver [11], with appreciable activity also present in kidney [88]. The optimal pH for HGO is about 7 [102,103], and it is very specific for HGA. Such closely related compounds as *o*-hydroxyphenylacetic acid, *p*-hydroxyphenylacetic acid, and gentisic acid are not

oxidized [103], nor are homogentisic acid ethyl ester and homogentisic acid lactone. The quinone formed by oxidizing HGA (benzoquinoneacetic acid) does not appear to be an intermediate in the oxidation [103]. In fact, this quinone is a strong inhibitor of HGO [6,103]. The requirement for ferrous iron cannot be satisfied by other bivalent metals, such as Co^{++}, Zn^{++}, Mg^{++}, or Mn^{++} [102].

Metabolism of Homogentisic Acid

One might expect elevated plasma levels of HGA in alkaptonuric patients in view of the large quantities of this acid excreted each day. Neuberger et al. [25] found, however, that in a 7-year-old alkaptonuric girl, the fasting plasma level was not more than about 3 mg/100 mL plasma, and this level did not increase significantly following the oral administration of 3 g L-phenylalanine. Nevertheless, within 6 hours approximately 85% of the administered amino acid could be accounted for as extra HGA in the urine. Neuberger et al. [25] made very significant observations in regard to the excretion of HGA during their investigation. The plasma clearance data indicated that unless a large fraction of the urinary HGA was both synthesized and excreted within the kidney, the glomerular filtration rate alone could not possibly account for the efficient rate of HGA excretion. Indeed, the renal clearance of HGA approached 400 to 500 mL/min, approximately equal to the rate of renal blood flow. Even though it is very unusual for a normal intermediary metabolite to be actively secreted by the kidney, this is obviously true for HGA. This conclusion is in accord with the earlier observation of Katsch and Metz [117] that giving intravenous HGA to an alkaptonuric subject did not increase the plasma concentration of this acid significantly. Experiments using an enzymatic assay to estimate plasma HGA concentrations have also confirmed these conclusions [31]. Alkaptonuric and nonalkaptonuric individuals were both found to excrete HGA very rapidly after oral administration, and the renal clearance indicated active secretion by the kidney.

It appears that two factors help to keep the plasma and presumably the tissue concentrations of HGA at a low level: the great capacity to metabolize this acid in the liver and kidney, and the rapid renal tubular secretion of HGA. In the alkaptonuric patient, the renal secretory mechanism is sufficient to keep the plasma HGA level very low, even when extra HGA is given. This renal defensive mechanism is very significant, and probably explains why it takes so many years before ochronosis appears in alkaptonuria subjects. It is quite possible that the tissues are only occasionally flooded with HGA and that this must be repeated many times over the years before tissue pigmentation occurs to a significant extent. Benzoquinoneacetic acid may be an intermediate in this process.

EXPERIMENTAL ALKAPTONURIA AND OCHRONOSIS

Genetic Animal Models

Spontaneous alkaptonuria has very rarely been reported in species other than human beings. A Cynomolgus male monkey caught in the wild was found to excrete appreciable amounts of HGA, positively identified by gas chromatography-mass spectrometric analysis [118]. Another report describes a mutant mouse with undetectable liver HGO and homogentisic aciduria. However, no ochronotic pigment deposits were present in the tissues. The authors suggested that the lack of ochronosis might be explained by the relatively high concentrations of endogenously produced ascorbic acid in the mouse [119]. In contrast, an earlier paper by Lewis [120] described a rabbit with urine that darkened on exposure to air and showed some other qualitative tests for HGA. Unfortunately, HGA was never isolated or positively identified, and the rabbit died without leaving offspring. In addition, there are a few reports of generalized ochronosis in the bones and connective tissues of cattle, dogs, and horses in which the tissues are described as being as black as coal; again, HGA was never positively identified in the urine of any of these animals [17,121,122].

Induced Alkaptonuria in Animals

Experimental alkaptonuria has been produced in rats and mice by feeding large quantities of phenylalanine or tyrosine. It has also been reported that vitamin C-deficient guinea pigs fed extra phenylalanine [123,124] or tyrosine [123] excreted HGA, as well as *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid, but the methods used in these studies for urinalysis do not distinguish between *p*-hydroxyphenylpyruvic acid and HGA [125]. Other workers have found only the two *p*-hydroxy compounds and no HGA in similar experiments with vitamin C-deficient guinea pigs [126].

Experimental alkaptonuria has, as noted, been produced in rats and mice by feeding large quantities of phenylalanine and tyrosine. There is also one report of transitory HGA excretion after feeding a human volunteer large amounts of L-tyrosine [127]. Another type of experimental alkaptonuria has been induced in rats by a diet deficient in the sulfur-containing amino acids [128], which was not corrected by giving ascorbic acid but was reversed by giving cysteine [129]. In this type of experimental alkaptonuria, proportionately less *p*-hydroxyphenylpyruvic acid is excreted than in the type that responds to ascorbic acid.

In addition to the above methods, the fact that HGO requires ferrous iron has been exploited. Inhibition of HGO by the injection of α,α′-dipyridyl and feeding extra tyrosine were used by Suda and Takeda [102] to induce experimental alkaptonuria in guinea pigs. The excretion of HGA in these animals was not corrected by giving vitamin C.

It can be concluded that most of the methods of producing experimental alkaptonuria have relied on either direct inhibition of HGO or the creation of an imbalance in the various enzyme reactions sufficient to cause an accumulation of HGA and its urinary excretion. Attempts to produce experimental alkaptonuria and ochronosis in animals by feeding special diets have met with only limited success. In most instances the inhibition of HGO has been inadequate, and only a small fraction of normal HGO activity is sufficient to prevent the accumulation of HGA and deposition of ochronotic pigment.

Prolonged feeding of L-tyrosine has produced some degree of ochronotic pigmentation of the connective tissues in animals. Bondurant and Henry [130] maintained rats on a diet supplemented with 12% tyrosine for 40 days. Gross examination of the dissected knee and hip joints revealed no structural abnormalities, but these tissues showed the deposition of pigment in the articular cartilage of the head of the femur and in opposing tibial and patellar surfaces of the knee joint. Microscopic examination showed focal accumulations of dark-brown pigment in the cytoplasm of chondrocytes in the epiphyseal cartilage in some animals.

Blivaiss et al. [131] induced experimental ochronosis in 4-week-old rats by supplementing their diet with 8%

tyrosine for up to 28 months. After this time there were ochronotic-like pigment deposits in cartilage, such as the joint capsules, condyles, sternum, and trachea. Pathologic changes were found in the articular cartilage, which included abnormal alignment of chondrocytes with pigment inclusion, fibrillation, and fragmentation, as well as bone denudation. By histochemical techniques, they found an increase in nonsulfated glycosaminoglycans in the connective tissues. The histochemical changes were similar to those observed in the patella from a patient with alkaptonuria.

HEREDITARY ASPECTS

The first description of the inheritance of alkaptonuria was by Garrod in 1902 [9]. He presented evidence that this condition was congenital and familial, and that it occurred more often in families in which there were consanguineous marriages. He suggested that alkaptonuria was transmitted as a simple recessive Mendelian trait, and believed that HGA arose in the normal course of tyrosine metabolism. In 1902, Bateson and Saunders [132] also suggested that the inheritance of a rare recessive factor might explain the incidence of alkaptonuria. These studies on the mode of transmission of alkaptonuria were among the very first of any on human hereditary metabolic disorders. During the subsequent years, several examples were recorded of direct transmission of alkaptonuria (i.e., parent and offspring both affected), but Garrod suggested that these were examples of a heterozygous individual mating with a homozygous alkaptonuric, rather than representations of a dominant form of the disorder. As more family histories were described, the general conclusion about the recessive nature of the disease remained unchallenged.

In 1932, Hogben et al. [133] carefully reviewed all the known cases of alkaptonuria reported up to that time. The recessive character of the disease was confirmed in nearly all the families, and at least half of all affected individuals were offspring of consanguineous matings. Although there was an unequal sex distribution in their cases — 100 males and 46 females — the authors did not interpret this as an indication that the condition was semilethal in females. They noted that males were more often the probands in affected families and suggested that the higher incidence in males might be explained by the more frequent medical examination of males. They also noted that among infants there were slightly more females than males, in agreement with this explanation.

Among the families summarized by Hogben et al. [133] were a few in which a dominant form of alkaptonuria had to be considered. In one family studied by Pieter [134], the author felt compelled to conclude that a dominant type of alkaptonuria existed. Such a conclusion depends on the predicted opportunity for marriage between homozygous and heterozygous individuals, and the frequency of the heterozygote in the population sample under study. The incidence of alkaptonuria in the general population can only be roughly estimated. It is reasonable to assume, however, that it is less rare than was believed 50 years ago. A study in Northern Ireland by A.C. Stephenson reported the incidence of alkaptonuria to be from 3 to 5 per million individuals (see [135]). This would give a considerably higher incidence of heterozygous individuals in the general population than assumed by Hogben et al. [133]. At present, most, if not all, cases appear to represent the inheritance of a single autosomal recessive gene. This is supported by the biochemical finding that a single enzyme system is inactive in this condition and that only one clinical form of alkaptonuria is known.

An unusually high incidence of alkaptonuria has been observed in the Trencin District of Czechoslovakia, near the Slovakian-Bohemian border [136]. Nearly 200 alkaptonuric individuals have been identified among the 16,000 inhabitants [137,138]. Genetic analysis of the affected families shows a concentration of alkaptonuric patients within specific localities, and the authors propose that this is due to a founder effect, with genetic drift and inbreeding in the specific isolated hamlets of that region [139].

Rare diseases with recessive inheritance are regularly encountered more frequently in selected inbred populations, and this complicates any estimation of the incidence of heterozygotes in the general population. It would be helpful if a diagnostic test were available to detect the carrier state. One would hope to find approximately half the normal amount of enzyme in the tissues of heterozygous individuals, but obligatory heterozygous carriers of alkaptonuria do not excrete HGA after an oral loading dose of L-tyrosine [140], and measurements of the ability to metabolize HGA by an oral HGA tolerance test have so far shown no differences between presumed carriers and normal controls. These results are not unexpected in view of the tremendous capacity of the liver to metabolize HGA. Even if this reserve were reduced to one-half, it might not be exceeded by any oral tolerance test. It is interesting to calculate the potential reserve capacity to metabolize HGA: assuming a liver weight of 1500 g and also assuming that the liver HGO is as efficient *in vivo* as found under assay conditions *in vitro*, it may be calculated that the normal adult liver should be able to metabolize over 1600 g of HGA per day.

MOLECULAR BIOLOGY OF THE GENETIC DEFECT

Considerable progress has been made within the last few years in our understanding of the genetic defects in alkaptonuria at the molecular level, and this has been well summarized by Scriver [141]. On the basis of the coinheritance of alkaptonuria with other Mendelian diseases, chromosome 3 was suspected to be the likely location of the gene causing alkaptonuria. Homozygosity mapping with polymorphic markers in two consanguineous families suggested further that the most likely region was 3q2 (combined lod score = 4.3) [142]. Another laboratory also assigned the alkaptonuria gene (*AKU*) to human chromosome 3q [143]. Further supporting evidence came from quite another source, when the murine alkaptonuria gene (*Aku*) was mapped to mouse chromosome 16 in a region of synteny between human chromosome 3 and that mouse chromosome [144]. The structural gene for human HGO was cloned in 1996 [145]. The investigators concerned also demonstrated that specific mutations of the *HGO* gene were associated with the alkaptonuria phenotype, and that these mutations followed the expected pattern within alkaptonuric family pedigrees. The same laboratory also showed that the *HGO* gene mapped to human chromosome 3q21–q23, the location of the *AKU* locus [145]. Their unusual approach to cloning the human *HGO* gene began by first cloning the homologous *hmgA* gene in *Aspergillus nidulans* after growing the mold on phenylalanine [146]. As described by Scazzocchio [147], success with the *Aspergillus* genetic analysis led to similar success cloning the human *HGO* gene, using probes derived from the mold *hmgA*

and a spectrophotometric method to measure HGA by its absorption after conversion to maleylacetoacetic acid [148].

The human *HGO* gene has 14 exons encoding 445 amino acid residues which extend over nearly 60 kb of DNA. The gene is expressed in the liver, prostate, colon, and small intestine in particular, and there seems to be only one copy of the gene [145]. Two pedigrees were analyzed for mutations that might be expected to result in the loss of catalytic function. One mutation, Pro230Ser, was present in both parents in family M, and in one parent in family S. Another mutation, Val300Gly, was present in the other parent in family S. The affected children all had either a double dose of Pro230Ser, or were compound heterozygotes with both of these mutations. Expression experiments in *E. coli* of the wild type and Pro230Ser mutant forms of human HGO demonstrated that this single amino acid change in the protein structure caused a complete loss of enzymatic activity [145].

A more complete description of the human *HGO* gene has recently been published by the same laboratory [149], with further details about the regulatory regions, and the intronic and exonic characteristics. Several simple sequence repeats (SSRs) in the introns were polymorphic in their Spanish population sample, and these markers should be very useful in tracing particular *HGO* mutations in different ethnic groups and geographic areas in the future.

Researchers at the University of Würzburg recently reported the cloning of the mouse *HGO* gene [150]. In collaboration with Slovak investigators at the Komensky University they used the high degree of homology (93%) between human and mouse *HGO* to clone independently the human *HGO* gene [151]. They found the same 14 introns and coding sequence published by the Spanish group [145]. In four alkaptonuric patients from the Slovakian region they found two novel mutations: Gly481Ala, in exon 8, and a single base insertion in exon 7 (454–457insG), which leads to a stop codon, 21 codons farther downstream [151]. The relatively high prevalence of alkaptonuria in Slovakia (up to 1 in 19,000) will be explored with these new molecular probes to look for founder effects and the distribution of *HGO* mutations within this special population.

The Madrid laboratory has reported on the *HGO* mutations in 14 representative alkaptonuric subjects from six European countries, Algeria and Turkey. Point mutations believed to produce nonfunctional enzyme have been identified in 11 of the 14 exons, and several intronic defects are also believed to be responsible for this metabolic disorder phenotype [152]. They have recently added six additional mutations, bringing the total of known *HGO* mutations at this time to 27 [155]. These mutations and those found in the Slovak individuals and other locations are illustrated in Figure 8.

The associated polymorphisms and repetitive sequences found associated with the *HGO* gene and its mutations permit the designation of several distinct haplotypes, five of which are the most representative. These haplotypes will be very useful in exploring the long history and migration of the alkaptonuria mutations in different populations throughout the world.

DNA tests may also be used in the future in the detection of carriers among relatives of identified alkaptonuric patients, as well as the identification of variant forms of the disorder which would be expected to show some clinical differences from the standard phenotype.

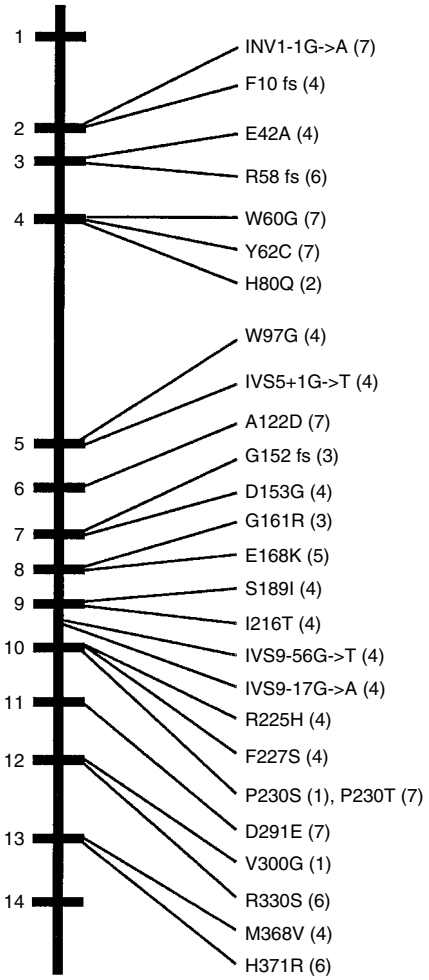


Figure 8. Locations of mutations causing alkaptonuria in the human homogentisic acid oxidase (*HGO*) gene. Mutations reported in [145] are indicated by (1); in [149] by (2); in [151] by (3); in [152] by (4); in [153] by (5); in [154] by (6); and in [155] by (7). The 14 exons of the *HGO* gene are represented roughly to scale.

TREATMENT

Attempts to treat alkaptonuria have been directed either toward correcting the underlying metabolic defect or preventing or reversing the pigmentation and arthritic changes. Galdston et al. [40] administered several vitamins, brewers' yeast, tyrosinase, insulin, and adrenocortical extract without altering the daily amount of HGA excreted by an alkaptonuric patient. Several groups have studied the effectiveness of vitamin C [40,156,157]. Although ascorbic acid corrects the alkaptonuria induced in guinea pigs by feeding large amounts of tyrosine [158], it does not change the defect in the hereditary type of human alkaptonuria. Other agents, such as vitamin B12 [159], and cortisone [160,161] are also without benefit, as far as correcting the metabolic defect is concerned.

At times, it has been suggested that dietary phenylalanine and tyrosine be reduced to decrease the output of HGA, but a severe restriction in the intake of these amino acids is not practical except for brief periods, and probably would

be dangerous to the alkaptonuric patient if continued for very long.

Since, from a practical standpoint, the important consequence of this metabolic disorder is that it leads to pigmentation and arthritis, an effective therapeutic measure could be aimed at preventing or correcting these outcomes. It is possible, as Sealock et al. pointed out [23], that increased tissue concentrations of ascorbic acid might prevent or greatly reduce the deposition of ochronotic pigment, even though this treatment would have no impact on the basic enzymatic defect.

The observation by Murray et al. [67] that ascorbic acid protects lysyl hydroxylase from inhibition by HGA (possibly via benzoquinoneacetic acid [162]), leads to the obvious suggestion that prolonged maintenance of relatively high tissue concentrations of ascorbic acid might delay, and possibly reduce, the degree of pathologic changes in the connective tissues. At least, extended clinical experience giving a gram or so of ascorbic acid per day in divided doses is worth investigating [163,164]. It has been claimed that an alkaptonuric patient with arthritis showed symptomatic improvement after taking 2 g of ascorbic acid per day [165]. Megadoses of ascorbic acid, however, should probably be avoided and could be counterproductive, since, at very high levels, ascorbic acid may compete with and compromise the active secretion of HGA by the kidney.

NTBC (mesotrione), [2-(2-nitro-4-trifluormethylbenzoyl)-cyclohexane-1,3-dione], a potent inhibitor of *p*-hydroxyphenylpyruvate oxidase (*p*HPPO), is used to treat hereditary tyrosinemia (MIM 276700). Recently, this inhibitor has also been suggested as a possible treatment for alkaptonuria, since it should theoretically shift the metabolic block from HGO to the preceding metabolic step, *p*HPPO (see Fig. 7), and prevent the formation of HGA. However, many uncertainties remain, such as the safety of long-term use of NTBC, at what age the treatment of alkaptonurics should start, and whether a complete metabolic block would be necessary. Although metabolic inhibitors for treating severe disorders, like tyrosinemia, have gained ready acceptance because of the dire consequences of no treatment, great care must be taken in the chronic treatment of disorders that are less

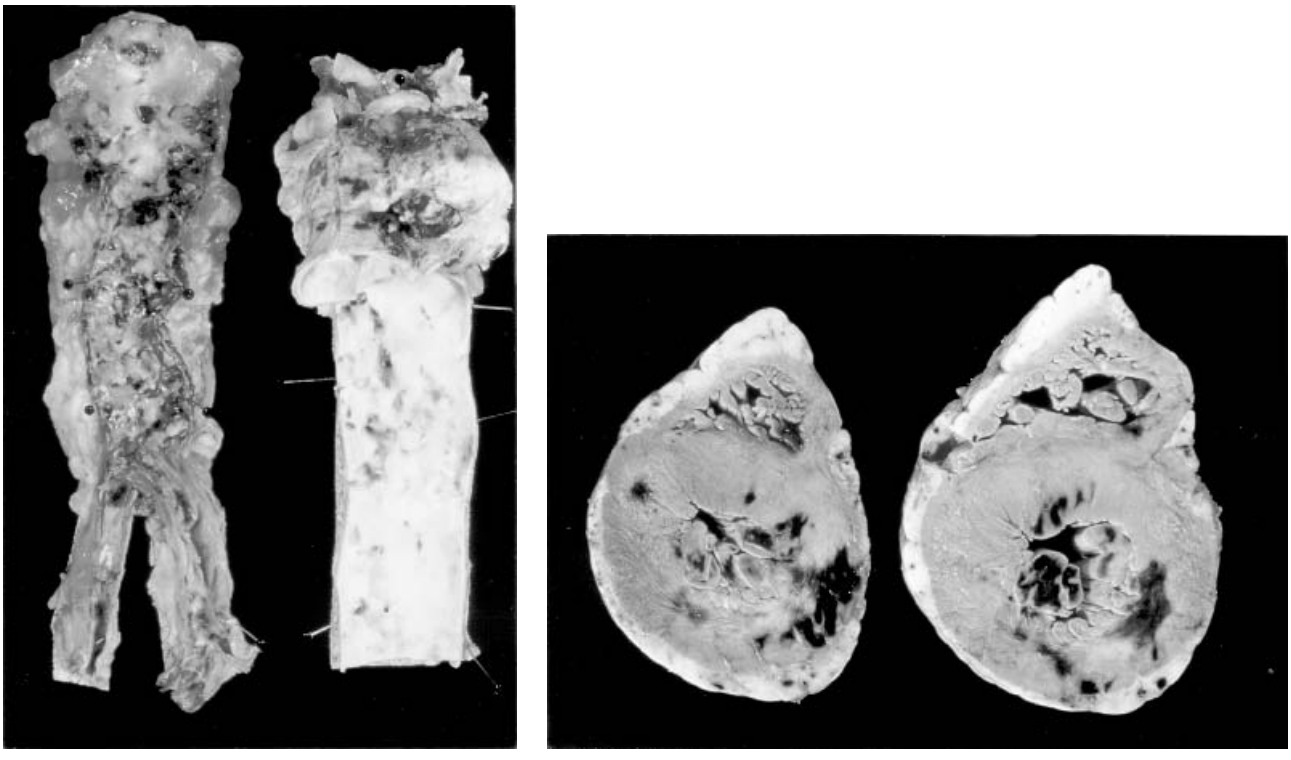
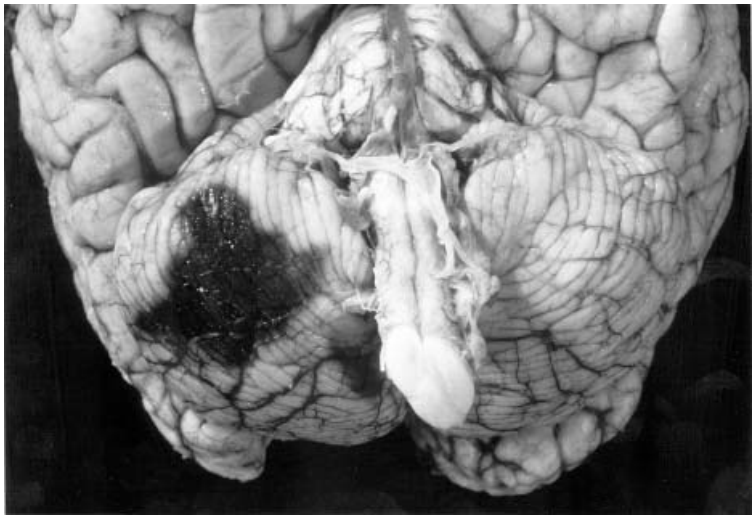


Figure 9. The 68-year-old woman whose tissues are shown, died of cardiac insufficiency 2 weeks after an aortocoronary bypass operation and aortic valve replacement for coronary heart disease and severe aortic stenosis. Both mammary arteries had to be used for the procedure because the leg veins were damaged as a result of the multiple orthopedic operations she had previously undergone (see below). Dissections of both mammary arteries, probably due to alkaptonuria-related vessel wall changes, were found at autopsy. The patient had a long-standing history of generalized arthritis, and total prostheses had been implanted in both knee and both hip joints. Endarterectomy of both carotid arteries, as well as percutaneous transluminal angioplasty of the right femoral artery for symptomatic arteriosclerosis, had been performed on different occasions within the previous 15 years. She reported that her urine darkened in contact with air and her underwear often displayed dark stains. On clinical examination marked blueness of the sclerae as well as dark conchae and anthelixes could be seen. Marked arteriosclerosis of the abdominal aorta with dark pigmentation on the left, compared with the less severely affected thoracic aorta on the right (**A**). Pigment deposition in the infarcted myocardium (**B**) and in the cerebellum at infarcted areas which showed histologic signs of cellular organisation (**C**). The surface of the kidneys was irregular and presented dark spots (**D**). (For alkaptonuric spine changes in the patient, see Fig. 6, left panel.) (Courtesy of Dr. B. Bode, Department of Pathology, University Hospital, Zurich.)



C

D

Figure 9. (*Continued*)

severe and have slowly acquired pathology, like alkaptonuria. It is probable, however, that this approach for treating alkaptonuria will be tried and evaluated during the next few years.

The recent advances in our understanding of the molecular defect in alkaptonuria will, no doubt, encourage the application of genetic engineering to replace the missing enzyme by recombinant techniques. While it may be possible to achieve such a result at some time in the future, it is important now to be aware of the potential toxicity of some of the intermediary compounds that occur later in the tyrosine metabolism pathway, particularly maleylacetoacetic acid and fumarylacetoacetic acid. Extensive conversion of HGA to these later metabolites in inappropriate environments, where they may not easily be further metabolized, might result in even greater toxicity for the patient than that produced by HGA, itself [166].

RECENT DEVELOPMENTS

Details have been received of a 47-year-old man with alkaptonuria who exhibited progression of his ochronosis much more rapidly than his two affected siblings. In fact, his skin was extremely dark. It was determined that he suffered from diabetic nephropathy with glomerular insufficiency. His plasma HGA level was approximately twice that encountered in other alkaptonuric patients, while his urinary HGA level was typical of the disorder. After receiving a renal allograft from his homozygously normal sister, his renal function normalized, and his plasma HGA level fell by approximately one-half, as did his 24-hour urinary output of HGA. This suggests that the transplanted kidney provided secretory function, and may also have provided HGO activity; (personal communication, W. Introne, C. Phornphutkul, I. Bernardini, W. Gahl. 23 October 2001).

N.B. *With the permission of the author, the editors have added as Figure 9 a number of photographs that they felt might be of some clinical interest.*

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Chapter 21

Fibrodysplasia Ossificans Progressiva

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SUMMARY

- **Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant disorder of connective tissue.**
- **The classic phenotype of FOP includes the diagnostic triad of congenital malformation of the great toes; progressive heterotopic ossification of skeletal muscles, tendons, ligaments, and fascia in an endochondral process; and progression of disease activity in characteristic anatomic patterns (dorsal to ventral, axial to appendicular, cranial to caudal, and proximal to distal).**
- **Ectopic ossification in FOP usually begins in the muscles of the neck or back as soft tissue swellings that look like tumors. The lesions appear rapidly, tend to be asymmetric, and are variable in both size and shape, frequently leading to misdiagnosis of a neoplastic process.**
- **Cells from FOP patients show increased expression of bone morphogenetic protein-4 (BMP-4).**
- **A genome-wide linkage analysis using four families with inheritance of FOP revealed consistent linkage of FOP with the 4q27-31 region of chromosome 4. The *BMP4* gene is not within this locus, but genes involved in the BMP signalling pathway, as well as other candidate genes, have been identified in this interval.**
- **There is no established medical treatment for FOP. Creative use of BMP technology will likely have important applications in the inhibition of heterotopic ossification in diseases such as FOP. Soluble BMP receptors, dominant negative receptors, and BMP antagonists may show promise in binding and physiologically inactivating BMP where it is not needed or wanted.**

INTRODUCTION

Fibrodysplasia ossificans progressiva (FOP) (MIM 135100) (synonym: myositis ossificans progressiva) is a rare autosomal dominant disorder of connective tissue in which severe disability due to progressive soft tissue ossification accompanies characteristic skeletal malformations. Early case reports were provided by Guy Patin in 1692 [1] and by John Freke in 1736 [2], but it was not until 1879 that Helferich described the characteristic associated malformation of the big toes [3]. By 1918, Rosenstirn [4] was able to identify 115 definite cases of FOP in the world literature. The number of reported cases now exceeds 600 and includes several large series [5–10] in addition to multiple single case reports. Two attempts at complete ascertainment have been made. A minimum point prevalence of 1 patient per 1.64 million of the population was found in the United Kingdom [11], and a similar point prevalence was recently found in France (Le Merrer, Hôpital Necker, Paris; personal communication). The sex ratio is equal, and FOP has been reported in all ethnic groups.

CLINICAL FEATURES

The principal clinical features of FOP are skeletal malformations and soft tissue ossification. Although malformations may be demonstrated radiologically at a number of sites in the skeleton, they are evident clinically only in the hands and feet [7]. Characteristically (90% of patients), the big toes are short with malformations of the first metatarsals, and contain only a single phalanx which may be deviated laterally (Fig. 1). Less commonly (10% of patients), the big toes are of normal length, but are stiff from early childhood or become rigid in adolescence. Rarely, severe reduction defects of all toes are present [7,8].

Short thumbs, due to short first metacarpals, are seen in about 50% of FOP patients, and incurved little fingers are seen in a similar percentage of individuals. Occasionally, shortening or stiffening of other digits may be apparent. All of these digital malformations in FOP are bilaterally symmetrical [7,8].

The pregnancy, birth history, and birth weight of FOP patients are unremarkable. The shortened big toes may be noted at birth, but tend to be misdiagnosed as isolated congenital hallux valgus. The child is otherwise asymptomatic until soft tissue ossification begins.

Ectopic ossification in FOP usually presents as a series of lumps in the muscles of the neck or back [8,12] (Fig. 2). Typically, these lumps are first noted at 2–6 years of age,

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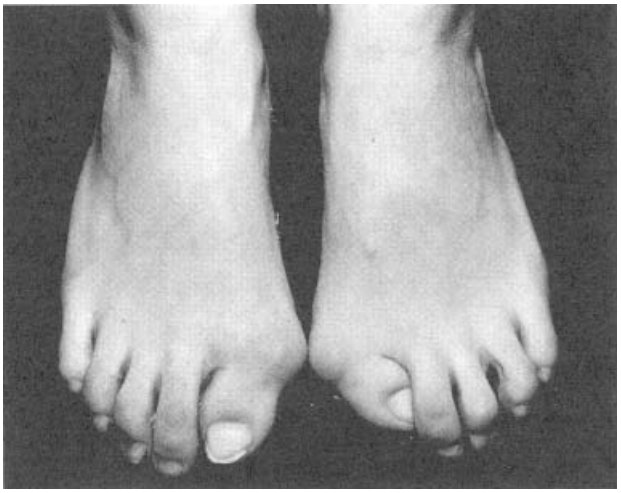


Figure 1. Short big toes with valgus deviation.

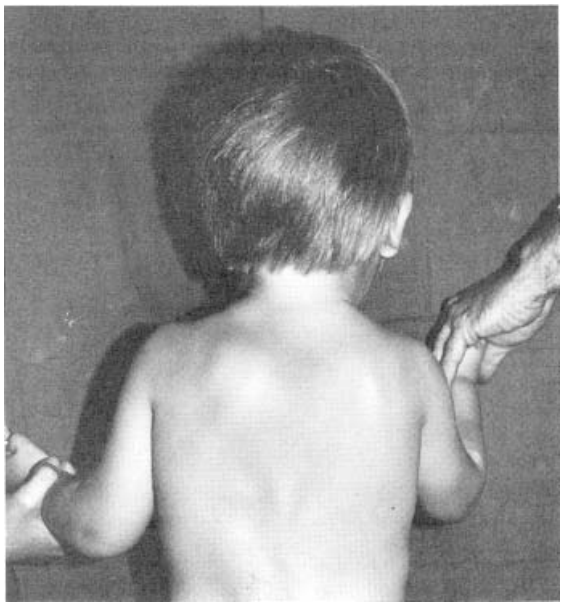


Figure 2. Early paraspinal ossifying lumps.

but they may occasionally be present at birth, or be first noted only after adolescence [9,13,14]. Soft tissue swellings that look like tumors appear over several hours, tend to be asymmetric, and are variable both in size and shape leading to suspicion of a neoplastic process [15]. They may or may not be painful and the overlying skin may be warm and reddened, leading to suspicion of an infective process [10]. Lumps are usually firm in texture and gradually shrink in size over several days or weeks. They may be succeeded by other lumps, and this often gives the patient the mistaken impression that the lumps are moving about [8]. Occasionally, fever or malaise accompanies the appearance of new lumps, but in general constitutional disturbance is absent [16]. The lumps themselves cause stiffness, which improves as they subside, but the disease process continues, and when ectopic bone forms, irreversible limitation of movement becomes apparent [9]. Mature heterotopic bone replaces skeletal muscle, tendon, ligaments, and fascia, locking joints in place, and rendering movement completely impossible [13] (Fig. 3). Flare-ups in limb musculature and soft tissues can often be mistaken for thrombophlebitis [17].

The majority of episodes of ectopic ossification occur spontaneously, although local trauma may result in an exacerbation. Local trauma may include intramuscular injections [18], lump biopsy [19], operations to excise ectopic bone [10], accidental injury [20], falls [21], dental therapy [22,23], and careless venepuncture.

Ectopic bone in FOP has a predilection for certain sites. The axial musculature bears the brunt of the disease. Limb involvement tends to be most marked proximally; with proximal muscle involvement preceding distal involvement, axial involvement preceding appendicular involvement, and dorsal involvement preceding ventral involvement [9]. Limited jaw opening is also extremely common [22–25]. In contrast, involvement of the muscles of the face is uncommon. Certain muscles, including the tongue, the extraocular muscles, the diaphragm, heart, sphincter muscles, and visceral smooth muscle are never involved [8,9].

Ectopic bone formation also occurs in ligaments, fasciae, aponeuroses, tendons, and joint capsules [5]. Skin is not directly involved, as in a related condition, progressive osseous heteroplasia (POH) (MIM 166350), but ulceration over a projecting mass of bone may occur [19].

Stiffness of the cervical spine is a common early finding [26]. Later, fixation of multiple major joints in a variety of positions results in a progressive loss of mobility [13]. Ultimately, the patient becomes virtually immobilized, with residual movements possible only in the distal limbs, tongue, face, and eyes [13]. Submandibular swelling can be a life-threatening complication especially when associated with massive anterior neck swelling and difficulty in swallowing [22]. Special measures to decrease swelling, including a course of glucocorticoids may be warranted [22].

Hearing impairment, first noted by Lutwak [27], is a common feature of FOP [6,8] occurring in 52% of patients [28]. The onset is usually in adolescence or early adult life, and develops over a period of some weeks, but is then nonprogressive. The hearing loss is usually conductive and may be due to middle ear ossification, but in the only patient who has had surgical exploration of the middle ear, a mobile ossicular chain was found with no evidence of stapedius ossification [29].

Ocular problems do not occur in FOP and mental development is usually normal. Two patients in the series of Connor and Evans [8] were mildly mentally retarded and, interestingly, these two also had the most severe hand and foot malformations with reduction defects of all digits.

Other highly variable features include diffuse scalp baldness, excessive skin seborrhea, menstrual irregularities, and premature menopause [8]. Sexual development is usually normal, although lack of breast development in females appears to be a common feature of the condition. Longitudinal growth appears to be normal, although severe scoliosis may develop, especially when asymmetrical heterotopic ossification of the spine occurs early in life [30].

RADIOLOGICAL FEATURES

The radiological findings in FOP illustrate the combination of skeletal malformations, ectopic ossification, and skeletal changes secondary to immobilization and activity [31].

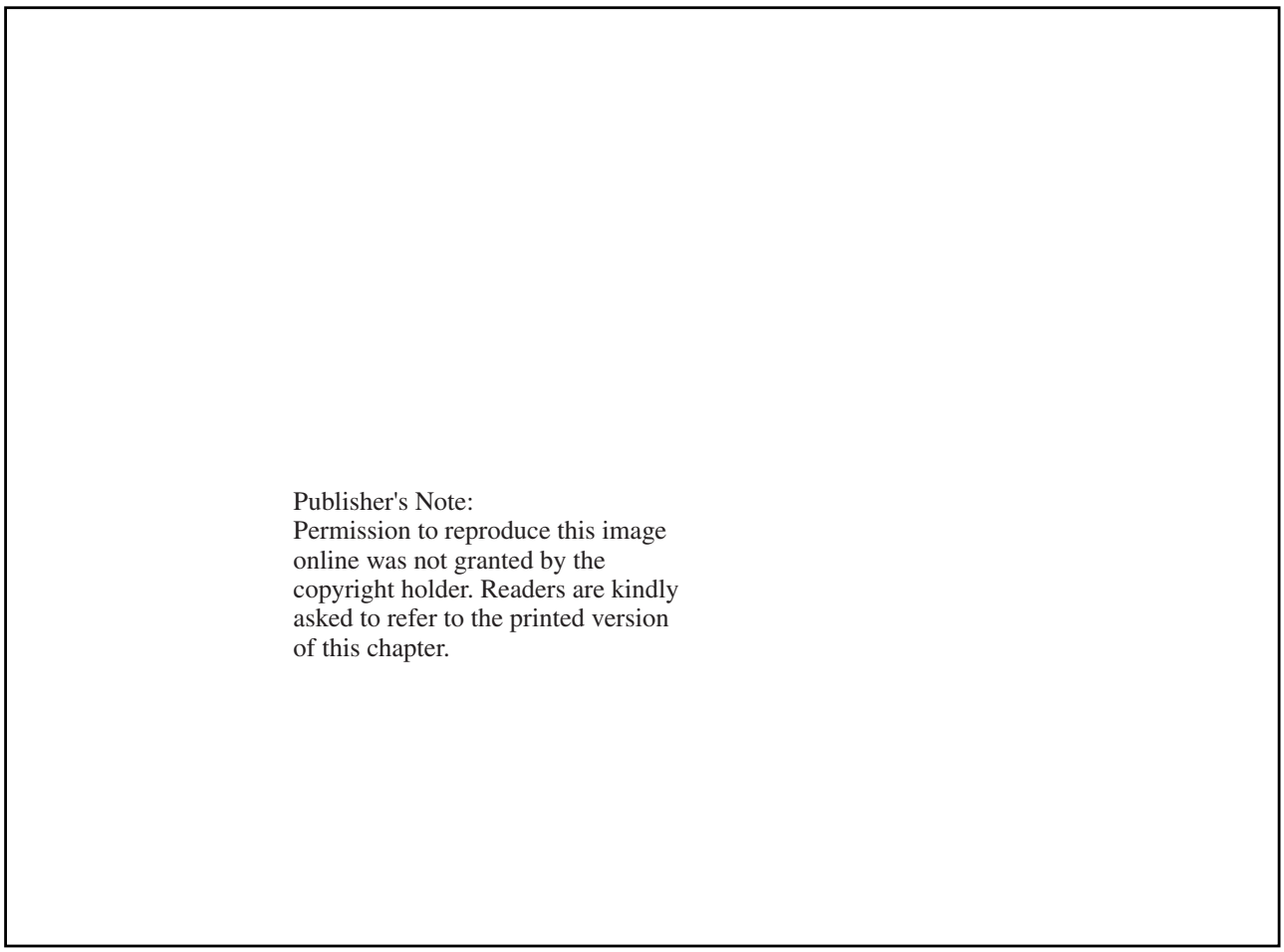


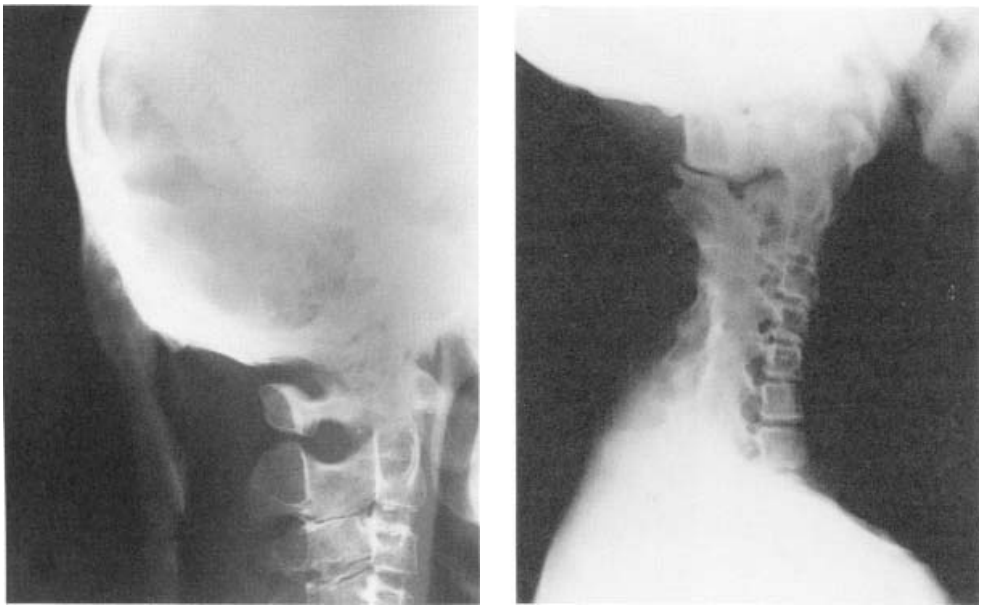
Figure 3. Clinical photograph and skeleton of a man with fibrodysplasia ossificans progressiva. The rigid posture noted in this 25-year-old man with fibrodysplasia ossificans progressiva is due to ankylosis of the spine, shoulders and elbows. Plates and ribbons of ectopic bone contour the skin over the back and arms (**A**), and can be visualized directly on the skeleton (**B**) (following death from pneumonia at age 40 years). Courtesy, Mutter Museum, College of Physicians of Philadelphia. (Reprinted, with permission, from Shafritz et al. [47] Copyright© 1996, Massachusetts Medical Society. All rights reserved).

In addition to malformations of the digits, other skeletal malformations may include the following: short, broad, femoral necks; shallow acetabula; small mandibles and osteochondromas, especially along the medial borders of the proximal tibiae; a narrow anteroposterior lumbar spinal canal; a decreased humeral epicondylar angle; and misshapen cervical vertebrae [12,32,33]. The malformations of the cervical spine are characteristic and consist of small vertebral bodies, enlarged pedicles, and large spinous processes. Progressive fusion of both the bodies and the apophyseal joints may occur and may actually represent segmentation defects [26] (Fig. 4).

In the hands, common findings are short first metacarpals and malformed middle phalanges of the fifth finger, occasionally with an accessory epiphysis. The changes in the feet are more variable. Commonly, only a single phalanx is present in each big toe, though in early childhood there may also be an accessory epiphysis near the head of the first metatarsal, which later fuses with either the metatarsal or the single phalanx [7] (Figs. 5, 6).

Some patients have two malformed phalanges in each big toe, which later fuse (Fig. 7), while occasionally the initial radiographs of the feet are entirely normal. Synostosis of the phalanges, both of the big toe and of the thumb, often occurs in later childhood and may also occur in the other toes [7]. Patients occasionally have severe reduction defects of all digits (Fig. 8).

Ectopic bone is apparent on radiographs as early as six weeks after the initial appearance of an ossifying lump. Ectopic bone matures through an endochondral process, develops normal trabecular architecture, and eventually develops secondary synostoses to the normotopic skeleton [31] (Figs. 9–11). Radionuclide bone scans show increased uptake at ossifying sites before new bone can be documented radiologically [34,35]. Reinig et al [36] have investigated 12 FOP patients with serial computerized



a b

Figure 4. (**a**) Malformed cervical vertebrae with early fusion and ossification of the ligamentum nuchae in a 12-year-old girl. The vertebral arches and spinous processes are impressive, whereas the vertebral bodies are relatively small. The ligamentum nuchae shows as a broad, bony connection between the occiput and the back. (Reprinted, with permission, from Giedion [105].) (**b**) Extensive cervical fusion at 10 years of age. (Reprinted, with permission, from Connor and Smith [26].)

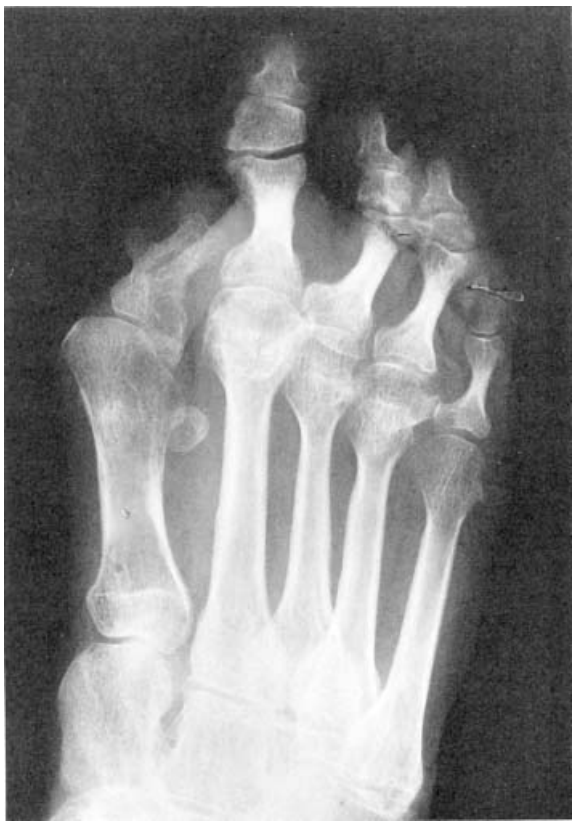


Figure 5. Short monophalangic big toe with valgus deviation in a 35-year-old female.

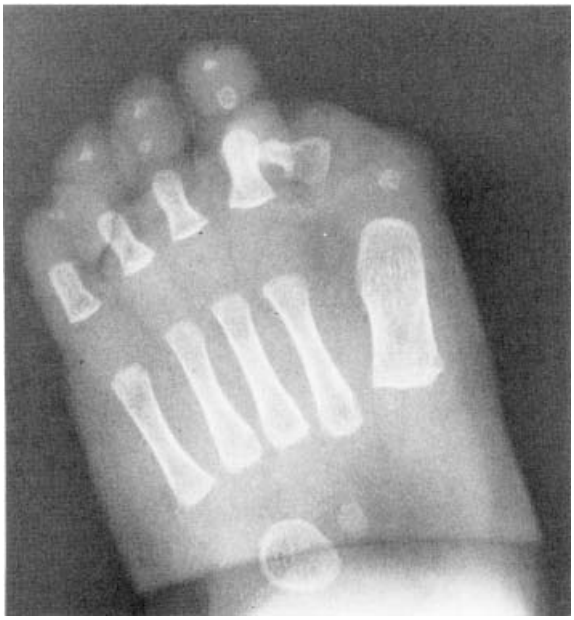


Figure 6. Accessory epiphysis in the monophalangic big toe of a 5-month-old boy. (Reprinted, with permission, from Giedion [105].)

tomography (CT) scans. Initially during a flare-up, swelling is observed in the muscle fascial planes (corresponding to the active phase on the bone scan) and subsequently ectopic bone is seen.

Benign chondro-osseous neoplasms, including osteochondromas and enchondromas, have been reported [12,37].

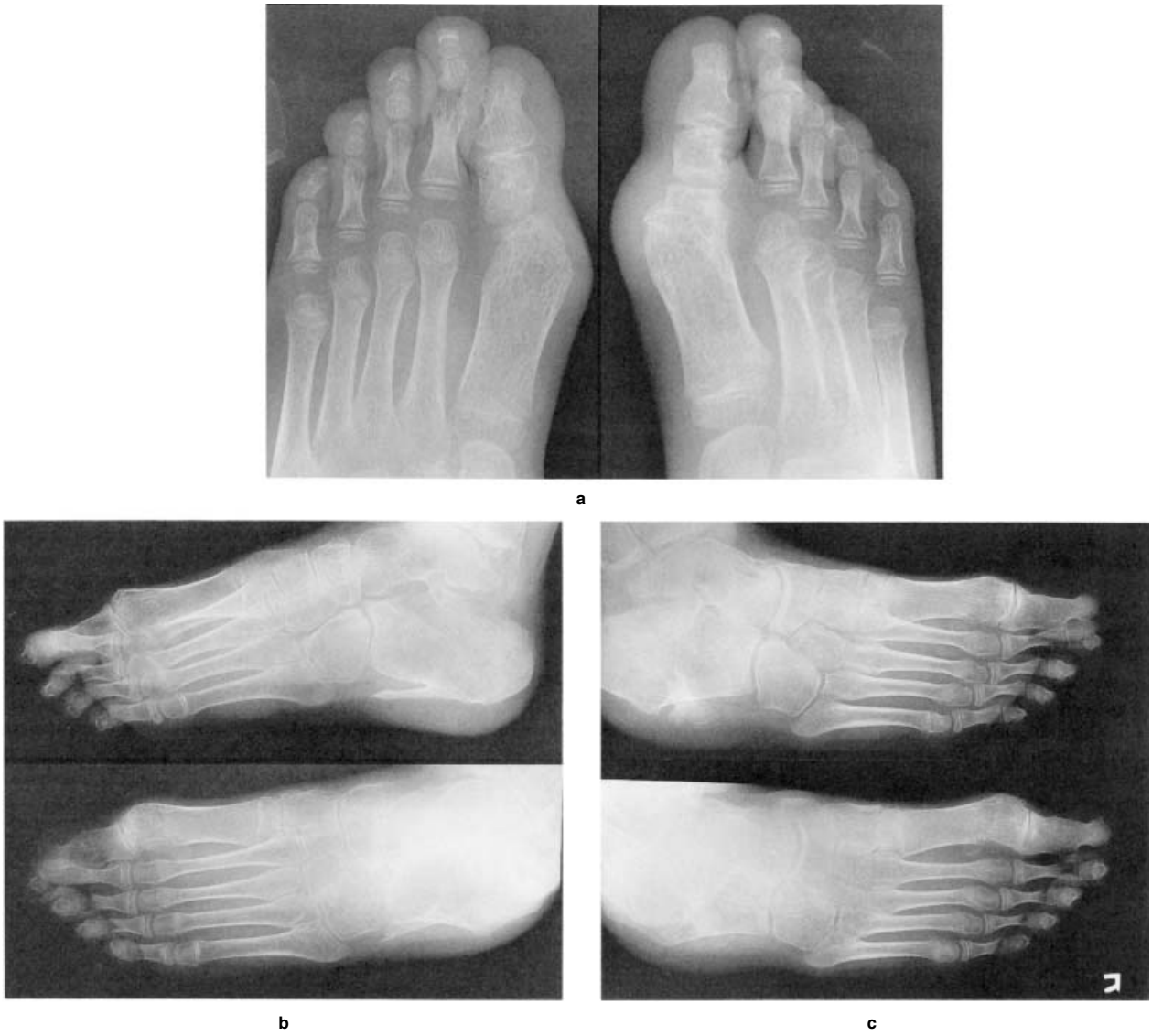


Figure 7. Progressive fusion of two malformed phalanges in the big toes at 7 years (**a**) and at 14 years (**b**). Note ossification of the ligamentous insertion of a muscle or aponeurosis into the calcaneum of the left foot, and of the soft tissue beneath the calcaneum of the right foot.

Immobilization results in disuse osteoporosis, and this is most marked in the region of ankylosed joints [31] (Fig. 11). Therapy may also produce radiological changes, e.g., osteoporosis in patients on corticosteroids, and widening of the physis and disorganized metaphyseal bone growth in patients on long-term sodium etidronate [38].

LABORATORY STUDIES

Routine biochemical and hematological investigations are normal in FOP, as is urinary excretion of hydroxyproline [10,39]. Abnormal electrocardiograms are often noted and include bundle branch blocks, ST segment changes, and evidence of right ventricular dysfunction [40,41]. Pulmonary function tests, even in childhood, reveal a restrictive ventilatory defect and reduced lung volumes, but arterial blood gases have been normal in the few patients in whom they have been measured, until late in the course of the disease when mild chronic carbon dioxide retention has been noted [40,41].

NATURAL HISTORY

Erratic progression of disability is usual in FOP. Patients typically have long periods of apparent disease inactivity, but, in general, limitation of the spine and both shoulders is present by 10 years of age. One or both hips are generally involved by 20 years of age, and most patients are chair-bound or bed-bound by 30 years of age [8,9,13]. Ectopic ossification, though generally less frequent in adults, does not stop completely and continues into old age [8,13]. The ultimate level of disability does not appear to be related to the patient's sex, age of onset of heterotopic ossification, or type or extent of skeletal malformations. Once present, disability is permanent [13].

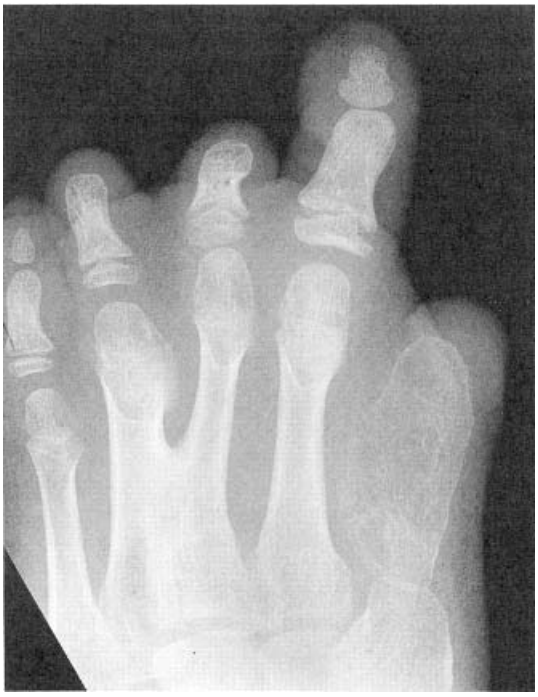


Figure 8. Reduction defects of all digits, with synostosis of the third and fourth metatarsals, in a 15-year-old female.

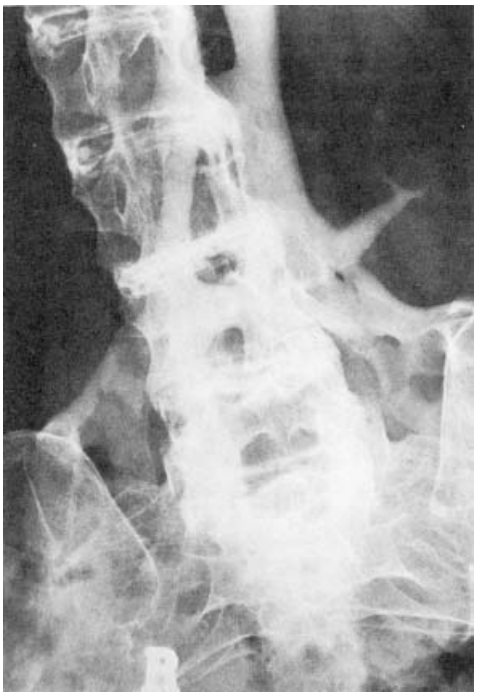


Figure 10. Paraspinal ossification of ligaments and muscle connective tissue in a 50-year-old female. (Reprinted, with permission, from Connor [42].)

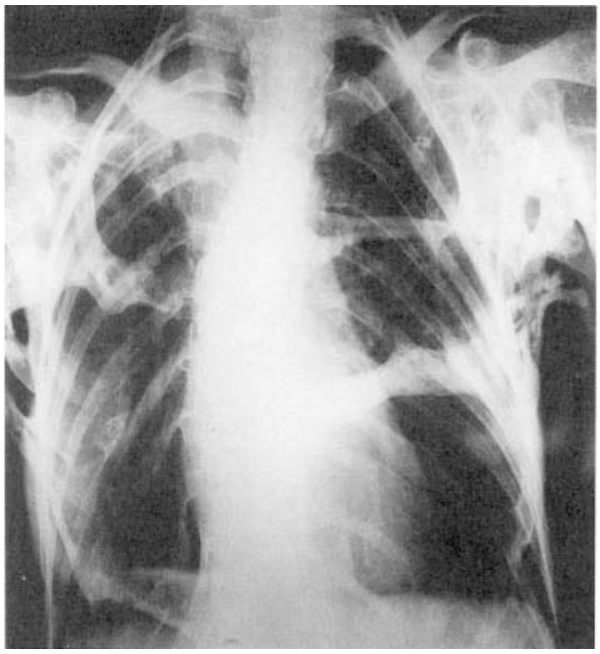


Figure 9. Extensive ectopic ossification. (Reprinted, with permission, from Connor et al. [40].)

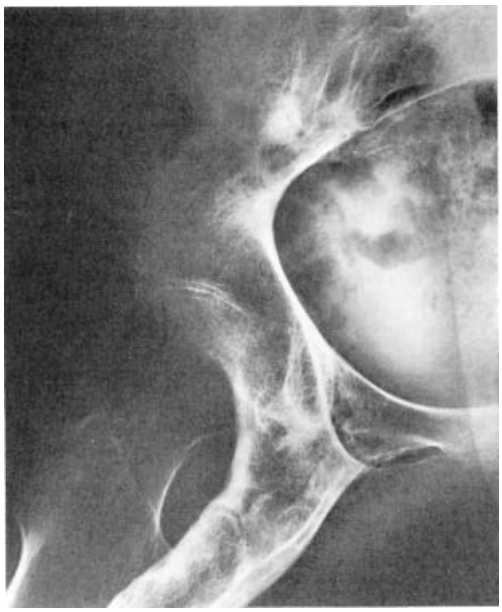


Figure 11. Ankylosis of the hip in a 24-year-old female. Disuse osteoporosis of the femur is contrasted dramatically with ossification of the adductor muscle, which shows well-formed cortical bone. (Reprinted, with permission, from Giedion [105].)

Although life span is reduced in FOP, most patients will reach adult life. The average age at death of 33 patients was 34.7 years [42] and several patients have survived into old age [14]. Pneumonia has been reported to be the usual terminal illness and this is promoted by the chest wall restriction. However, the more recent anecdotal experience of the authors suggests a high incidence of sudden death syndrome associated with sleep apnea, late in the course of the disease. Formal sleep studies with prospective

measurement of blood gases have not been performed, but patients should be cautioned not to use oxygen therapy in an unmonitored setting, especially during sleep. Some patients have died from starvation secondary to jaw fixation, brain injury following a fall, or suicide [21]. Although lesional biopsies are commonly misdiagnosed as sarcomas, there does not appear to be an increased incidence of malignant disease in FOP patients [15].

PATHOLOGICAL STUDIES

Biopsy specimens of FOP lesions are exceedingly rare. Tissue samples are available only rarely from nonelective surgical procedures or from biopsies taken prior to the diagnosis of FOP.

Early FOP lesions exhibit an intense perivascular lymphocytic aggregation followed by invasion of lymphocytes into the surrounding muscle with subsequent myocyte death. Interfascicular fibroproliferative tissue follows with extensive neovascularity [43]. Levels of basic fibroblast growth factor (bFGF), an extremely potent angiogenic peptide, are markedly elevated in the urine of patients with FOP during times of disease flare-up [44], correlating with the appearance of the vascular fibroproliferative lesion. A role for hematopoietic cells in heterotopic osteogenesis has been suggested [45]. Immunohistochemical evaluation of lymphocyte markers revealed a predominance of perivascular B-lymphocytes and a mixed population of B-lymphocytes and T-lymphocytes weakly positive for bone morphogenetic protein-4 (BMP-4) invading the skeletal muscle [43]. Whether the early lymphocytic infiltration is a causative or reactive event, or both, cannot be determined from the observations in the small sample of patients examined.

Intermediate stage FOP lesions cannot be distinguished histologically from aggressive juvenile fibromatosis, a condition that does not progress to form bone [15], but immunohistochemistry can be useful [12,46]. BMP-4 expression has been detected in cultured fibroproliferative cells and in intact tissue specimens from pre-osseous FOP lesions, but not in aggressive juvenile fibromatosis lesions [15,47]. Later stage FOP lesions show characteristic features of endochondral ossification including chondrocyte hypertrophy, calcification of cartilage, and formation of lamellar bone with marrow elements, almost identical to the pattern seen in a normally developing growth plate or in fracture repair.

INHERITANCE

Genetic Transmission

Most described cases of FOP result from new mutations, with the mutation rate estimated to be $1.8\ (SE \pm 1.04) \times 10^{-6}$ mutations per gene per generation [11]. The rarity of FOP suggests the likelihood of a single mutated locus causing the disorder in all or most individuals, and recent genome-wide linkage analysis in four small multi-generational families suggests linkage to a region on the long arm of human chromosome 4 [48].

Reproductive fitness in FOP is low, and only a few examples of inheritance of FOP within a family have been documented [5,11,20,27,40,49–51]. Previous major reviews of FOP reported no clear examples of dominant transmission [6,8]. The autosomal dominant inheritance of FOP was first suggested in 1886, in a 7-year-old boy with characteristic features of FOP, whose father had the same congenital deformity of the great toes but no other features of this disorder [52]. Similar occurrences were reported soon after [53–56].

Kaplan et al. [20] reported a father, two daughters, and a son affected by FOP. This family showed little variability in phenotype between affected individuals. Connor et al. [57] reported a three-generation family with FOP with a wide range of phenotypic severity, ranging from disabling ectopic bone formation and premature death to an asymptomatic adult whose only manifestation was a malformation of the big toes.

Two case reports of FOP in identical twins [58,59] have been noted. The parents had no sign of the condition. The first suggestion of gonadal mosaicism in FOP was reported in two half-sisters with the same unaffected mother and different unaffected fathers [60], raising the possibility that the mother had a mutant gene for FOP in multiple ova but that the mutant gene was present in few or no somatic cells. Gonadal mosaicism is a proven cause of recurrence in sibs of other autosomal dominant disorders [61].

Although variable expressivity of the FOP phenotype is widely accepted, there is little evidence that the condition is nonpenetrant [11]. However, a report of one family with affected cousins does suggest the remote possibility of nonpenetrance [5,49].

Risk of Inheritance

There is a 50% percent chance that any child of a person with FOP will themselves have the condition. There is no documentation of successful prenatal diagnosis of FOP [62]. Theoretically, ultrasound may be able to identify skeletal changes, but it is unknown whether these are apparent early enough in the pregnancy when termination is generally considered acceptable. An increased paternal age has been suggested as an associated risk factor for occurrence of FOP [11,63,64].

Counseling a family that has one child with FOP requires that both parents be examined thoroughly. Examples of a parent with mild features, such as short, laterally deviated great toes, with a child showing the complete FOP phenotype have been observed [52,53,57]. The possibility of gonadal mosaicism [60] means that the parents cannot be absolutely reassured, but can be given a very low recurrence risk.

MOLECULAR GENETICS

Candidate Gene Approach

Positional cloning was, until recently, impractical for FOP due to the small number of affected individuals and the lack of multigenerational families showing inheritance of the disease. The candidate gene approach has been pursued as an alternative indirect method in an attempt to identify the mutated gene. In selecting a candidate gene for FOP, the main diagnostic criteria (congenital malformations of the great toes, heterotopic endochondral ossification, temporal and spatial patterns of ectopic bone formation) must be considered. The candidate gene for FOP must be functional during normal embryonic development (to account for the malformations of the big toe), as well as being active postnatally to induce heterotopic ossification in tendon, ligament, fascia, and skeletal muscle.

Bone Morphogenetic Protein (BMP) Genes

The genes that best fit the criteria of an FOP candidate gene are those that encode the bone morphogenetic protein (BMP) family and other components in the BMP signal transduction pathways [65–72].

The BMP genes, members of the larger transforming growth factor beta (TGF-β) superfamily of peptides, have been highly conserved throughout evolution [65], and genes with a high degree of homology with members of the mammalian BMP family have been found in the fruit fly, *Drosophila melanogaster* [65,69,71]. The *BMP2* and *BMP4* genes, which produce proteins that are about 90% homologous to each other, are also homologous to the *Drosophila decapentaplegic* (*dpp*) gene. The DPP protein shows ~75% amino acid identity to BMP-2 and BMP-4 in the mature carboxyl-terminal region, which is the mature functional domain of these proteins.

In *Drosophila*, the *dpp* gene is an essential gene in early embryonic development, as well as later in development when it provides necessary information for limb formation [65]. The pattern of *dpp* expression is analogous to the expression of BMP-2 and BMP-4 in vertebrate development [65,69,71]. These BMPs play critical roles both in early embryogenesis and in skeletal formation, important criteria for FOP candidate genes. BMP-4 and DPP both appear to function by directing cell fate [65,73]. The absence of BMP-4 in a transgenic knockout mouse is lethal in early embryogenesis, resulting in little or no mesodermal differentiation, and no hematopoiesis [70,74]. BMP-4 has also been implicated in patterning of the developing mouse and human limb [75–77]. Over-expression of BMP-4 in the chick embryonic limb bud is associated with ectopic osteogenesis and polarizing defects in limb formation [78].

BMP Expression in FOP

Although early FOP lesions are histologically identical to those of aggressive juvenile fibromatosis, these two disorders can be distinguished by immunohistochemistry with BMP-2/4 antibodies [15]. Tissue from aggressive juvenile fibromatosis lesions (which do not progress to form bone) shows no binding of BMP-2/4 antibody, while FOP lesional tissue binds the antibody, indicating the presence of the BMP proteins within early stage FOP lesions that will progress to endochondral ossification.

Cells derived from a pre-osseous FOP lesion and from immortalized lymphoblastoid cell lines established from FOP patients showed increased expression (by Northern analysis and ribonuclease protection assay) of BMP-4 but not BMP-2 compared to controls. In addition, correlation of BMP-4 expression with FOP was observed in a family showing inheritance of FOP: the affected father and three affected children expressed BMP-4, while the unaffected mother did not [47]. Further studies have verified that BMP-4 protein is synthesized in lesional cells from patients who have FOP [15].

Steady-state levels of mRNA expression for BMP-4 and the BMP receptors were evaluated using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and documented the presence of Type I and Type II BMP-4 receptor mRNAs in FOP lesional tissue as well as in unaffected muscle tissue [79]. In lymphoblastoid cell lines of affected individuals in a family that exhibited autosomal dominant inheritance of FOP, the previous finding of elevated steady-state levels of BMP-4 mRNA was confirmed [79], but no differences in the steady-state levels of mRNA for either the Type I or Type II BMP-4 receptors were observed between affected and unaffected individuals in the same family.

Recent results have indicated that the increased levels of BMP-4 mRNA in FOP cells result from an increased rate of transcription of the *BMP4* gene [80]. The increased activation of BMP-4 in FOP cells may be due to a mutation within the *BMP4* gene itself or to a mutation in another genetic locus that causes over-expression of BMP-4 in the cells of FOP patients. The structure and function of the human *BMP4* gene is currently being examined in order to understand how it is regulated [81]. An examination of the exon-coding sequences of the *BMP4* genes of patients with FOP has identified no mutations in these transcribed regions [82].

Linkage analysis of two small families with autosomal dominant transmission of FOP has been performed using a highly polymorphic microsatellite marker near the *BMP4* gene locus [83], in order to determine whether the *BMP4* gene is consistent with or excluded from linkage with FOP in these two families. In one family [20], which showed the classic phenotypic features of FOP (as described above), the results were consistent with linkage to *BMP4* and the lymphoblastoid cells of affected members expressed high levels of BMP-4 mRNA. In the second family [57], in which affected members showed mild phenotypic features of FOP, linkage of FOP and *BMP4* was excluded; interestingly, BMP-4 expression levels were not elevated in affected members of this family.

Recently, a genome-wide linkage analysis using four families showing inheritance of FOP revealed consistent linkage of FOP with the 4q27-31 region of chromosome 4 [48]. The *BMP4* gene is not within this locus, but genes involved in the BMP signaling pathway, as well as other candidate genes, have been identified in this interval.

Genetic mutations in FOP could reside anywhere in the BMP-4 signaling pathway [48], or in other molecular pathways that affect the level of BMP-4 expression. Additional information about the cellular and molecular events that occur during the progression of FOP lesion formation, as well as a better understanding of the events that induce bone formation during normal embryonic development and fracture healing, will both expand the list of candidate genes and focus on the most likely causes of this disorder.

ANIMAL MODELS OF FOP

A fibrodysplasia ossificans progressiva-like condition has been recognized in cats, and six sporadic cases have been reported [84–87]. The disease occurs in both males and females, and is seen in both the domestic short-hair and domestic long-hair cat. At diagnosis, affected cats ranged from 10 months to 6 years of age. Unlike the disease in man, affected cats do not have congenital malformations of the distal limbs. Radiography revealed multiple foci of heterotopic ossification within affected muscles. Intense perivascular lymphocytic infiltration at the advancing edge of fibroproliferative lesions was nearly identical to that seen in human FOP lesions. There is marked proliferation of connective tissue followed by cartilage and bone formation within epimysium, tendons, and fasciae. The clinical course of the feline disease is rapid, with the development of severe disability within weeks to months. The FOP-like disease in the cat closely mimics FOP in humans, and may serve as an animal model for this disorder. Unfortunately, all evaluations performed to date have been post-mortem studies on pet cats, and no live animals are currently available for examination.

In a compelling transgenic animal model, murine embryonic overexpression of the *c-fos* proto-oncogene leads to postnatal heterotopic chondrogenesis and osteogenesis, with phenotypic features similar to those seen in children who have FOP [88]. The over-expression of Fos protein

in embryonic stem cell chimeras leads to heterotopic endochondral osteogenesis mediated, at least in part, through a BMP-4 signal transduction pathway. In contrast, early FOP lesions express abundant BMP-4 without over-expression of c-Fos, suggesting that the primary molecular defect in FOP may be independent of sustained Fos effects on chondrogenesis and osteogenesis [88,89].

DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

Patients with FOP are frequently diagnosed incorrectly, most often with aggressive juvenile fibromatosis, prior to the correct diagnosis of FOP (MIM 135100). The diagnosis of FOP should be suspected from the appearance of the toes at birth and from the emergence of soft tissue swellings on the neck and back during the first decade of life, even before the appearance of radiographically demonstrable heterotopic ossification. If the correct identity of the disease eludes the observer and an unfortunate biopsy is performed, the appearance of a highly vascular fibroproliferative lesion should provoke suspicion of FOP. The radiographic appearance of segmentation defects of the cervical spine, or the clinical appearance of asymmetric fibroproliferative swelling in a sternocleidomastoid muscle could mistakenly provoke diagnostic consideration of Klippel-Feil syndrome (MIM 148900) or idiopathic torticollis (MIM 128100; MIM 602124), respectively.

In children who present with heterotopic ossification of the skin and progressive involvement of deep connective tissue and skeletal muscle, the diagnosis of progressive osseous heteroplasia (POH) must be considered (MIM 166350). POH is a recently described genetic disorder of mesenchymal differentiation characterized by dermal ossification during infancy and by progressive heterotopic ossification of cutaneous, subcutaneous and deep connective tissues during childhood [90]. The disorder can be distinguished from FOP by the presence of cutaneous ossification, by the absence of congenital malformations of the skeleton, by the absence of tumor-like swellings, by the asymmetric mosaic distribution of lesions, by the absence of predictable regional patterns of heterotopic ossification, and by the predominance of intramembranous rather than endochondral ossification. POH can be distinguished from Albright's hereditary osteodystrophy (AHO) (MIM 103580) by the progression of heterotopic ossification from skin and subcutaneous tissue into skeletal muscle, by the absence of morphologic features associated with AHO, and by the presence of normal endocrine function.

PREVENTION AND MANAGEMENT

Preventive measures are directed at avoiding trauma that might stimulate the induction of a new lesion. Once FOP is diagnosed, all intramuscular injections and all dental blocks using local anesthetic must be avoided [18,23]. Assiduous attention should be directed to dental hygiene to decrease the necessity for therapeutic dental intervention [23,91].

Falls are a common cause of severe morbidity in patients with FOP [21]. The head is a common site of injury in FOP, and the injury profile includes traumatic brain injury, intracranial hemorrhage, and death. Deficiencies in coordinate gait and upper limb protective function likely account for the severity of head injuries in the FOP population. Precautions are intended to minimize the risk of injury without compromising a patient's functional level or independence. These include limitation of high-risk activities, the use of protective head gear, and the institution of safety improvements in living environments.

There is no established medical treatment for FOP [92–94]. The rarity of the disorder, its variable severity, and the fluctuating clinical course are substantial difficulties in evaluating potential therapies.

Physical therapy may be harmful and may provoke new lesions [11]. Surgical release of joint contractures is generally unsuccessful and risks new, trauma-induced heterotopic ossification [11]. Osteotomy of ectopic bone to mobilize a joint is usually counterproductive because of robust heterotopic ossification at the operative site. Spinal bracing is ineffective, and surgical intervention is associated with numerous complications [30]. Dental therapy should avoid mandibular blocks and stretching of the jaw [23,95]. Newer dental techniques for focused administration of anesthetic are available. Guidelines for general anesthesia have been reported [96]. All intramuscular injections should be avoided [18].

Adrenocorticotrophic hormone, corticosteroids, binders of dietary calcium, intravenous infusion of ethylenediaminetetraacetic acid (EDTA), nonsteroidal anti-inflammatory agents, radiotherapy, oral disodium etidronate, and warfarin (to inhibit gamma-carboxylation of osteocalcin) are generally ineffective [97,98]. Two recent studies suggest possible, very limited, benefits from a course of intravenous etidronate [99] or prophylactic use of 13 *cis*-retinoic acid [99,100]. Accordingly, medical intervention is currently supportive.

Creative use of BMP technology will likely have important applications in the inhibition of heterotopic ossification in diseases such as FOP. Soluble BMP receptors, dominant negative receptors, and BMP antagonists may be promising in binding and physiologically inactivating BMP where it is not needed or wanted [101]. The hope for an effective treatment for FOP has been increased by the recent discovery of BMP-4 overexpression in the condition [47,102,103]. "With so much being discovered about how the BMPs act," says Brigid Hogan, a developmental geneticist at Vanderbilt University in Nashville, Tennessee, "it might be possible to develop drugs that would block some part of the BMP-4 pathway — and therefore prevent the progression of what is a horrible nightmare disease" [104].

RECENT DEVELOPMENTS

Identification of the Genetic Cause of FOP

Because of the typically debilitating nature of FOP, few examples of inheritance from parent to child are known. Four small multigeneration families were identified, however, and have been used in a genome-wide linkage analysis [106]. This analysis linked the FOP phenotype to markers in a 36 cM region of 4q27–31 (with a lod score of 3.10 at recombination fraction 0), supporting an earlier study [83] that excluded linkage of FOP to the *BMP4* gene on chromosome 14. Several candidate genes for FOP have been identified within the chromosome 4q27–31 linkage interval and the coding regions of several of them, including *SMAD1*, have been examined for mutations in FOP patients; however, mutations that correlate with the disorder have not been identified.

The BMP antagonist noggin had been considered as a candidate gene for FOP, but genetic studies have indicated the absence of linkage of the noggin gene to FOP [107]. There are two reports [108, 109] of mutations in the noggin gene in patients with FOP, but extensive mutational analysis in some of the identical patients, as well as in more than 30

additional FOP patients, failed to detect any mutations in the noggin gene ([107]; own unpublished data).

BMP Antagonists in FOP

Differential mRNA and protein expression of several BMP pathway components, including BMP antagonists, has been examined in FOP-derived and control cells to try and uncover additional clues to the identity of the genetic defect in FOP [110].

BMP-4 mRNA and protein are uniquely overexpressed in lymphocytes and lesional cells from patients with FOP. The cellular effects of BMPs are determined in part in a dose-dependent fashion by tightly-regulated morphogenetic gradients of BMPs and secreted antagonists such as noggin, chordin, and gremlin. Although the various BMP antagonists are unique proteins, they share the functional property of binding specifically to extracellular BMPs, preventing them from interacting with their transmembrane receptors. Several studies have indicated that BMP-4 upregulates the expression of noggin and gremlin, thereby establishing an autoregulatory negative feedback loop. A defect in the feedback pathway between BMP-4 and one or more of its extracellular antagonists could plausibly contribute to elevated BMP-4 activity in FOP and, therefore, basal and BMP-4-induced expression of noggin, chordin, and gremlin mRNA in control and FOP lymphoblastoid cell lines was investigated [111]. Control cells exhibited marked increases in noggin and gremlin mRNA expression in response to a BMP stimulus (as measured by RT-PCR), whereas patient-derived cells exhibited a dramatically attenuated response. This suggests that a loss of negative feedback due to an insufficient BMP antagonist response may account in part for increased BMP-4 activity in FOP. The findings support the importance of a critical balance between an inductive morphogen and its secreted antagonists and suggest the potential of BMP antagonist-based strategies in the therapy of FOP.

Mast Cells in FOP Lesions

Postnatal heterotopic ossification in FOP is often heralded by hectic episodes of severe post-traumatic connective tissue swelling and intramuscular edema, followed by an intense and highly angiogenic fibroproliferative mass. The abrupt appearance, intense size, and rapid intrafascial spread of the edematous pre-osseous fibroproliferative lesions implicates a dysregulated wound response mechanism, and suggests that cells and mediators involved in inflammation and tissue repair may be conscripted in the growth and progression of FOP lesions. The central and coordinate role of inflammatory mast cells and their mediators in tissue edema, wound repair, fibrogenesis, angiogenesis, and tumor invasion led to the investigation of the potential involvement of mast cells in the pathology of FOP lesions and the discovery that inflammatory mast cells are present at every stage of the development of FOP lesions, and most prominent at the highly vascular fibroproliferative stage [112]. Mast cell density at the periphery of FOP lesional tissue is 40 to 150-fold greater than in normal control skeletal muscle or in uninvolved skeletal muscle from FOP patients, and 10 to 40-fold greater than in any other inflammatory myopathy examined. These findings document the mobilization and activation of inflammatory mast cells in the pathology of FOP lesions, and provide a novel and previously unrecognized target for pharmacologic intervention.

Influenza B Infection and FOP

Influenza B and other viral infections are known to cause a broad range of myopathies, especially in children. These muscle syndromes range from mild, self-limiting myalgias to life-threatening rhabdomyolysis. The etiology of muscle injury in influenza infection is unclear. Possibilities include direct invasion of muscle by the virus, activation of pro-inflammatory transcription factors, and autoimmune processes. FOP flare-ups may be stimulated by blunt soft tissue injury, surgical trauma, intramuscular injections, or myotoxic local anesthetics.

Mounting anecdotal evidence from patients, families, and physicians has suggested that a relationship exists between viral infections (such as influenza) and FOP flare-ups. A case study has been conducted of a family in which all three sisters (two affected with FOP and one unaffected) developed influenza symptoms (fever, cough, sore throat, headache, and myalgias) [113]. Both of the sisters with FOP experienced signs of a FOP flare-up (severe soft tissue swelling, erythema, warmth, and pain) within 6–12 hours of the onset of influenza symptoms. These are the first cases in which a documented viral infection has served as a trigger for an FOP flare-up. A larger retrospective study investigating the temporal relationship between influenza infection and FOP flare-ups further supports a connection between influenza infection and the progression of FOP.

Metalloproteinase (MMP) Activity in FOP

Although heterotopic angiogenesis is important in the pathogenesis of FOP lesions, its mechanism in FOP patients is not well understood. MMP-2 and MMP-9 (see also Chapter 7, this volume) have been shown to be critical for the extracellular matrix degradation that is necessary for endothelial cell invasion and for new capillary sprouting in wound healing and a variety of diseases. The role of MMP-2 and MMP-9 in the angiogenesis of pre-osseous lesions in FOP has been examined [114]. Urine samples from FOP patients experiencing flare-ups exhibited levels of MMP-9 activity significantly higher than in normal controls, suggesting that MMPs may play a role in angiogenesis during the development of FOP heterotopic ossification, thereby possibly representing a target for anti-angiogenic therapy.

Therapy

The ultimate goal of research into FOP is the development of treatments that will prevent, halt, or even reverse the progression of the condition. An updated and comprehensive review of the management of FOP can be found on the IFOPA website at *www.ifopa.org* under "Research/Treatment Guidelines" (see also "Patient Support and Information", below).

PATIENT SUPPORT AND INFORMATION

The International Fibrodysplasia Ossificans Progressiva Association (IFOPA) is a nonprofit organization that supports research and education for patients with FOP. The IFOPA was founded in 1988 by Jeannie Peeper, an adult with FOP, in order to end the social isolation imposed by this rare and debilitating disease. Today, the IFOPA has nearly 200 members in over 15 countries. The IFOPA's home page on the World Wide Web contains information about FOP, the IFOPA, and the international collaborative research project. "What Is *FOP*? A Guidebook for Families" is also available on the website. The address for the site is *www.ifopa.org* Those using email can contact the IFOPA at ifopa@vol.com.

The address of the IFOPA is: Ms. Jeannie Peeper, President, IFOPA, PO Box 196217, Winter Springs, FL 32719-6217.

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Chapter 22, Part I

Disorders of Lysosomal Enzymes: General Considerations

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INTRODUCTION

The connective tissues consist of two main components, the collagens and the proteoglycans. Both are situated in the extracellular compartment and are secreted by resident cells of the connective tissues. The proteoglycans of the extracellular matrix are molecules with a molecular weight of several million (see Chapter 4, this volume). They consist of core proteins to which glycosaminoglycan (GAG) subunits are covalently linked in a fern-like pattern. The core proteins themselves are noncovalently bound to stringlike molecules of hyaluronan (hyaluronic acid). The presence of numerous anionic groups such as carboxyl and sulfate groups is responsible for the acid characteristics of glycosaminoglycans and for their property of binding certain dyes such as toluidine blue. These dyes change their colors metachromatically into colors of longer wavelength. In spite of their high molecular weight, the proteoglycans are readily water-soluble and therefore frequently escape histochemical visualization because they are lost into the aqueous phase during tissue fixation. Therefore, the histochemical demonstration of extracellular proteoglycans is difficult and requires special methods.

Connective Tissue Homeostasis

Similarly to all other structures in the organism, the extracellular constituents of the connective tissue matrix are continuously renewed. In part, the metabolism of these high molecular weight structures occurs extracellularly through the activity of enzymes such as hyaluronidase, which are secreted by the connective tissue cells into the extracellular matrix. Through the action of hyaluronidase, the proteoglycans are depolymerized. The next degradation step consists of the degradation of core proteins by proteinases, which are also secreted by the cells. An uncontrolled degradation of matrix constituents is prevented by several types of inhibitors of proteinases present in the extracellular space. Extracellular matrix degradation is discussed extensively in Chapter 7 (this volume). The glycosaminoglycan chains and polypeptides resulting from partial extracellular degradation and/or the soluble proteoglycans are finally taken up into the cells of the connective tissues and degraded in the lysosomes, their degradation occurring in a step-wise manner through several lysosomal hydrolases. These maintain a strict order in the sequence of their actions, which cannot be bypassed. For this reason, any deficiency of a single individual lysosomal hydrolase, usually the result of a genetic defect or, rarely, of inhibition by a lysosomotropic drug (e.g., chloroquine), stops lysosomal degradation of the substrate at the point where the missing enzyme should act. This is the reason for the existence of as many different genetic mucopolysaccharidoses and oligosaccharidoses as there are enzymatic steps in the degradation of glycosaminoglycans and oligosaccharides.

Regulation of the rates of extracellular matrix degradation and synthesis by the resident cells of the connective tissues is responsible for there being an equilibrium between the secretion of newly synthesized material and the uptake and degradation of older, previously secreted material [1]. Recently, new developments in our understanding of the regulation of connective tissue homeostasis have been described. Heparan sulfate proteoglycans at cell surfaces and in the extracellular matrix bind diverse molecules, including growth factors and cytokines, and it is suggested that the activities of such molecules may be regulated by the metabolism of heparan sulfate [2]. Proteoglycans seem to be involved in feedback signaling from the extracellular matrix to the cells synthesizing it. Various cytokines and growth factors have been found able to penetrate the extracellular matrix and to inhibit or stimulate proteoglycan biosynthesis. The degradation of proteoglycans can also be enhanced or inhibited by several different cytokines. Proteoglycans may function as receptors, protectors, inactivators, and storage ligands of cytokines. Agonistic or antagonistic cytokines are both involved in the regulation of proteoglycan turnover during balanced or unbalanced metabolism in normal and pathological situations, respectively [3].

Extensive data on glycosaminoglycan (GAG) composition and the distribution of collagen fibril diameters have been collected for a diverse range of connective tissues. It has been shown that tissues with collagen fibrils of smaller diameter (average diameter less than 60 nm) have high concentrations of hyaluronan while those with larger diameter collagen fibrils (average diameter approximately 200 nm) have high concentrations of dermatan sulfate. It has thus been suggested that the lateral growth of fibrils beyond a diameter of about 60 nm is inhibited by the presence of an excess of hyaluronan, but that this inhibitory effect is diminished by an increasing concentration of dermatan sulfate. It has also been postulated that high concentrations of chondroitin sulfate will inhibit fibril growth beyond an average diameter of approximately 150 nm. Such an inhibition may, in turn, be diminished by

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an increasing concentration of dermatan sulfate, such that this becomes the dominant GAG present in the tissue [4].

Invasion by nonresident cells such as macrophages, granulocytes, and other cells mediating inflammation, or of tumor cells, may severely disturb the equilibrium between synthesis and degradation. In most instances this disturbance results in increased degradation, because the invading cells secrete lysosomal enzymes such as hyaluronidase or cathepsins as well as inflammatory cytokines. This process often results in a diminution of the extracellular substance and the formation of defects. On the other hand, resident cells may start to divide and multiply as a reaction to local inflammation due to the secretion of cytokines, e.g., IL-6 (interleukin 6). The increase in cell number, or the reactions of the cells to factors resulting from local inflammation, may mediate an increased secretion of both types of molecule that form the matrix: collagens and proteoglycans. This may result in an excess of extracellular material and/or in a qualitative change of the composition of matrix components, offering a probable explanation for the formation of connective tissue scars and the so-called keloids of the skin, or for the formation of callus in bone and osseous fibrosis. Again, the regulation of these mechanisms is mostly unknown. In the formation of keloids, which often occur after dermal burns, genetic factors may play a role, since they are more frequent and prominent in dark-skinned individuals than in the white population [5].

Equally unknown and unclear is the cause of the disturbed regulation of the metabolism of the extracellular matrix in the mucopolysaccharidoses. In these disorders, the lysosomal storage of nondegradable glycosaminoglycans is explained by specific enzyme deficiencies. However, the increase of these compounds extracellularly, e.g., in the skin, bones, and cartilage, which is often the basis of the clinically visible symptoms in patients, still remains to be adequately explained. Gene expression and the synthesis of extracellular matrix components might directly or indirectly be related to lysosomal storage. In aspartylglucosaminuria, a lysosomal storage disease, the ultrastructure of collagen fibrils in the skin of patients has been shown to be abnormal. *In vitro*, fibroblasts from these patients show reduced synthesis of collagens due to decreased steady-state concentrations of mRNA and an imbalance in proteoglycan synthesis [6].

INTRACELLULAR HANDLING OF CONNECTIVE TISSUE COMPONENTS

Reasons for, and Specificity of, Cellular Lysosomal Accumulation

The composition of the proteoglycans in the extracellular matrix is different in different types of connective tissue. In particular, there are differences between the loose connective tissue of skin and the highly organized structures of the matrix in bone and cartilage. As a consequence, skin, bone, cartilage, tendon, and fascia may be differently affected depending on the specific type of lysosomal enzyme defect. Genetic defects of lysosomal enzymes that catalyze degradation steps common to all glycosaminoglycans, such as β-glucuronidase, may result in morphological changes in many different tissues and locations. Again, a deficiency of an enzyme that is responsible for a step in the degradation of just one type of glycosaminoglycan, but one that is present in many different types of connective tissues, e.g., dermatan sulfate, will also express itself in a broad range of clinical symptoms. On the other hand, if a genetic defect results in the deficiency of a lysosomal enzyme that catalyzes one step in the degradation of a glycosaminoglycan that is only a minor component of the connective tissue matrix, e.g., heparan sulfate, the involvement of skin, bone, and cartilage may be minimal, as in Sanfilippo disease. Heparan sulfate, however, plays an important role in the brain and, therefore, the clinical expression of this disorder, in which heparan sulfate degradation is disturbed, is mainly linked to progressive behavioral and intellectual deterioration.

Lysosomal Function

Lysosomes are cellular organelles that maintain a pH of between 4.5 and 6.5 by an ATP-dependent proton pump. They contain a large number of hydrolytic enzymes that have a pH optimum in this acidic range that are able to disassemble macromolecules such as proteins, glycopeptides, glycosaminoglycans, and glycogen, as well as nucleic acids and complex lipids, into their individual constituents, e.g., monosaccharides and their derivatives, amino acids, fatty acids, and cholesterol, as well as inorganic acids like phosphate and sulfate. These low molecular weight molecules either diffuse through the lysosomal membranes or are transported across them by energy-dependent mechanisms into the cytosol. They may either be released from cells into the extracellular fluid or be reutilized by them for energy production or macromolecular synthesis.

The substrates for lysosomal degradation originate either from the cells themselves during the process of intracellular renewal of macromolecules and membrane lipids, or may arise from the extracellular environment from which they are internalized into the cells by pino- or phagocytosis.

In this chapter are described heritable genetic diseases that originate from single or multiple deficiencies of lysosomal enzymes that are normally responsible for the degradation of the glycosaminoglycan components of the extracellular matrix. The dysfunction of lysosomal degradation on the basis of such enzyme deficiencies inevitably results in an arrest of sequential lysosomal substrate degradation and the accumulation of nondegraded or partially degraded glycosaminoglycans within the lysosomes. This storage of substrates leads to vacuolization and swelling of the cells and to changes in homeostasis of the extracellular matrix.

Intracellular Uptake of Substrates

The intracellular uptake of components of the connective tissue matrix is the prerequisite for intracellular lysosomal degradation. The mechanism comprises cellular incorporation, intracellular vesicular transport, incorporation of the substrates into the lysosomes, and, finally, lysosomal degradation.

Intracellular Incorporation

The uptake of macromolecular components of the extracellular matrix usually occurs by pinocytosis, rarely by phagocytosis. Both are energy-requiring, temperature-dependent processes. Extracellular proteinase activity, together with extracellular hyaluronidase activity, results in the disassembly of the large glycosaminoglycan structures that then become soluble and can be taken up by resident connective tissue cells. During endocytosis, extracellular molecules enter the cell interior in vesicles that are formed by the invagination and inward fusion of plasma membranes. These macromolecules may be in solution, loosely bound to the membrane, or bound to specific receptors at this stage [7]. Receptor-mediated endocytosis is effectively used by many cell types to internalize macromolecules for

lysosomal breakdown. The macromolecular ligands bind to specific cell surface receptors that then move to, and cluster within, specialized regions of the plasma membrane termed "coated pits." Once receptors bound to appropriate ligands are within the coated pits, coated vesicles are formed that are internalized. As soon as the coated pits have lost their connection with the extracellular space, the clathrin coat that specifies the coated vesicles disassembles, and the smooth-surfaced uncoated vesicles, together with their contents of ligands and receptors, are now delivered into the endosomal compartment [8,9]. The degree of binding of molecules to the plasma membrane regulates their rate of uptake and is paralleled by the rate of degradation of molecules like proteins, some glycoconjugates, and proteoglycans in the endocytosing cells [7].

Little is known about the control of endocytosis and pinocytosis. Rates of endocytosis vary according to the cell cycle, nutritional conditions, and in response to certain hormones, such as growth factors [7], but, on the whole, the rate of degradation of endocytosed molecules correlates with variations in the rate of uptake. In conditions in which the intracellular vacuolar system becomes enlarged, the availability of receptors at the cell surface will be decreased and, therefore, the uptake of a particular ligand may be decreased.

There are examples of feedback inhibition that may control ligand entry during endocytosis into the cells and lysosomes, and there may be secondary feedback inhibition of endocytosis, as a consequence of lysosomal storage or the resecretion of endocytosed material or the inhibition of endosomal/lysosomal fusion. All of these mechanisms may result in a disequilibrium of connective tissue homeostasis, as is found in many of the lysosomal connective tissue disorders [10].

Intracellular Transport of Endocytosed Substrates

Endosomes are small plasma membrane vesicles which may have a shuttle function between the larger vacuoles in the interior of a cell and the plasma membrane, in both directions. The endosomal compartment is composed of a network of tubules and vesicles which may form a reticulum within the peripheral cytoplasm of the cells. In this complex structure, ligands dissociate from their receptors, and receptors sort themselves from each other. The interior and the material contained within endocytic vesicles, as well as the interior of the coated vesicles previously mentioned, are acidified by proton pumps that exist within the membranes of the vesicles. Acidification seems to be responsible for the dissociation of ligands and receptors and plays a central role in redirecting the receptors back to the plasma membranes by means of receptosomes [8].

Mode of Access of Macromolecules to the Lysosomal Interior

One of the controlling steps in lysosomal function is the entry of substrates into the lysosomal compartment. Lysosomes contain numerous hydrolytic enzymes capable of degrading biological macromolecules taken up by endocytosis, pinocytosis, or phagocytosis. In the process of remodeling hypertrophic cartilage and bone, however, lysosomal enzymes are also secreted by clastic cells into a localized acidic extracellular compartment [11]. For most macromolecules (proteins, nucleic acids, or glycosaminoglycans), entry into the lysosomal compartment involves membrane fusion. Acidification of the intracellular vesicles and endosomes seems to be a prerequisite for subsequent fusion with lysosomes. This fusion can be inhibited by low temperature and by weak bases such as amines [12]. Such weak bases may also interfere with the processing of lysosomal enzymes, as a consequence of which they may be secreted into the extracellular space instead of being incorporated within the lysosomes. Significant progress has been made in recent years toward elucidating the pathways by which lysosomal membrane proteins are delivered to late endosomes and lysosomes. While some lysosomal membrane proteins follow the constitutive secretory pathway and reach lysosomes indirectly via the cell surface and endocytosis, others exit the *trans*-Golgi network in clathrin-coated vesicles for direct delivery to endosomes and lysosomes. Sorting from the Golgi or the plasma membrane into the endosomal system is mediated by signals encoded by the short cytosolic domains of these proteins [13].

In the regular process of endocytosis, most of the endosomal membranes are rescued and recycled back to the plasma membrane, although some may enter the lysosomal compartment and become degraded. As the flow from endosomes to lysosomes by vesicle/lysosome fusion is bidirectional, a certain proportion of the endocytosed molecules may leave the cells by a secretory process accompanying membrane recycling [14]. This may be important in lysosomal storage diseases, in which the intracellular storage of material may favor this secondary secretory route into the extracellular fluid and interfere with extracellular matrix homeostasis.

Intralysosomal Degradation

After the fusion of the endocytic vesicles containing endocytosed material with primary or secondary lysosomes, hydrolytic degradation commences. As well as proteoglycans that have been taken up from the extracellular compartment, newly synthesized glycosaminoglycans and proteoglycans are also diverted from the secretory route into lysosomes. Both *in vivo* and *in vitro* it has been shown that more than half of newly synthesized proteoglycans fail to be secreted from the cells, and are segregated into lysosomes and degraded [15]. This degradation of newly synthesized material may represent another important function devised to regulate net synthesis and secretion and ensure homeostasis of the extracellular matrix.

SYNTHESIS, SORTING, AND TRANSPORT OF LYSOSOMAL ENZYMES

The targeting of lysosomal enzymes from their site of synthesis in the rough endoplasmic reticulum to their final destination in lysosomes is directed by a series of protein and carbohydrate recognition signals on the enzymes.

Lysosomal enzymes act only within a relatively narrow acid pH range of between 4.5 and 6.5. At neutral pH they may be mostly inactive. It is generally the case that lysosomal enzymes mediate only degradative processes. However, there are certain exceptions. One of these is an enzyme (acetyl-CoA-α-glucosaminide N-acetyltransferase) that catalyzes the N-acetylation of amino sugars within heparan molecules, which at the time of their entry into lysosomes are not acetylated. Acetylation of amino sugars seems to be a prerequisite for hydrolytic release from their linkage with uronic acid to be achieved. This N-acetylating lysosomal enzyme is deficient in Sanfilippo disease type C [16].

It is generally assumed that lysosomal enzymes of different types are present together in the same lysosomal vacuoles.

Activities are very stable at the low lysosomal pH, but the enzymes are very sensitive to irreversible inactivation at alkaline pH, both *in vivo* and *in vitro*. They are greatly resistant to tryptic digestion and, in general, proteinases that rapidly degrade nonlysosomal proteins do not act against undenatured lysosomal enzymes, many of which remain quite stable and have half-lives of days to weeks. They also seem to be stabilized by the presence of their substrates. All of the properties mentioned may contribute to the fact that the pattern of lysosomal enzyme activity differs greatly from cell type to cell type and between different organs.

Synthesis of Lysosomal Enzymes

Lysosomal enzymes are synthesized on membrane-bound polysomes in the rough endoplasmic reticulum (RER). Lysosomal enzymes, along with secretory and plasma membrane proteins, contain amino-terminal signal sequences that direct the vectorial discharge of the nascent proteins into the lumen of the RER [17]. The three classes of proteins also share a common peptide signal for asparagine glycosylation involving the transfer of large, preformed oligosaccharide-dolichol-diphosphates to the nascent polypeptides. The responsible enzyme (dolichyl-P-Glc:$Man_9GlcNAc_2$-PP-dolichyl glucosyltransferase) is deficient in carbohydrate-deficient glycoprotein syndrome (CDGS type V) [18]. In the RER the signal peptide is cleaved, and the processing of the asparagine-linked oligosaccharides begins with the excision of three glucose and one mannose residues. The proteins then move by vesicular transport to the Golgi stack, where they undergo a variety of post-translational modifications [19,20]. The next signal is unique to lysosomal enzymes and permits their high-affinity binding to a specific phosphotransferase that catalyzes the formation of a mannose 6-phosphate recognition marker. This carbohydrate determinant allows binding to specific receptors that translocate the lysosomal enzymes from the Golgi complex to an acidified prelysosomal compartment. There the lysosomal enzymes are discharged for final packaging into lysosomes. Two distinct mannose 6-phosphate receptors have been identified, and cDNAs encoding their entire sequences have been cloned. An analysis of the deduced amino acid sequences of the receptors shows that each is composed of four structural domains: a signal sequence, an extracytoplasmic amino-terminal domain, a hydrophobic membrane-spanning region, and a cytoplasmic domain. The entire extracytoplasmic region of the small receptor is homologous to the 15 repeating domains that constitute the extracytoplasmic portion of the large receptor [21].

The acquisition of phospho-mannosyl residues occurs through the concerted action of two enzymes. First, UDP-N-acetylglucosamine-1-phospho-transferase transfers N-acetylglucosamine-1-phosphate from the nucleotide sugar UDP-N-acetylglucosamine to selected mannose residues on the lysosomal enzyme to give rise to a phosphodiester intermediate. Then, another enzyme, N-acetylglucosamine-1-phosphate, α-N-glucosaminidase, removes the N-acetylglucosamine residue. The resultant mannose 6-phosphate phosphomonoester serves as a central recognition marker that leads to high-affinity binding to mannose 6-phosphate receptors in the Golgi complex [22,23]. After addition of the recognition marker, the sorting and diversion of lysosomal enzymes take place through the binding of the enzymes to mannose 6-phosphate receptors, which are concentrated in *cis*-Golgi cisternae, coated vesicles located near the *cis*-most Golgi cisternae, and endosomes or lysosomes, and also at the cell surface of some cell types in coated pits [24]. The *cis*-Golgi cisternae, coated vesicles, and endosomes or lysosomes are assumed to represent the sorting site, carrier vesicles, and delivery site, respectively, for lysosomal enzymes.

By this mechanism, enzymes directed toward lysosomes are segregated from homologous proteins that are destined for secretion. The ligand (lysosomal enzyme)-receptor complex is delivered to the prelysosomal staging area, where dissociation of the ligand occurs through acidification of the compartment. The receptors then cycle back to the Golgi apparatus to pick up further ligand molecules, whereby the lysosomal enzymes are packaged into lysosomes. Small amounts of the lysosomal enzymes are secreted into the extracellular space before their delivery to lysosomes. They may bind to mannose 6-phosphate receptors at the cell surface and be internalized again, to be delivered to the lysosomes together with substrates. This secretion-recapture mechanism may account for 5–10% of total lysosomal enzymes delivered to the lysosomes. This alternative route to the lysosomes provides the rationale for lysosomal enzyme therapy in cells and in patients with lysosomal enzyme deficiency. Its practical use so far has been restricted to enzyme therapy with recombinant glucocerebrosidase in visceral Gaucher disease patients and to bone marrow replacement therapy in mucopolysaccharidosis patients.

Some of the lysosomal enzymes, such as acid phosphatase and β-glucosidase, and lysosomal membrane proteins, may be transported to the lysosomes by a mannose 6-phosphate-independent mechanism [25,26].

Intracellular Turnover of Lysosomal Enzymes

Simultaneously with oligosaccharide processing, lysosomal enzymes undergo proteolytic processing. They are synthesized as pre-proenzymes with amino-terminal extensions. The pre-piece, which is the signal sequence, is cleaved immediately after transport into the endoplasmic reticulum. The pro-piece is cleaved later, usually over the course of several hours to several days. This processing appears to be initiated in the prelysosomal compartment and continues in the lysosomes [27]. Most lysosomal enzymes are already active as proenzymes. In some cases, however, such as cathepsin D, the pro-sequence functions as an activation peptide. In the other lysosomal enzymes that are synthesized as active enzymes, the function of this processing is not clear. The pro-piece may have a stabilizing effect and may contain sorting information for the targeting of lysosomal enzymes by the mannose 6-phosphate-independent pathway.

The degradation of lysosomal enzyme polypeptides begins during their biosynthesis [28]. Once the lysosomal enzymes reach the lysosomes, they undergo decay according to first-order kinetics. The turnover of lysosomal enzymes is heterogeneous, the lifetimes of individual enzymes being different from one another and from the turnover of lysosomal membrane proteins, but being of the order of several days to several weeks [29]. In some lysosomal disorders, the degradation of mutated but metabolically active enzyme protein may be greatly accelerated, resulting in a functional enzyme deficiency [30]. An excessive degradation rate with the loss of functional enzyme activity may also be due to the lack of a stabilizing protein, as in the combined deficiency of β-galactosidase and glycoprotein sialidase. Loss of enzymatic function may result from difficulty in hydrolase packaging in the prelysosomal compartment, as suspected for the late-onset form of glycogenosis type II [31]. The longevity of lysosomal

enzymes may also be altered by defects in the synthesis of their carbohydrate moiety, as in CDGS, a hypothesis rendered feasible by experiments with drugs interfering with glycosylation such as tunicamycin [32].

LYSOSOMAL ENZYME DEFICIENCIES

Genetic deficiency of a lysosomal enzyme leads to the lysosomal accumulation of its nondegraded substrate. Usually, an enzyme deficiency is caused by failure to synthesize a catalytically active form of a particular enzyme, due, for example, to a point mutation. Another reason for a missing lysosomal enzyme activity is an inability to transport the active enzyme from its site of synthesis to the lysosomes [22,23,27].

Glycogenosis type II was the first genetic disorder to be identified as a lysosomal storage disease [33], since when about 40 different lysosomal storage diseases have been identified as being due to single or multiple enzyme deficiencies [34].

Those enzyme deficiencies in which there are clinical manifestations of lysosomal dysfunction can be attributed to mutations within the genes coding for one or other of the polypeptide chains of lysosomal enzymes. This may lead to an abortive polypeptide chain that is instantly degraded and never reaches the lysosomes, an enzyme lacking catalytic activity, or an enzyme that is more sensitive to inactivation. Lysosomal enzymes are glycoproteins and the formation and trimming of carbohydrate side chains is responsible for the targeting of lysosomal enzymes to the lysosomes. The formation of mannose 6-phosphate groups in the oligosaccharide side chains mediates the binding of lysosomal enzymes to specific receptors. For this recognition marker two enzymes are required: N-acetylglucosamine-phosphotransferase and phosphodiester-glycosidase. The deficiency of the phosphotransferase is the basic defect underlying I-cell disease and pseudo-Hurler polydystrophy (Part II, this chapter).

A deficiency of the phosphodiester-glycosidase has been discovered in one particular family [35]. The absence of mannose 6-phosphate groups in lysosomal enzymes may also be due to mutations in the genes for the lysosomal enzymes themselves, as has been shown for α-glucosidase [36]. Defects interfering with the correct structure of the carbohydrate chains in lysosomal enzymes result in a multiple enzyme deficiency of all enzymes that are dependent on the mannose 6-phosphate pathway for reaching the lysosomes. Not all lysosomal enzymes, however, require the presence of the mannose 6-phosphate recognition group and binding to the appropriate receptors in order to be transported into the lysosomes. Some of them, which are not absent or deficient in I-cell fibroblasts, such as acid phosphatase and β-glucosidase, seem to require a conversion of the high-mannose type to complex type oligosaccharide to be transported to the lysosomes [37].

In summary, the following mechanisms may lead to deficiencies of lysosomal enzymes [34,38]:

1. The precursor of the enzyme is not synthesized, or is synthesized at a diminished rate.
2. Following synthesis of a normal amount of the precursor, rapid degradation of the unstable enzyme occurs.
3. The precursor lacks the mannose 6-phosphate recognition marker, resulting in deficient targeting to lysosomes. This may be due to: (a) N-acetylglucosamine-1-phosphotransferase deficiency; (b) N-acetylglucosamine-1-phosphodiester-N-acetylglucosaminidase deficiency; (c) change of the Ser/Thr–X–Asn sequence functioning as glycosylation site as a result of amino acid substitution.
4. Either the precursor or the mature enzyme may have altered physicochemical and/or enzymological properties.
5. The enzyme may be degraded too rapidly because of the absence of protective protein required for its stabilization.
6. Absence of a factor required for normal enzymic activity.
7. The product accumulating as a result of a deficiency of one enzyme may inhibit the activity of (an) unrelated enzyme(s).

PATHOGENESIS OF LYSOSOMAL DISORDERS OF CONNECTIVE TISSUE

Any condition that interferes with the intralysosomal activity of an enzyme involved in disassembling connective tissue components (glycoproteins and GAGs), as well as the abnormal extracellular secretion of one or more lysosomal enzymes, will eventually lead to a connective tissue disorder. This will manifest itself primarily in organs that contain large proportions of collagen and proteoglycans such as skin, bone, and cartilage. Other organs in which proteoglycans may play a functional role, such as the developing brain, may also be involved, as may be organs and tissues such as the heart and cornea, of which an essential part is comprised of connective tissue components.

The quantity and composition of proteoglycans, particularly keratan sulfate, in organs may change with age [39]. This may, in part, explain why the urinary excretion of keratan sulfate in MPS IV and oligosaccharide excretion in patients with oligosaccharidoses may be reduced or even disappear with increasing age. It also may provide an explanation for the observation that the enlarged liver in patients with mannosidosis may normalize with increasing age.

Reasons for Central Nervous System Involvement

Glycosaminoglycan (GAG) metabolism seems to play an important role in neuronal maturation and synaptogenesis [40,41]. Any imbalance may interfere with neuronal migration, differentiation, and function, especially in the early stages of development. This explains why any rational treatment, for example, enzyme substitution, should be initiated as early as possible, preferably before the patient presents major symptoms of central nervous system involvement. However, this may create severe ethical problems, since the available and developing treatments are by no means without acute risk.

The repertoire of the lysosomal system extends beyond its function in degrading biological macromolecules for energy and recycling purposes. Controlled shifts in lysosomal activity help neurons to regulate their cytoplasmic volume and to remodel local cellular domains [42]. Therefore, the pathogenesis of brain damage in lysosomal disorders may originate from as yet unexplored aspects of lysosomal function.

Lysosomal accumulation of GAGs may also interfere with the orderly metabolism of other components of

the brain. It has been shown that these accumulating substances inhibit the activity of β-galactosidase, which is responsible for the degradation of gangliosides. The accumulation of gangliosides in neurons is manifested by the typical "zebra" bodies seen in gangliosidosis. Some glycosaminoglycan storage products may be more inhibitory, and this may be why brain function, for example, is more disturbed in Hunter disease than in Hurler disease, and may even be spared in milder cases as a result of residual activity.

Thickening of Bones and Skin, and Hirsutism

Thickening of the skin in some of the mucopolysaccharidoses may well be explained by an excess of GAGs in the skin, this being the expression of an imbalance between synthesis, secretion, and degradation of GAGs interfering with the orderly array of collagen fibrils. Hirsutism is frequently observed in older patients with MPS I, II, VI, or VII. This may be the result of the direct effect of a changed microenvironment on hair growth [43] or due to the increased binding of androgenic steroids from the adrenal glands by GAGs.

In MPS I, II, and VII, there seems to be a surplus of GAGs in connective tissues resulting in thickening of the skin and bones, while in others, such as MPS IV, there is, in contrast, something of a shortage due to an imbalance of keratan sulfate and heparan sulfate or dermatan sulfate. Not much is known of the collagen counterpart that may also be increased or reduced.

Glycosaminoglycans are constituents of larger proteoglycans that may or may not be associated with collagens. The glyco-moieties of the proteins contain glucose, galactose, mannose, fucose, and sialic acid as well as amino sugars. This explains why the oligosaccharidoses on the one hand are phenotypically connective tissue disorders, while on the other hand they involve noncollagen structures in other organs, particularly the CNS.

Reasons for Phenotypic Variation

Identical lysosomal enzyme defects may vary greatly in their phenotypic expression, this phenomenon usually being thought to be linked to different residual functional enzyme activities.

The more is known about the molecular genetics of lysosomal enzyme deficiencies, the more it becomes evident that point mutations, even at the same locus, may result in different phenotypes. Polymorphism within genomic DNA may modulate the severity of a point mutation by interfering with enzyme processing and intracellular transport, thereby determining the amount of enzyme present at the intralysosomal site and thus influencing its residual activity. Another reason for the differential involvement of cells and organs in lysosomal disorders may stem from the fact that processing and routing of lysosomal enzymes may vary among different cells (see, for example, I-cell disease, Part II of this chapter). Thus, different lysosomal enzyme activity patterns may ensue, and different residual enzyme activities may protect or damage cells.

Enzyme deficiencies due to single base mutations within the same triplet may vary greatly in the severity of their clinical phenotypes because of variations in transcription; for example, the mutation may lead to another amino acid with consequent inhibition of dimer formation, loss of a glycosylation site, or a stop codon.

PROSPECTS AND OUTLOOKS FOR NEW TREATMENTS

The prenatal diagnosis of lysosomal storage disorders may acquire new relevance in the light of the efficacy of early treatment. Once a diagnosis is confirmed in an index case, either by enzyme analysis of a variety of available specimens of fetal origin, by ultrastructural observation, or by molecular genetic studies, pseudodeficiency of a lysosomal enzyme must be excluded before early therapy or elective abortion can be instituted. A combined approach to prenatal diagnosis involving biochemical, molecular genetic, and morphological studies is recommended [44].

Enzyme replacement for genetic defects of lysosomal hydrolases should be evaluated in animal models first. Substantial progress in this area has been achieved. In mice and dogs lacking β-glucuronidase, homologous to human MPS VII (Sly disease), autologous implants of genetically modified cells (fibroblasts, myoblasts) embedded in collagen lattices (neo-organs) have been implanted for the continuous *in vivo* production of enzyme. All animals expressed β-glucuronidase from the vascularized neo-organs that developed after implantation and the lysosomal storage lesions in their livers and spleens greatly improved [45–47].

In a canine model of MPS I, infusions of recombinant human α-L-iduronidase resulted in decreased lysosomal storage in liver, kidney, spleen, lymph nodes, synovium, adrenals, and lungs. Detectable enzyme activity was present in brain and decreased glycosaminoglycan storage was apparent, although histologic improvement was not evident. Cartilage and heart valve did not show any detectable improvement [48]. Animal models occur naturally, but may also specifically be created by knocking out the gene responsible for a certain lysosomal enzyme as with the targeted disruption of the murine α-L-iduronidase gene in mice [49].

Another feasible approach may be to correct cells from patients *in vitro* using viral vectors to result in overexpression of a deficient enzyme, and then to reimplant the enzyme-secreting cells in the patients [50,51].

Allogeneic bone marrow transplantation for the mucopolysaccharidoses has been explored, and visceral features in patients so treated were ameliorated after transplantation. Transplant-related mortality was 10% if an HLA-identical sibling marrow donor was available and 20–25% if mismatched tissue was used. Bone marrow transplantation was not effective if severe neurological symptoms were already present at the time of transplantation [52]. Microglia invading the brain may have been responsible for successful enzyme replacement in brain after bone marrow engraftment [53].

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Chapter 22, Part II

Disorders of Lysosomal Enzymes: Clinical Phenotypes

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SUMMARY

- Both the structural integrity and normal function of connective tissues are progressively affected in many lysosomal enzyme deficiency disorders. Hydrolases are normally involved in the metabolism of mucopolysaccharides (glycosaminoglycans), oligosaccharides, lipids, and even some proteins. In many of the "lysosomal disorders" connective tissue disease is associated with the adverse effects of substrate storage in brain and visceral organs. Target connective tissues include the skeletal system in general and metaphyseal cartilage and bone in particular, in addition to joints, ligaments, tendons, fasciae, meninges, cardiac valves, skin, and gingivae.
- The mucopolysaccharidoses are a group of lysosomal storage disorders caused by deficiency of one or other of the lysosomal hydrolases involved in glycosaminoglycan metabolism. Consequently, storage of dermatan sulfate and/or heparan sulfate or keratan sulfate and their excessive urinary excretion are observed in patients. Identification of the compound(s) excreted remains of orienting value for the diagnostic assay of the deficient enzyme and ultimately for the characterization of the encoding gene mutation.
- The mode of inheritance of all mucopolysaccharidoses is autosomal recessive, except for mucopolysaccharidosis (MPS) II, Hunter disease, an X-linked recessive trait. Several clinical features are common to all mucopolysaccharidoses and thus of little diagnostic value. All disorders have a progressive clinical course and many a fatal outcome between early infancy and mid-childhood. Even in the milder clinical types morbidity is considerable and life expectancy significantly reduced. Mental deficiency is important in MPS IH, MPS IIA, and MPS VII but in none of these is as early and severe as in the four genetically distinct, clinically indistinguishable types of Sanfilippo disease (MPS III). Mental retardation is absent or of minor consequence in the Morquio diseases (MPS IV A and B) and Maroteaux-Lamy disease (MPS VI). Facial and general body features show gradual coarsening. Progressive skeletal abnormalities noticeably different from the dysostosis multiplex expressed with variable severity in the other entities are specific only to Morquio type A disease (MPS IVA).
- For each of the mucopolysaccharidoses the mutant gene has been mapped and cloned, and its nucleotide sequence partially or fully determined. Most often patients are compound heterozygotes for two different mutations.
- In multiple sulfatase deficiency, several lysosomal and microsomal sulfatases are deficient. They are nonfunctional because a specific type of post-translational modification, common to all sulfatases, does not occur. A particular cysteine residue in the nascent glycoprotein is not transformed into 2-amino-3-oxopropionate, thus precluding the maturation of catalytically active sulfatases.
- The oligosaccharidoses (OS) result from the defective metabolism of carbohydrates in glycoproteins and glycolipids. Excessive urinary excretion of oligosaccharides is the common hallmark of all. Clinically these disorders resemble both the mucopolysaccharidoses and the lipidoses.
- The sialidoses are due to significantly decreased activity of glycoprotein sialidase. Even the most common type, childhood dysmorphic sialidosis, formerly called mucolipidosis type I, is rare. Normosomatic sialidosis, or cherry-red spot myoclonus syndrome, shares a slowly progressive clinical course with the former but lacks dysmorphic features. The latter designation points to decreasing visual acuity and progressive nervous system involvement. In neonates, sialidosis may cause nonimmune hydrops and lead to early fatal outcome.
- Among the four clinical phenotypes delineated in patients with isolated deficiency of β-D-galactosidase, three are known as G_{M1}-gangliosidosis, the fourth as Morquio disease type B (MPS IVB). Except for the latter, it is preferable to view the clinical phenotype as continuously variable, ranging between the severe infantile form with early fatal outcome and the chronic late or adult onset form. Dysostosis multiplex is severe in infantile G_{M1}-gangliosidosis and mild or absent in the adult type patient. This is in contrast to the radiographic findings in MPS IVB in which the mutant enzyme retains some activity toward G_{M1}-gangliosides but none toward keratan sulfate and oligosaccharide substrates, thus explaining the mild central nervous system effects and the serious connective tissue disease.

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- **In the mannosidoses the clinical course is slow and connective tissue involvement mild. α-Mannosidosis is the result of a severe deficiency of the lysosomal acid α-mannosidase. Genetic heterogeneity is more extensive than the variability among phenotypes. β-mannosidosis has an inconsistent phenotype in the few patients reported. The human gene encoding β-D-mannosidase has been cloned and sequenced.**
- **The central nervous system is involved much more prominently than connective tissue in fucosidosis, a quite rare but more severe oligosaccharidosis with infantile onset. A quickly evolving disease (type l) and an almost nonprogressive albeit equally severe disorder (type 2) have been discerned. Patients with both phenotypes have been observed in the same pedigree.**
- **Aspartylglucosaminuria has a high incidence in Finland. It is a chronic disorder not easily recognizable before the age of 5 years, with mental deficiency a major component and a gradually coarsening facies and inguinal herniae signs of connective tissue disease. Most patients live well into adulthood. A number of mutations in the gene encoding aspartyl-β-glucosaminidase have been identified. Schindler disease, a chronic but very rare oligosaccharidosis, due to a deficiency of N-acetyl-α-D-galactosaminidase causes mainly severe neurologic deterioration similar to neuroaxonal dystrophy.**
- **Both I-cell disease (mucolipidosis II) and the milder pseudo-Hurler polydystrophy (mucolipidosis III) are due to a deficiency of N-acetylglucosamine-phosphotransferase by which intracellular transport and compartmentalization of the majority of lysosomal hydrolases is considerably disturbed. The cultured fibroblasts of patients contain numerous dense inclusions and are termed inclusion cells (I-cells). Clinically, I-cell disease is reminiscent of Hurler disease, but has an earlier onset and more severe morbidity. The onset of pseudo-Hurler polydystrophy occurs in early childhood. The disorder has a more chronic course. Growth rate, severely deficient in the patient with I-cell disease, is less affected in pseudo-Hurler polydystrophy.**
- **Galactosialidosis encompasses three disorders with a clinical resemblance to G_{M1}-gangliosidosis and sialidosis. Both glycoprotein sialidase and β-D-galactosidase are deficient. In all instances, galactosialidosis is a severe disease with consistent involvement of the nervous system, the retina, the skeleton, and other organ systems. The primary defect is a deficiency of the lysosomal 32 kDa β-D-galactosidase protective glycoprotein and/or its 54 kDa precursor, because of which sialidase is not activated, β-D-galactosidase is left unprotected against intralysosomal degradation and cathepsin A catalytic activity is abolished.**
- **The primordial metabolic defect in pyknodysostosis, a rare osteochondrodystrophy, is cathepsin K deficiency. Many different mutations have been identified in the cathepsin K gene in the few unrelated patients already studied.**
- **Gaucher disease type I (almost 80% of Gaucher disease patients), the most prevalent lysosomal storage disease, is the only sphingolipidosis with significant skeletal involvement. The central nervous system is spared. Glucosylceramide accumulates in typical reticuloendothelial cells, called Gaucher cells, as the result of glucosylceramide-β-glucosidase (glucocerebrosidase) deficiency. A number of different mutations, mostly single nucleotide substitutions, have been identified. The disease occurs with high frequency in Ashkenazi Jewish subjects in whom the large majority carries one of four more common mutations. Significant therapeutic progress has been achieved in Gaucher type I disease.**

INTRODUCTION

Many of the lysosomal disorders were originally delineated as storage disorders of childhood rather than as disorders of connective tissue. In fact, most such entities are both. In some, the lysosomal accumulation of macromolecules and the ensuing cell and parenchymal organ damage, including that to brain, is of major clinical importance. In others, the extracellular connective tissue is most adversely affected. In this chapter, only those lysosomal disorders with significant involvement of connective tissues such as bone, cartilage, and soft tissue will be discussed.

THE MUCOPOLYSACCHARIDOSES

Recognized as disorders of connective tissue as early as 1960 [1], the mucopolysaccharidoses and their study have played an important role in promoting clinical and scientific knowledge about amorphous connective tissue. In Table 1, the milestones in our understanding of the glycosaminoglycan (GAG) storage disorders are set out. Because, in each, elucidation of the enzyme defect has preceded insight into the gene mutation responsible, the

TABLE 1. Milestones in Understanding the Mucopolysaccharidoses (MPS)

1917; 1919	Clinical descriptions by Hunter and Hurler
1952	Discovery of storage of acid mucopolysaccharides (glycosaminoglycans[GAGS])
1957	Excessive urinary excretion of acid mucopolysaccharides noticed in patients; urinary test for group diagnosis
1962–63	Additional MPS entities delineated: Scheie, Sanfilippo, Maroteaux-Lamy diseases
1965	First McKusick classification; toluidine blue metachromasia observed in fibroblasts
1968–74	''Corrective factors'' in metabolism of GAGs; main primary enzyme deficiencies known
1975–90	Assignment of lysosomal enzyme genes to chromosomal locations; molecular biology of lysosomal enzymes involved in the mucopolysaccharidoses; monoclonal antibodies; processing; intracellular routing
1990–	Molecular biology of genes; molecular diagnosis; genotype-phenotype correlation; animal models: experimental gene therapy; bone marrow transplantation; enzyme substitution strategies

sequence of events outlined represents a classic example of functional genetics, in which knowledge of a gene product and its function precedes and contributes to the identification, cloning, and characterization of the gene.

The mucopolysaccharidoses (MPS) are a group of heritable disorders characterized by intralysosomal storage of GAGs, previously termed acid mucopolysaccharides. In each entity, this is due to the deficiency of a single specific lysosomal enzyme involved in the hydrolytic breakdown of GAGs. All of the disorders have a slow course but interfere significantly with normal life expectancy. Except for Hunter disease with its X-linked recessive mode of inheritance, all types of MPS are autosomal recessive disorders.

At present, at least a dozen different clinical entities characterized by excessive urinary excretion of GAGs have been formally delineated. They are listed in Table 2. Several of the fully developed phenotypes, though superficially similar, can be differentiated clinically. The features most useful for this differentiation, as well as signs and symptoms rather common to all diseases, are shown in Table 3.

Mucopolysaccharidosis I

Hurler Disease; MPS IH (MIM 252800)

Hurler disease, MPS IH, has served as a point of reference in the process of detection and delineation of several clinically related disorders. This prototype among the mucopolysaccharidoses, due to α-L-iduronidase deficiency, can be diagnosed clinically in the first 6 months of life, even though most of its features in infancy are but retrospectively recognized as relevant components of the syndrome. The neonate or young infant with Hurler disease has noisy breathing and is hypotonic. In males, often postoperative scars are indicative of earlier surgical treatment of inguinal herniae or hydroceles, which may recur. Restricted motion in the shoulders is observed from infancy. Corneal cloudiness, if equivocal on clinical inspection, is confirmed by slit lamp examination. As infancy progresses, the head becomes more scaphocephalic, and its circumference enlarges excessively. There is frontal bossing. Between 6 and 15 months of age, the child's growth and weight gain are excessive, resulting in plots which may cross over onto higher centile curves. Upper respiratory infections and otitis recur frequently. Some reduction in the range of motion of the large and small joints and some broadening of the wrists and hands may already be apparent. The abdomen becomes increasingly prominent, and hepatosplenomegaly is a regular finding. There is a moderate degree of hirsutism. Delay in psychomotor development is only moderate. An umbilical hernia, a constant feature, is rarely of clinical importance before the age of 3 years (Fig. 1). From 18 months onward, growth decelerates considerably and usually ceases during the fourth year of life, from which time also the head circumference no longer increases. Independent walking is usually achieved with delay. Speech, often with a hoarse voice, remains elementary. As toddlers, Hurler disease patients continue to make slow progress in psychomotor development, but in

TABLE 2. Catalog of the Mucopolysaccharidoses (MPS) [1]

			Primary Metabolic Defect				
Abbreviation	Eponym	MIM No.	Enzyme	Gene Symbol	EC No.	Main Metabolite Affected	Location of Gene on Chromosome
MPS IH	Hurler	252800	α-L-iduronidase	*IDUA*	3.2.1.76	DS, HS	4p16.3
MPS IS	Scheie	252800	α-L-iduronidase				
MPS IHS	''Compound''	252800	α-L-iduronidase				
MPS II A MPS II B	Hunter	309900	Iduronate 2-sulfatase	*IDS*	3.1.6.13	DS, HS	Xq27-q28
MPS III A	Sanfilippo A	252900	Heparan-N-sulfate sulfatase	*HSS*	3.10.1.1	HS	17q25.3
MPS III B	Sanfilippo B	252920	N-acetyl-α-D-glucosaminidase	*NAGLU*	3.2.1.50	HS	17q21
MPS III C	Sanfilippo C	252930	AcCoA: α-glucosaminide N-acetyltransferase	*GNAT*	2.3.1.3	HS	—
MPS III D	Sanfilippo D	252940	GlcNAc-6-sulfate sulfatase	*GNS*	3.1.6.14	HS GlcNAc-6-S	12q14
MPS IV A	Morquio A	253000	GalNAc-6-sulfate sulfatase	*GALNS*	3.1.6.4	KS, CS GalNAc-6-S	16q24.3
MPS IV B[2]	Morquio B	253010	β-D-galactosidase	*GLB1*	3.2.1.23	KS	3p21.33
MPS VI A MPS VI B	Maroteaux-Lamy	253200	GalNAc-4-sulfate sulfatase (arylsulfatase B)	*ARSB*	3.1.6.1	DS, CS GalNAc-4-S	5q13-q14
MPS VII	Sly	253220	β-D-glucuronidase	*GUSB*	3.2.1.31	DS, HS, CS	7q11.21-q11.22

DS, dermatan sulfate; HS, heparan sulfate; KS, keratan sulfate; CS, chondroitin sulfate.

[1] Allied to the mucopolysaccharidoses is mucosulfatidosis or multiple sulfatase deficiency (MSD); MIM 272200; see text.

[2] In still a third type of MPS IV the enzyme defect remains unknown.

TABLE 3. Mucopolysaccharidoses (MPS): Clinical Features of Differential Diagnostic Importance

	MPS I[1]			MPS II[1]		MPS III[2]	MPS IV		MPS VI[1]		MPS VII[4]
Symptoms	Hurler	Scheie	Compound	A	B		A[3]	B	A	B	
Neonatal problems	Several	None	None	Few if any	None	None	None	None	Several	None	Few if any
Final stature	Severely dwarfed	Nearly normal	Reduced	Dwarfed	Reduced	Almost normal	Severely dwarfed	Reduced	Severely dwarfed	Reduced	Dwarfed
Coarse facies	Severe; family features eliminated	Slight	Flat, receding chin	Moderate to severe	Mild	Mild or absent	Mild or absent	Absent	Moderate to severe	Mild	Severe
Cornea	Cloudy	Cloudy	Cloudy	Clear	Clear	Clear	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy
Kyphosis	Frequent, dorsolumbar	None	None	Rare, mild	None	None	Constant, dorsal	Mild, rare	Frequent. dorsolumbar	Rare, mild	Frequent, dorsolumbar
Psychomotor retardation	Severe	None	Probably none	Severe	None to mild	Extreme	None	None	None to mild	None	Moderate
Behavior	Calm, friendly	Normal	Normal	Stubborn, destructive	Normal	Restless, with-drawn, early dementia	Normal	Normal	Normal	Normal	Normal
Dysostosis	Early, severe	Minimal	Moderate	Severe	Moderate	Minimal	Severe, typical	Moderate	Early, severe	Mild to moderate	Moderate to severe

Several important clinical features, though usually of consistent severity within one syndrome, are of less or no differential diagnostic value. They are macrocephaly, herniae, hepatosplenomegaly, cardiac and great vessel valve disease, hirsutism, upper respiratory infections, otitis and/or hearing loss, restriction of motion in all joints.

[1]The clinical entities first delineated represent but reference types in a continuous spectrum of severity; frequency spectrum skewed toward the more severe phenotypes.

[2]Four genetic entities known within one overall phenotype with inconsistent pattern of variation.

[3]Besides the MPS IVA outlined, both an intermediate and a mild phenotype exist.

[4]Several clinical forms distinguishable as more patients are reported, ranging from neonatal immune hydrops to late childhood onset disease.

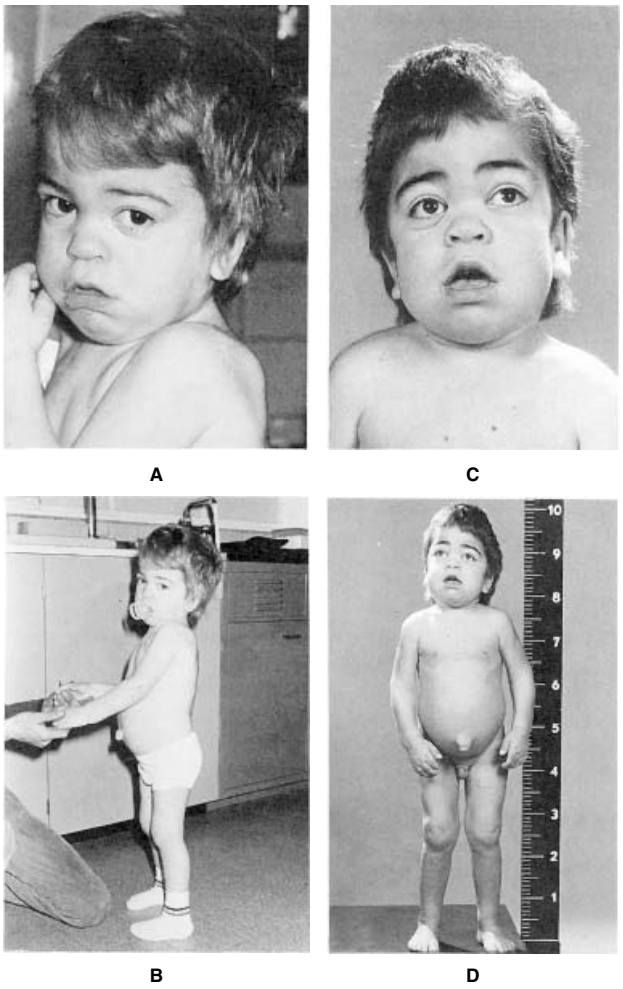


Figure 1. A,B: Patient with Hurler disease (MPS IH) at age $3\frac{7}{12}$ years, with rather mild abnormality of facial and general body configuration, hirsutism, persisting epicanthal folds, a low nose bridge, broad hands, and moderate restriction of joint mobility range, an umbilical hernia, and a prominent abdomen. **C,D**: Same patient at age $7\frac{3}{12}$ years. Features of MPS IH have progressed considerably. Final standing height (101 cm [3rd centile: 113 cm]) is rather tall in this mucopolysaccharidosis. Clouding of corneae not visualized photographically.

subsequent years increasing physical disability, conductive hearing loss, and respiratory infections represent additional hindrances impairing their progress. From the moment unaided sitting is achieved, or even before, a thoracolumbar kyphosis may be evident. It soon becomes fixed and rarely requires orthopedic treatment.

Mean life expectancy is about 10 years. Before such fatal outcome due to cardiac valve disease and mainly mitral stenosis and insufficiency and to bronchial and lung sclerosis, facial features have become increasingly grotesque, their resemblance to Gothic gargoyles leading to the now-obsolete term "gargoylism". The skin has become thick and stiff. Increasing deafness becomes more of the sensorineural type. Mental function and social contacts have meanwhile gradually declined. Finally, in the last stage, ambulation becomes limited, partly because of increasing stiffness of the large joints with fixation in flexion, and partly because of cardiorespiratory insufficiency and lack of mental and social initiative.

The radiographic features of the Hurler patient's skeleton have been termed succinctly dysostosis multiplex [2]. They are evident from birth and evolve from mild to severe by the time growth ceases. All bones are affected. The most obvious abnormalities already evident at the age of clinical onset of the disease are listed in Table 4. Dysostosis multiplex, defined as a particular pattern of radiographic changes, is not pathognomic for Hurler disease. Qualitatively similar abnormalities are observed in some of the other mucopolysaccharidoses, and in several of the oligosaccharidoses. In these entities, dysostosis multiplex is either more or less severe and evolves either more quickly or more slowly. In each instance, the following radiographs should preferentially be taken: skull (lateral), spine (lateral), pelvis and hips (anteroposterior), hands and wrists, and at least one example of a long bone. Fig. 2 illustrates various aspects of dysostosis multiplex in a patient with fully developed Hurler disease. Inherent to the recognition of the autosomal recessive mode of inheritance of Hurler disease, was the original view that all patients are homozygous for a mutant MPS IH gene with severe phenotypic effect [3]. The advent of molecular biology has meanwhile shown that most of them are compound heterozygotes for different mutations in the α-L-iduronidase gene. (See below).

Scheie Disease; MPS IS (MIM 252800)

The second phenotype due to α-L-iduronidase deficiency is represented by the patient with Scheie disease, which is a milder condition than Hurler disease. The first complaints arise in late childhood and relate to fine motor clumsiness in the hands due to decreasing motility of the finger joints. Clinical examination brings to light a decreased range of motion at the shoulders and slight clouding of the corneae, which may become troublesome and require corneal transplants that, however, provide only temporary relief. Often pes cavus is observed. Inguinal herniae may have been repaired earlier in life. Growth and intelligence are not significantly impaired. Subclinical to moderate hearing loss is noticed. Interference with life expectancy is usually

TABLE 4. Early Signs of Dysostosis Multiplex: Radiographic Features at the Clinical Onset of Hurler Disease[1]

Calvarium	Large, elongated (scaphocephalic head)
Spine	Ovoid vertebrae and /or hook-shaped hypoplastic vertebra(e) T_{12}, L_1, L_2
Pelvis, hips	Hypoplastic narrow iliac body; shallow acetabula
Long bones	Failure of tubulation (wide diaphyses)
Ribs	Broad
Hands (metacarpals)	Pointed bases, widened diaphyses, coarse trabeculation; wide proximal and middle phalanges

[1] Dysostosis multiplex, quantitatively different or similar, is observed in several other mucopolysaccharidoses (MPS); in MPS IV it differs qualitatively. For similarities and differences in the oligosaccharidoses, see text and [2]

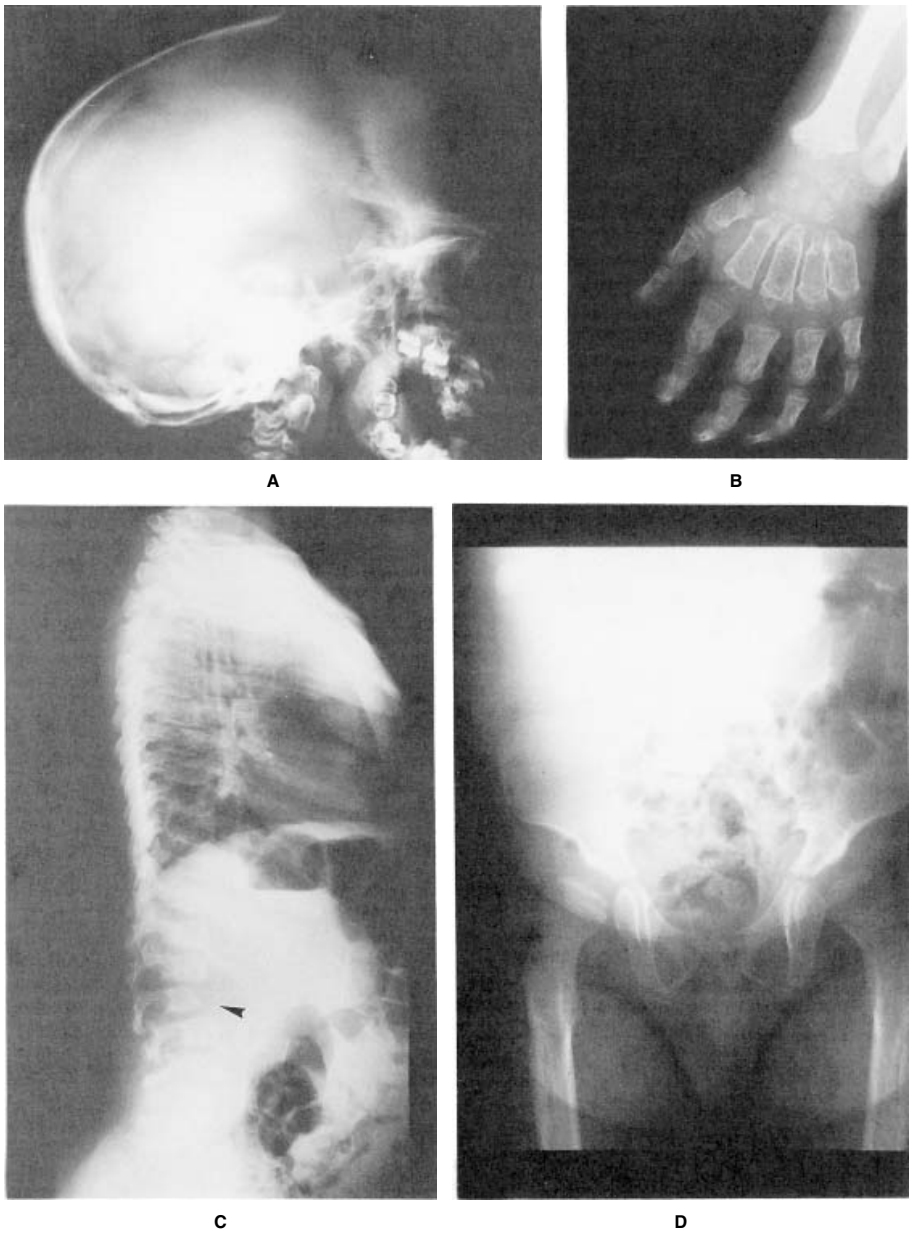


Figure 2. Roentgenographic dysostosis multiplex in fully developed MPS IH as documented at 7 3/12 years in the patient shown in Figure 1. **A**: Enlarged cranial vault with elongated sella turcica. **B**: Left hand and wrist: tilted, irregular distal radial and ulnar metaphyses; dysplastic small carpal bones; metacarpals with proximal pointing, widened diaphyses, and coarse trabeculation; phalanges shortened and widened; claw hand deformity shown by flexion of fingers. **C**: Spine; small L_3, with anterior–inferior beaking (arrow). **D**: Pelvis: narrow iliac bodies and flaring wings; oblique, shallow acetabula; coxa valga; flattened proximal femoral epiphyses.

mild in Scheie disease, except when aortic valve thickening and stiffening leading to stenosis and insufficiency causes progressive cardiac failure. Complications include retinopathy, glaucoma, and, rather often, carpal tunnel syndrome. On radiographic examination, only mild dysostosis multiplex is noticed, even in patients with rather advanced Scheie disease.

Following the discovery of the same enzyme defect as in Hurler disease [4–6], Scheie disease patients, originally classified as representing MPS V, were at first considered as being homozygous for a MPS IS mutant gene with only mild effect [3], but have more recently been recognized as compound heterozygotes [7–9].

MPS IHS (Formerly Hurler-Scheie Compound MPS) (MIM 252800)

This third phenotype among the α-L-iduronidase mutants is intermediate between MPS IH and MPS IS with regard to both time of appearance and severity of signs and symptoms. The majority of patients survive until young adulthood (Fig. 3). Final stature, though below normal, is significantly taller than in the Hurler disease patient. Despite considerable physical limitations, patients may be successful pupils in

Figure 3. Patient with MPS IHS at 18 years of age, and a successful high school student; corneal clouding severe but barely visualized photographically; prominent maxillar part of facies.

regular grade and high school. The term "compound form of MPS I" was coined on the basis of the hypothesis that these patients are compound heterozygotes for the allelic mutations, MPS IH and MPS IS, at the MPS I locus [10], even though MPS IHS patients born to consanguineous parents have been observed [3]. Meanwhile, in the majority of compound MPS-IHS patients heterozygosity has been proved [7–9].

Molecular Defect in MPS I and Diagnosis

Patients with Hurler disease (MPS IH), Scheie disease (MPS IS), or MPS IHS excrete excessive amounts of dermatan sulfate and heparan sulfate in the urine. In the cytoplasm of lymphocytes, large vacuolar structures, when stained with toluidine blue, show red metachromasia, demonstrating the glycosaminoglycan nature of their contents. Since the 1970s, formal confirmation of the clinical diagnosis has been achieved by the finding of nearly absent activity of α-L-iduronidase in leukocytes and cultured fibroblasts [5,6]. Assay of this enzyme in chorionic villi or cultured amniotic fluid cells also allows the prenatal diagnosis of MPS I [5]. In Fig. 4, the role of α-L-iduronidase in the physiologic degradation process of glycosaminoglycans is schematically presented. Although but one of many lysosomal acid hydrolases involved, its deficiency precludes their concerted catabolic action in the patient. Although the severely affected Hurler disease patients usually lack both immunodetectable α-L-iduronidase protein and α-L-iduronidase activity, a correlation between residual enzyme activity and severity of the phenotype has only been partially possible [11,12].

Knowledge of the α-L-iduronidase (*IDUA*) gene and its mutations may be useful in establishing genotype-phenotype correlations. The *IDUA* gene maps to chromosome 4p16.3 [13] in close proximity to the genes for Huntington disease and fibroblast growth factor receptor 3 (FGFR3), which is mutated in achondroplasia and related osteochondrodystrophies [14] (see Chapter 23, Part IV, this volume). The gene is approximately 19 kb in length and contains 14 exons. Exons 2 and 3 are separated by a large intron of nearly 13 kb, which contains an *Alu*-repeat element and a highly polymorphic VNTR repeat. This latter was an important point of reference in the identification of the gene itself. The promoter region of the *IDUA* gene contains several possible transcription start sites consistent with the maintenance of low levels of transcription. At its 5′ end, the *IDUA* gene is flanked by another *Alu* repeat sequence [14–16].

α-L-iduronidase mRNA has a length of only 2.3 kb. Alternative splicing of exons 2 and 3 has been reported [14], but its clinical significance remains unclear at present. The 1959-bp open reading frame in *IDUA* is represented by 653 amino acids in a single polypeptide containing six potential N-glycosylation sites [15]. A signal peptide of 25 amino acids is cleaved off upon leaving the endoplasmic reticulum, placing the N-terminus at alanine 26.

Genotype-Phenotype Correlation in MPS I

To the present, several hundred mutations including nonsense, missense, and splice site mutations and small deletions or insertions have been identified within the *IDUA* gene [7–9,14,17–19]. In addition to cataloging the *IDUA* mutants [14], the earlier described and most frequently encountered ones have been given allelic variant subnumbers of MIM 252800 [20]. Intragenic polymorphisms have also been found. They may be helpful in identifying founder effects as they account for high frequencies of alleles in some ethnic groups and not in others.

Some genotypes reliably predict the clinical phenotype. Null mutations and the effects of either premature termination of the gene product, frameshifts, or major sequence rearrangements, invariably cause severe disease (MPS IH) in the homozygous patient, and usually also in the compound heterozygote. This is true for the mutations Trp402Stop and Gln70Stop accounting for approximately 50% of patients of Northern European origin [7–9]. Because of the degree of allelic heterogeneity, not only patients with MPS IHS but also many with Hurler or Scheie phenotypes are genotypically compound heterozygotes. It is now well understood that rather than the discontinuous set of three clinically delineated phenotypes — severe, intermediate, and mild — a continuous spectrum of severe to mild clinical expression more accurately describes the variability of phenotypes among MPS I patients and reflects the expected consequence of multiple alleles at the *IDUA* locus. Obviously intrafamilial clinical variability is significantly more restricted than the wide overall variability. Unless a large proportion of mildly affected Scheie type patients had not been ascertained, the distribution of MPS I patients would have been considerably skewed toward the severe Hurler phenotype.

The consequences of simple amino acid substitutions are inconsistent, as some produce a severe phenotype while others result in intermediate or even mild disease. Some of the amino acid polymorphisms probably modulate the directly deleterious effect of amino acid substitutions. The mutation Arg89Gln found with high frequency in Japan, but not in Europe, produces reduced stability and activity of the mutant α-L-iduronidase. It is found often in Scheie disease, and may have its deleterious effect potentiated by the presence of the Ala361Thr polymorphism and thus produce an intermediate phenotype. Besides the steady improvement in the molecular documentation of genetic heterogeneity in MPS I, detailed genotype-phenotype correlation with high predictive value is

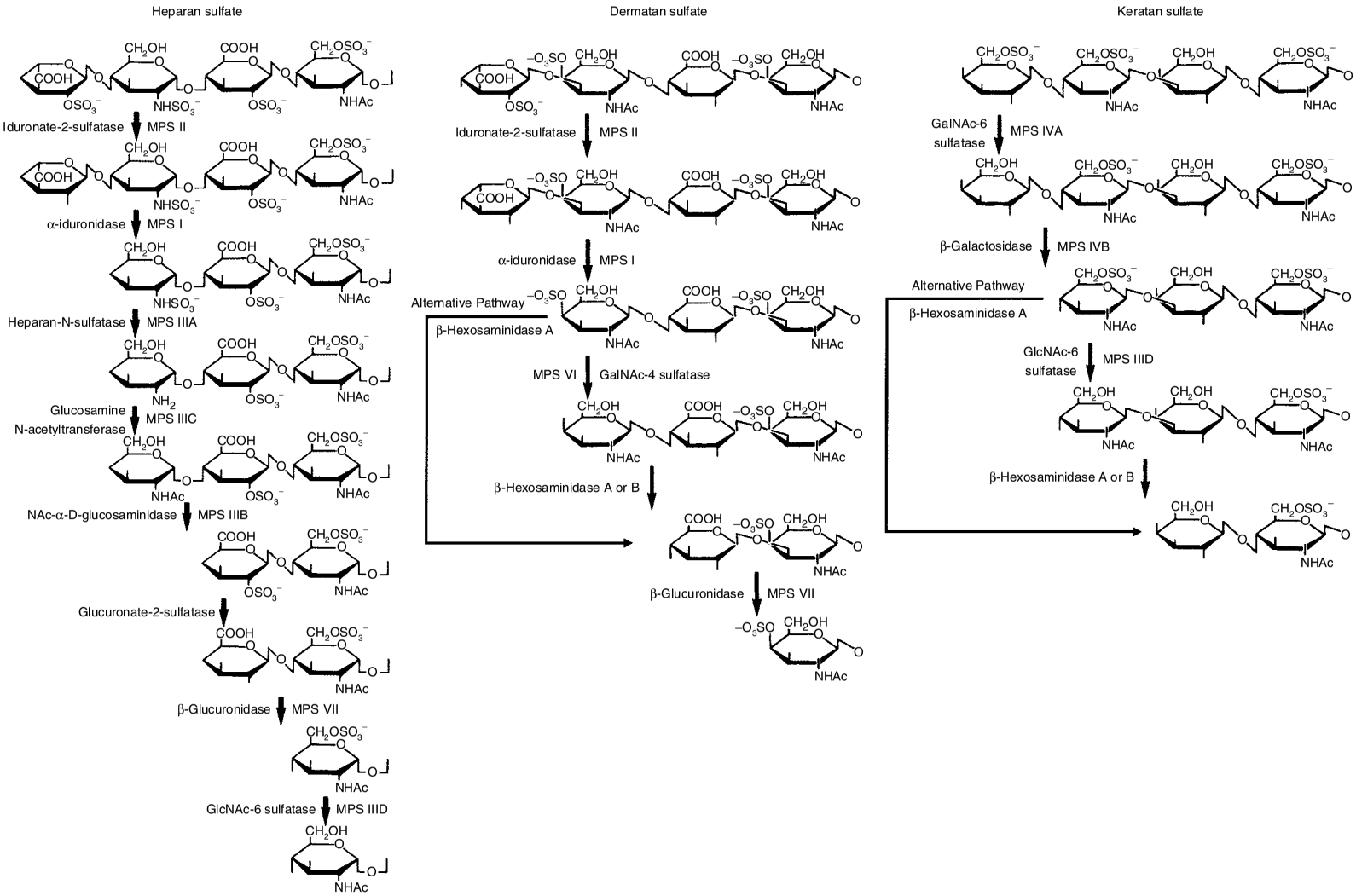


Figure 4. Schematic degradation of glycosaminoglycans by the concerted catalytic action of exoglycosidases and sulfatases having the effect of stepwise removal of glycosyl moieties and sulfate groups and provision of the appropriate substrate for the next enzyme action. Normal catabolism is specifically interfered with in each mucopolysaccharidosis by the primary enzyme defect indicated. For abbreviations, see text and Tables 2 and 3. Structures shown are qualitative representations only as the relative proportions of iduronate/glucuronate moieties, the degree of hexosamine acetylation, and the type and number of sulfate groups differs from tissue to tissue. **Dermatan sulfate (DS)**: polymer of disaccharide repeats of 2-sulfated iduronate (glucuronate) α 1,3 (β 1,3) residues linked to GalNAc sulfated at C4; each repeat is joined by β 1,4 linkage to the next. **Keratan sulfate (KS)**: polymer of disaccharide repeats of galactose joined by β 1,4 linkage to GlcNAc; each repeat is joined by β 1,3 linkage to the next; nearly all GlcN moieties are sulfated at C6. **Heparan sulfate (HS)**: polymer of α-glycosidically linked disaccharide repeats consisting of iduronate (glucuronate), some of it 2-sulfated, joined by α 1,4 (β 1,4) linkage to GlcNAc, itself sulfated at C6 and/or at its free NH-substituent on C2. Glycosaminoglycans are components of large proteoglycan complexes in cartilage matrix and other connective tissues, mostly O-linked onto proteins. For further information, see Chapter 4 (this volume). (Figure adapted from [116].)

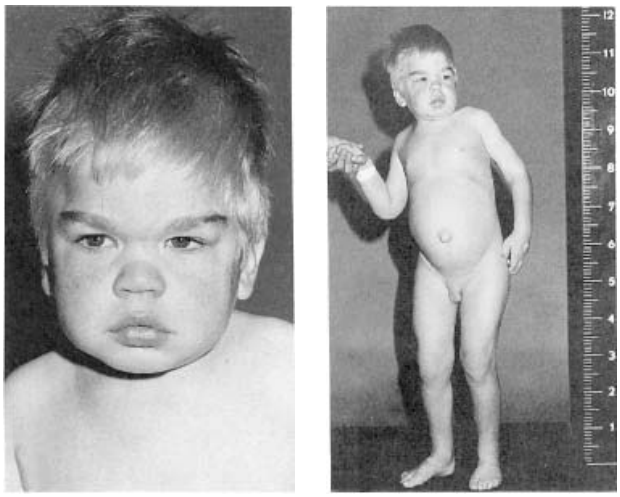
still to be achieved. In this respect, the lack of systematic description of phenotypes, including their natural courses, is at present at least as large a flaw as the lack of knowledge of the amount and quality of α-L-iduronidase mRNA produced and the incomplete understanding of enzyme processing, intracellular routing and kinetics, the rate of substrate accumulation in different tissues, and residual specific organ catalytic activity *in vivo*. The strategy and success of attempts at enzyme and gene therapy will much depend on such knowledge about the individual patient.

Matters of genetic heterogeneity at the *IDUA* locus are further complicated by the existence of rare pseudodeficiency alleles. Such mutations result in low or unmeasurable α-L-iduronidase enzyme activity when assayed with artificial substrates, but produce a normal phenotype. Irrespective of the unknown physiopathologic significance of this additional molecular heterogeneity, its existence has implications in particular in prenatal diagnosis [21].

Hunter Disease; MPS II (MIM 309900)

Usually the patient with Hunter disease does not come to medical attention before his third birthday, when parents may be concerned about slow development of speech and short attention span. Often, recurrent respiratory infections, including bouts of otitis, will have troubled the affected toddler. Rather frequently he will already have been operated upon for inguinal herniae. He has quite noisy breathing and excessive nasal discharge. The patient is described as increasingly disobedient, destructive, and stubborn. Complaints about loose stools soon become regular. Upon clinical examination, the boy has coarse facial and stocky body features. The head is large, but often not scaphocephalic. The bridge of the nose is low. The corneae are clear. At this early stage of the disorder the patient's height is tall, and sometimes exceeds the 97th centile. The abdomen is prominent. There is hepato- and often also splenomegaly. If cooperation is adequate, limitation of the range of movement at the shoulders can be demonstrated. The hands are broad and the fingers slightly stiff (Fig. 5).

For the inexperienced clinician, the early diagnosis of Hunter disease, of great importance for the purpose of prevention, is a major challenge. At 3 or 4 years of age, physical features in the Hunter disease patient are much milder than those in Hurler disease, which, at that age, is already fully developed. However, the completely developed clinical phenotype of MPS II becomes quite similar to that of MPS IH, hence the obsolete term Hurler-Hunter syndrome. Whereas Hurler disease patients remain good-natured and of calm disposition, Hunter disease boys have a short attention span and are impatient and agitated. Their growth slows down from about 4 years of age, but does not come to a complete halt until about 6 or 7. Deafness is of major importance. Initially of the conductive type, by age 5 it is compounded by an increasing sensorineural component, which thereafter becomes the sole reason for total lack of hearing. Facial features gradually coarsen and mental and motor handicap and joint stiffness increase gradually. The eyebrows become heavy, the hair more coarse. Hirsutism increases over the back and the limbs. Kyphosis is rather rare in Hunter disease. Patients may show patches of nodular skin. Heart murmurs due to thickened and stiffened cardiac valves are often heard in older patients. Hunter disease frequently has a fatal outcome, usually between the ages of 10 and 15 years, due to either cardiorespiratory complications refractory to treatment, or brain stem disease,



A B

Figure 5. A, B: Patient with Hunter disease type A (MPS IIA) at the age of $5\frac{10}{12}$ years. A stocky, strong boy with coarse hair and facial features, a low nose bridge, increasing limitation of range of movement in large and small joints, a claw hand deformity, and a prominent abdomen with a small umbilical hernia. Growth decelerating but stature still at 50th centile.

or both. Dysostosis multiplex in Hunter disease does not differ qualitatively from that in Hurler disease, but is less pronounced at any age [2].

In a minority of patients, Hunter disease has a much milder clinical course. In such patients, its clinical onset can be at a variable age, but is usually not after mid-childhood. Deafness, respiratory infections, and stiffening of the hands are the most frequent initial complaints. Survival to adulthood is the rule. Growth, although adversely affected, leads to short stature rather than true dwarfism. Dysostosis multiplex remains quite mild. Usually, patients can attend regular grade school and even high school in some instances. The majority of patients with the milder form of the disorder die in young adulthood of acute complications of slowly progressive cardiac valve disease.

It is obvious that the severer and milder forms of the disease, previously often called MPS IIA and MPS IIB, respectively [20], are but the extremes of a continuum of overall clinical variation. However, the phenotypes of affected boys within a single family or pedigree are rather consistent.

Molecular Defect in MPS II and Diagnosis

Iduronate 2-sulfatase (IDS) deficiency is the primary metabolic defect in Hunter disease [22]. The role of the enzyme in glycosaminoglycan degradation is illustrated in Fig. 4. No *in vitro* complementation has been observed between cultured fibroblast strains derived from patients with severe and mild disease, confirming that all *IDS* mutations are functionally allelic. It is clear, however, that besides the effects of mutations affecting the iduronate sulfatase glycoprotein itself, its intracellular processing and compartmentalization, already well known in human fibroblasts [23], must depend on still other genes. The *IDS* gene spans 24 kb near the telomeric region of the X-chromosome at Xq27-Xq28. Its transcriptional orientation is from telomere to centromere. [20,24–26].

Carrier detection in clinically normal females, a major challenge in any X-linked disorder when relying on enzyme specific activity, becomes straightforward provided the mutation in the proband has been identified. If not, hair root enzyme studies [27] or *in vitro* inhibition of intercellular cross-correction by fructose 1-phosphate [28] must still be resorted to. Occasionally, full Hunter disease has been reported in girls with a normal karyotype. Most such observations have been explained by nonrandom inactivation of the X-chromosomes probably induced by either autosomal translocation, Xq deletion [29,30], or the zygotic process of twinning [31].

The isolation, cloning and sequencing of a 2.3 kb iduronate 2-sulfatase cDNA represented a major step in effectively achieving the molecular diagnosis of Hunter disease [32,33], demonstrating its extensive genetic heterogeneity, and providing answers to questions of genotype-phenotype correlation. The *IDS* gene contains 9 exons, the complete base sequences of which have been elucidated [34,35]. Patients in whom the *IDS* gene has been deleted completely or grossly rearranged invariably have the severe form of Hunter disease [29,36–38]. They may represent as many as 20% of all *IDS* gene mutations. As many as half of all patients have missense or nonsense mutations. A large majority of these are severely affected, while the remainder have either an intermediate form or are more mildly affected. Mutations with a severe clinical effect usually involve amino acids that are conserved between human and murine iduronate sulfatases. They do not usually involve amino acids identified as homologous in several other sulfatases. As expected, mutations in patients with mild disease are compatible with slight residual iduronate sulfatase activity. However, in more than 20% of MPS II patients with a deletion of even a single or two or three base pairs, the majority of which lead to a frameshift and premature polypeptide termination, a total loss of enzyme function is found. These are therefore called null mutations. Also, several splice site mutations have been documented, mostly causing relatively small insertions or deletions in the mRNA [34–38].

A second gene, called *IDS2*, has been identified. It is presumed to be a pseudogene containing base sequence stretches related to exons 2 and 3 and introns 2,3, and 7 of the *IDS* gene [39]. Its location is telomeric to the latter. It is probable that in about 10% of the more severely affected Hunter disease patients, a *cis* intergenic (gene-pseudogene) recombination with the primary *IDS* gene has occurred, resulting in the disruption and inversion of the intervening DNA within the primary *IDS* gene. In the rearranged gene, pseudogene intron 3- and intron 7-related sequences are found [40]. This apparent "hot spot" type of chromosomal rearrangement is probably based on crossing over between fairly homologous stretches of nucleotides.

The entire *IDS* region telomeric to the *IDS2* gene contains a number of as yet unknown genes that may also be deleted in the more severely affected Hunter disease patients, in particular those with atypical additional symptoms such as ptosis of the eyelids, obstructive sleep apnea, and seizures. Thus, such patients may be affected by a contiguous gene deletion syndrome of which Hunter disease and a disorder causing some type of epilepsy are components [33,37,41–43].

In one patient with moderate to severe Hunter disease, no intragenic *IDS* mutation but a large deletion 178 bp upstream of *IDS* exon 1 was detected spanning an *IDS* promoter and resulting in the total loss of IDS function [44]. The continuously enlarging list of mutations within and around the *IDS* gene [45] makes systematic tabulation of allelic variants an increasingly formidable task [20]. If, in the original reports, systematic description of the corresponding phenotypes remains scanty at best, any attempt at genotype-phenotype correlation remains a daunting assignment.

Sanfilippo Disease; MPS III A (MIM 252900), III B (MIM 252920), III C (MIM 252930), and III D (MIM 252940)

As shown in Table 2, no less than four different enzyme defects have been demonstrated in various patients with Sanfilippo disease, all of whom excrete mainly or exclusively large amounts of heparan sulfate in the urine. For the specific role of each of the enzymes involved in glycosaminoglycan catabolism, see Figure 4. Clinically and chemopathologically, all types of MPS III can hardly be considered disorders of connective tissue, and, therefore, they are not discussed here at length. Their main clinical features are listed in Table 3. The heads of these patients are large, but skeletal growth remains approximately normal. Nevertheless, the thickening of the mitral valve leaflets and chordae tendineae, mitral regurgitation, mild mitral stenosis, asymmetric septal hypertrophy, and aortic insufficiency known to occur in the natural course of any type of MPS are frequently apparent also in MPS III patients. In some they lead to congestive cardiomyopathy, as has been pointed out by Dangel [46]. These findings known in MPS I, II, and the rest of the entities are the more surprising in MPS III, which is known to affect mainly and foremost the central nervous system. For extensive discussion of clinical phenotypes, pathology, and chemopathology, the various enzyme defects, and molecular aspects known to date, the reader is referred to excellent reviews elsewhere [47,48].

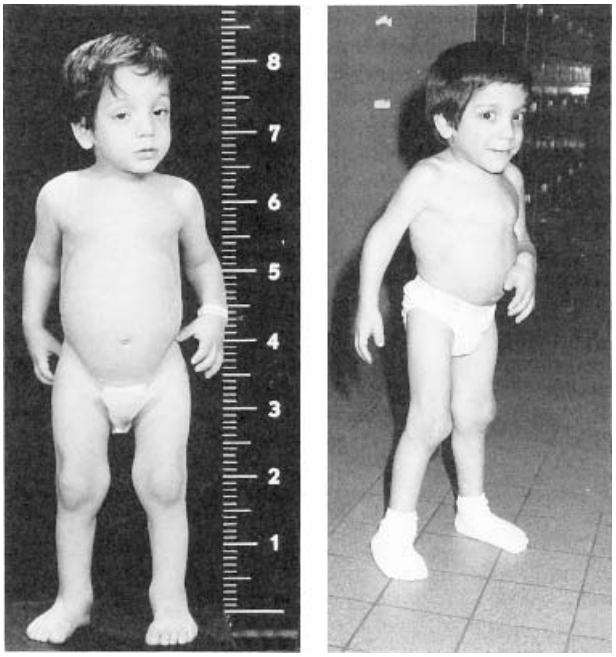
Morquio Disease; MPS IV

MPS IVA (MIM 253000)

The clinical features of this severe short-trunk type dwarfism do not appear until the second or third year of life, when pectus carinatum and growth failure become parental complaints and medically observable (Fig. 6). Genua valga and thoracolumbar kyphosis may be the presenting symptoms. Throughout its clinical course, Morquio disease does not affect mental functioning. Before the age of 6 years, the full clinical picture has developed and consists of a rather large head consistently held in slight retroflexion; facial features that, except for a rather prominent maxilla and mandible, are rather unremarkable; cloudy corneae, at least upon slit lamp examination; widely spaced teeth with hypoplastic enamel; a short neck; protruding sternum; joint laxity; conductive hearing loss; progressive physical disability and easy fatigue for both mechanical and neurological reasons. Vibration sense and muscular tone in the lower limbs become inadequate. Multiple arthroses also compound the clinical picture in older patients [10,20,48,49].

Life expectancy within each type of Morquio disease is determined by the probability of complications, mainly cervical myelopathy, which is unavoidable if careful orthopedic fusion of the cervical spine is not performed sufficiently early in childhood. Luxation of the first cervical vertebra due to odontoid hypoplasia and hyperlaxity of ligaments can cause sudden death due to major damage to the lower brain stem [50].

The radiographic features are those of a spondyloepiphyseal dysplasia with coxa valga. They may also be recognized



A **B**

Figure 6. A: Patient with Morquio A disease (MPS IVA) at age $2\frac{4}{12}$ years. Rapidly decelerating growth. Prominent knees with mild limitation of range of movement without stiffness of weak connective tissue. Rather proportional body configuration. Slight cloudiness of corneae. **B**: Same patient at $4\frac{8}{12}$ years of age. Increase in stature has been minimal. Progressive deformation of sternum and thoracic cage, wrists with radial deviation, and knock knees. Many bouts of upper respiratory infection throughout.

as an unusual form of dysostosis multiplex with, as main added features, odontoid dysplasia and ensuing atlantoaxial instability leading to increased risk of cervical spinal cord compression; moderate to severe platyspondyly; and marked shortening of tubular bones without diaphyseal widening.

Molecular Defect in MPS IVA and Diagnosis. The excessive urinary excretion of keratan sulfate, initially of orienting value in diagnosis, decreases considerably after adolescence. A deficiency of N-acetylgalactosamine-6-sulfate sulfatase (GalNAc-6-sulfate sulfatase) is detected not only in severely affected patients but also in those with an intermediate or even mild phenotype [51–53]. How the enzyme deficiency interferes with keratan sulfate metabolism may be deduced from Fig. 4. The mild form of MPS IVA is to be distinguished in the laboratory from that caused by a nonallelic mutation at the β-D-galactosidase locus (see Morquio disease type B, below).

In normal subjects, GalNAc-6-sulfate sulfatase is synthesized as a precursor glycopeptide of 120 kDa. After the removal of the N-terminal signal peptide and some carbohydrate modification, the precursor is enzymatically cleaved giving rise to the mature heterodimeric enzyme consisting of two subunits of 40 kDa and 15 kDa, respectively [54]. As might be predicted from the clinical variability of MPS IVA, various mutations in the GalNAc-6-sulfate sulfatase (*GALNS*) gene affect to varying degrees either the synthesis, maturation, stability, subunit association and/or kinetic properties of the enzyme [55].

Genomic sequences and full length cDNA encoding N-acetylgalactosamine-6-sulfate sulfatase have been cloned and characterised [56,57]. The gene, mapped by *in situ* hybridization, is located at 16q24.3 [58,59]. It is approximately 35 kb in length and contains 14 exons. The open reading frame in the cDNA consists of 1566 bp encoding 522 amino acids [57]. In addition to multiple polymorphisms useful in haplotype identification [60], the several types of gene mutation detected include not only missense, frameshift, and splice-site mutations, but also large structural alterations such as a rather common double gene deletion, 8.0 and 6.0 kb in size, respectively [61], so far found only in Japanese patients [60]. The 8.0-kb deletion results from an *Alu-Alu* recombinational event, while the 6.0 kb deletion involves intragenic recombination between short incomplete direct repeats at the two breakpoints. Although mutation analysis of the *GALNS* gene demonstrates rather extreme allelic genetic heterogeneity, a characteristic mutation common to each ethnic population studied appears to emerge, except in the case of patients of continental European origin [62,63]. In Colombia, 16 out of 19 patients (68%) have been shown to carry the Gly301Cys missense mutation [64]. Two mutations, Ile113Phe and Thr312Ser, represented 32% of a sample of patients from Ireland and Australia [63]. The former has not yet been found in Japan. The skewed prevalence in some of the populations studied and the results of haploid type analysis within the *GALNS* gene point at ethnic group-specific founder effects. Emerging also is the impression that missense mutations resulting in the replacement of glycine by amino acids interfering with the tridimensional conformation of the enzyme and those in regions of the gene conserved among all sulfatases, have a severe clinical effect. Some mutations have consistently been associated with a mild phenotype. Patients with an intermediate phenotype are often compound heterozygotes for two *GALNS* mutations. At the present time, any conclusions relating to phenotype-genotype correlations must remain tentative.

MPS IVB (MIM 253010)

This is a milder form of Morquio disease, radiographically resembling moderate spondyloepiphyseal dysplasia [64]. In most patients, direct involvement of the central nervous system cannot be demonstrated. In some, however, mental regression is observed [65]. The condition is due to deficiency of β-galactosidase [66–68] (Fig. 4). The mutations involved are known to be allelic to the β-galactosidase mutations causing the various types of G_{M1}-gangliosidosis (Table 6). They affect mainly the affinity of β-galactosidase for keratan sulfate and oligosaccharides and not, or only slightly, that for gangliosides and other lipid-soluble substrates [66]. Cell hybridization studies have shown that MPS IVB and the various forms of G_{M1}-gangliosidosis belong to the same complementation group [67]. (See G_{M1}-gangliosidosis in "Disorders Due to Deficiency of β-D-Galactosidase", below).

MPS IV C; Non-Keratan Sulfate-Excreting MPS IV (MIM 252300)

In addition to the various clinical phenotypes of MPS IVA and MPS IVB patients, children and adolescents with mild manifestation of the disease and radiographic features also qualitatively within the range of MPS IV have been described, in whom no deficiency of either N-acetylgalactosamine-6-sulfate sulfatase or β-D-galactosidase has been found [69]. Thus, it is likely that in some patients with "Morquio disease," the primary enzyme defect has not yet been elucidated [52]. As pointed out by McKusick [20], many

types of spondyloepiphyseal dysplasia have been incorrectly labeled Morquio syndrome. Because lack of keratansulfaturia is common in the older MPS IVA patient, the unaffected function of both N-acetylgalactosamine-6-sulfate sulfatase and β-D-galactosidase stands as a requirement for the diagnosis of MPS IVC.

Maroteaux-Lamy Disease; MPS VI (MIM 253200)

Both severe (VIA) and mild (VIB) clinical phenotypes are generally recognized among patients with Maroteaux-Lamy disease, who are physically quite similar to patients with MPS 1H or children with pseudo-Hurler polydystrophy, discussed among the oligosaccharidoses below, respectively. As a rule, however, developmental retardation and mental deficiency are not features of Maroteaux-Lamy disease.

The MPS VIA patient comes to medical attention somewhat later in infancy and usually has milder craniofacial features than the Hurler patient. Growth is often equally deficient and dysostosis multiplex quite severe. Some patients may survive into early adulthood. Clinical recognition of the MPS VIB patient is often delayed until more than 5 years of age. Growth and facial features are only mildly affected and dysostosis multiplex is minimal. In both types of patient, almost exclusively dermatan sulfate is excessively excreted in the urine [49]. Metachromatic granules in lymphocytes and granulocytes are more easily demonstrated in MPS VI than in the other types of mucopolysaccharidoses.

Molecular Defect in MPS VI and Diagnosis

Both clinical forms of MPS VI are specifically associated with a severe deficiency of N-acetyl-galactosamine-4-sulfate sulfatase (arylsulfatase B), most likely due to functionally allelic mutations. In fact, the clinical expression of deficient arylsulfatase B activity represents a continuous range of phenotypes with regard to both intrinsic severity and clinical course between the better known MPS VIA and milder MPS VIB. The molecular basis for this phenotypic spectrum remains to be clarified but is clearly related to genetic heterogeneity. The enzyme defect was elucidated a quarter of a century ago [70,71]. Its metabolic role in dermatan sulfate degradation is depicted in Figure 4. In patients, arylsulfatase B protein is not only significantly decreased but also structurally altered. Its degree of modification shows some correlation with the severity of clinical presentation [72,73]. This trend toward a low level of cross-reacting material using polyclonal-based sandwich ELISA has also been confirmed by protein assay and immunochemical analysis in other types of MPS patients. Information on enzyme processing and targeting is, however, an essential prerequisite in correlating overall residual catalytic activity with clinical phenotype [74]. The arylsulfatase B enzyme has been purified from various sources [75]. It is synthesized as a 64 kDa precursor polypeptide, which is subsequently phosphorylated and enzymatically processed into two major mature forms. Only form I, composed of a 47 kDa and an 11.5 kDa polypeptide, appears to exert catalytic activity toward the artificial substrate *p*-nitrocathecol sulfate [76].

The gene has been assigned to chromosome 5q13-q14 by *in situ* hybridization [77]. The cloning and characterization of genomic DNA and full-length cDNA has established the human *ARSB* gene as containing 8 exons [78,79]. This has opened the route to accurate testing for the heterozygous genotype and to studies on genotype/phenotype correlation. Various gene mutations have already been documented, comprising mostly missense mutations, some nonsense nucleotide substitutions, and some single nucleotide deletions with frameshift consequences. Few have been observed in more than single families. In homozygous patients the degree of severity is rather consistent, but in heterozygous genotypes is difficult to predict. The biochemical phenotype does not necessarily reflect the intralysosomal molecular events that one would want to see correlated with the clinical features [73,80–82].

Sly Disease; MPS VII (MIM 253220)

This disorder was clinically delineated simultaneously with the discovery of the underlying defect of β-D-glucuronidase [83]. The patient with Sly disease does not differ clinically from the one with Hurler disease, although the clinical course starts somewhat later and may last well into adulthood. Rather extensive variability is noticeable among patients described, varying from the now classic MPS VII (Sly disease) patient to mildly affected individuals with asymptomatic thoracic kyphoscoliosis without organomegaly, corneal clouding or dwarfing, but with β-D-glucuronidase activity only 10% of normal in cultured fibroblasts [84,85]. At the opposite end of the phenotypic spectrum, several examples of nonimmune fetal hydrops have been observed before or at birth [86–88]. As glucuronic acid is a building block in several glycosaminoglycans, the deficiency of β-D-glucuronidase impairs the catabolic breakdown of heparan as well as dermatan sulfate (Fig. 4).

The lengthy process of determining the precise chromosomal location of the human gene at 7q11.21-q11.22, was completed relatively recently. The fluorescence *in situ* hybridization technique applied, simultaneously revealed two pseudogenes located at 5p13 and 5q13, respectively [89]. Cloning and molecular characterization revealed that the human β-D-glucuronidase gene (*GUSB*) is 21 kb long and contains 12 exons. Through alternative splicing, two different transcription products arise. In the shorter of the two types of mRNA, a major part of exon 6 is lacking [90]. Complete sequencing was achieved by Tomatsu et al. [91]. Once again, the expected genetic heterogeneity has been amply documented by the finding in homozygotes, severely affected compound heterozygotes, and hydropic neonates, of missense, frameshift, and premature splice site-introducing mutations [88,89,92].

Multiple Sulfatase Deficiency; Mucosulfatidosis (MIM 272200)

The clinical and pathological phenotype of this rare but severe disorder is a composite of features found in the mucopolysaccharidoses and metachromatic leukodystrophy [93,94]. First described by Austin et al. in 1965 [95], it is due to the deficiency of several lysosomal and microsomal sulfatases. The disease has its clinical onset in the first year of life, when psychomotor retardation, hepatosplenomegaly, recurrent respiratory infections, inguinal herniae in males, moderate dysostosis multiplex, and increased urinary excretion of glycosaminoglycans as well as sulfatides is observed. Craniofacial changes are less severe than in Hurler disease, but as neurodegenerative symptoms become overwhelming in the second year of life, the facial features are soon those of a child with severe mental retardation. By the third birthday, loss of the few acquired skills, progressive quadriplegia, ataxia, nystagmus, convulsions, and loss of vision and hearing are apparent. The corneae remain clear. In most patients, dermal scaling or moderate ichthyosis is observed as a result of steroid sulfatase deficiency, as in X-linked isolated steroid sulfatase deficiency (MIM 308100). Multiple sulfatase deficiency ends fatally between early and late childhood. Most

of the few clinical reports available agree closely with the average phenotype described. However, other clinical types, including a neonatal [96,97] and a late-onset variant [98], have been reported. Multiple sulfatase deficiency in the fetus is also a cause of low urinary estriol in pregnancy [99]. No less than five different phenotypes including metachromatic leukodystrophy and various types of MPS may be recognized initially at the time of the clinical onset of the disorder [94], yet all mutations causing multiple sulfatase deficiency are allelic. Formal diagnosis of the condition is achieved by the demonstration in leukocytes or fibroblasts of simultaneous deficiencies of the lysosomal arylsulfatases A (cerebroside sulfatase) (EC 3.1.6.8) and B (EC 3.1.6.1) and the microsomal arylsulfatase C (steroid sulfate sulfohydrolase) (EC 3.1.6.2). Other sulfatases, in particular those known to be primarily deficient in Hunter disease, Sanfilippo diseases A and D, and Morquio A disease, respectively (Table 2), are also equally and concomitantly deficient [93,100]. *In vitro* complementation studies have provided no indication that the genes for the sulfatases involved are affected [101]. However, in expression studies of sulfatase cDNA in multiple sulfatase-deficient fibroblasts, sulfatase polypeptides were found to have significantly reduced catalytic activity [102]. Subsequently, a genetic defect in posttranslational arylsulfatase modification has been demonstrated. The conversion of cysteine residues 69 in arylsulfatase A and 91 in arylsulfatase B into 2-amino-3-oxopropionic acid, which is required for the generation of catalytically active sulfatases, does not occur in multiple sulfatase deficiency. This lack of sulfatase posttranslational modification causes multiple sulfatase deficiency [103]. The cysteine residues in question are located within an amino acid sequence highly homologous among human sulfatases. This entire sequence is also conserved in four lower eukaryote sulfatases.

THE OLIGOSACCHARIDOSES

Because of the adversely affected metabolism of carbohydrate in glycoproteins and glycolipids of oligosaccharidosis patients, there is an excess of oligosaccharides in their tissues and body fluids. At present, this group of disorders is also called the glycoproteinoses because of the defective catabolism of glycoproteins (Fig. 7). Excessive urinary excretion of oligosaccharides is the common hallmark of all

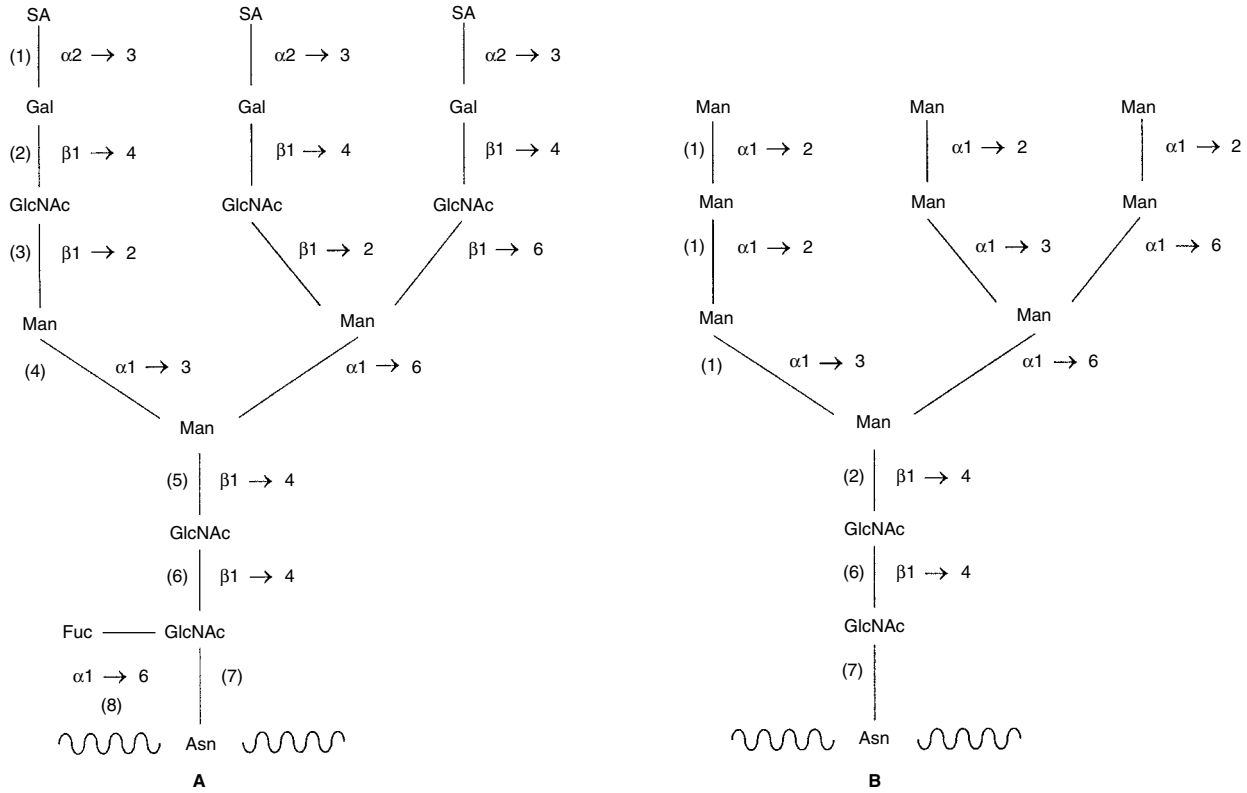


Figure 7. Catabolism of glycoproteins A: Complex N-linked (asparagine-linked) glycan in glycoproteins (lysosomal hydrolases) directed toward secretion into the extracellular fluid. (For biosynthesis, see refs. [105,115,116]. Concerted metabolism of the chemical bonds indicated occurs by stepwise catalytic action of (1) glycoprotein sialidase; (2) β-D-galactosidase; (3) β-hexosaminidase; (4) α-D-mannosidase; (5) β-D-mannosidase; (6) endo-β-N-acetylglucosaminidase; (7) aspartylglucosaminidase; and (8) α-L-fucosidase. **B**: Representative example of high-mannose type N-linked (asparagine-linked) glycan in glycoproteins (lysosomal hydrolases), fit for the phosphorylation reaction at the C6 position of the terminal mannose moiety. This phosphorylation is catalytically mediated by GlcNAc-phosphotransferase (see section on I-cell disease in text and legend to Figure 13) and required for recognition by the lysosomal hydrolase and binding to specific Man-6-P receptors in the prelysosomal compartment. Physiologic metabolism of the specified chemical bonds in this glycan structure is stepwise with the concerted catalytic involvement of (1) α-D-mannosidase; (6) endo-β-N-acetylglucosaminidase; and (7) aspartylglucosaminidase. Various types of hybrid glycan structures have been observed in lysosomal glycoproteins. SA, sialic acid; Gal, galactose, GlcNAc, N-acetylglucosamine; Man, mannose; Fuc, fucose; Asn, asparagine residue in polypeptide chain.

TABLE 5. Progress in Understanding the Oligosaccharidoses

1965–68	''Atypical mucopolysaccharidoses (MPS) or lipidoses'': clinical delineation: • G_{M1}-gangliosidosis [132] • I-Cell disease [247] • Aspartylglucosaminuria [210] • Pseudo-Hurler polydystrophy [279] • Mannosidosis [154] • LipoMPS [108] • Fucosidosis [183] Primary hydrolase deficiencies concomitantly elucidated: • α-D-mannosidase [154] • α-L-fucosidase [184] • β-D-galactosidase [129] • β-N-GlcNAc-aminohydrolase [215]
1970	''Mucolipidosis'': concept and group differentiation [104]
1970–80	• Excessive urinary excretion of oligosaccharides: chemopathological hallmark • Glycoprotein degradation disorders • Structural specificity of oligosaccharides: [107,113,114]
1971–86	Oligosaccharidos(i)/(e)s & secondary deficiencies of hydrolases
1971–81	Spin-off results from search for primary defect in I-cell disease and pseudo-Hurler polydystrophy • Heuristic hypotheses [251,255–258] • Glycoconjugate chemistry [115,116] • Post-translational enzyme processing • Intra-, intercellular mannose 6-phosphate-mediated routing • Sialidosis: primary deficiency is of glycoprotein sialidase [117,118]
1981	Primary defect in I-cell disease/Pseudo-Hurler polydystrophy: GlcNAc-1-phosphotransferase [261,262]
1971–86	Oligosaccharidos(i)/(e)s and deficient ''protective protein'': • Definition of the galactosialidoses [298–300,317–319,327] • Cathepsin A and ''protective protein'' [316]
1985–	• Hydrolase receptors [270–276] • Oligosaccharidos(i)/(e)s genes assigned to chromosomes; characterization; molecular diagnosis
1995–	GlcNAc-1-phosphotransferase genes [293–296]

oligosaccharidoses. This term is the preferred substitute for the name ''mucolipidosis'' proposed in the first comprehensive paper on a number of hereditary disorders clinically related to both the mucopolysaccharidoses and the sphingolipidoses [104]. Progress in understanding and delineating the oligosaccharidoses is summarized in Tables 5 and 6. All oligosaccharidoses are inherited as autosomal recessive traits and may be due to the deficiency of a single lysosomal hydrolase or to altered processing or protection of lysosomal enzymes. While awaiting effective treatment, management also of this group of disorders lies in prevention, with early diagnosis, genetic counseling, and prenatal diagnosis, available for all oligosaccharidoses, as main strategic measures. Comprehensive reviews are available [105,106].

The Sialidoses (MIM 256550)

At present, at least four different disease entities (Table 6) have been given the common name of sialidosis, coined in 1977 [107]. This term should be reserved exclusively for the clinical conditions discussed here and due to the isolated primary deficiency of glycoprotein sialidase (NAc-neuraminic acid hydrolase).

Childhood Dysmorphic Sialidosis

This disorder was originally called lipomucopolysaccharidosis by Spranger [108], who provided its clinical delineation. It was subsequently named mucolipidosis I [104]. Complaints of slow psychomotor development usually come in early childhood, when coarse facial features and thoracolumbar kyphosis are also noted. At first, a puffy face, depressed nasal bridge, and broad maxilla are reminiscent of MPS IH or hypothyroidism. Developmental milestones are reached with delay. Intellectual development and physical growth are slower than normal, with height being below the third centile between the ages of 3 and 5 years, when similarity of the facies to that in Hurler disease becomes more striking. The nose has a bulbous shape with anteverted nostrils. Gingival hypertrophy is mild to moderate. The teeth are widely spaced, the tongue is enlarged, and the maxillary part of the face prominent. The thoracic cage is barrel shaped, with pectus excavatum and thoracolumbar kyphosis. Hepatomegaly, although an early feature in some patients, is inconsistent or absent in others. Splenomegaly is rare. Herniae may be present. Sensorineural deafness is already present in early childhood. The clinical picture is

TABLE 6. The Oligosaccharidoses

Clinical Designation • Type	MIM No.	Metabolic Defect: Enzyme	Gene Symbol	EC No.	Metabolic Features	Gene Chromosomal Location
Sialidosis						
• Neonatal (hydropic) • Infantile, nephrosialidosis • Childhood dysmorphic • Normosomatic	256550	Glycoprotein sialidase	*NEU1*	3.2.1.18	—	6p21.3
G_{M1}-gangliosidosis						
• Infantile (1)	230500	β-D-galactosidase	*GLB1*	3.2.1.23	—	3p21.33
• Late Infantile (2)	230600				—	
• Adult (3)	230650				—	
• MPS IVB	253010				Keratan sulfate in urine	
α-Mannosidosis	248500	α-D-mannosidase	*MANB*	3.2.1.24	—	19p13.2-q12
β-Mannosidosis	248510	β-D-mannosidase	*MANBA*	3.2.1.26	Man-β-GlcNAc in urine	4q22-25
Fucosidosis	230000	α-L-fucosidase	*FUCA1*	3.2.1.51	—	1p34
Aspartylglucosaminuria	208400	1-aspartamido-β-N-GlcNAc-aminohydrolase	*AGA*	3.5.1.26	Aspartylglucosamine in urine	4q32-33
Schindler type neuroaxonal dystrophy	104170	N-acetyl-α-D-galactosaminidase	α-*NAGA*	3.2.1.49	—	22q13
I-cell disease (Mucolipidosis II) [104]	252500	GlcNAc-phosphotransferase α/β-subunit	—	2.7.8.17	Most acid hydrolases deficient in cells; excessive in body fluids	12q
Pseudo-Hurler polydystrophy (Mucolipidosis III)	252600	GlcNAc-phosphotransferase				
Variant A		α/β-subunit	—	2.7.8.17		12q
Variant C		γ-subunit	—			16p
Galactosialidosis						
• Neonatal (hydropic) • Late infantile • Juvenile, Adult	256540	''β-gal'' protective protein (cathepsin A)	*PPGB*	—	Deficiency of glycoprotein sialidase; β-D-galactosidase	20q13.1

only fully developed in late childhood, with the appearance of progressive ataxia and nystagmus, muscle wasting and loss of strength. By then ophthalmologic examination reveals a cherry-red macular spot, often strabismus, and, inconsistently, cataract and corneal opacity. Subsequently, a coarse tremor and myoclonic jerks complete the neurologic syndrome. Mental deterioration is not a feature and seizures have not been observed, but sensory deficits and decreased nerve conduction velocity are indications of peripheral nerve involvement. Of interest is that limitation of large joint movements is only minimal and that the small joints of the hands are not affected. Progression of dysmorphic sialidosis is slow. Patients become chair-bound from adolescence. Adverse effects of the neurologic deficit and pneumonia have been considered the causes of death in adolescence or early adulthood [109–112]. Careful consideration of the diagnosis of α-mannosidosis is, however, necessary in patients considered to have childhood dysmorphic sialidosis, because its clinical phenotype, including its natural course, may be similar [109]. The radiological manifestations of childhood dysmorphic sialidosis may be summarized as slowly progressing to a mild to moderate degree of dysostosis multiplex.

Peripheral lymphocytes contain abnormal vacuoles. In bone marrow smears, histiocytic cells have a foamy cytoplasm. Histopathologic changes are noticed in neurons, as well as in mesenchymal and visceral cells. Hepatocytes and Kupffer cells are filled with cytoplasmic vacuoles and granules. Electron micrographs show enlarged, membrane-bound lysosome-like organelles filled with reticulogranular material. These vesicular structures fill major portions of the cytoplasm [109]. The discovery of an increased urinary excretion of several oligosaccharides [113,114] stimulated their structural elucidation as glycoprotein-derived sialyloligosaccharides in which sialic acid residues occupy the terminal nonreducing position [115] (Fig. 7). The urinary oligosaccharide pattern in sialidosis patients [114] is similar to that found in patients with I-cell disease or pseudo-Hurler polydystrophy, and hardly differs from that in galactosialidosis patients. In contrast to the results obtained by routine diagnostic methods, the various compounds found in the urine differ in their detailed chemical structure depending on the particular enzyme defect, as may be shown by more sophisticated chemical and molecular analytic methods [115,116].

The demonstration in fibroblasts of a deficiency of glycoprotein acid sialidase active against water-soluble substrates establishes the diagnosis in the sialidoses. Here, this deficiency is associated with neither defective β-D-galactosidase (see "Galactosialidosis", below) nor with deficiency of several other lysosomal hydrolases (see I-Cell Disease and Pseudo-Hurler Polydystrophy, below). The parents of patients with childhood dysmorphic sialidosis have intermediate sialidase activity [117]. The enzyme defect in patients can also be demonstrated in freshly isolated leukocytes.

Normosomatic Sialidosis; Cherry-Red Spot-Myoclonus Syndrome

Almost simultaneously with the discovery of the primary metabolic defect in childhood dysmorphic sialidosis, a deficiency of glycoprotein acid sialidase was found also to be the enzyme defect in the cherry-red spot-myoclonus syndrome [118].

The designation, cherry-red spot-myoclonus syndrome, correctly refers to the main clinical features: a slowly progressive reduction of visual acuity, with onset in some patients before 10 years of age, and a crippling, often generalized action myoclonus appearing in the second decade of life. A macular cherry-red spot is almost consistently found, but the time of its first appearance cannot be determined without prospective observation in younger affected sibs of index cases. Although the absence of dysostosis multiplex, even in patients with mild scoliosis, the absence of psychomotor retardation and mental deficiency, except for terminal deterioration, the absence of corneal clouding even by slit-lamp examination, and the absence of facial coarsening make the inclusion of this entity among connective tissue disorders a matter for discussion, these features are important in differentiating patients from those with the juvenile or adult type of galactosialidosis.

As the enzyme diagnosis in patients with the normosomatic sialidosis or cherry-red spot-myoclonus syndrome is sometimes delayed until adulthood, oligosaccharidurie may no longer be detectable at that time. The residual activity of acid sialidase is higher in fibroblasts from juvenile normosomatic sialidosis patients than in those from patients with childhood dysmorphic sialidosis. The parents of patients have intermediate levels of sialidase activity. Somatic cell hybrids between childhood dysmorphic sialidosis and juvenile normosomatic sialidosis fibroblast strains do not show enzymatic complementation, providing evidence of allelism [119].

Infantile Sialidosis; Nephrosialidosis

This variant, described originally in two sibs with early appearing and severe clinical features of childhood dysmorphic sialidosis with an identical isolated deficiency of glycoprotein acid sialidase [120], may be called infantile sialidosis. It has subsequently been observed by Aylsworth et al. [121]. Because the glomerular nephropathy, considered an essential component of this type of sialidosis, was fatal in one of the original patients described by Maroteaux, it has also been termed nephrosialidosis [120].

Neonatal (Hydropic) Sialidosis

Glycoprotein sialidase deficiency may apparently result in fetal and/or neonatal non-immune hydrops, ascites, and hepatosplenomegaly. The findings of excessive excretion of urinary oligosaccharides and specific glycoprotein sialidase deficiency establish the diagnosis in these very sick infants [122,123].

Molecular Biology of Sialidase and Its Gene

Glycoprotein sialidase performs an essential role in removing terminal sialic acid residues from the complex carbohydrate side chains in mature sialoglycoconjugates. The allelic mutations responsible for sialidosis cause a continuously variable spectrum of phenotypes. The excessive urinary excretion of oligosaccharides in addition to the spectrum of phenotypes is rather similar to what is observed in patients with any type of galactosialidosis or with disorders due to GlcNAc-phosphotransferase deficiency, both of which are described further in this chapter. The two subunits of glycoprotein sialidase have been isolated and purified: the larger one, a 76 kDa polypeptide is associated with a smaller 32 kDa subunit. The latter is a trifunctional protein, which functions simultaneously as the activator of acid sialidase, as the "protective protein" for β-galactosidase, which is deficient in galactosialidosis, and as cathepsin A [124,125]. The gene coding for the 76 kDa subunit of glycoprotein sialidase has been mapped to 6p21.3. A full-length cDNA clone representing the gene and predicting a 415 amino acid polypeptide with considerable homology to other neuraminidases in nature has been characterized and

sequenced [126,127]. The gene coding for the protective protein with cathepsin A activity (PPCA) is located on chromosome 20 [128]. Already a few missense and nonsense mutations have been found to be associated with various types of sialidosis. Irrespective of their phenotype, most patients are compound heterozygotes for two different mutations [20].

Disorders Due to Deficiency of β-D-Galactosidase

Disorders due to acid β-D-galactosidase deficiency are better known as G_{M1}-gangliosidoses [129]. This term implies only defective ganglioside degradation and progressive brain disease, and is therefore not an appropriate label for the classification of patients with only or mainly a spondyloepiphyseal dysplasia such as Morquio disease type B.

Infantile G_{M1}-Gangliosidosis; Landing Disease; G_{M1}-Gangliosidosis Type 1 (MIM 230500)

The onset of this devastating disease is at or soon after birth. Feeding difficulty, hypotonia, edema of hands and feet, weak sucking and other primitive reflexes, poor weight gain, and/or failure to thrive are the usual early signs and complaints. Psychomotor development is minimal and head control remains poor. Sensory responses of and social contact with the patient are limited. The cranial and facial features are reminiscent of those encountered in both Hurler disease and I-cell disease (see below). The corneae are clear. Gingival hypertrophy and sometimes macroglossia are present. Cardiomyopathy, muscular weakness, and hypotonia are occasional manifestations. Recurrent respiratory infections complicate the clinical course throughout. Retinal cherry-red spots similar to those observed in Tay-Sachs disease are found in about half of the patients. Already in infancy, rather generalized angiokeratoma [130,131] may be observed, as illustrated in Figure 8. Hepatosplenomegaly is a regular feature, as are moderate thoracolumbar kyphosis and enlargement of most peripheral joints [131,132] (Fig. 8). Already before 1 year of age, generalized convulsions and spastic quadriplegia are found. In the second year of life, blindness, deafness, and decerebrate rigidity compound the clinical picture. Patients rarely survive beyond their second birthday, the fatal outcome being due to either brain stem dysfunction or infectious respiratory disease.

Radiographically, the skeletal changes are moderate to severe dysostosis multiplex [2], similar to that seen in I-cell disease. In the infant patient, temporary periosteal cloaking of the long bones is regularly seen, a feature shared with the I-cell disease patient in infancy.

In the urine there is excessive excretion of oligosaccharides, mainly those with terminal β-galactose residues [133]. The pathological changes due to storage of G_{M1}-gangliosides are not fully described here. They include neuronal lipidosis and general visceral histiocytosis due to intralysosomal

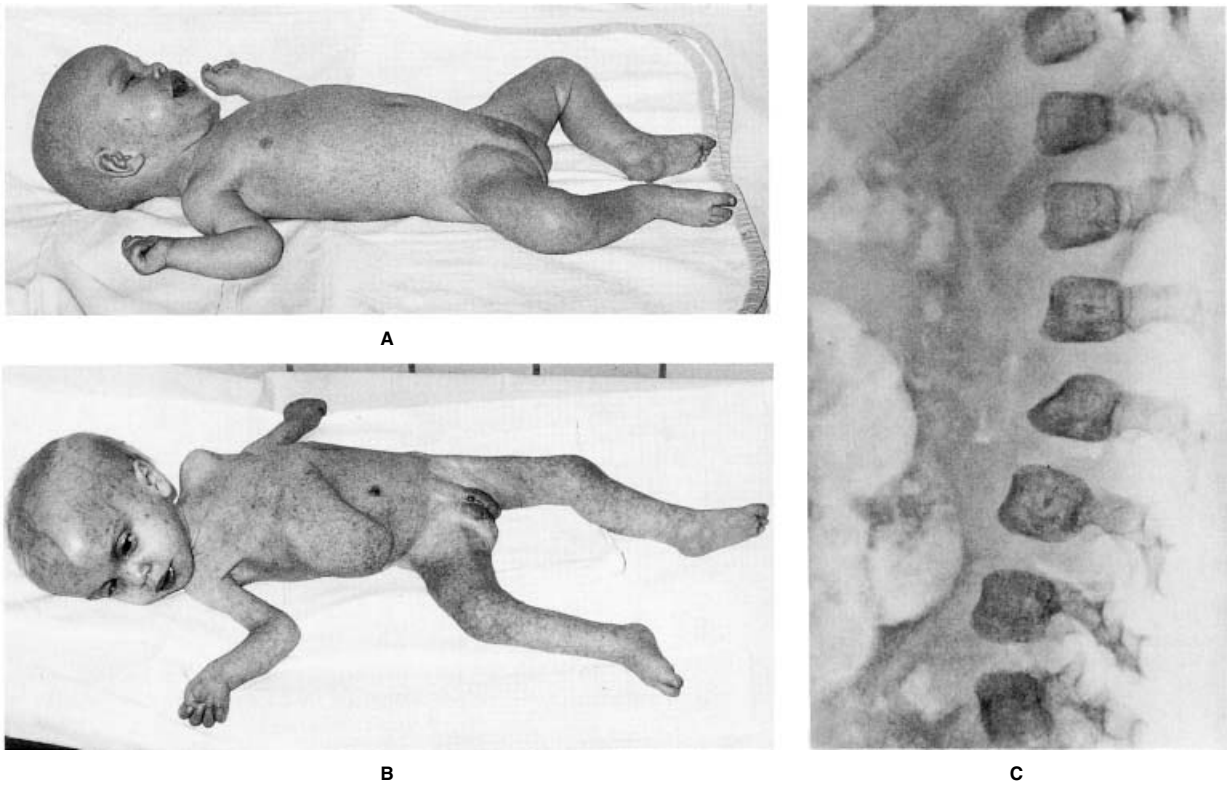


Figure 8. Patient with type 1 G_{M1}-gangliosidosis (Landing disease). **A**: General features at 9 months of age: macrocephaly; hypotonia; bilateral macular cherry-red spots in the retina; pronounced formation of angiokeratomas over large areas of skin. Head control never achieved; convergent strabismus; prominent mouth. **B**: Same patient at 18 months of age. Macrocephaly; extreme hypotonia and dystrophia; hepatosplenomegaly. **C**: Typical aspect of dysostosis multiplex in lumbar spine already apparent at 9 months of age. (Reprinted from [131] with kind permission of S. Karger AG, Basel, Switzerland.)

storage of loose heterogeneous material, obviously a tissue type-dependent range of galactoproteins in addition to gangliosides. The diagnosis is made formally by the finding of a severely deficient acid β-D-galactosidase [129] in leukocytes or cultured fibroblasts. The mutation apparently affects the catalytic activity of acid β-galactosidase toward all galactoconjugates in a similarly profound fashion [133]. Figure 4 and Figure 7 illustrate the role of β-D-galactosidase in connective tissue galactoconjugates.

Late Infantile or Juvenile G_{M1}-Gangliosidosis; G_{M1}-Gangliosidosis Type 2 (MIM 230600)

For details, the reader is referred to a review elsewhere [133]. The phenotype, also with considerable morbidity and a lethal outcome in middle to late childhood, has a later onset at around l year of age and features milder facial, skeletal, and visceral abnormalities. Cardiac involvement may be a regular albeit inconsistent feature in the late infantile as well as infantile type of G_{M1}-gangliosidosis [134,135]. Dysostosis multiplex is mild or may even be absent. Neurological dysfunction, known to be the rule in infantile G_{M1}-gangliosidosis, gradually but consistently becomes also here part of the clinical syndrome as it fully develops. The mutant β-galactosidase is qualitatively similar to the abnormal enzyme in the infantile form. It has, however, preserved two to five times greater residual activity toward all natural substrates [136], which consequently are stored at a slower rate. Confirmation of diagnosis is by assay of acid β-D-galactosidase as in the infantile form.

Adult G_{M1}-Gangliosidosis; G_{M1}-Gangliosidosis Type 3 (MIM 230650)

A few patients have been documented in whom a childhood-onset neurological disorder with slowly progressive paralysis, dystonic posture, cerebellar dysfunction, and, years later, gradual loss of intellectual and social functioning as main components, is apparently due solely to a deficiency of acid β-D-galactosidase [137]. As connective tissue appears to be uninvolved, the responsible mutation apparently produces an enzyme with catalytic activity toward galactoproteins sufficiently preserved but with that toward gangliosides seriously impaired.

Molecular Biology of β-D-Galactosidase and Its Gene

Although the activity of β-D-galactosidase is relatively deficient in any patient tissue, cultured fibroblasts, and body fluid, the extent of substrate storage is directly correlated with the severity of the disorder and inversely so with residual enzyme activity [138]. Comparison of data from different laboratories is, however, difficult. The enzyme in question is different from galactosylceramidase (EC 3.2.1.46), which is deficient in globoid cell leukodystrophy (Krabbe disease), and the gene for which is on chromosome 14. The mature β-galactosidase occurs in tissues in either monomeric, dimeric or multimeric form, the latter comprising aggregates of the monomer [66]. In fibroblasts, the enzyme is synthesized as a high molecular weight precursor of 85 kDa that is processed via an intermediate form to the 65 kDa mature protein that contains about 8% carbohydrate. Most of the latter is of a complex oligosaccharide nature. In the lysosome, β-D-galactosidase is stabilized through its association with its protective lysosomal glycoprotein, which also activates glycoprotein sialidase. For more about protective protein with cathepsin A activity and its gene *PPGB* (Table 6) see under "Galactosialidosis", below. The catalytic hydrolysis of G_{M1}-gangliosides and other lipid substrates by β-galactosidase also depends on the presence of the low molecular weight sphingolipid activator protein 1 (SAP-1), also called saposin B, a physiologic detergent that facilitates enzyme-substrate interaction [133].

In the MPS IVB patient (previously considered under the mucopolysaccharidoses), β-D-galactosidase activity has been found reduced to about 5% of its normal level using both artificial substrates and G_{M1}-gangliosides. However, its hydrolytic activity toward keratan sulfate and oligosaccharides from patients' urine samples is totally lacking [68,139]. In liver and fibroblasts from many patients with G_{M1}-gangliosidosis or Morquio B disease, cross-reacting material has been demonstrated in immunologic studies using antisera against purified human β-galactosidase [67,140]. Gradually, the *in vitro* effect of specific mutations has become more specifically related to enzyme phosphorylation, processing, sorting to lysosomes, aggregation into multimeric form, binding to protective protein, catalytic properties, optimum pH, thermostability, and electrophoretic mobility [133]. The gene coding for β-D-galactosidase has been fine mapped to 3p21.33 by *in situ* hybridization [141], although it had previously been assigned to chromosome 3 over 20 years ago [142]. The gene coding for lysosomal β-galactosidase protective protein is located on chromosome 20 (see under "Galactosialidosis", below).

Human nascent placental β-galactosidase consists of 677 amino acids, including 23 signal sequence residues. This is the *in vitro* functional expression product of cDNA in COS cells and virally transformed mutant fibroblasts [133,143,144]. The human gene coding for β-galactosidase extends over 60 kb and contains 16 exons [145]. A steadily increasing number of mutations is being identified, providing one more example of genetic heterogeneity [133,146]. The mutations are classified either as "allelic variants" [20] or according to their main phenotypic effect [133]. Most frequently, single nucleotide substitutions are observed in either homozygous or compound heterozygous genotypes. The clinical effect is mostly severe and evident from early infancy. The more chronically evolving late infantile and adult G_{M1}-gangliosidoses are less frequent phenotypic consequences. Interestingly, the Arg457Stop (*C*GA to *T*GA transition) nonsense mutation in exon 14 in the homozygous state introduces a premature stop codon and results in severe infantile G_{M1}-gangliosidosis, whereas the Arg457Gln (CGA to C*A*A transition) missense mutation combined in the heterozygote with Ile51Thr yields the chronic adult phenotype. A few larger structural rearrangements within the gene, including duplications and insertions, have also been detected. The largest group of patients with known mutations is from Japan, probably due to the keen and effective work of Japanese scientists rather than a significantly higher prevalence of β-D-galactosidase-related disease in that part of the world. At least five different missense mutations causing MPS IV B have also already been documented [20,133,147].

α-Mannosidosis (MIM 248500)

A severe clinical form with late infantile onset and a less severe, slower evolving type of α-mannosidosis may be discerned. The precise age of onset of the latter, which occurs in late childhood or adolescence, is often uncertain because of its slow and gradual nature. Mannosidosis also comprises a wide range of phenotypes, rendering delineation of separate clinical disorders rather irrelevant, in particular because variability of clinical course has been observed even among sibs [148,149].

Clinical Phenotype

The neonatal period is uneventful in α-mannosidosis, but even during the patient's first year of life, recurrent respiratory infections and otitis require hospital admissions. Psychomotor progress slows in the second year and walking is usually achieved with delay. Belated onset of speech and psychomotor retardation are the usual reasons for clinical evaluation. In childhood, signs and symptoms are normal stature, macrocephaly, a prominent forehead, a coarse facies, clear corneae, persistent epicanthic folds, a low nasal bridge, noisy breathing, thick lips, and a prominent jaw and mouth. Joint mobility is minimally impaired. The abdomen is protuberant with an umbilical hernia as a rule. In boys, inguinal herniae or hydroceles are common. Hepatosplenomegaly is mild to moderate in young patients, but disappears in adolescence. Mild kyphosis is common. Hearing loss of both the conductive and the sensorineural type, a clumsy broad-based gait and a deficiency of motor skills are almost always present from later childhood on [150,151]. Speech and language remain at an elementary level. The patient's disposition is usually agreeable, but sudden episodes of anger or aggressiveness and periods of sleep disturbance are regularly reported. Ophthalmologic examination does not reveal any retinal changes but regularly detects cataracts. Infectious complications are the main reasons for a fatal outcome in mid-childhood.

In most patients, the onset occurs some time in mid-childhood. The clinical course is rather mild and barely progressive, with slowly coarsening facial and general body features. A patient is shown in Figure 9. Data on life expectancy are scarce, but most patients apparently survive well into adulthood. In sibs reported from Israel [152], corneal clouding was apparent. These patients also showed marked limitation of joint mobility. Hearing loss has remained moderate in the patient shown (Fig. 9), but from the age of 21 she has shown severe mental deterioration without significant physical disability. The radiographic findings can be summarized as mild, representing slowly progressive dysostosis multiplex.

Figure 9. Girl with α-mannosidosis at the age of 10 years with normal general body features, a minor reduction of range of movement in large and small joints, and a decreasing growth rate. Mildly coarse facies; large skull; hearing aid reveals moderate sensorineural deafness. Patient, a young adult in 1998, has intermittent but lengthening periods of dementia.

Molecular Defect in α-Mannosidosis and Diagnosis

α-Mannosidosis is an oligosaccharidosis as demonstrated by the excessive urinary excretion of mannose-containing oligosaccharides in simple screening tests using silica gel thin-layer chromatography [113,114]. Many of these mannose-rich compounds have been isolated and structurally characterized [105] (see also Fig. 7).

Vacuolization in peripheral lymphocytes is prominent in all patients reported. In liver tissue, numerous clear vacuoles are seen in hepatocytes and Kupffer cells. The cytoplasmic inclusions also exist in histiocytes in bone marrow, endothelial cells of sinusoid vessels, and lymphocytes in spleen and lymph nodes. Throughout the central nervous system the cytoplasm of neurons is distended, being packed with an abundance of single, membrane-bound vacuoles with remarkably clear, sparsely dispersed reticulogranular material [151]. The severe morphologic changes in hepatocytes and neurons are not due to storage of large macromolecules but to the accumulation of abnormal amounts of mannose-containing oligosaccharides derived from improperly degraded carbohydrate chains in glycoproteins.

The diagnosis of mannosidosis is based on the demonstration in tissue or body fluids of a severe deficiency of lysosomal α-D-mannosidase, which has optimal activity near pH 4.5 [153,154]. The activity of α-D-mannosidase can be determined most easily in total homogenates of leukocytes or cultured fibroblasts, where, at pH 4.2, interference by neutral isozymes is negligible, and the specific activity of α-D-mannosidase in mannosidosis is but a few percent of that in controls. Similar results in cultured amniotic fluid cells and postmortem organs, respectively, establish prenatal diagnosis and confirmation of mannosidosis. α-D-mannosidase exists in two forms termed A and B, which have identical catalytic and immunological properties. They are products of the same gene [105]. In human plasma or serum, an "intermediate" or "neutral" form of α-D-mannosidase is predominant, and the lysosomal "acid" enzyme much less abundant. The former is thermolabile at 56 °C and most active at pH 5.5–6.0 [155], but the latter is thermostable. Serum or plasma lysosomal mannosidase can be measured at its pH optimum of 4.6 most reliably only after thermoinactivation (56 °C) of samples dialyzed against phosphate-buffered saline [156,157]. The mannosidase activity in tissues and body fluids of patients with mannosidosis is mainly due to unaffected isozymes with neutral or intermediate pH optima. The kinetic and physicochemical properties of the residual mutant enzyme studied in many patients may differ from those of the wild-type enzyme. Changes encountered especially in mutant tissues with high-residual mannosidase activity may be particular to specific patients and thus document enzymatically the existence of genetic heterogeneity. Often thermostability is decreased and Km values for artificial substrates increased [158,159].

Molecular Biology of α-D-Mannosidase and Its Gene

The synthesis of lysosomal α-mannosidase has been studied in normal and mannosidosis fibroblasts by immunoprecipitation [160–162]. Results may differ according to tissue studied, but may also be influenced by proteolytic degradation of the gene product. The enzyme is synthesized as a

large, 110 kDa precursor protein. Subsequently, proteolytic processing results in 65 and 27 kDa subunits.

The gene encoding α-mannosidase, termed *MANB*, has been mapped to the region 19p13.2-q12 [163]. It was assigned to chromosome 19 by studies in mouse-human hybrid cells [164]. The first cDNA clone representing the *MANB* gene was isolated from retina/muscle cDNA libraries [165] and subsequently differentiated from cDNA derived from spleen, lung, and fibroblasts [166,167]. Riise et al. [168] have established the overall structure and organization of the *MANB* gene which spans 21.5 kb and contains 24 exons. The corresponding transcription initiation sites have been sequenced for a few hundred nucleotides upstream from the gene. By Northern blot analysis, a single mRNA species of approximately 3.5 kb was apparent and a corresponding 962 amino acid-containing polypeptide identified. This has led to the detection of a heterogeneous collection of nonsense and missense mutations [20,67,169,170]. Homozygous as well as compound heterozygous patients have been described. Thus, α-mannosidosis is recognized as another lysosomal enzyme disorder in which clinical heterogeneity is matched by genetic heterogeneity. More mutations need to be known before questions regarding genotype-phenotype correlation can be answered.

β-Mannosidosis (MIM 248510)

First recognized as causing a severe affliction of the central and peripheral nervous systems with a rapidly fatal outcome in goats [171], a comparable human β-mannosidosis was not detected until 1986. Then, a boy with a slowly coarsening facies, mild bone disease, and developmental retardation on the one hand [172], and adults with a mild and slowly evolving condition on the other [173], were reported to have profound β-mannosidase deficiency. By sheer coincidence, the boy reported by Wenger et al. [172] apparently also had Sanfilippo disease and therefore may be phenotypically rather atypical. To date, only about a dozen patients have been described, mostly at various stages of the disorder's natural course. In infancy, a disease-free interval is usually observed, although one instance has been reported of rapidly evolving CNS disease with status epilepticus by 1 year and a fatal outcome by 15 months of age [174]. In another infantile patient, seriously delayed and impaired speech development has been documented [175]. More often, gradually coarsening facial features, consistent mental deficiency and sometimes neuropathy [176] have been reported. In older patients, behavioral problems have been encountered as have angiokeratomata [173,177]. Hearing loss is also a feature in the chronic disease [178]. Organomegaly has not yet been reported, and cerebral atrophy has been observed inconsistently on MRI scan.

Routine oligosaccharide urinary screening does not easily detect β-mannosidosis. Therefore, quantitative analysis of disaccharide excretion, in particular of the specific Manβ(1–4)GlcNAc, is required. This compound accumulates in patients with β-mannosidase deficiency because only the innermost mannosyl residue in any N-linked oligosaccharide in glycoproteins is in β-linkage [172,179] (Fig. 7).

Molecular studies of the caprine gene encoding β-mannosidase [180] have contributed to the molecular biological investigation of its human counterpart. The human gene is located in the region 4q22-q25. The entire coding region of this *MANBA* gene has now been sequenced and shown to consist of 3293 nucleotides, of which 2640 represent the true coding region. One acceptor splice site mutation that upon translation yields a truncated enzyme in the sib patients reported by Kleijer et al. [181] has already been identified [182].

Fucosidosis (MIM 230000)

This disorder also, more a disease of the nervous system than of connective tissue, was originally recognized as an atypical mucopolysaccharidosis with normal urinary excretion of glycosaminoglycans [183]. With the demonstration that the vacuoles visible in peripheral lymphocytes and Kupffer cells are abnormally swollen lysosomes, a search among the lysosomal hydrolases for the responsible metabolic defect led to the finding of a profound deficiency in tissues and body fluids of α-L-fucosidase [184].

Clinical Phenotype

An initial symptom-free period of 6–12 months is followed by frequent upper respiratory tract infections at the time when delay in psychomotor development becomes overt. Physical examination at this stage reveals a slightly coarse facies, thickened lips and tongue, thick skin, and generalized hypotonia with depressed deep tendon reflexes. Some patients never learn to walk alone. Abundant sweating with increased salinity of the sweat has been noticed in young patients. Slowing of linear growth occurs from about the age of 2 years [183,185,186]. Mild hepatosplenomegaly, inconsistently observed in early childhood, is absent in older patients. Mild thoracolumbar kyphosis and cardiomegaly are more often radiographic than clinical findings. The corneae are clear. Before 3 years of age, and earlier in some patients, the clinical course is characterized by progressive loss of motor skills, apathy, hypertonia and spasticity with hyperreflexia, sometimes seizures, increasing mental deterioration, and finally dementia and decerebrate rigidity. A minority of patients with this natural course, which extends over only a few years to a slightly longer period, succumb to complications of their neurodegenerative disease. The phenotype described has been termed fucosidosis type 1.

The observation of several patients with severe mental deficiency, an almost nonprogressive disease, mild radiographic abnormalities, angiokeratoma, and telangiectasia, reported when in their teens or twenties [186–190], prompted the suggestion of a second phenotype, fucosidosis type 2. These patients, surviving beyond adolescence and into early adulthood, represent the majority of cases. Late in their disease they may have recurrent attacks of dehydration due to inability to control body temperature [186].

As more information on the natural course and extent of the clinical spectrum of fucosidosis becomes available, the hypothesis of two distinct clinical phenotypes cannot be maintained. Patients with both types of disease have been observed in one large pedigree [191] and in single sibships [192,193]. In somatic cell hybridization experiments with fibroblast cultures derived from both types of patient, no mutual complementation is observed.

Radiographic changes are either absent or mild in young patients. Skeletal age lags behind the patient's chronological age. Lumbar vertebrae are either slightly hypoplastic or normal in the presence of dorsolumbar kyphosis. Thus, true dysostosis multiplex is not encountered in fucosidosis. Instead, mild spondyloepiphyseal dysplasia is often found in longer surviving patients.

Molecular Defect in Fucosidosis and Diagnosis

Fucose-containing oligosaccharides are excreted excessively in the urine [113,114]. Simple screening by thin-layer

chromatography can distinguish the pattern of oligosaccharide excretion in fucosidosis from that in other oligosaccharidoses, provided the appropriate control samples are included in the study [105,113].

No less than 25 fucosylglycoasparagine-containing compounds in the urine of fucosidosis patients have been separated and characterized. Their consistent feature is the $\alpha(1 \rightarrow 6)$ fucose to GlcNAc linkage [194] (Fig. 7). This is precisely the one which through lack of functional α-L-fucosidase remains unhydrolyzed in patients. One of the glycolipids accumulating in the CNS and other tissues of patients is the H-antigen glycolipid (Fuc $(\alpha 1 \rightarrow 2)$ Gal $(\beta 1 \rightarrow 4)$ GlcNAc-α-Gal-ceramide). The relationship between the clinical severity of fucosidosis and the quantity and quality of the fucosyltransferase(s)-dependent blood group antigens (H, Le^a, Le^b) remains largely unresolved.

Assay of α-L-fucosidase in leukocytes using the fluorescent 4-methylumbelliferyl-α-L-fucopyranoside as a substrate remains the method of choice for establishing or excluding the diagnosis of fucosidosis [195]. The profound deficiency of α-L-fucosidase found in tissues [186,189,190] is similar both in patients with rapidly advancing disease and in those with a more protracted course. Determination of α-L-fucosidase activity in serum is of no value in the diagnosis of fucosidosis because in about 12% of normal individuals the average serum enzyme activity is only about 5% of that in the rest of the population [196,197]. In these normal individuals with variant α-L-fucosidase in serum, the enzyme in leukocytes has normal activity.

Molecular Biology of α-L-Fucosidase and Its Gene

The lysosomal enzyme, α-L-fucosidase, hydrolytically removes fucose from the non-reducing end of many types of glycoproteins and glycolipids. The enzyme occurs predominantly in a homotetrameric form but also exists in multiple other combinations of its common subunit of 50–60 kDa. It has been purified from a variety of tissues. Glycosylation of the enzyme appears to be quite important, as a 51 kDa precursor molecule is processed to the mature 59–60 kDa end product [198]. From studying the mutant residual fucosidase, genetic heterogeneity among patients has been inferred: in many patients, barely any or no cross-reacting material is detectable, while in some the precursor protein remains unprocessed [199].

Fucosidosis is worldwide a rare disorder. Often parental consanguinity (40% of cases) is observed [200]. In both Italy and the United States, more patients have been observed than in any other country, which finding may not entirely be related to prevalence.

By the use of somatic cell hybrids, the gene locus (*FUCA*) for human α-L-fucosidase was located on chromosome 1 [201] and later on its short arm at band p34 [202]. The existence of a pseudogene of *FUCA* has also been established [203]. This latter was confirmed and more precisely mapped to 2q31-q32 by fluorescence *in situ* hybridization and confocal microscopic measurement [204].

The gene for human fucosidase comprises 8 exons spanning 23 kb. The nucleotide sequence in the pseudogene is 80% identical to the *FUCA* cDNA but has no open reading frame [205]. By Northern blot analysis, an mRNA of 2.3 kb has been demonstrated. These results have led to the identification and characterization of a variety of mutations in the *FUCA* gene. These are listed as allelic variants [20], and include base transitions or transversions, each leading to a premature stop codon, and single- and two-base deletions resulting in frameshifts. At least one splice site mutation and a large deletion involving two exons have been characterized [200,206–208]. Most of the patients concerned had an early onset and a clinically severe course of their disease. Only one of these mutations, Gln351Stop [206], has been observed in several families, while the others have been identified only in isolated patients or pedigrees. One recently characterized missense mutation resulting in the substitution of the conserved leucine 405 by arginine (Leu405Arg) [209] was observed in an adult with fucosidosis. Although her longevity is unusual, her disease, however, is one of extreme mental and physical severity.

Aspartylglucosaminuria (MIM 208400)

Although discovered in a British sibship [210] and observed worldwide as a rare metabolic disorder [20], aspartylglucosaminuria has the particularly high minimal incidence of 1/18,500 in Finland [211]. It is thus of no surprise that the detailed phenotypic description, including natural course, as well as the metabolic and molecular elucidation of aspartylglucosaminuria has been contributed mainly by Finnish clinicians and scientists.

Clinical Phenotype

The disorder is rarely recognized in infancy, although hypotonia and weak sucking are sometimes recorded retrospectively. In toddlers and young children, frequent upper respiratory and middle ear infections and umbilical and inguinal herniae are the most prominent complaints. The infections decrease in frequency after the age of 6 years. Aspartylglucosaminuria is not an easy clinical diagnosis before the age of 5 years [212]. Mental retardation, a consistent feature from later childhood on, is not usually recorded formally before the diagnosis of aspartylglucosaminuria is made. Gross motor development proceeds within normal limits, but delay in speech development, clumsiness, short attention span, and restlessness are the usual sources of initial concern. Besides the gradually coarsening facial features and the herniae, abnormal connective tissue signs are hardly noticeable. Developmental delay is gradually more apparent. From adolescence, the loss of mastered skills is observed, and is followed by a more consistent decline from early adulthood. Middle-aged adult patients have adaptive skills inferior to those in the average mentally retarded patient. In one quarter of cases, bouts of behavioral unrest may compound the clinical picture [213,214].

Molecular Defect and Diagnosis

This oligosaccharidosis results from the deficiency of aspartyl-β-glucosaminidase, a lysosomal amidase that hydrolyzes the amide bond between asparagine and N-acetyl-glucosamine in glycoproteins [215]. As a consequence, 2-acetamido-1-L-β-aspartamido-1,2-dideoxy-β-D-glucose, trivially named aspartylglucosamine (GlcNAcAsn), accumulates in tissues and is excreted excessively in patients' urine [216] (Fig. 7). First trimester prenatal diagnosis of aspartylglucosaminuria is possible [217]. For routine use the abnormal metabolite is easily discerned by high voltage electrophoresis/ninhydrin technology. A highly sensitive liquid-chromatographic method for the detection of GlcNAcAsn in urine and a fluorometric enzyme assay for aspartylglucosamine in lymphocytes have been developed [218,219].

Molecular Biology of Aspartyl-β-Glucosaminidase and Its Gene

The gene for aspartyl-β-glucosaminidase (*AGA*) has been mapped to 4q32-q33 by deletion mapping [220] and *in situ* hybridization [221]. Purification of the enzyme led

ultimately to the isolation and cloning of cDNA and its complete nucleotide sequencing [222,223]. The overall 13 kb sequence of the human *AGA* gene has an open reading frame distributed over 9 exons [224]. The growing list particularly of point mutations resulting in either single amino acid substitutions or reading frameshifts, but also of small deletions, and splice site nucleotide substitutions resulting in larger deletions involving complete exons, is ever more impressive [212,225–227]. The most common "Finnish" mutation located in exon 4 and resulting in a Cys163Ser substitution eliminates an all important intrachain disulfide bond, with resultant misfolding and faulty compartmentalization of the enzyme [228]. The mature enzyme normally forms a heterotetrameric (2α,2β chain) structure, following cleavage of the nascent precursor into α and β subunits [229]. In addition to *in vitro* mutagenesis, the identification of mutations has contributed substantially to understanding their steric effects on enzyme structure as well as the important posttranslational regulation of aspartyl-β-glucosaminidase production and function [230,231]. The considerable genetic heterogeneity notwithstanding, significant phenotypic differences among aspartylglucosaminuria patients have not been recorded.

Schindler Disease; Neuroaxonal Dystrophy Schindler Type; Kanzaki Disease; N-Acetyl-α-D-Galactosaminidase Deficiency (MIM 104170)

Schindler disease is characterized by neurologic deterioration, progressive joint contracture and the appearance of subcutaneous and submucous nodules, which lead to progressive and characteristic hoarseness [232]. Schindler disease was first observed in two German male sibs of consanguineous parents [233]. The clinical onset was in late infancy, starting with the cessation of development, hyperacusis, and the subsequent regression and loss of skills acquired earlier. Other early manifestations included general and axial hypotonia, grand mal seizures, poorly coordinated movements, visual impairment, strabismus and nystagmus. This period of rapid regression resulted by 4 years of age in considerable mental and neurologic deficit compounded by cortical blindness, myoclonus, rigidity and flexion contractures, and by decorticate posturing. The subsequent course was but a stable vegetative state with nearly complete lack of contact with the environment. More recently, two Dutch sibs have been reported: a girl with a milder neurodegenerative disorder and her younger brother, considered still to be presymptomatic, had the same enzyme defect [234,235].

Almost simultaneously with the original report, an adult, mildly retarded Japanese female with disseminated angiokeratoma and glycopeptiduria was shown to have numerous cytoplasmic vacuoles in kidney and skin cells [236]. The angiokeratoma lesions had appeared on the lower trunk and spread slowly over the body surface. This patient had consanguineous parents, but she had two unaffected children of her own. Telangiectatic lesions were found also in the ocular conjunctiva and in the fundus of the eye. Endoscopy demonstrated telangiectasia in the gastric mucosa. The clinical picture of this patient was completely different from that found in Schindler disease. It has been called Kanzaki disease [20,106]. That it is also different from any other known oligosaccharidosis or mucopolysaccharidosis, and also from Fabry disease and fucosidosis, was confirmed enzymatically. Similar patients have since been reported [237]. Neuropathological findings show that Schindler disease is principally a disorder of the central and peripheral nervous systems with a remarkable lack of any visceral abnormality [232,233]. The clinical course and the similar axonal ultrastructural findings are rather reminiscent of the Seitelberger type of infantile neuroaxonal dystrophy (MIM 256600) or of some aspects of Hallervorden-Spatz disease (MIM 234200). It is not unlikely that some patients reported earlier with either of these disorders and in whom no enzyme studies were performed may have been examples of Schindler disease.

The abnormal thin-layer chromatographic urinary oligosaccharide profile was the first indication pointing to a metabolic disorder. The blood group A trisaccharide, GalNAc α (1 → 3) Gal (2 → 1) α Fuc, represented a major compound among other glycoconjugates excessively present, all of which had terminal α-NAc-galactosaminyl moieties. The latter were found in both the N- and the O-linked accumulated glycopeptides, glycoproteins, and glycosphingolipids. In addition, keratan sulfate was found stored in various biopsied tissues and cultured fibroblasts. Therefore, a specific deficiency of lysosomal α-NAc-galactosaminidase was predicted and confirmed in Schindler disease [233,238]. It was subsequently demonstrated also in Kanzaki disease [239]. Residual activity was well below 2% of normal in the former, and below 5% of normal in the latter. Immunoblotting using monospecific rabbit anti-human enzyme antibody showed an absence of cross-reacting material in patients. Studies on the biosynthesis of normal and the Schindler mutant enzyme showed that the mutation did not interfere with the synthesis of the 52 kDa precursor protein, and probably also not with the generation of the mature 48 kDa enzyme protein. The latter was, however, more readily denatured in the German patients than in the Kanzaki-type patients. An interesting account of the relationship between α-N-acetylgalactosaminidase, first recognized as α-galactosidase B, and the true α-galactosidase A is found in the review by Desnick and Wang [232]. The enzyme had initially been considered an isozyme of α-galactosidase A, deficient in Fabry disease (MIM 301500) [240].

The gene encoding α-NAc-galactosaminidase is located at 22q13 [241]. The full-length 2.2-kb cDNA and subsequently the entire genomic DNA encoding the enzyme has been isolated, sequenced, and expressed in COS-1 cells [242,243]. Remarkable similarity with α-galactosidase A has, indeed, been established. The gene contains 9 exons and an open reading frame of 1236 bp encoding 411 amino acids.

Specific point mutations have been detected in the Schindler disease patients and in Kanzaki disease. The German patients were homozygous for a G → A transition at nucleotide 973 resulting in a lysine for glutamic acid substitution at position 325 (Glu325Lys) [242]. In the cDNA of the Kanzaki proband, also, a missense mutation was found at amino acid position 329. A C → T transition at nucleotide 985 resulted in an arginine to tryptophan substitution (Arg329Trp) [244]. At present, the reason for the large phenotypic difference between the two types of patient harboring a functionally allelic mutation only 3 amino acids apart in the defective enzyme remains unresolved.

Disorders Due to N-Acetylglucosamine-Phosphotransferase Deficiency

I-Cell Disease (Mucolipidosis II) (MIM 252500)

I-cell disease is a slowly progressive disorder with clinical onset at birth and a fatal outcome in childhood. The neonate with I-cell disease has a low birth weight, a plump and

swollen facies, and skin that is thick and particularly stiff about the ears. Congenital herniae are consistently present in males. One or more orthopedic abnormalities are common: e.g., clubfoot, dislocation of the hip(s), thoracic deformity, or kyphosis. Despite generalized hypotonia, the range of movement in the shoulders is already limited.

Unlike Hurler disease (MPS IH), there is no temporary period of excessive skeletal growth during the first and second years of life. Instead, the poor growth decelerates fast within 6 months of birth and often ceases before 15 months. Growth failure is always severe and a final height of 80 cm is rarely exceeded. The head is not scaphocephalic. Stiffening of all joints occurs from the first year of life. Psychomotor retardation is extreme in some patients, but rather mild in others. Upper respiratory infection and otitis recur frequently, and breathing is noisy. The facies is rather flat. It resembles that of patients with MPS IH but shows consistent differences: small orbits and hypoplastic supraorbital ridges, prominence of the eyes, a tortuous pattern of periorbital veins, telangiectatic capillaries over the midface and cheeks, impressive gingival hyperplasia, and a prominent mouth. Hepatosplenomegaly is moderate or absent. The corneae are clinically clear, and haziness is found only on slit-lamp examination [245]. In the longer surviving patients there is a coarsening of facial features and a broadening of the hands and wrists. Cardiac murmurs appear gradually. The abdomen is protuberant with an umbilical hernia often present (Fig. 10). Bronchopneumonia and congestive heart failure are the usual causes of death.

The radiological abnormalities in I-cell disease are qualitatively indistinguishable from those in MPS IH and G_{M1}-gangliosidosis type 1. Excessive periosteal new bone formation along the long tubular bones before 1 year of age is a feature shared with patients with infantile G_{M1}-gangliosidosis. This phenomenon is mainly a radiographic observation and has been called periosteal cloaking (see also G_{M1}-gangliosidosis, above). In I-cell disease, dysostosis multiplex is quantitatively more severe at any given time in the clinical course than in any of the mucopolysaccharidoses or oligosaccharidoses [2,246].

Molecular Defect in I-Cell Disease and Secondary Deficiency of Hydrolases. The name "I-cell" disease originated from the observation in the phase-contrast microscope of a large number of cytoplasmic granular inclusions in patients' skin fibroblasts cultured *in vitro*. These cells were called "inclusion cells" or "I-cells" (Fig. 11), and the corresponding disorder I-cell disease [247]. It has also been called mucolipidosis II [104].

In I-cells, the activity of a large number of lysosomal acid hydrolases is considerably reduced or absent [248–250]. The activity of the same acid hydrolases is greatly increased in the culture medium of I-cells and also in the patients' extracellular fluids [251,252]. In postmortem tissues, the specific activities of β-D-galactosidase and glycoprotein sialidase are reduced, but those of other acid hydrolases are within normal limits [249,250]. Findings in leukocytes are inconsistent but rather similar. The *in vitro* findings mentioned are also to be found in amniotic fluid and cultured amniocytes as well as in chorionic villi, and have been used successfully in the prenatal diagnosis of I-cell disease [106]. The secondarily impaired degradation of glycoproteins is sufficiently extensive *in vivo* to result in excessive urinary excretion of several sialyloligosaccharides [113,114] (Fig. 7). It should be noted that the excretion pattern in the sialidoses is qualitatively similar.

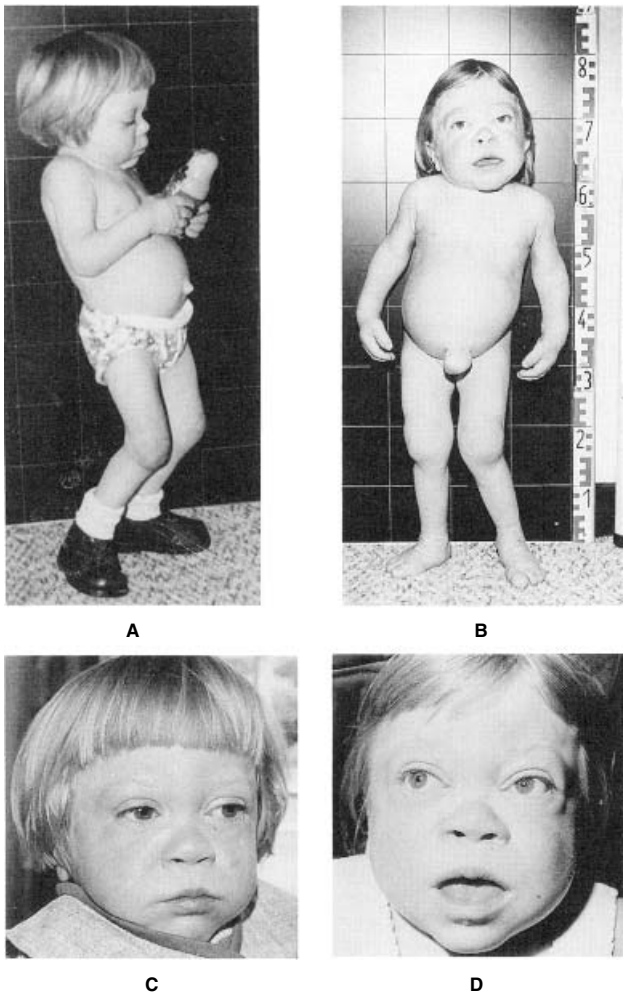


Figure 10. Girl with I-cell disease. **A**: Lateral view at $3\frac{1}{2}$ years of age. **B**: Frontal view at $8\frac{1}{2}$ years. Growth had ceased completely at 2 years (scale in dm). Progression of clinical features as described in text, readily apparent. **C**: Facies of same patient at $3\frac{1}{2}$ years and (**D**) at $8\frac{1}{2}$ years of age. Puffy eyelids; proptosis of eyes and shallow orbits; epicanthic folds; low nose bridge; prominent mouth; mental handicap rather mild.

In I-cell disease, connective tissue cells are primarily affected. Whereas in cultured fibroblasts the cytoplasmic granules are filled with pleiomorphic material, the corresponding *in vivo* inclusions in mesenchymal cells are either empty or contain only sparse granulofibrillar material. The single most characteristic pathological feature is the presence of a large number of cytoplasmic, unit membrane-bound vacuoles in connective tissue cells, irrespective of the organ in which they are located. Such abnormal cells are particularly abundant in skin, gums, heart valves, and the zones of endochondral and membranous bone formation. Pericytes of capillaries, adventitial cells, and Schwann and perineural cells are affected. In the renal glomeruli, there is foamy transformation in only the visceral and not the parietal cells of Bowman's capsule. Changes in neurons and glial cells are minimal, inconsistent, and possibly of a secondary nature [253,254].

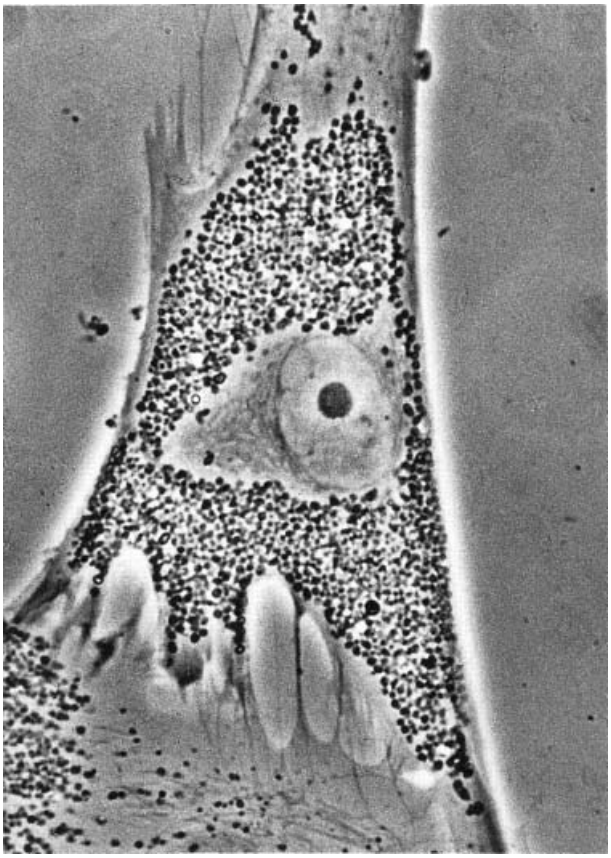


Figure 11. Phase-contrast micrograph of living "inclusion" cell (I-cell): normal nucleus; numerous optically dense granular inclusions fill the cytoplasm except for the juxtanuclear hypertrophic Golgi apparatus. All cells in fibroblast cultures derived from I-cell disease and pseudo-Hurler polydystrophy patients have similar phase-contrast features.

The primary metabolic defect in I-cell disease is responsible for the lack in lysosomal hydrolases of a common recognition marker essential for their binding to specific receptors and subsequent proper intracellular routing. The carbohydrate nature of the common recognition marker was shown by Hickman et al. [255]. Subsequently, mannose 6-phosphate and phosphorylated mannans were found to be effective inhibitors of adsorptive pinocytosis of hydrolases, and pretreatment with alkaline phosphatase abolished receptor-mediated pinocytosis of hydrolases, underscoring the role of phosphate in the recognition marker [256]. In I-cells, phosphorylation does not occur or is only partially achieved. Also, processing of the newly synthesized precursor proteins is incomplete. The enzymes encountered in I-cell disease culture medium are unphosphorylated, and are also of larger molecular size than the mature intracellular hydrolases. They possess no phosphate-containing recognition marker [257,258], which is needed to gain access to the lysosomal compartment or to enter the cells by absorptive pinocytosis. In normal fibroblasts, a major proportion of $^{32}P_i$ groups in newly phosphorylated high mannose-type oligosaccharides in biosynthetic intermediates of lysosomal enzymes are blocked by N-acetylglucosamine, rendering the mannose 6-phosphate-containing carbohydrate chains insensitive to alkaline phosphatase [259,260]. The mere finding of these phosphodiester-structures in the nascent intracellular glycoproteins suggested their formation by enzymatic transfer of N-acetyl-glucosamine-1-phosphate to C_6 of mannose. This reaction is highly deficient in fibroblasts and parenchymatous organs from patients with I-cell disease or pseudo-Hurler polydystrophy (see below), and constitutes the primary metabolic defect in these disorders: i.e., there is a deficiency of UDP-N-acetylglucosamine: lysosomal hydrolase N-acetyl-glucosaminyl-1-phosphotransferase [261–263], for which the term GlcNAc-phosphotransferase is adopted here as a trivial name (Fig. 12; see also the section on synthesis of lysosomal enzymes in Part I of this chapter). The enzyme has been formally adopted by the "Enzyme Commission" as EC 2.7.8.17. A phosphodiesterglucosidase, unaffected in both I-cell disease and pseudo-Hurler polydystrophy, subsequently removes the N-acetyl-glucosamine moiety and uncovers the phosphate, thus rendering the mannose 6-phosphate recognition marker functional [264] (Fig. 12). Normal lysosomal enzymes, having acquired the active recognition marker, can be recognized by specific receptors that direct their transfer to lysosomes. There are two distinct mannose 6-phosphate receptors, which have been purified and structurally characterized by cloning of the corresponding cDNAs [265–268].

Both receptors are normal in I-cell disease and pseudo-Hurler polydystrophy. This conclusion is presumed because of the observation that the internalization of normal lysosomal hydrolases by I-cells is normal [269]. The pathway of enzyme transport from the cell surface to the lysosomes, although discovered first, has since been shown to be of only secondary importance in the routing of these enzymes. Both mannose 6-phosphate receptors are integral membrane proteins containing an aminoterminal extracytoplasmic domain, a unique transmembrane region, and a cytoplasmic carboxyl-terminal domain. One is a large molecule (275 kDa), comprising 15 homologous domains

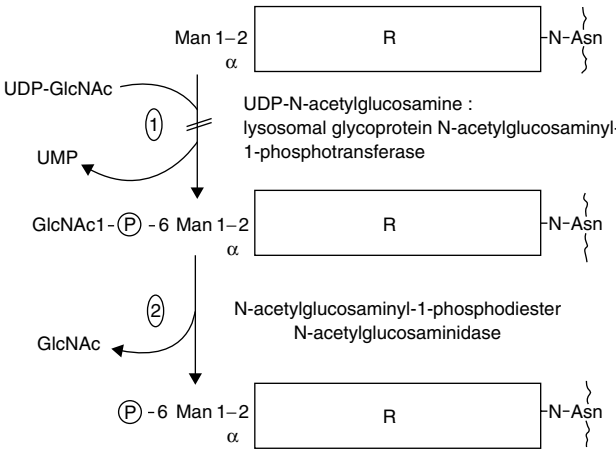


Figure 12. Enzymatic biosynthesis of the mannose 6-phosphate (P) recognition marker in oligomannosyl-type N-linked oligosaccharides (R) in glycoproteins. The step 1 enzyme is totally or partially deficient in I-cell disease and pseudo-Hurler polydystrophy, respectively [261–263]. The step 2 phosphodiesterglycosidase is unaffected. The normal two-step process results in the formation of a 6-mannose phospho-monoester, the central component of the recognition marker leading to high-affinity binding to mannose 6-phosphate receptors in the Golgi complex and thus ensuring proper routing to the lysosomal compartment.

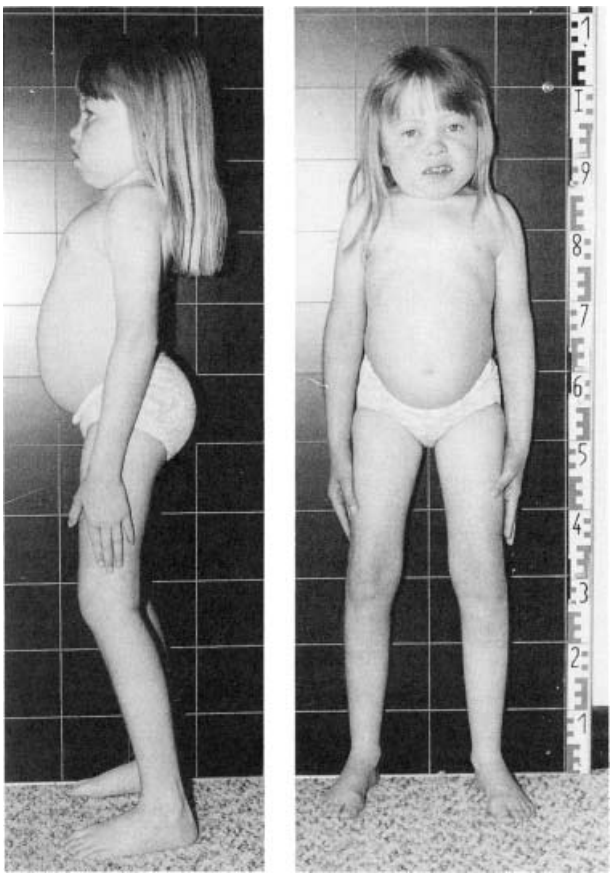
each of about 154 amino acids in the extracellular domain, while the other is much smaller (46 kDa) and contains only a single domain, homologous to those in the larger receptor. The smaller receptor occurs mainly in homodimer form. The larger receptor binds mannose 6-phosphate markers irrespective of the presence of divalent cations. It is, therefore, called the cation-independent mannose 6-phosphate receptor (CI-MPR). The smaller receptor requires cations for the optimal binding of ligand (Man-6-P) and is referred to as the cation-dependent mannose 6-phosphate receptor (CD-MPR). The molecular biology of the mannose 6-phosphate receptors has been reviewed elsewhere, and their primary role in targeting newly synthesized lysosomal enzymes to the lysosomal compartment in fibroblasts and in their own intracellular recycling demonstrated [270,271]. Their three-dimensional structure has also been elucidated [272]. Observations in mutant mice have proved that either type of receptor can only partially compensate for the functional absence of the other, and that the absence of both is barely compatible with survival [273–276]. The ligand binding of either type of receptor has some degree of specificity, the CI-MPR being the most effective [277].

The delineation of I-cell disease and the discovery of the primary and secondary enzyme deficiencies have been of major importance in allowing the elucidation of the mannose 6-phosphate/mannose 6-phosphate receptor-dependent intracellular routing of lysosomal hydrolases, at least in fibroblasts, and probably in other connective tissue cells [248–252]. Although the primary deficiency of GlcNAc-phosphotransferase is found in all tissues examined [263], the secondary enzymatic [249,250] and pathologic [253,254] features are only partially or not at all demonstrable in lymphoid cells and tissues and in parenchymal organs such as liver and spleen. Therefore, alternative pathways for intracellular trafficking of lysosomal enzymes to lysosomes must exist besides the mannose 6-phosphate/mannose 6-phosphate receptor–dependent pathway [263,278]. Such alternative mechanisms of inter- and intracellular transport of lysosomal enzymes have been noted in Part I of this chapter.

Pseudo-Hurler Polydystrophy (MIM 252600)

This disorder was delineated and named pseudo-Hurler polydystrophy by Maroteaux [279]. It has also been called mucolipidosis III [104]. Complaints of physical slowness, joint stiffness, and slow mental development around the age of 3 years mark the clinical onset. The patient's facies is coarse, and the range of movement in shoulders and hips already reduced. The growth rate is slower than normal and the head circumference remains proportional to stature, which is well below normal in adults (Fig. 13). The corneae are clear by inspection but show opacities on slit-lamp examination. The liver and spleen are not usually enlarged [280,281]. Mental deficiency in pseudo-Hurler polydystrophy patients is mild and nonprogressive. The course of the disease is slow and characterized by increasing joint stiffness and by joint pain, in particular of the hips. Pseudo-Hurler polydystrophy patients of advanced age have been reported [280], but comprehensive data on life expectancy are not yet available.

There is generalized osteoporosis and bone age is considerably delayed. Dysostosis multiplex, though variable between patients, is generally mild, except for the severe and progressive lesions in the hips [2,280], where secondary arthrotic changes represent a considerable problem. Stiffness in the shoulders is of soft tissue origin. Urinary excretion of glycosaminoglycans is normal, while that of sialyl-oligosaccharides is excessive, complex, and indistinguishable from that found in I-cell disease urine [113,114]. This finding is nevertheless of orienting value for diagnosis.



A B

Figure 13. A,B: Patient with pseudo-Hurler polydystrophy at 10 years of age: prominent maxillar part of facies. Severe hip dysplasia with hips fixed in partial flexion; compensatory exaggerated sagittal curvature of back, small stature (scale in dm); normal slender hands; intelligence low normal range.

The I-cell phenomenon is apparent in pseudo-Hurler polydystrophy fibroblasts, as in I-cell disease cultures [282]. The activity of many acid hydrolases is considerably reduced in the cells and elevated in culture medium [283]. The residual activities of β-D-galactosidase [284] and sialidase [285] are higher in "I-cells" from pseudo-Hurler polydystrophy patients than in those from I-cell disease donors. Contrary to findings in I-cell disease, in oligosaccharides isolated from pseudo-Hurler polydystrophy acid hydrolases, some degree of phosphorylation has occurred [258]. These findings correlate with the milder clinical phenotype in pseudo-Hurler polydystrophy. Of direct diagnostic importance is the fact that in the serum of patients there is also greatly increased activity of many acid hydrolases.

The dividing line between pseudo-Hurler polydystrophy patients and "I-cell disease" patients with mild mental handicap and protracted course may not be clear, but differences in growth rate, final height, and degree of dysostosis multiplex are the better criteria for distinguishing

between the two disorders. However, several patients have been reported who demonstrate the continuously varying distribution of clinical severity of which I-cell disease and pseudo-Hurler polydystrophy are but the points of reference [286].

The I-cell phenomenon in fibroblasts derived from either type of patient has pointed from the start toward a common pathogenesis for both disorders, as was subsequently underscored by the finding of the same enzyme defect [262].

Molecular Biology of GlcNAc-Phosphotransferase and Its Genes

The phenotypic heterogeneity of patients with GlcNAc-phosphotransferase deficiency initially led to the delineation of I-cell disease on the one hand and pseudo-Hurler polydystrophy on the other [245,279]. More recently, the clinical heterogeneity has rather been interpreted as a continuum of severity with regard to age of clinical onset, natural course and final outcome. The degree of secondary deficiency of hydrolases has also been correlated with the clinical variability encountered [284–287]. In addition, the primary GlcNAc-phosphotransferase deficiency is more complete in I-cell disease than in pseudo-Hurler polydystrophy [258,287,288]. However, even in pseudo-Hurler polydystrophy patients there is heterogeneity of GlcNAc-phosphotransferase activity. Because of the consistency of the abnormal phenotype in multiply affected sibships, the phenotypic variability must reflect genetic heterogeneity of either an allelic or a nonallelic nature, indicating that I-cell disease and pseudo-Hurler polydystrophy may be disorders resulting from mutations in different genes.

That at least pseudo-Hurler polydystrophy must be due to more than one nonallelic gene mutation has been implied for some time by the results of *in vitro* complementation tests [287,289,290]. Complementation in heterokaryons following pairwise *in vitro* fusion of two different mutant fibroblast strains results in the restoration of intracellular lysosomal hydrolase activity and the correction of qualitatively abnormal physicochemical properties. Complementation has been observed to result also in the restoration of GlcNAc-phosphotransferase activity [291].

Three complementation groups (A, B, and C) were recognized in pseudo-Hurler polydystrophy [287,292]. Complementation group A cell strains had considerably reduced GlcNAc-phosphotransferase activity irrespective of whether the phosphate acceptor substrate used was a purified lysosomal hydrolase or the artificial α-methylmannoside. Only one, rather atypical patient represented complementation group B. Complementation group C comprised variant pseudo-Hurler polydystrophy donors with deficient GlcNAc-phosphotransferase activity when assayed with lysosomal enzymes or glycoproteins as phosphate acceptors, but normal activity of the enzyme when the α-methylmannoside acceptor was used [287–289]. Thus, GlcNAc-phosphotransferase must be an heteromeric enzyme with a catalytically active subunit and, in addition, a subunit for substrate recognition. In pseudo-Hurler polydystrophy type C patients, the causal mutation yields an enzyme subunit defective in recognizing its natural substrates but apparently does not affect the catalytic subunit. On the other hand, the enzyme in I-cells of pseudo-Hurler polydystrophy group A is catalytically defective, even when it properly recognizes its natural acceptor substrates. The same applies to the majority of I-cell disease patient cell strains.

More recently, bovine GlcNAc-phosphotransferase has been isolated and purified from lactating mammary glands and its subunit structure determined [293,294]. The enzyme is a 540 kDa complex of disulfide-linked homodimers, each of which is composed of a 166 kDa α subunit and a 51 kDa γ subunit. With these dimers, two identical, 56 kDa β subunits are noncovalently associated. The enzyme complex may be symbolized as $\alpha 2\beta 2\gamma 2$.

This important stride led to the isolation of the human GlcNAc-phosphotransferase γ subunit cDNA and its localization to chromosome 16p, in the study of a large Druze pedigree in which the variant or type C pseudo-Hurler polydystrophy was segregating [295]. The entire sequence of the 1219 bp cDNA has been elucidated. It contains an open reading frame of 915 bp, predicting a mature protein product of 281 amino acids. Transient expression of the human GlcNAc-phosphotransferase γ polypeptide (35 kDa) has been achieved in transfected COS cells, in which it also forms disulfide-linked homodimers as has been found for the γ subunit in the bovine enzyme complex. Following RT-PCR amplification and sequencing of the cDNA from type C pseudo-Hurler polydystrophy patients in two families, the insertion of a single cytosine at codon 167 has been detected. It results in frameshifted translation and premature termination of the polypeptide [295].

The purification and molecular characterization of the bovine GlcNAc-phosphotransferase has also been of major importance for the isolation of human cDNA clones encoding the α and β subunits of this oligomeric enzyme. These clones were shown to be derived from a single cDNA clone, itself the reverse transcribed product of a 6.2 kb α/β transcript. Following translation into an α/β precursor polypeptide, the α and β enzyme subunits are generated by proteolytic cleavage at a lysine-asparagine bond. The N-terminal α subunit, the larger of the two, consists of 928 amino acids, while the β subunit, C-terminal in the α/β precursor, contains 328 amino acids [296]. The α and β subunits are weakly homologous to yeast chitin synthetase 2, strongly suggesting that they represent the catalytic portion of the enzyme. In fibroblasts from four I-cell disease patients no α/β-mRNA was found by Northern blot analysis, while in I-cell strains derived from two pseudo-Hurler polydystrophy patients (complementation group A) this α/β-transcript was present in significantly reduced amounts. In both types of fibroblast strain, the 1.3 kb γ subunit transcript was found to be normally present [296]. The human α/β-subunit gene has been shown to reside on chromosome 12q. It spans 76 kb and is genomically organized in 22 exons. Polymerase chain reaction amplification has demonstrated that all individual exons are present in all patients so far studied. Small insertions and deletions causing frameshifts and substitutions that disrupt splice junctions have been found. These either prevent transcription or alter the stability of the message [296] as the causal factors in I-cell disease and pseudo-Hurler polydystrophy patients.

Galactosialidosis (MIM 256540)

The designation "galactosialidosis" [297] has been applied to three more or less clinically delineated "disorders" or types, with resemblance, both clinical and metabolic, to generalized G_{M1}-gangliosidosis and sialidosis, but genetically distinct from each. With similar oligosaccharidiuria and β-D-galactosidase deficiency established in patients, early reports already either stressed their unique combination of clinical features [298] or made attempts at classifying cases with a long follow-up into different groups [299]. Objective classification became feasible following the discovery by

Wenger et al. that in this type of patient glycoprotein sialidase was deficient in addition to β-D-galactosidase [300].

In addition to the most frequent, juvenile (adult) type of galactosialidosis, so far mostly reported from Japan [297,301–303], both late infantile [298,304] and neonatal (hydropic) [305–309] forms have been discerned. Galactosialidosis is a disease with severe manifestations in nearly all patients, who, in fact, represent a continuum of phenotypes heterogeneous with respect to age of onset (pre- or perinatal until late adolescence), rate of progression, severity, and prognosis. For clinical purposes and for genotype-phenotype correlation studies, the alternative division into a type I form with generalized and severe manifestations and perinatal or early infantile onset and a type II form with later (late infantile, juvenile, adult) onset and protracted course, has at least an equally heuristic value [302,303]. The latter type then needs further subdivision into subtypes IIA (severe expression) and IIB (mild expression). Galactosialidosis is a rare metabolic disorder with a frequency distribution of phenotypes, at least in Japan, skewed toward the later onset and chronic course types.

Juvenile (Adult) Type Galactosialidosis

The clinical onset of this form occurs from late childhood until early adult life [299,301–303]. Intrafamilial variation between patients is smaller than interfamilial differences. The composite clinical picture of the fully developed syndrome consists of the following signs and symptoms: (1) slowly progressive cerebellar ataxia, tremor, and action myoclonus, pyramidal but no extrapyramidal signs, and no nystagmus; (2) decreasing visual acuity and cherry-red spot in the macula; (3) mild dysostosis multiplex, mild coarsening of facial features, and moderately short stature; and (4) moderate intellectual impairment, which is unusual in the initial stages of the disease. Any one of the components may be of prime and/or early concern or may instead be lacking. Angiokeratoma is frequently observed in older patients. On slit lamp examination most patients show corneal opacities. Hearing loss is occasionally present. In some instances, mild facial coarsening, neuromotor developmental delay, slow physical growth and physical impairment or discomfort may be found retrospectively before "the age of onset" [310]. Cardiac abnormalities have been observed in juvenile galactosialidosis [311]. This type of galactosialidosis corresponds to type II in the clinical division proposed by the Japanese authors [302].

Late Infantile Galactosialidosis

Only a few patients with clinical onset of galactosialidosis during or before the second year of life have been reported [297,298,303,305]. In them, coarse facial features, hepatosplenomegaly, dysostosis multiplex, and developmental delay are noted initially, without ophthalmologic or neurologic signs which may develop further along in the natural course, which usually is quite prolonged. Too few detailed and long-term follow-up reports are available. One patient followed over more than 18 years has developed fibrotic thickening of the cardiac valves and required aortic valve replacement [304]. In this group of patients, the rate of clinical progression of galactosialidosis is remarkably heterogeneous [312]. The rather consistently protracted course makes the older patients indistinguishable from those with the juvenile (adult) onset type of galactosialidosis.

Neonatal (Hydropic) or Early Infantile Galactosialidosis

Neonates presenting with a condition clinically almost indistinguishable from G_{M1}-gangliosidosis type 1 but with extensive edema and ascites have been diagnosed as having a combined deficiency of glycoprotein sialidase and β-galactosidase. They have a coarse facies, a depressed nasal bridge, cloudy corneae, a macular cherry-red spot, and conjunctival telangiectasia with similar lesions over the lower abdomen. Some succumb in early infancy [303,305–307,309]. Some show severe nonimmune hydrops *in utero* or at birth with early fatal outcome [306,307]. In the longer surviving patients, respiratory infection recurred frequently and both congestive heart failure and renal failure were the compounding causes of death, in addition to anemia and thrombocytopenia. Radiographically, the skeletal changes were identical to those in G_{M1}-gangliosidosis and I-cell disease [306]. The classification of galactosialidosis patients according to the Suzuki group of authors [302,303], places the neonatal (hydropic) or early infantile cases into their galactosialidosis type I.

Pathological Features and Diagnosis

Patients with any type of galactosialidosis have vacuolated peripheral lymphocytes, aligning them with all other storage disorders due to a lysosomal hydrolase deficiency [309]. Detailed reports on post-mortem examinations of galactosialidosis patients remain few in number [313,314]. Numerous cytoplasmic membrane-bound vacuoles are readily apparent in hepatocytes, Kupffer cells, Schwann cells associated with peripheral nerves, and fibroblasts. Under the electron microscope, concentric or wavy lamellar as well as fine granular material is found within the swollen lysosomal structures in neurons throughout the central nervous system and in connective tissue cells in parenchymal organs. Similar abnormalities are detectable in cultured fibroblasts. In the central nervous system, the storage material encountered within neurons is reminiscent of what is found in G_{M1}-gangliosidosis. However, G_{M1} and other ganglioside species are less abundant. The several oligosaccharides in glycoproteins or glycolipids accumulating are of the N-acetyllactosamine type, with the mannose β 1–4 GlcNAc sequence preserved at their reducing termini (see Fig. 7). The structures of the oligosaccharides excreted are very variable, but quite similar to those of the compounds excreted excessively in the urine of patients with sialidosis [315].

As indicated in the introduction, the demonstration of the combined deficiency of β-D-galactosidase and glycoprotein sialidase in fresh leukocytes and cultured fibroblasts provides objective confirmation of the clinical diagnosis of galactosialidosis. A deficiency of the former enzyme is not found in all tissues, nor in plasma, and, where detectable, is less profound than in patients with G_{M1}-gangliosidosis. In obligate heterozygotes, glycoprotein sialidase activity is intermediate but β-D-galactosidase is normal [316]. The significance of cathepsin A specific activity for diagnostic purposes is discussed below.

The Metabolic and Molecular Defect

The enzyme deficiencies encountered in each type of galactosialidosis do not mutually complement one another *in vitro* and thus must be caused by allelic mutations. Immunoprecipitation with anti-β-galactosidase antibody has revealed that the 85 kDa β-galactosidase precursor polypeptide is synthesized as in normal fibroblasts, but is only partially processed into its 66 kDa monomeric form.

The defective product does not aggregate into high molecular weight complexes and has a much faster than normal turnover following its synthesis [317–319]. In the pulse-chase experiments performed in these initial studies, attention was paid to three polypeptides of 54 kDa, 32 kDa, and 20 kDa, respectively, the first apparently being the precursor of the latter two species, because all three were absent in fibroblasts from patients with the early infantile type of galactosialidosis.

The primary defect in galactosialidosis is the absence or profound deficiency of the lysosomal 32 kDa β-galactosidase protective protein and its 54 kDa precursor. It is normally required for the aggregation of the 66 kDa β-galactosidase monomers and their protection against intralysosomal degradation. This protein is an essential factor also for the activation of glycoprotein sialidase [317–321]. The molecular characterization of the 32 kDa protective protein was greatly aided by the cloning of its corresponding cDNA [322], from which its amino acid sequence was deduced. A hydrophobic signal peptide of 28 amino acids is proteolytically cleaved from the N-terminus of the 298 amino acid sequence of the 32 kDa component that represents the larger of two functional domains within the 54 kDa precursor, or proform, of the protective protein. The 32 kDa polypeptide is separated by endoproteinase cleavage from the N-terminus representing the 20 kDa polypeptide. Subsequently, the larger polypeptide is trimmed starting from its COOH-terminus. The two proteins are held together as a heterodimer by the formation of disulfide bridges. Glycosylation of the two asparagine residues is completed before the polypeptide complex dimerizes (95 kDa) and acquires its multifunctional role [323]. Only the glycosylation site in the 32 kDa polypeptide becomes phosphorylated and thus acquires the mannose 6-phosphate recognition marker needed for correct routing toward the prelysosomal vesicles [324]. Part of this maturation process needed for activation takes place in the lysosomal compartment itself. This sequence of events has been elucidated by studying the transient expression of human protective protein cDNA in COS-1 cells.

Extensive molecular homology is found between protective protein and two yeast carboxypeptidases, including, in particular, the conserved amino acids which, although belonging to different subunits, constitute the active enzymatic site in the human protein. The protective protein was found to have cathepsin A activity [322]. In fibroblasts derived from infantile galactosialidosis patients less than 1% of normal cathepsin A activity is detected [316], whereas there is 2–5% residual activity in cells from patients with the late infantile or juvenile/adult types. Heterozygote values for cathepsin A activity were, on average, half of normal. This carboxypeptidase shows amply sufficient specific activity in chorionic villi and amniocytes to allow it to be used in prenatal diagnosis. A sign of warning against the possibility of a mutation interfering substantially with the protective function of the protective protein without affecting its cathepsin A activity must, however, be posted. Thus, demonstration of the coexisting secondary enzyme deficiencies may remain the more reliable assay method for diagnostic purposes [321].

Molecular Pathology: Genetic Heterogeneity

Somatic segregation of human chromosomes from man-mouse hybrid cell lines allowed localization of the gene encoding the protective protein (PPGB) to chromosome 20 [325] and its more precise assignment to 20q13.1 [326]. Immunoprecipitation with specific antibody has shown that in fibroblasts from patients with the early infantile type of galactosialidosis, there is either a marked reduction or absence of the 52 kDa precursor species and a total lack of protective protein itself [318,327]. There is even an absence of the corresponding mRNA [322]. It appears that in at least some patients the precursor protein is wholly or partially retained in the endoplasmic reticulum. Recently elucidated simple point mutations may have this effect for various reasons, such as the prevention of phosphorylation of the protective protein and subsequent disturbed enzyme routing and early 32 kDa breakdown, as caused by the Val104Met, Leu208Pro, and Gly411Ser substitutions [324]. In the juvenile (adult) onset type of galactosialidosis, an increased amount of 52 kDa precursor was actually found, but there was no active 32 kDa protein, suggesting a block in the maturation process. Frequent among the mutations in Japan is the deletion of exon 7 in the *PPGB* gene, symbolized as IVS7DS, A-G +3, Ex7del, which is due to a splice site alteration 3 bp downstream from exon 7. Homozygotes for this mutation have a mild, adult type of galactosialidosis, probably due to some alternative production of mRNA and some residual mature protective protein. This mutation results in a much earlier clinical onset if in compound heterozygous constellation with the missense Trp65Arg mutation. In fibroblasts from patients with the late infantile type of galactosialidosis with early onset and chronic course, accumulation of the immature 52 kDa precursor was often also noticed. It was, however, accompanied by a much reduced but measurable amount of active 32 kDa glycoprotein. Treatment of the fibroblast strain with the protease inhibitor, leupeptin, led to an increase of the active protective protein/cathepsin A species [327]. Such a form of galactosialidosis is found in the case of a Phe412Val substitution. The patients reported by Chitayat et al. [310] and Strisciuglio et al. [304] are of this type. In Japan, the homozygous state for the missense mutation Tyr395Cys is also common among severely affected patients, although the mutation results in a somewhat milder clinical course in the compound heterozygotes with Tyr395Cys/Ex7del [303]. Clinical severity and early age of onset appear to correlate reasonably well with the amount of active protective protein available. Thus, clinical heterogeneity relates partially to heterogeneity in molecular pathology, but both appear to be largely surpassed by genotypic heterogeneity.

PYKNODYSOSTOSIS; CATHEPSIN K DEFICIENCY (MIM 265800)

Named and first described by Maroteaux and Lamy in 1962 [328], pyknodysostosis is an osteochondrodystrophy clinically apparent from early childhood, characterized by short-limb type shortness of stature, a disproportionately large calvaria with frontal and occipital bossing and late or never closing fontanelles, a small nose, and receding chin. In addition, patients have various dental anomalies, short and broad hands, and increased bone fragility.

The radiographic abnormalities are at first mild and hardly typical. They become more complete in later childhood. The cranial sutures and large fontanelle remain wide open. Wormian bones are found. The mandibular angle is obtuse, the facial bones relatively small. Bone density increases gradually. There is some metaphyseal undermodeling of tubular bones, and often coxa valga deformity and partial hypoplasia with irregular osteolytic lesions in the terminal phalanges of the fingers and toes. The acromial ends of

the clavicles are usually hypoplastic. Often signs of old or more recent bone fractures are found in radiographic surveys [2,329].

Pyknodysostosis is inherited as an autosomal recessive trait. That the condition is rare, is reflected by the high degree of parental consanguinity encountered in reported series [329–331].

Recent progress in identifying and characterizing the pyknodysostosis gene is the result of positional cloning based on homozygosity mapping and linkage analysis which showed it to be located on 1q21 [332–334]. By the candidate gene approach, some genes known to be localized in the same chromosomal region, such as the interleukin-6 receptor gene and the cathepsin S gene, although known to be highly expressed in osteoclasts, were effectively ruled out as candidates for the pyknodysostosis gene [332–334]. On the other hand, the cathepsin K gene (*CTSK*), which maps to the same region and is also highly expressed in osteoclasts, was shown to be defective in pyknodysostosis and to carry an A to G transition at nucleotide 1095, leading to a change of the normal termination codon 330 into one for a tryptophan residue (Stop330Trp) and therefore to the elongation of the C-terminus of cathepsin K by 19 amino acids. This mutation cosegregated with pyknodysostosis in all affected individuals in the pedigree reported by Edelson et al. [329,334]. In addition, nonsense and missense mutations were also discovered in other patients. The human cDNA for cathepsin K has been cloned and sequenced [334,335] and shown to encode a 329 amino acid–containing protein. This work was facilitated by the great homology of human cathepsin K with its rabbit homolog. Its amino acid sequence is about half identical also to human cathepsin S, the gene for which is located in the same vicinity and possibly the accompanying product of a tandem duplication of an ancestral cathepsin. Cathepsin K is a cysteine protease of major importance in bone resorption and remodeling mainly mediated by osteoclasts. It has the highest type I collagenolytic activity among the cysteine proteinases. Osteoclasts viewed by light microscopy appear normal in pyknodysostosis, but their pericellular zone of demineralization is enlarged. Under the electron microscope, these osteoclasts contain large, abnormal cytoplasmic vacuoles containing fibrils, probably of collagen I, the main fibrillar component of the intracellular matrix of bone. Pyknodysostosis has thus been shown to be a lysosomal disorder for which defective tissue-specific expression and pericellular secretion of cathepsin K is the pathogenetic mechanism [332,334]

GAUCHER DISEASE TYPE I; ACID β-GLUCOSIDASE DEFICIENCY (MIM 230800)

Gaucher disease, a cerebroside (glucosylceramide) lipidosis and, as such, a member of the group of sphingolipidoses, is the most prevalent lysosomal storage disorder and may have its clinical onset at any age. Characterization of the compound stored excessively has contributed significantly to the gradual elucidation of the specific enzyme deficiency of glucosylceramide β-glucosidase (EC 3.2.1.45) by several authors [336,337]. Phenotypic heterogeneity is notorious in Gaucher disease, leading to the delineation and conventional classification of three clinical types. The chronic or adult form of Gaucher disease is most frequent (70–80% of all patients). It has been termed Gaucher disease type I, and is the only form in which connective tissue is adversely involved. There is no involvement, however, of the central nervous system in this most frequent type. At least two-thirds of patients are of Ashkenazi Jewish extraction. In some groups the heterozygote frequency is estimated to be about 1 in 16 [338].

Symptoms may arise in early childhood and are usually present by adulthood (Fig. 14). They include splenomegaly, with evidence of hypersplenism and pancytopenia. Splenectomy is often indicated, but is often followed by intensifying bone disease. There is sometimes hepatomegaly. The skeleton is often involved, with bony lesions consisting of either mere expansion of the space normally occupied by the bone marrow (Erlenmeyer flask deformity of the femur), or osteopenia, cortical thinning and osteonecrotic lesions with pathological fractures (Fig. 14c). The latter are often refractory to orthopedic surgical or conserving measures. Painful bone crises lasting several days are common in this stage of Gaucher disease type I. Skeletal surveys and ultrasonographic studies may also detect intrathoracic and/or abdominal lymphadenopathy. The lungs may become affected with, as consequences, progressive dyspnea and sometimes cyanosis, failing gas exchange, and clubbing of fingers and toes. Unexplained febrile episodes are observed. In a few patients, renal failure may become a troublesome component. In addition, an abnormal increase in γ-globulin content is regularly found in Gaucher disease serum. Cardiac involvement, mostly of the calcified constrictive pericarditis type, has been reported in Gaucher disease type I patients. This phenomenon may be associated with a specific missense mutation [339]. Although the central nervous system is not primarily affected in Gaucher disease type I, complications may arise as a consequence of systemic vascular or bone disease [340].

The pathologic hallmark in Gaucher disease is the Gaucher cell, a disease-altered reticuloendothelial (monocyte/macrophage lineage) cell which, under the light microscope, contains rather typical fibrillary cytoplasmic material. This type of cell is observed mainly in the bone marrow, but also in lymph nodes and visceral organs.

As many thousands of patients have been diagnosed and studied worldwide, the phenotypic heterogeneity of Gaucher disease, even within each of the "classic types," continues to expand. Type II Gaucher disease represents but 15% of patients. It is a fulminant disorder of the central nervous system, starting in infancy and ending fatally, usually before 2 years of age. The reader is referred to authoritative reviews elsewhere [338,340,341]. Type III Gaucher disease, encountered mainly but not exclusively in Sweden, has its onset in infancy. Although neuromotor and mental development may continue normally until later childhood, intellectual and neurologic deterioration becomes manifest and progresses inexorably until the fatal outcome before adolescence (see the reviews cited above). In type II and type III Gaucher disease, Gaucher cells are readily demonstrable. They are also found in the perivascular regions. However, in these forms of the disorder, neuronal glucocerebroside storage and necrosis are the prevailing pathologic features.

Glucosylceramide accumulates in Gaucher cells in all types of Gaucher disease, but not in the central nervous system in the type I form under discussion here. The residual activity of glucosylceramide β-glucosidase, also called glucocerebrosidase (EC 3.2.1.45), is higher in type I Gaucher disease — levels of 10–20% of normal may be found–than in the infantile neuronopathic type II, in which it is often less than 1% of normal. The enzyme is usually assayed as acid β-glucosidase. Cross-reacting material is found in the spleens of all three types of patient. However, acid β-glucosidase mRNA

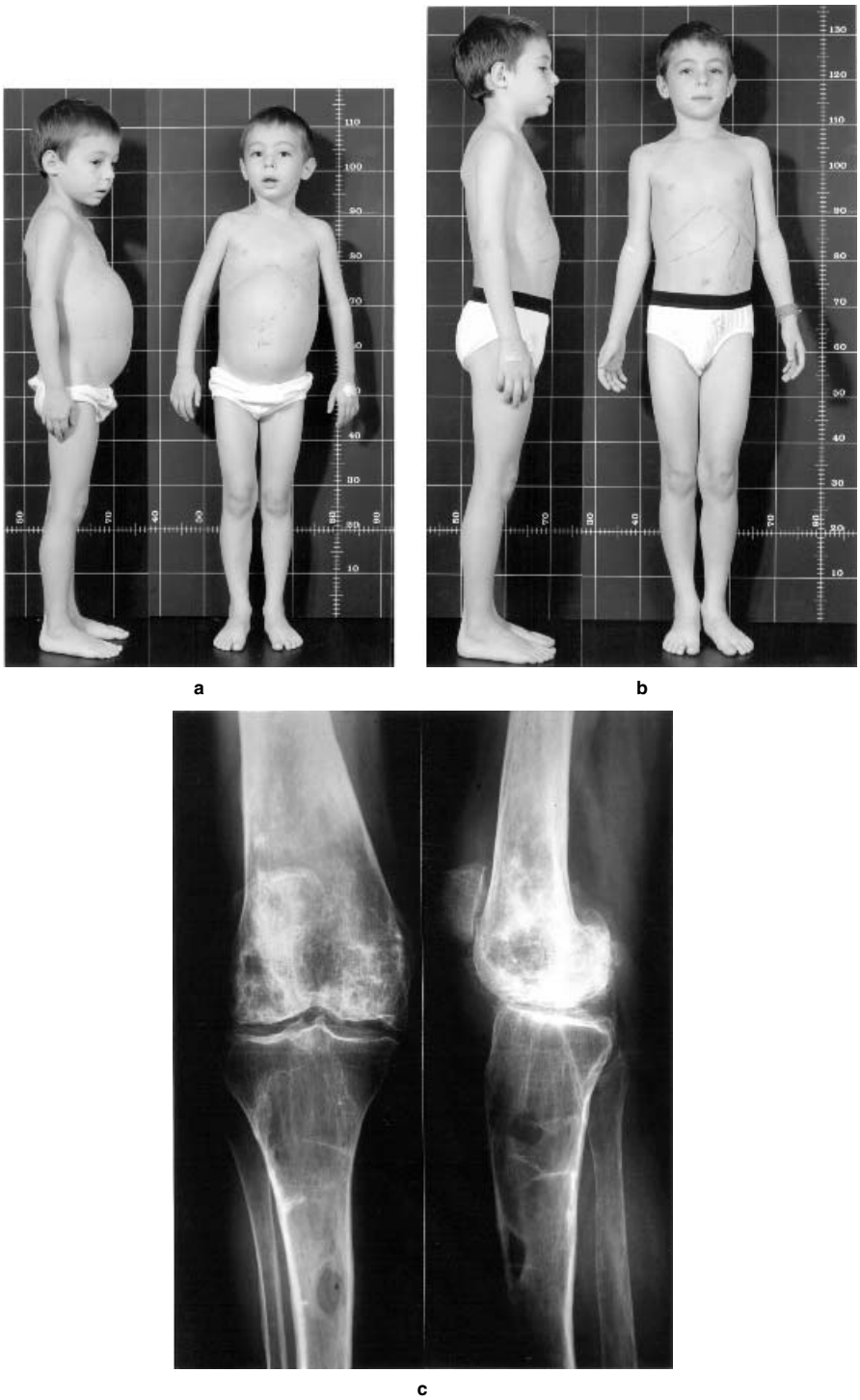


Figure 14. Gaucher disease. (**a**) 6.5-year-old boy (A.S.) with protuberant abdomen and hepatosplenomegaly, short stature (3rd centile), anemia and thrombocytopenia before enzyme replacement therapy and (**b**) 2.2 years later after enzyme replacement therapy: note marked regression of hepatosplenomegaly and catch-up growth (50th centile). The boy is presently 15 years old, his height and weight are on the 50th centiles, his liver and spleen are only mildly enlarged, and his hemoglobin and thrombocyte counts are normal (courtesy of Drs. R. Gitzelmann and A. Superti-Furga, Zürich). (**c**) Radiographs of the right knee of a 53-year-old woman (R.R.) show diffuse osteopenia and osteolytic defects (osteonecrosis) of the distal part of the femur and the proximal parts of the tibia and fibula as well as narrowing of the knee and patellofemoral (right panel) joints. Note the typical Erlenmeyer-like shape of the distal femur due to undertubulation (left panel). Her spleen had been removed at age 4.5 years and at age 53 years the right hip joint had to be replaced (courtesy of Dr. B. Steinmann, Zürich).

is present in normal amounts in fibroblast extracts [342]. The mutant enzyme is most often altered at single amino acid residues, interfering only with catalytic efficiency. *In vitro* complementation between fibroblasts from neuronopathic and non-neuronopathic Gaucher disease patients has not been observed [343], thus providing evidence that all forms of Gaucher disease are allelic. Studying the characteristics of residual acid β-glucosidase activity had previously led to the conclusion of biochemical heterogeneity even among type I patients [344,345].

The gene encoding acid β-glucosidase has been located at 1q21 [338,346,347]. It contains 11 exons and spans 8 kb. A pseudogene (*psGBA*) transcribable into mRNA but not producing any active enzyme, is located 16 kb downstream [348]. Cloning and characterization of the *GBA* gene has allowed the identification of an increasing number of mainly point mutations and gene alterations, showing once again that genotypic heterogeneity still largely surpasses the heterogeneity of Gaucher disease phenotypes. In Ashkenazi Jewish patients with type I Gaucher disease, more than 95% carry one of four so-called frequent or common mutations. These are Asn330Ser, 84insG, Leu444Pro, and IVS 2-G[+1]A, mostly in the compound heterozygous state. Among non-Jewish type I patients, allelic heterogeneity is considerably greater, many mutations being found only in a single patient or family [349]. Fluorescence-assisted methodology has allowed the discovery of further novel mutations in a large French sample of Gaucher disease type I patients [350]. Many patients are compound heterozygotes for two different *GBA* mutations, often carrying one common and one "personal" allele. The Asn370Ser mutation predicts a mild phenotype, while the Leu444Pro allele predicts the more severe type III phenotype. However, even identical compound heterozygotes and sib patients may turn out to have strikingly different degrees of severity of disease. Such clinical differences are difficult to explain, and must be due to environmental factors. In non-sib genotypically identical patients a multifactorial explanation may be offered. Such observations must induce caution with regard to the predictive value of the mutations identified, not only in genetic counseling but also in terms of forecasting success in substitution therapy. Over one third of all mutations known today in the *GBA* gene have been listed as allelic variants and their clinical effects summarized. The mutation Asp409His, MIM allelic variant 230800.0006, is apparently associated with hemorrhagic pericarditis [20].

ANIMAL MODELS

The successful delineation of human lysosomal storage disorders by objective enzyme assays more than two decades ago, created a scientific climate favorable to the discovery of the first models of these disorders, such as α-mannosidosis in Angus cattle [351], and arylsulfatase B and iduronidase deficiency in cats [352,353]. Although, over subsequent years, the number of natural animal models of lysosomal diseases has increased considerably, their rarity, in both nonprimates and humans, is an indication of higher order levels of genomic homology among the species involved. This homology not only concerns structural but also critical regulatory genes. The animal models in the following paragraphs are highly valuable because they contribute to understanding the correlation between the one-dimensionally complex genotype and the resulting multidimensionally complex phenotypes of the lysosomal disorders. Because of the extensive homology of contiguous linkage groups, the localization of animal genes encoding lysosomal enzymes has facilitated considerably the precise mapping of the homologous human genes. One well-known example is the conserved close synteny of the Huntington gene and the *IDUA* gene in mouse and man [354]. In addition to disease models arising in nature, animal models have also been created by targeted gene disruption techniques, such as the iduronidase knockout mouse [355]. As well as their value in the assessment of the main and side effects of any type of treatment, animal models are particularly useful in studying clinical aspects of the natural course of a disease, its pathogenesis and chemo- and anatomopathology, e.g., murine Hurler disease [356].

MPS VI and MPS VII

MPS VI and MPS VII also stand out with respect to the number of animal models detected and studied. The former is known in both cats and mice. The cat model has been used for both the evaluation of strategies of enzyme replacement therapy and genotype-phenotype correlation studies [357]. A mouse model of MPS VI has also been produced by targeted gene disruption [358]. A mutant mouse with β-glucuronidase deficiency (MPS VII) has also been described. Its encoding gene has been mapped and the mutation characterized as a single base-pair deletion [359]. This mutant model has been used in the demonstration of long-term, low-level *in vitro* expression of β-glucuronidase activity in mutant cells following retroviral vector-mediated *gusb* gene transfer [360]. The treatment of newborn MPS VII mice with recombinant mouse β-glucuronidase has been shown to be more effective the earlier it is administered. Early enzyme replacement by itself or followed by syngeneic bone marrow transplantation has long-term therapeutic effects in murine MPS VII, and such early treatment is significantly more effective than therapy initiated at an adult age [361]. Several other strategies of β-glucuronidase substitution in mutant mice have been more or less successfully tried and adequately documented [361,362]. The more recent availability of recombinant products, which may be administered intravenously to the newborn, has renewed interest in enzyme replacement therapy and met with some success [362]. The mutation in the β-glucuronidase-deficient dog has been found by sequencing *GUSB* cDNA to be a single base pair substitution leading to an arginine to histidine substitution [363].

β-D-Galactosidase Deficiency

β-D-galactosidase deficiency has been documented in sheep [364]. It has also been generated in the mouse using embryonic stem cell technology and gene targeting involving homologous recombination between the normal gene and a mutant homologue carried within introduced vector DNA. Although considerable accumulation of G_{M1}-gangliosides occurred in the brain, neurologic abnormalities were not observed before the age of 5 months in the mutant animals, raising a note of caution about ignoring pathogenetic differences between species and possibly overemphasizing the similarities [365].

Mannosidosis

In addition to the observation of α-mannosidosis in cattle [351], the equivalent of this human disease has also been found in cats. These animals treated by bone marrow transplantation showed no disease progression, measurable amounts of α-mannosidase activity in brain tissue, and an absence of storage in neurons [366]. Thus, bone marrow

transplantation can contribute significantly to substituting the missing enzyme in the central nervous system and preventing the effects of the disorder. Both the feline enzyme and the bovine show greater than 80% identity with human α-mannosidase [367,368].

Species differences in the clinical effects of "homologous" gene mutations exist between caprine and human β-mannosidosis. In goats, this disorder severely affects the central nervous system, whereas in man it is milder and more slowly evolving [171]. The disease has also been observed in cattle [369]. Complete cDNA sequencing demonstrated greater than 95% amino acid identity between the caprine and bovine genes. In the former species, the mutation is a single base-pair deletion that results in a frameshift and generation of a premature stop-codon [180].

Other Oligosaccharidoses

Fucosidosis encountered in rat and dog has so far not been an important animal model, but the mutations affect homologous genes with a high degree of structural identity [370]. There is, as yet, no demonstration of an animal model of the human combined deficiency of β-D-galactosidase and glycoprotein sialidase involving the lysosomal protective protein. Observations in sheep suggest a primary mutation of the acid β-galactosidase gene. The first example of "I-cell" disease in a cat has been reported [371]. For more general information on genetic homology between mouse and man the reader may consult the review by Searle et al. [372].

PREVENTION AND TREATMENT

Genetic Counseling and Prenatal Diagnosis

With causal therapy not yet available or unequivocally effective in most of the lysosomal disorders of connective tissue, in general, their conceptual and strategic management remains the goal of preventive medicine. Paradoxically, prevention finds its retrospective foundation in early diagnosis in the proband and its practical strategies in genetic counseling, prenatal diagnosis or, perhaps in the future, preimplantation diagnosis. Fetal monitoring either by assay of the relevant lysosomal hydrolases or confirmation or exclusion of a gene mutation in chorionic villi or cultured amniotic fluid cells is available. Elective termination of pregnancy remains an option for parents. However, prospective preventive measures like mass population screening continue to be unrealistic, although molecular genotyping in probable heterozygotes is becoming increasingly effective as responsible gene mutations become known in more affected individuals. Such tests are much more reliable, even in autosomal recessive disorders, than laborious attempts to demonstrate intermediate lysosomal enzyme activity, because of the overlap usually found between normal individuals and heterozygous carriers.

Supportive and Symptomatic Management

If cure is, as yet, unrealistic for many lysosomal disorders, providing comfort and care can and must be the physician's primary goal. This includes supportive and symptomatic management. This type of "treatment" has been spelled out in detail for patients with the various mucopolysaccharidoses [48,373], but can, however, be called comprehensive only if guidance to a patients' parents and healthy sibs is included. In addition, it should provide information and training to all concerned with the educational and social needs and basic right to human happiness of children and adults with one or other of the slowly progressive multisystemic disorders.

Enzyme Replacement

Infusion Therapy

Methods of enzyme replacement therapy deserve renewed attention, even if only because of recent success in treating type I Gaucher disease patients by administration of alglucerase, a glucocerebrosidase purified from placenta and modified in its carbohydrate side-chains to enhance its uptake by enzyme-deficient macrophages [374] (Fig. 14b). A recombinant acid β-glucosidase, called imiglucerase, is currently under large-scale production and available for treatment [375]. Unfortunately, the success story does not include the neuronopathic type II Gaucher disease.

Attention must also be given to the carefully controlled enzyme replacement therapy performed in the canine model of MPS I using recombinant, properly processed, purified α-iduronidase, following its overexpression in a recombinant Chinese hamster ovary (CHO) cell line, the results of which were impressive [376,377]. Similar treatment protocols in which human α-iduronidase is administered to patients have commenced [378].

Bone Marrow Transplantation

As the lysosomal disorders are systemic in nature, substitutive transplantation of the most affected organ is not a realistic therapeutic option for the provision of functional enzyme. Bone marrow transplantation and thus stem cell donation from the hematopoietic system of a suitable donor to a young patient has proved to be a clinically effective way of treating patients with Hurler disease and some other lysosomal disorders, provided engraftment is obtained. Results have been multisystemic in nature and clinically impressive. They provide a considerably improved quality of life for patients and their families, but are not curative. Remarkably, the central nervous system may benefit from bone marrow transplantation as iduronidase apparently penetrates the blood-brain barrier because some stem cells populate the brain as microglial secretory elements. Dysostosis multiplex and other connective tissue abnormalities do not consistently improve or worsen. In proposing this treatment to parents, its immunosuppressive and myeloablative components and the limits to its success must be explained, in addition to its benefits [379–381].

Gene Therapy

In principle, the lysosomal disorders are among the more suitable candidates for somatic cell gene therapy. Their cause is monogenic and, in nearly all instances, the responsible genes have been isolated and the corresponding specific mutations fully characterized. The underlying pathogenesis has become better understood as has the processing and intra- and intercellular routing of the hydrolases involved. The roles and functions of the plasma membrane receptors are more fully appreciated, and so are the modifications of oligosaccharides in the glycoprotein hydrolases. The chemical modification of exogenous β-glucosidase in the treatment of type I Gaucher disease patients, mentioned above, is an example. Obligate heterozygotes are clinically normal, their sometimes low hydrolase specific activity notwithstanding, which indicates that only a small fraction of normal enzyme activity may be sufficient to prevent lysosomal disease.

However, curative treatment of most lysosomal disorders of connective tissue is not soon expected to be available. Levels of potential therapeutic intervention are, first, the

gene mutation itself; second, the ensuing enzyme deficiency; third, the macromolecular substrates accumulated. Unlike the more or less effective dietary measures that may be adopted to minimize small central nervous system–intoxicating molecules in the newborn with either phenylketonuria or galactosemia, direct means for reduction of macromolecular storage and accompanying tissue damage are unknown. While it is hoped that gene therapy will become of major importance in the future, it is by no means a component of the clinician's armamentarium at present, the main challenge currently being to achieve efficacy. Gene therapy should not require a more rigorous preclinical evaluation than any other novel medical treatment, except for unprecedented applications or in respect of issues such as new viral vectors, gene therapy in fetuses, or approaches involving germ line alteration [382]. The presently known vectors for gene delivery are at best of an experimental nature, their defects and residual biological properties being mostly assets to safety but flaws to long-term effectiveness. An essential prerequisite is the ability to control expression of a newly introduced gene in animal models, because in addition to adequate expression, examples of overexpression of transduced human corrector genes have been demonstrated in autologous cultured cells. *Ex vivo* expression of the wild-type iduronate 2-sulfatase gene transduced within a retroviral vector has been shown to be significantly greater than normal in lymphoblasts of Hunter disease patients [383]. The natural murine MPS VII model has been used for the *in vivo* functional evaluation of implants of genetically modified cutaneous fibroblasts, bone marrow cells, and myoblasts. β-Glucuronidase was secreted by the "organoid" implants for almost 6 months and demonstrated in several organs, with subsequently declining lysosomal storage [384,385].

Application of practical gene therapy for human lysosomal diseases of connective tissue, however, must still await the clarification of several fundamental problems, such as improving the extent of initial transfection, maintaining stable expression of the transgene, and avoiding rejection by the recipient's immunodefense system [360–362].

RECENT DEVELOPMENTS

Mucopolysaccharidoses

Hurler Disease; MPS 1H

This reference mucopolysaccharidosis must now also be ranked among the group of secondary elastic fiber disorders as it has been demonstrated that the accumulation of at least one of the galactose-containing GAGs, dermatan sulfate, results in the functional inactivation of elastin-binding protein (EBP), and hence prevents the normal assembly of elastic fibers by mutant fibroblasts [386] (see also Morquio Disease Type B, below). The defective elastogenesis helps to explain the progressive cardiac valve hardening and subsequent dysfunction characteristic of Hurler disease. Moreover, it helps to understand the obstructive coronary arteriopathy that occurs in some Hurler disease patients. This has been shown to be similar to the elastin arteriopathy in patients with supravalvular aortic stenosis, an example of a primary disorder of elastin fiber synthesis (see Chapter 10, this volume). This secondarily inflicted tissue damage is partially refractory to iduronidase replacement therapy, irrespective of the route of administration. It is likely that other MPS entities also belong to the category of secondary elastic-fiber disorders.

Hunter Disease; MPS II

The number of different mutations discovered within the *IDS* gene now stands at well over 200. Studies in patients from different geographic and/or ethnic backgrounds confirm that most missense mutations result in intermediate or severe phenotypes. About 75% of MPS II patients have severe clinical expression. Small mutations consistently outnumber large deletions and major rearrangements involving the gene by a margin of 4 to 1 [387–390]. A fusion transcript comprising sequences of both the contiguous gene *W* and the *IDS* gene has been identified [391]. Direct dye primer sequencing of PCR products is of great value in carrier testing, even in families without a surviving patient, as shown by the finding of mixed nucleotides in an obligate heterozygote [392].

Morquio Disease Type A; MPS IV A

The observation of a two generation family with intermediate Morquio disease in the younger generation (compound heterozygosity for Arg94Gly/Arg259Gln) and mild disease in the older generation (homozygosity for the Arg259Gln substitution) of the pedigree [393], indicates that compound heterozygotes may be more severely affected than patients homozygous for Arg259Gln. In a patient with both Morquio disease type A and deficiency of adenine phosphoribosyltransferase (APRT), with the expected concomitant urolithiasis, a 16q24.3 deletion apparently involving both contiguous genes has been found [394]. Such a combined enzyme deficiency may be more common in Morquio disease type A patients than statistically anticipated as APRT deficiency may go unnoticed. A structural model of human GalNAc-6-sulfate sulfatase has been proposed based on the total lack of transient *in vitro* expression in "severe" mutants and the considerable residual activity in cells from patients with mild Morquio disease type A phenotypes [395].

Morquio Disease Type B; MPS IV B

This entity is rather frequently associated with the missense mutation Trp273Leu in the *GLB1* (β-galactosidase) gene in both the homozygous and the compound heterozygous genotype [396]. The significant skeletal deformities and connective tissue abnormalities in patients with this terminal β-galactose oligosaccharide storage disorder occur not only as a result of the impaired breakdown of glycoproteins and complex galactose-containing GAGs by the defective β-galactosidase. In addition, the progressive physical handicap is also related to the defective formation of mature elastic fibers. Under normal circumstances, the early stages of elastogenesis are controlled by the 67 kDa EBP which has a galactolectin domain that can bind galactose-bearing moieties (references in [397]). EBP, a cycling molecular chaperone, protects the highly hydrophobic tropoelastin molecules and facilitates their orderly assembly upon the microfibrillar scaffold of growing elastic fibers. The pericellular accumulation of galactosugar-binding molecules can disrupt elastogenesis. EBP has turned out to be a catalytically inactive variant form of acid β-galactosidase encoded by the minor 2 kb transcript of the *GLB1* gene. This protein, a major component in the non-integrin cell surface receptor complex, lacks β-galactosidase activity. It is also called S-gal, and arises following alternative splicing of pre-mRNA which results in the deletion of exons 3, 4 and 6 and the addition of 32 amino acids encoded according to a different reading frame by exon 5. Unlike the regular lysosomal glycoproteins, it is not almost exclusively sorted

into the endosomal compartment. Fibroblasts from patients with infantile G_{M1}-gangliosidosis due to nonsense mutations in the *GLB1* gene and those from patients with Morquio disease type B, in both of which the different molecular forms of β-galactosidase are deficient, are unable to assemble elastic fibers. In contrast, G_{M1}-gangliosidosis fibroblasts with missense mutations that result in the inactivation only of acid β-galactosidase, are able to assemble elastic fibers. The skeletal and connective tissue abnormalities in severe G_{M1}-gangliosidosis are also most probably related to EBP dysfunction [397]. The cardiac involvement recently highlighted in these patients may have a similar molecular basis [398].

Oligosaccharidoses

Sialidosis

Since the cloning of *NEU1*, encoding glycoprotein sialidase [126,127], more than a dozen mutations have been characterized in patients with various clinical types of sialidosis. Among them, the missense mutations Phe260Tyr, Leu270Phe and Ala298Val, clustered in the same region on the surface of the sialidase macromolecule, have been shown to dramatically reduce catalytic activity and cause rapid intralysosomal degradation of the expressed protein, as determined by transgenic *in vitro* expression in cultured mutant cells. Although correctly processed and sorted in patient fibroblasts, the mutant protein was shown not to associate with the multienzyme complex [399,400], the formation of which is required for the proper functioning of glycoprotein sialidase, β-galactosidase, and cathepsin A [124,125,401]. Based on these and similar observations, molecular models of acid sialidase have been proposed [399–401]. In addition, acid sialidase has turned out to be an important signaling factor required for the production of the cytokine interleukin-4 by activated T lymphocytes, on the surface of which catalytic activity is increased. The mechanism of sorting sialidase activity to the plasma membrane must be related to the internalization signal, consisting of the C-terminal tetrapeptide 412TyrGlyThrLeu415, found in lysosomal membrane proteins targeted to endosomes via clathrin-coated pits. Redistribution of sialidase from lysosomes to the activated T lymphocyte surface is accompanied by increased reactivity of the enzyme with anti-phosphotyrosine antibodies. This may indicate that phosphorylation of Tyr 412 inhibits sialidase internalization upon activation of the lymphocytes. Meanwhile, these data indicate that the intracellular distribution of lysosomal sialidase is controlled by the internalization signal in its cytoplasmic tail [402].

G_{M1}-Gangliosidosis

New data about β-galactosidase processing indicate that the C-terminal precursor fragment is an essential domain of the enzyme. During the molecular maturation process it remains associated with the 65 kDa moiety previously thought of as the mature enzyme, instead of being degraded. Thus, β-galactosidase is now recognized as a two-subunit protein in which the C terminal domain is essential for catalytic activity [403].

α-Mannosidosis

Six patients between the ages of 7 and 26 years, including two sets of siblings, have been shown to have an immunodeficiency at both the humoral and the cellular level in a matched case-control study, with sex and age as matching variables [404].

Aspartylglucosaminuria

Knowledge of the molecular pathogenesis of aspartylglucosaminuria is increasing steadily as the effects on the maturation, transport, sorting, stability, and catalytic activity of aspartyl-β-glucosaminidase of 18 different mutations, including the Finnish founder mutation (AGU_{Fin}), Cys163Ser, and six novel mutations, have been better "dissected", making use of the three-dimensional model structure of the enzyme. Mutations at the dimer interface interfere unfavorably with αβ dimerization in the endoplasmic reticulum or prevent it altogether. Active site mutations not only destroy catalytic activity but also interfere with precursor maturation. According to their molecular effects, mutations may be subdivided into groups designated mild, average (includes AGU_{Fin}) and severe [405]. However, detailed clinical follow-up will be required to discern specific genotypes within the aspartylglucosaminuria phenotype which has, until now, been considered rather uniform.

Pyknodysostosis

Recent studies have indicated an expanding role for cathepsin K and its regulatory factors. Dysfunctional osteoclasts resulting in osteosclerosis are found not only in human pyknodysostosis, but also in mice with osteopetrosis with either a dominant or a recessive mode of transmission. Recessively inherited osteopetrosis in mice is associated with homozygous or compound heterozygous genotypes with mutations in the gene for cathepsin K. The phenotype resulting from dominant inheritance, more syndromic in nature, includes microphthalmia, an absence of neural crest-derived pigment cells, and mast cell defects, and is apparently due to heterozygous mutations in the gene encoding Mitf, a member of a helix-loop-helix transcription factor subfamily which also contains TFE3, one of several potential dimerization factors. Mitf also modulates age-dependent changes in osteoclast function. Cathepsin K has been identified as a transcriptional target of Mitf and TFE3 via three consensus elements in its promoter. Cathepsin K mRNA and protein are deficient in osteoclasts with mutant Mitf. Overexpression of wild-type Mitf significantly up-regulates endogenous cathepsin K expression in cultured human osteoclasts [406]. It should be noted that cathepsin K expression has been discovered also in a significant proportion of human breast cancers (references in [406]) and could play a role in breast cancer invasiveness. The enzyme has become of interest in relation to the treatment of osteoporosis and its inhibitors may be explored as potential therapeutic agents [406].

Animal Models

MPS VII

MPS VII missense mutant models produced in the mouse with phenotypes of varying severity include the active site nucleophile replacement Leu76Phe, the commonest human mutation, Glu540Ala, and Glu540Gln. The Glu540Ala mouse has a severe phenotype and no β-glucuronidase activity at all. Following the introduction of both the human *GUSB* Glu540Ala cDNA transgene into intron 9 and the active site Glu540Ala mutation into the endogenous *Gusb* gene, the resulting MPS VII mice display ubiquitous expression of inactive human β-glucuronidase and tolerance to immune challenge with the human enzyme. This observation may serve as a model for studying the immunologic consequences of either long-term enzyme therapy with human β-D-glucuronidase or future gene therapy with vectors expressing the enzyme [407].

Mannosidosis

A natural α-mannosidosis model in the guinea pig which closely resembles the human disease has been described [408]. It will be convenient for investigating the effect of future therapeutic strategies for α-mannosidosis, in particular, and probably neuronal storage diseases in general. It is particularly relevant because the purification and molecular characterization of recombinant human lysosomal α-mannosidase has now been achieved [409].

Treatment

MPS-I

In view of the favorable assessment of recombinant α-iduronidase replacement in the canine model of MPS I [376,377] and the improved availability of the recombinant enzyme [410], a 52-week enzyme replacement study has been carried out in 10 patients (9 with MPS IHS and 1 with MPS IS). In the prepubertal children growth rate increased, as did the range of motion in the shoulder and elbow joints, in all patients. Hepatosplenomegaly decreased significantly. After only two weeks of weekly intravenous enzyme administration the urinary excretion of GAGs decreased. Episodes of sleep hypopnea were less frequent and cardiac functional scores improved, although changes in heart valve function were inconsistent. In general, the ability to perform daily activities and endurance became steadily better. Hypersensitivity reactions and IgG-mediated immune responses to the enzyme infusions were moderate and manageable with premedication and adjustments of the infusion rate. However, null genotype MPS IH patients may be expected to have a greater immune response [378,411]. None of the patients in this trial had Hurler disease (MPS IH) and were thus not mentally handicapped. The recombinant enzyme is not expected to cross the blood-brain barrier and prevent progression of neuronal damage. At present, a second multicenter clinical trial, designed to last two years and to involve more patients with moderate or mild disease, is nearing completion. Preliminary indications are that this type of enzyme replacement therapy is not likely to benefit the most common and most severely affected MPS IH patients.

MPS VI

Based on the observation that enzyme replacement therapy with recombinant human N-acetylgalactosamine 4-sulfate sulfatase (rhASB) was effective in an MPS VI model and had few side effects, a randomized, two dose, blinded study of weekly intravenous treatment with rhASB is currently under way. Following at least 12 weeks of therapy, neither allergy nor serious adverse reactions have been noted [412]. The final report on the efficacy of the treatment in these patients without mental retardation is eagerly awaited.

Aspartylglucosaminuria

Although previous reports may have brought a more optimistic message, bone marrow transplantation cannot now be encouraged for the treatment of aspartylglucosaminuria patients after infancy. In a report on bone marrow transplantation in five patients of between 1 and 10 years of age with the disorder, severe subsequent complications included autoimmune hemolytic anemia. No neurological or mental improvement was observed, although long-term further physical health has been judged good [413]. The ever increasing molecular knowledge of the various *AGA* mutations has not yet led to favorable therapeutic intervention in the disease, which is often not diagnosed before early childhood, especially outside Finland.

Gaucher Disease

The clinical successes of enzyme replacement therapy are being confirmed in an ever growing number of Gaucher disease type I patients. However, the generalized osteopenia that exists in most treated adults is often refractory. A double blind, two arm, placebo-controlled trial of bisphosphonate (alendronate) disodium treatment in adults who had received enzyme replacement therapy for at least 24 months showed a significant increase in bone mineral density and indicates that anti-resorptive therapy is a useful adjunctive measure for Gaucher disease-related osteopenia in adults [414].

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Chapter 23, Part I

Chondrodysplasias: General Concepts and Diagnostic and Management Considerations

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INTRODUCTION

The chondrodysplasias are rare, inherited disorders of skeletal development and linear growth. By definition, they involve disturbances of the cartilage components of the growing skeleton; however, other tissues are often also affected. They occur in all vertebrates, and well over 100 distinct disorders, or, more accurately, clinical phenotypes have been described in man. Their prevalence is estimated at about 1 in 4,000 births. They are characterized clinically by skeletal deformities and varying degrees of dwarfism, typically with disproportion between the lengths of the trunk and the limbs. Some chondrodysplasias are apparent at or before birth, whereas others do not become evident for several years. Their severity ranges from those incompatible with postnatal life to those that are so mild that they are difficult to detect. Thus, the chondrodysplasias are an extremely heterogeneous group of connective tissue disorders.

HISTORICAL PERSPECTIVE

Short persons with abnormal body proportions can be identified in European and American art from ancient times [1]. However, it was not until 1878 that Parrot coined the term "achondroplasia" to distinguish these individuals from short persons with normal body proportions. Fifty years later, Morquio in France and Brailsford in England separated them into two groups, those with predominantly short trunks and those with predominantly short limbs [1]. While the term achondroplasia was retained for the latter group, Morquio-Brailsford syndrome, or simply Morquio syndrome, was applied to the former group. By the middle of the 20th century, it had become evident that considerable heterogeneity existed, and the 1950s through the 1970s saw the identification of many "new" chondrodysplasias, bringing the number to over 100 [2–9]. Delineating the diagnostic features (clinical manifestations, radiographic findings, inheritance patterns, morphology of the growth plate, etc.) received the greatest attention during this period. However, as this was accomplished, a gradual change occurred, bringing the focus of the 1980s to defining the natural history and variability of the disorders [10–15] and that of the 1990s to elucidating the responsible mutations and characterizing the pathogenetic mechanisms by which they disrupt bone growth [16–22].

NOMENCLATURE, CLASSIFICATION, AND ETIOLOGY

The nomenclature of the chondrodysplasias is confusing. They have often been called chondrodystrophies, as well as skeletal and bone dysplasias. Chondrodysplasia is the most appropriate term, because it implies abnormal development or growth (dysplasia) and distinguishes the conditions from the osteodysplasias, such as osteogenesis imperfecta (see Chapter 8, this volume), which involve bone abnormalities. The term "osteochondrodysplasias" refers to both groups and is synonymous with the designations bone and skeletal dysplasias.

Many different schemes have been employed to name individual chondrodysplasias [23,24]. Greek terms have been used, e.g., thanatophoric (death bringing), diastrophic (twisted), and metatropic (changing) dysplasias. Eponyms have been commonly utilized, such as Kniest or Ellis-van Creveld dysplasia. Many conditions have been named after the part of the skeleton or particular bone most affected, e.g., epiphyseal and metaphyseal dysplasias. Even the histologic appearance of growth plate cartilage has been employed on occasion, e.g., fibrochondrogenesis. A common phenomenon has been the "splitting" of what was originally thought to be one condition into two or more. Modifier terms, such as congenita, tarda, dominant, recessive, X-linked, and pseudo-, have been employed to distinguish such disorders as well as to connote particular features. Finally, most disorders are called dysplasias, but some are referred to as syndromes.

Many schemes have also been used to classify the chondrodysplasias. One of the earliest was based on whether it was the limbs or the trunk that was predominantly affected. Conditions were designated short-limbed or short-trunked chondrodysplasias. Other schemes have relied on the age at which the manifestations become apparent, i.e., neonatal versus late-onset chondrodysplasias. In 1964, Rubin published

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a book entitled *Dynamic Classification of Bone Dysplasias*, in which the location of radiographic changes provided the basis for classification [25]. There were epiphyseal dysplasias, metaphyseal dysplasias, spondyloepiphyseal dysplasias, and so on. Although this provided a convenient way to divide the chondrodysplasias into general categories, it was an oversimplification, and many disorders had to be "forced" into one category or another. Nevertheless, this approach has been the most widely used system until recently, and international conferences were held in the 1970s through the 1990s to refine it for both naming and classifying chondrodysplasias [26].

The concept of chondrodysplasia phenotype families grew out of the need to develop a more manageable scheme to name and classify the chondrodysplasias. As advanced by Spranger in the late 1980s and early 1990s, it grouped disorders with similar quantitative manifestations into chondrodysplasia families [27–29]. The concept implied that common phenotypes arise from common pathogenetic mechanisms. Accordingly, members of a chondrodysplasia phenotype family were predicted to result from a common pathogenesis and potentially from mutations of the same gene. This concept was proven correct to a large extent as the genes harboring chondrodysplasia mutations were identified (Table 1). In fact, by the end of the 1990s, it had become apparent that a large proportion of disorders result from mutations in a relatively small number of genes, the chondrodysplasia genes [20,29,30].

An International Working Group on Bone Dysplasias was convened in 1997 to address the impact of molecular genetics on nomenclature and classification. The group constructed a hybrid scheme using genetic etiology, i.e., gene locus, as the primary basis for classification [31]. This resulted in many changes from earlier classifications since, in several instances, disorders previously thought to be different were grouped together and disorders previously thought to be related were separated. If genetic etiology is unknown, similarities in clinical and radiographic manifestations are employed. A caveat of the scheme is that it is dynamic. As the genetic etiology for a condition is identified, the basis for classification switches from phenotype to genotype. Another consequence of this dynamic, gene-based classification is that it directly interfaces with Online Mendelian Inheritance in Man (OMIM), which provides extensive references for disorders listed in the classification as well as links to a wide range of informational services [32].

The recent delineation of chondrodysplasia genes and their mutations has provided important insights not previously appreciated regarding the etiology of the chondrodysplasias. Allelism is common, and graded series of disorders that range from very severe to very mild are found in the better defined groups, such as those due to mutations of *COL2A1* (type II collagen), *FGFR3* (fibroblast growth factor receptor 3), and *DTDST* (diastrophic dysplasia sulfate transporter) [19,20,33]. This may well turn out to be the case for most of the groups as more mutations are found and the full spectrum of clinical phenotypes associated with mutations of a given gene is defined. These observations have changed the way these disorders are viewed. Previously, they were considered distinct entities with unique clinical, radiographic, and genetic features. The emerging view is that they are clinical phenotypes distributed along spectra of phenotypic abnormalities associated with mutations in particular genes. For mutations in some genes, such as *COL2A1*, the distribution is fairly continuous, with clinical phenotypes merging into one another across a broad range. There is much less clinical overlap for mutations of some other genes, such as *FGFR3*, in which the distribution is discontinuous.

MOLECULAR GENETICS

A number of genes that harbor mutations causing chondrodysplasias were identified in the 1990s (Table 1). Links to references for specific disorders are given in OMIM [32]. These genes encode several categories of proteins including cartilage matrix proteins, transmembrane receptors, ion transporters, and transcription factors. The number of gene loci identified to date is much smaller than had been anticipated from the number of clinical phenotypes delineated earlier. Indeed, the vast majority of patients have disorders that map to fewer than 10 loci, and mutations at two loci (*COL2A1* and *FGFR3*) account for well over half of all patients [19]. This implies that there is a limited number of genes whose function is critical to skeletal development, especially linear bone growth, and that mutations in these genes give rise to a wide range of chondrodysplasia clinical phenotypes.

Mutations at the *COL2A1* and *FGFR3* loci demonstrate quite different genetic characteristics. *COL2A1* mutations are distributed throughout the gene with very few instances of recurrence in unrelated persons [22]. In contrast, *FGFR3* mutations are restricted to a few locations within the gene and the occurrence of new mutations at these sites in unrelated individuals is the rule [34]. There is a strong correlation between clinical phenotype and mutation site for *FGFR3*, but not for *COL2A1* mutations (see also this Chapter, Parts II and IV).

PATHOGENESIS

The pathogenesis of the chondrodysplasias must be considered at at least two levels: disturbances in the behavior of a gene product resulting from a mutation and the consequences of such disturbances to skeletal development and growth. The pathogenesis of specific chondrodysplasias, or groups of chondrodysplasias, is covered in much more depth in subsequent parts of this chapter. This discussion is intended to provide a brief overview.

Molecular Disturbances

Chondrodysplasia mutations act through different mechanisms. For example, most mutations involving cartilage matrix proteins cause disease when only one of the two alleles of the relevant gene is mutated, i.e., in the heterozygous state [22,35]. They typically act through a dominant negative mechanism in which the protein products of the mutant allele interfere with the assembly and function of multimeric molecules that contain the protein products of both the normal and mutant alleles. Mutations of type II collagen illustrate this phenomenon [16,17,36]. The type II collagen molecule is a triple helical entity comprised of three collagen α chains, each of which is the product of the type II collagen gene, *COL2A1*. When α chains from both normal and mutant alleles are combined to form triple helices, most molecules contain at least one mutant α chain. In fact, seven of the eight possible combinations contain at least one mutant α chain. It is not known how many mutant chains are required to produce a dysfunctional molecule, but, depending on the mutation, it could theoretically be as few as one.

Heterozygous mutations involving type X collagen act, at least in part, differently from the model just described [37]. Those known to date map to the region of the α chain that

TABLE 1. Chondrodysplasia Genes and Their Products

Gene Locus	Chromosomal Location	Gene Product	Protein Function	Clinical Phenotype	MIM No.	Inheritance	Mechanism
COL2A1	12q13.1–q13.3	Type II collagen α1 chain	Cartilage matrix protein	Achondrogenesis II	200610	AD	Dominant negative
				Hypochondrogenesis	120140.0002	AD	Dominant negative
				SED congenita	183900	AD	Dominant negative
				Kniest dysplasia	156550	AD	Dominant negative
				Late onset SED		AD	Dominant negative
				Stickler dysplasia	108300	AD	Haploinsufficiency
COL9A2	1p32.2–p33	Type IX collagen α2 chain	Cartilage matrix protein	MED	600969	AD	Dominant negative
COL9A3	20q13.3	Type IX collagen α3 chain	Cartilage matrix protein	MED	600969	AD	Dominant negative
COL10A1	6q21–q22.3	Type X collagen α1 chain	Hypertrophic cartilage matrix protein	Schmid metaphyseal chondrodysplasia	156500	AD	Haploinsufficiency
COL11A1	1p21	Type XI collagen α1 chain	Cartilage matrix protein	Stickler-like dysplasia	184840	AD	Dominant negative
COL11A2	6p21.3	Type XI collagen α2 chain	Cartilage matrix protein	Stickler-like dysplasia	215150	AR	Loss of function
COMP	19p12–p13.1	Cartilage oligomeric matrix protein	Cartilage matrix protein	Pseudoachondroplasia	177170	AD	Dominant negative
				MED	600969	AD	Dominant negative
DTDST	5q32–q33	DTD sulfate transporter	Transmembrane sulfate transporter	Diastrophic dysplasia	222600	AR	Loss of function
				Achondrogenesis 1B	600972	AR	Loss of function
				Atelosteogenesis II	256050	AR	Loss of function
FGFR3	4p16.3	FGF receptor 3	Tyrosine kinase receptor for FGFs	Thanatophoric dysplasia I	187600	AD	Gain of function
				Thanatophoric dysplasia II	187610	AD	Gain of function
				Achondroplasia	100800	AD	Gain of function
				Hypochondroplasia	146000	AD	Gain of function
PTHR	3p21–p22	PTHrP receptor	G protein-coupled receptor for PTH and PTHrP	Jansen metaphyseal chondrodysplasia	156400	AD	Gain of function
SOX9	17q24.3–q25.1	SRY box 9	Transcription factor	Campomelic dysplasia	114290	AD	Haploinsufficiency
CBFA1	6p21	Core binding factor α subunit	Transcription factor	Cleidocranial dysplasia	119600	AD	Haploinsufficiency
LMX1B	9q34.1		Transcription factor	Nail patella dysplasia	161200	AD	Haploinsufficiency
CTSK	1q21	Cathepsin K	Enzyme	Pycnodysostosis	265800	AR	Loss of function

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; SED, spondyloepiphyseal dysplasia; MED, multiple epiphyseal dysplasia; DTD, diastrophic dysplasia; FGF, fibroblast growth factor; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; SRY, sex-determining region of the Y chromosome. (See also ''Recent Developments''.)

is responsible for chain recognition, which is the first step in the assembly of collagen molecules. The mutations disrupt this process resulting in a failure of the mutant α chains to be incorporated into molecules and the loss of function of the products of the mutant allele, in the process referred to as haploinsufficiency. Haploinsufficiency is also thought to be the genetic mechanism in chondrodysplasias involving transcription factors.

Mutations involving ion transport genes, such as the diastrophic dysplasia sulfate transporter, also act through a loss of function of a transporter [33] (see also this Chapter, Part III). However, in this case, the functional loss of one allele does not cause disease; loss of function, or more accurately, a substantial loss of function of the product of both alleles is required. The severity of clinical phenotypes probably reflects the combined level of residual function of the two alleles [38,39].

In contrast, mutations of transmembrane receptors studied to date act through a gain of function; that is, the mutant receptors initiate signals in a constitutive manner independent of their normal ligands [34,40]. As with loss of function mutations, the severity of clinical phenotype is thought to reflect the extent of constitutive receptor activation.

Consequences for Skeletal Development and Growth

Regardless of genetic mechanism, the mutations responsible for the chondrodysplasias disrupt skeletal development. More specifically, they disrupt the growth phase of skeletal development, that is, the phase during which formed elements of the embryonic skeleton grow to reach their final adult size. For the most part, mutations that disturb earlier events in skeletal development are lethal and/or are not recognized as chondrodysplasias. By definition, chondrodysplasia mutations disrupt the function of the skeletal growth plate, which is where postembryonic bone growth occurs.

The reader is referred to Chapter 1, Part II, in this volume, for a discussion of the growth plate. Briefly, it is a dynamic structure that resides near the ends of immature bones. Growth occurs because cartilage template is produced at the leading edge of the structure and converted into bone at its trailing edge. The process is extremely complex and incompletely defined. It is orchestrated largely by differentiating chondrocytes but requires the participation of other cell types including perichondrial cells, vascular and perivascular cells, osteoblasts and osteo(chondro)clasts. Regulatory circuits involving locally synthesized growth factors and their receptors control key cellular events, such as chondrocyte proliferation and terminal differentiation and vascular invasion of the cartilage template, ultimately to govern bone growth. Relevant growth factors identified to date include fibroblast growth factors (FGFs), parathyroid hormone-related protein (PTHrP), bone morphogenetic proteins (BMPs), and Indian hedgehog (Ihh). Matrix proteins also appear substantially to influence cellular events in the growth plate through direct interactions with membrane receptors and through their interactions with growth factors.

A number of mechanisms have been proposed to explain how mutations disturb these phenomena, although most are still at the speculative stage. Mutations involving cartilage matrix proteins such as types II and XI collagen probably act in part by altering the mechanical properties of cartilage as a template [16,19,21]. They may also affect the extent to which growth factors can diffuse through cartilage matrix to reach target cells. Mutations may also alter signals initiated by matrix protein-receptor interactions in the growth plate. Another proposed mechanism involves the toxic effects of accumulated proteins on growth plate cells. In pseudoachondroplasia, chondrocytes are filled with dilated endoplasmic reticulum containing cartilage oligomeric matrix protein (COMP). This may adversely affect the behavior of cells and even their survival [41]. Thus, mutations of matrix genes may potentially disturb growth plate function through many mechanisms ranging from disturbances of the mechanical properties of cartilage matrix to disturbances in the biologic properties and behavior of growth plate cells.

Many of the mechanical properties of cartilage reflect the abundance of the matrix proteoglycan, aggrecan. This molecule is very rich in sulfated glycosaminoglycan side-chains. The consequence of mutations of DTDST (the diastrophic dysplasia sulfate transporter) is that growth plate chondrocytes are unable to synthesize properly sulfated aggrecan, which presumably adversely affects the template functions of cartilage matrix [38,39]. Such disturbance could potentially disrupt the physiologic functions of cartilage matrix as with the collagen mutations.

Mutations that activate transmembrane receptors, such as FGFR3 and PTHR (parathyroid hormone-related protein receptor), exaggerate the physiologic signals normally transduced by these receptors [34,40,42]. Both FGFR3 and PTHR transmit inhibitory signals. FGFR3 appears to inhibit growth plate chondrocyte proliferation and possible terminal differentiation, while PTHR is thought to block terminal chondrocyte differentiation. Constitutive activation of either would be expected to reduce the number of terminally differentiated chondrocytes produced during a given period of time, and thereby to reduce the rate of bone growth.

GENERAL DIAGNOSTIC CONSIDERATIONS

Despite the emphasis in this chapter on dealing with the genetic and biologic aspects of the chondrodysplasias, clinicians must address the clinical aspects of these disorders. A few chondrodysplasias can be diagnosed at first glance, but most require a careful evaluation based on clinical history, physical examination, skeletal radiographs, family history, and laboratory testing. In the end, the process involves recognizing complex patterns characteristic of the different disorders. Specific features of each disorder are listed in subsequent parts of this chapter, and detailed references are also available through OMIM [32].

Clinical Features

The hallmark of the chondrodysplasias is disproportionate short stature [1,23,24,43]. Although this term refers to a disproportion between the limbs and trunk, most disorders exhibit some shortening of both, and subtle degrees of disproportion may be difficult to appreciate. Skeletal disproportion is usually accompanied by short stature, i.e., length/height below the 3rd percentile, but these measurements are occasionally within the low-normal range early in the course of certain conditions. There may also be disproportionate shortening of different segments of the limbs; the particular pattern may provide clues toward specific diagnoses.

With some notable exceptions, there is a strong correlation between the age of onset and the overall clinical severity. Many of the so-called lethal neonatal chondrodysplasias are evident by the time routine ultrasound examinations are

performed at the end of the first trimester of gestation. Gestational standards exist for long bone lengths, and discrepancies are often detected between biparietal diameter of the skull and long bone lengths. Many disorders become apparent around the time of birth, whereas others show up during the first year. A number of disorders become manifest in early childhood, a few in late childhood or later.

Although growth deficiency typically dominates the clinical picture, most patients also have problems unrelated to growth. For example, skeletal deformities such as abnormal joint mobility, angular deformities, etc., are common and usually symmetrical. Sometimes skeletal abnormalities adversely affect nonskeletal tissues, e.g., impaired growth at the base of the skull and of vertebral pedicles reduces the size of the spinal canal in achondroplasia. Short ribs reduce thoracic volume, which may compromise breathing in short trunk chondrodysplasias. Cleft palate is common to many disorders, presumably reflecting defective palatal growth.

In some instances, manifestations are unrelated to the skeleton, and reflect the expression of mutant genes in nonskeletal tissues. Examples include retinal detachment in spondyloepiphyseal dysplasia congenita, sex reversal in campomelic dysplasia, congenital heart malformations in Ellis van Creveld syndrome, immune deficiency in cartilage-hair hypoplasia, and renal dysfunction in asphyxiating thoracic dystrophy. These nonskeletal problems provide valuable clues to specific diagnoses.

Family and Reproductive History

A careful family history is designed to identify relatives with the condition manifested by the patient. Sometimes a Mendelian inheritance pattern can be elicited. However, because the clinical presentation may vary substantially in some disorders, one must search not only for features observed in the patient, but also for features that might be related to those observed in the patient. Special attention should be given to mild degrees of short stature, disproportion, deformities and other manifestations, such as precocious osteoarthritis, that may have been overlooked by the family. Physical examination of relatives may be helpful, as may be review of photographs, X-rays, and medical records of family members.

A reproductive history may be informative in as much as previous stillbirths, fetal losses, and other abnormal pregnancy outcomes may indicate a skeletal dysplasia in the family. Pregnancy complications, such as polyhydramnios or reduced fetal movement, are also common in bone dysplasias, especially neonatal lethal ones.

Even though almost all of the skeletal dysplasias are genetic, it is common for there to be no history of the disorder in a family. New mutations to normal parents are common for autosomal dominant disorders, especially chondrodysplasias that are lethal in the perinatal period, e.g., thanatophoric dysplasia. The majority of achondroplasia cases result from new mutations. Germ cell mosaicism, in which one parent harbors clones of mutant germ cells in their gonads, has been observed in some dominant disorders. A negative family history is common for recessively inherited disorders.

Radiographic Features

X-ray evaluation of a chondrodysplasia should include plain films of the entire skeleton. Efforts should be made to identify which bones and which parts of bones (epiphysis, metaphysis, and diaphysis) are most affected. If possible, films taken at different ages should be examined, because the radiographic changes evolve with time. Films taken before puberty are generally much more informative than those taken afterwards, because "closure" of the epiphyses obliterates many of the signs relied upon for radiographic diagnosis.

Diagnosis

Once it is established that a patient has a probable chondrodysplasia, establishing a diagnosis depends on matching the observed clinical picture with clinical phenotypes of well-documented disorders. There are a number of reference texts to facilitate matching phenotypes [3,6–8,44–50]. The online database OMIM provides frequently updated information about the disorders and comprehensive lists of current references [32].

With a few exceptions, laboratory testing has not been useful for diagnosing chondrodysplasias. However, this may change as molecular genetic testing for chondrodysplasias becomes more feasible and established in the commercial arena. Such testing would be most useful for disorders in which recurrent mutations occur, such as achondroplasia [51]. However, it should be emphasized that the diagnosis of achondroplasia can usually be made clinically. The greatest utility of such testing may be in prenatal diagnosis for couples in which both parents have typical (heterozygous) achondroplasia. They are at 25% risk for transmission of the much more severe homozygous achondroplasia, which can be detected by mutation analysis. Another example is in disorders due to mutations of *DTDST* [38]. These disorders are inherited in an autosomal recessive manner, and a limited number of mutant alleles have been found. If the mutations are identified in a patient, then they should be detectable in the parents and potentially useful for prenatal diagnosis. In contrast to the situation with these disorders, most chondrodysplasia mutations tend to be dispersed throughout host genes. This phenomenon makes their detection more difficult and thereby reduces the usefulness of such testing for diagnostic purposes, at least in the near future.

Many of the chondrodysplasias have distinct histologic changes of the skeletal growth plate [4,9,49,50]. Sometimes such tissue obtained at biopsy or discarded from a surgical procedure is helpful diagnostically. However, it is uncommon to make a diagnosis histologically if not already suspected on clinical grounds. An exception is for the lethal neonatal chondrodysplasias, in which an aborted fetus or newborn is difficult to evaluate clinically and radiographically.

MANAGEMENT

The first step in managing the care of a patient with a chondrodysplasia is to establish the correct diagnosis. This allows prediction of a prognosis and anticipation of the medical and surgical problems associated with a particular disorder. Establishing a diagnosis is especially important when trying to distinguish between what have been called "lethal disorders" and nonlethal disorders in a premature or newborn infant. For example, the poor prognosis for long-term survival would argue against initiating extreme lifesaving measures for thanatophoric dysplasia or achondrogenesis types Ib or II, whereas they may buy time for infants with SED (spondyloepiphyseal dysplasia) congenita or diastrophic dysplasia, which carry a good prognosis if they survive the newborn period.

There is no definitive therapy to normalize bone growth in any of the conditions. Consequently, management is

focused on preventing and correcting skeletal deformities, treating nonskeletal complications, genetic counseling, and helping patients and families cope with the disorders. Each disorder has its own unique set of problems and consequently management must be tailored to each disorder. Nevertheless, there are many problems common to many chondrodysplasias for which general recommendations can be made [4,8,10,11,13,49,52,53]. For instance, children with most chondrodysplasias should avoid contact sports and other activities that cause injury or stress to joints. Good dietary habits should be established in childhood to prevent or minimize obesity in adulthood. Dental care should be started early to minimize crowding and malalignment of teeth. Because of their small pelvis, pregnant women with various of the chondrodysplasias should be followed in high-risk prenatal clinics and have Cesarean section to deliver their babies. Prenatal diagnosis may be possible by ultrasonography if limb bone lengths differ enough from established norms. However, there are many conditions in which the growth deficiency is not sufficient to be distinguished from normal early in the pregnancy. Children and relatives should be given the opportunity to participate in support groups, such as the Little People of America and the Human Growth Foundation [54,55].

Two approaches have been used to date to increase bone length. Both are controversial. Surgical limb lengthening has been employed for a few disorders [56–60]. Its greatest success has been in achondroplasia in which nonskeletal tissues tend to be redundant and easily stretched. The procedure is usually done during the teen years. Injections of human growth hormone in pharmacologic doses comparable to those used to treat Turner syndrome have also been tried in several disorders; the results have been equivocal [61–65].

RECENT DEVELOPMENTS

Several new "chondrodysplasia genes" were incorporated into the International Nosology and Classification of Constitutional Disorders of Bone at a meeting of the International Working Group on Bone Dysplasias held in Oxford, England, in September 2001. The most notable of these are *SEDL*, which encodes a protein named sedlin that is thought to participate in protein secretion, and is mutated in X-linked SED (spondyloepiphyseal dysplasia) tarda [66]; *RMRP*, which encodes a nontranslated RNA component of a mitochondrial RNA-processing endoribonuclease, and is mutated in cartilage-hair hypoplasia [67]; *HSPG2*, which encodes the heparan sulfate-containing pericellular proteoglycan perlecan, and is mutated in dyssegmental dysplasia — Silverman-Handmaker type [68] and Schwartz-Jampel syndrome [69]; and *MATN3*, which encodes the cartilage matrix protein matrilin 3, and is mutated in MED (multiple epiphyseal dysplasia) [70].

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Chapter 23, Part II

Chondrodysplasias: Disorders of Cartilage Matrix Proteins

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SUMMARY

- **A major subset of the human chondrodysplasias are due to mutations of genes that encode cartilage matrix proteins, including types II, IX, X, and XI collagens and cartilage oligomeric matrix protein (COMP). The nomenclature and classification of these disorders has undergone substantial revision as clinical phenotypes have been matched to mutations at specific gene loci.**
- **Mutations of the gene for type II collagen, *COL2A1*, produce a broad spectrum of clinical phenotypes that fall under the general designation, spondyloepiphyseal dysplasia (SED). These autosomal dominant disorders range in severity from achondrogenesis type II, which is lethal at or before birth, to late-onset SED, the major feature of which is precocious osteoarthritis. Disorders of intermediate severity include SED congenita, SED Strudwick, and Kniest dysplasia. The mutations are thought to act through a dominant negative mechanism to reduce the number of collagen fibrils in cartilage matrix.**
- **Stickler dysplasia has been divided into three types. Classic Stickler dysplasia (type I), which involves eyes, ears, and the skeleton, results from a subset of mutations of *COL2A1* that lead to haploinsufficiency. The nonocular form of Stickler dysplasia (type II) and Stickler dysplasia type III are due to mutations of *COL11A2* and *COL11A1*, respectively. Mutations of type XI collagen genes have also been found in otospondylomegaepiphyseal dysplasia (OSMED), Weissenbacher-Zweymuller syndrome (WZS) and Marshall syndrome.**
- **Schmid metaphyseal chondrodysplasia, a relatively mild disorder affecting mainly the lower extremities, results from mutations of the gene encoding type X collagen, *COL10A1*. The mutations are heterozygous and cluster in the carboxyl terminal nonhelical NC1 domain of the molecule.**
- **Mutations of the gene for cartilage oligomeric matrix protein (COMP) have been found in patients with pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED). COMP is a member of the thrombospondin family of proteins. A 3 bp deletion that maps to a highly conserved region of thrombospondins involved in binding calcium accounts for more than 30% of the total COMP mutations. The mechanisms by which COMP mutations act remain unknown.**
- **Mutations of genes for the α2(IX) and α3(IX) collagen chains, *COL9A2* and *COL9A3*, respectively, have been detected in patients with MED.**
- **A number of mouse strains have been genetically engineered to serve as models for human chondrodysplasias due to *COL2A1* and *COL10A1* mutations. The naturally occurring mouse mutants, *Dmm* and *Cho*, which involve mutations of *Col2a1* and *Col11a1*, respectively, have been proposed as models for Stickler dysplasia types I and III, respectively.**

INTRODUCTION

This chapter primarily addresses chondrodysplasias due to mutations of genes that encode cartilage matrix proteins. The disorders fall into three categories, reflecting the distribution and function of the proteins. The first group contains disorders that result from mutations of types II and XI collagen genes. These collagens constitute the major and minor elements, respectively, of collagen fibrils of hyaline cartilage. The second group includes disorders due to mutations of cartilage oligomeric matrix protein (COMP), which resides in the matrices of many connective tissues, and of type IX collagen, which contributes to cartilage collagen fibrils. The third category is comprised of disorders of type X collagen, the distribution of which is restricted to the hypertrophic zone of the growth plate. Several other disorders for which the genetic basis has not been identified are included here because their clinical phenotypes resemble those due to cartilage matrix protein defects.

DISORDERS DUE TO MUTATIONS OF THE GENES FOR TYPES II AND XI COLLAGENS

The disorders in this category exhibit an extremely wide range of severity. Indeed, the clinical phenotypes range from

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profound dwarfism, lethal *in utero*, to phenotypically normal individuals with precocious osteoarthritis in adulthood. From severe to mild, the disorders include achondrogenesis type II, hypochondrogenesis, spondyloepiphyseal dysplasia congenita (SED congenita) and its variants, SED Strudwick, Kniest dysplasia, late-onset SED, and Stickler dysplasia.

Nomenclature and History

The nomenclature of these conditions has, historically, been confusing. For example, achondrogenesis refers to a lethal chondrodysplasia phenotype with a short trunk and very short extremities. It was initially divided into two types: type I, designated Parenti-Fraccaro (MIM 200600), and type II, termed Langer-Saldino (MIM 200610) [1–4]. Type I was subsequently subdivided into type IA (Houston-Harris) and type IB [5]. During the same period, another condition was reported and named hypochondrogenesis [6]. As infants with severity intermediate between achondrogenesis type II and hypochondrogenesis were ascertained, it was suggested that hypochondrogenesis was simply a mild form of achondrogenesis type II [7]. This was supported by histologic and biochemical evidence showing similar abnormalities in infants with the two conditions [7,8]. Further confusion arose when Whitley and Gorlin proposed the division of achondrogenesis into types I–IV based on radiographic severity [9]. Whitley-Gorlin type I corresponded to the original type I. Whitley-Gorlin type II was the severe form of the original (Langer-Saldino) type II achondrogenesis, whereas Whitley-Gorlin type III corresponded to the typical Langer-Saldino achondrogenesis type II, and Whitley-Gorlin type IV to hypochondrogenesis. It is now clear that achondrogenesis type II (Langer-Saldino) and hypochondrogenesis constitute the severe end of the spectrum of disorders resulting from type II collagen mutations, sometimes called type II collagenopathies. In contrast, achondrogenesis type 1B has turned out to be the most severe form of a series of disorders that result from mutations of the diastrophic dysplasia sulfate transporter gene (see Part III, this chapter).

Spondyloepiphyseal dysplasia, or SED, refers to a clinical phenotype manifesting radiographic abnormalities primarily of the spine and epiphyses. The term is sometimes used generically to refer to chondrodysplasias that exhibit such changes. At other times it is used for specific disorders, such as SED congenita, which is apparent at birth, and SED tarda, the onset of which is in later childhood. Although it was originally thought that these disorders were related because of the similarity of involvement, they are not, as the former phenotype results from type II collagen mutations, whereas the latter is due to mutations of an X-linked gene distinct from the type II collagen gene. The term SED tarda has occasionally been applied to chondrodysplasias due to type II collagen mutations that become manifest in childhood or later. We prefer to employ the term late-onset SED for this clinical phenotype, reserving SED tarda for the X-linked disorder.

Clinically distinct phenotypes have been defined that overlap SED congenita. They are due to mutations of type II collagen and thus fall within the type II collagenopathy spectrum, yet they can be distinguished from typical SED congenita. The two most notable conditions in this category are Strudwick dysplasia and Kniest dysplasia.

Stickler dysplasia refers to a clinical phenotype usually dominated by eye abnormalities accompanied by degenerative arthritis, hence the alternative designation, hereditary arthro-ophthalmopathy. Type II collagen mutations are found in some patients, making it a type II collagenopathy. However, mutations of genes encoding type XI collagen chains have been found in other families. Currently, three forms of Stickler dysplasia are recognized according to the mutant gene locus. The term Stickler dysplasia type I is used when mutations involve type II collagen, whereas the terms Stickler dysplasia types II and III are used when the mutations map to genes encoding the α2 and α1 chains of type XI collagen, respectively. As discussed below, patients with the type II variant lack the eye component of Stickler syndrome. From linkage exclusion, it has been suggested that a fourth locus exists for Stickler dysplasia [10].

Originally described as Pierre Robin syndrome with fetal chondrodyplasia, Kelly suggested in 1982 that the Weissenbacher-Zweymuller syndrome (WZS) (MIM 108300) is the neonatal expression of Stickler dysplasia [11,12]. When three infants with manifestations of WZS were found to develop the ocular and auditory findings of Marshall syndrome (MIM 154780), Winter proposed that WZS, Marshall syndrome, and Stickler dysplasia were related [13]. Subsequent studies showed that WZS and Stickler were separate entities, but revealed overlap between WZS and otospondylo-megaepiphyseal dysplasia (OSMED), which has also been called Nance-Insley syndrome (MIM 215150) and Nance-Sweeney chondrodysplasia [14–17]. OSMED occurs in both heterozygous and homozygous forms [18]. The confusion has now been largely resolved by the finding of *COL11A2* mutations in patients with WZS and OSMED as well as with Stickler dysplasia type II. Thus, the three conditions are allelic. Marshall syndrome has subsequently been linked to mutation of the *COL11A1* locus [19]. Controversy exists over whether Marshall syndrome is part of the Stickler dysplasia spectrum or a separate nosologic entity [16,19–21]. Spranger has suggested that the term Stickler dysplasia type III be used for all phenotypes resulting from *COL11A1* mutations [22].

The clinical manifestations of Wagner syndrome (MIM 143200) are dominated by degenerative changes of the vitreous and retina similar to those seen in Stickler dysplasia. Even though patients lack the arthropathy typical of Stickler dysplasia, there has been debate over whether Wagner syndrome is nosologically distinct from Stickler syndrome. It now appears that there are two forms of Wagner syndrome. One can result from mutations of *COL2A1* [23], while linkage to *COL2A1* is excluded in the second form, which maps to chromosome 5q13-14 [24].

Mutations

COL2A1

A mutation of the type II collagen gene (*COL2A1*) was first reported in 1989 by Lee et al. in affected members of a family with SED congenita [25]. The number of *COL2A1* mutations associated with SED and SED-like phenotypes has subsequently grown to over 40, as compiled by Kuivaniemi et al. in 1997 [26]. Table 1 lists mutations reported to date, including references. All have been heterozygous mutations and most map to the triple helical domain of the type II collagen α chain, where they are dispersed fairly evenly. The most frequent mutations involve the substitution of a bulky amino acid for glycine, which normally occupies every third position. Glycine to serine substitutions are the most common mutations.

There is a tendency for mutations residing toward the carboxyl terminus of the triple helix to produce more severe manifestations than those that map toward the amino terminus of the helix. This phenotypic gradient resembles

TABLE 1. Reported Mutations of *COL2A1*

Phenotype	Type	Mutation	Reference
Achondrogenesis type II	Missense	Gly310Asp	[235]
	Missense	Gly691Arg	[65]
	Missense	Gly769Ser	[64]
	Missense	Gly817Val	[236]
	Missense	Gly943Ser	[237]
Hypochondrogenesis	Missense	Gly574Ser	[238]
	Missense	Gly604Ala	[239]
	Missense	Gly805Ser	[235]
	Missense	Gly853Glu	[240]
	Missense	Gly910Cys	[241]
	Missense	Gly943Cys	[241]
	Missense	Gly988Arg	[28]
	Missense	Gly499Ile	[242]
SED congenita	Missense	Gly154Arg	[243]
	Missense	Gly175Arg	[242]
	Missense	Arg789Cys	[31,32]
	Missense	Gly895Ser	[34]
	Missense	Gly997Ser	[33,242]
	Splice	IVS20 +5 G>T	[244]
	Splice	IVS35 Del 4 bp	[241]
	Splice	IVS39 Del 3 bp	[245]
	Deletion	Del 9 bp exon 44	[242]
	Insertion	Ins 45 bp exon 48	[78]
	Deletion	Del exon 48	[25]
SED Strudwick	Missense	Gly292Val	[81]
	Missense	Gly304Cys	[81]
	Missense	Gly709Cys	[81]
Kniest dysplasia	Missense	Gly103Asp	[246]
	Missense	Ala102Val	[247]
	Splice	IVS12 Del 28 bp	[248]
	Splice	Skip Ex12	[249]
	Splice	Skip Ex14	[241]
	Splice	Skip Ex15	[241]
	Splice	Skip Ex 15	[250]
	Splice	IVS18 Del 16 bp	[57]
	Splice	IVS22 +1 G>A	[90]
	Splice	IVS24 +5 G>A	[89]
	Deletion	Del 18 bp exon 49	[251]
SED—late onset	Missense	Arg75Cys	[28,30, 252]
	Missense	Gly247Ser	[253]
	Missense	Arg519Cys	[27–29]
	Missense	Gly976Ser	[28]
Stickler dysplasia type I	Nonsense	Arg9Ter	[254]
	Missense	Gly67Asp	[23]
	Insertion	Ins 10 bp exon 4	[255]
	Splice	IVS12 −1 G>A	[38]
	Deletion	Del 1 bp exon 13	[38]
	Splice	IVS17 −2 A>G	[256]
	Deletion	Del 1 bp exon 20	[255]
	Nonsense	Glu506Ter	[38]
	Deletion	Del 1 bp exon 34	[38]
	Missense	Arg704Cys	[257]
	Nonsense	Arg732Ter	[258]
	Splice	IVS39 −1 G>T	[38]
	Deletion	Del 1 bp exon 40	[259]
	Deletion	Del 25 bp exon 40	[38]
	Deletion	Del 1 bp exon 43	[260]
	Insertion	Ins 1 bp exon 48	[255]
	Deletion	Del 1 bp exon 49	[38]
	Deletion	Del 1 bp exon 50	[261]
	Deletion	Del 1 bp exon 50	[38]

SED, spondyloepiphyseal dysplasia.

that observed for *COL1A1* and *COL1A2* mutations in osteogenesis imperfecta (see Chapter 8, this volume). However, as with *COL1A1* and *COL1A2* mutations, there are many exceptions. For instance, SED congenita mutations are distributed along the entire length of the triple helix, overlapping with mutations responsible for Kniest dysplasia and achondrogenesis type II. Thus, there is not a high correlation between the region to which a mutation maps and the specific phenotype that is produced. However, in the few instances in which specific mutations have recurred in different families, the resulting phenotype is similar.

Identical mutations do not generally recur in *COL2A1*. One notable exception is the Arg519Cys mutation found in five unrelated families with late-onset SED with precocious osteoarthritis [27–29]. Studying the genetic background from which this mutation arose in these families, Bleasel et al. identified three distinct haplotypes and concluded that the Arg519 codon may constitute a hot spot for mutations in *COL2A1* [29]. Recurrences have also been observed for Arg75Cys, Arg789Cys, and Gly997Ser mutations [28,30–34].

Most *COL2A1* mutations are missense mutations in exons that lead to amino acid substitutions, most often of glycine in the type II collagen α1 chain. In-frame duplications and deletions have been observed in SED congenita. Mutations that disrupt splicing, producing small in-frame deletions have also been found, especially in Kniest dysplasia. In contrast, splice mutations and deletions that shift the reading frame, leading to premature termination of translation, are characteristic of Stickler dysplasia type I, as are missense mutations that create premature stop codons.

COL11A1 and COL11A2

Mutations at these loci (Table 2) are less common than those of *COL2A1*, and they tend to be heterogeneous. For instance, *COL11A2* mutations associated with nonocular Stickler dysplasia type II include a 27 bp deletion, a splicing mutation that leads to an 18 amino acid deletion and a glycine to arginine missense mutation [35,36]. The patients were heterozygous for the first two mutations and homozygous for the mutation in the third instance. Heterozygosity for a glycine to glutamate (Gly955Glu) mutation has been found in WZS [37], while homozygosity for a missense mutation has been detected in OSMED [18].

A splicing mutation predicted to delete 18 amino acid residues from the triple helical region of the α1(XI) chain has been reported in a large family with Marshall syndrome [19]. Mutation analysis of 30 patients suspected of having Marshall syndrome or Stickler dysplasia revealed 15 novel mutations of *COL11A1* [38]. The majority of the mutations disrupted splice consensus sequences for 54 bp exons that mapped to the carboxyl terminal half of α1(XI). These patients exhibited phenotypes consistent with Marshall syndrome, i.e., moderate to severe hearing defect and less severe ocular findings.

Animal Models

There are a number of mouse strains that harbor mutations of genes encoding cartilage collagen chains. Most involve the *Col2a1* gene and most have been generated by transgenesis. The lower case *Col2a1* refers to the murine homolog of the human *COL2A1*. Despite the diversity of mutations, the resulting phenotypes are similar.

Col2a1

The semidominant, disproportionate micromelia (*Dmm*) mouse was detected in offspring of a male mouse whose

TABLE 2. Reported Mutations of *COL11A1* and *COL11A2*

Locus	Phenotype	Type	Mutation	Reference
COL11A1	Stickler dysplasia type III	Missense	Gly97Val	[96]
	Marshall syndrome	Splice	IVS +1 G>A	[19]
		Splice	IVS38 +2 T>C	[38]
		Splice	IVS38 +1 G>T	[38]
		Splice	IVS43 −2 A>G	[38]
		Splice	IVS47 −2 A>G	[38]
		Splice	IVS50 +3 A>C	[38]
		Splice	IVS50 +3 Ins T	[38]
		Splice	IVS50 +1 G>C	[38]
		Splice	Exon50/IVS50 Del 4 bp	[38]
		Splice	Del IVS52 −85 to IVS53 +23	[38]
		Splice	IVS54 +1 G>A	[38]
		Splice	IVS14 −2 Del A	[38]
		Missense	Gly148Arg	[38]
		Deletion	Del 18 bp exon 36	[38]
		Deletion	Del 9 bp exon 52	[38]
		Missense	Gly988Val	[38]
COL11A2	Stickler dysplasia type II	Splice	IVS +1 G>A	[35]
	OSMED	Missense	Gly175Arg	[35]
	Stickler dysplasia type II	Deletion	Del 27 bp exon 39	[36]
	WZS	Missense	Gly955Glu	[37]

OSMED, otospondylo-megaepiphyseal dysplasia; WZS, Weissenbacher-Zweymuller syndrome.

sperm were irradiated [39]. Heterozygotes exhibit short extremities and a blunt head. Homozygotes die at birth; they show severe dwarfism involving primarily the limbs. The growth plate is disorganized; the chondrocytes contain a distended rough endoplasmic reticulum and the matrix displays a paucity of collagen fibrils [40]. The mutation in *Dmm* is a 3 bp deletion that alters two codons, leading to the substitution of an asparagine residue for lysine and threonine residues, in a highly conserved region in the C-propeptide of type II collagen [41]. This is believed to disrupt the formation of intrachain disulfide bonds, which disturbs the association of chains and assembly of type II collagen molecules. *Dmm* has been proposed as a model for Stickler dysplasia type I, in which similar mutations have been found.

Several *Col2a1* mutations predicted to act in a dominant negative fashion have been targeted to cartilage in transgenic mice. Vanderberg et al. deleted exons 16–27 to produce a minigene designed to promote "protein suicide," a term used to describe the accelerated degradation of abnormally folded proteins [42]. The resulting phenotype was dominated by short tubular bones and included cleft palate, a short snout, delayed skeletal development, and a short spine. The growth plate was disorganized and cartilage matrix contained fewer than normal collagen fibrils. Taking into account some variability in severity, similar phenotypes have been produced in transgenic mice harboring Gly85Cys [43] and Gly574Ser [44,45] mutations, and a deletion of exon 7 [46]. Mice null for *Col2a1* exhibit a similar but more severe phenotype; cartilage collagen fibrils are markedly reduced and displayed an abnormal structure [47,48]. The common phenotype observed in these mice probably reflects the consequences of a reduced abundance of cartilage collagen fibrils on the formation and function of cartilage matrix as a template for skeletal growth. The mice serve as models for the SED group of human chondrodysplasias and may be useful for studying the non-growth-related manifestations of these disorders. For instance, evidence of osteoarthritis is found in older mice with the minigene [49], otic capsule abnormalities are detected in mice transgenic for the Gly574Ser mutation [50], and ocular abnormalities are observed in several transgenic strains [51]. Osteoarthritis and hearing and visual problems occur in patients with SEDs.

Col11a1

The recessively inherited chondrodysplasia (*cho*) mouse is characterized by short limbs and spine, a short snout, and cleft palate [52]. The growth plate architecture is disturbed and cartilage collagen fibrils are unusually thick [53]. A single base deletion that introduces a premature stop codon about 570 bp downstream of the translation initiation codon of *Col11a1* has been found in the *cho* mouse [54]. This would produce the equivalent of a *Col11a1* knockout. It is proposed that the absence of the α1(XI) collagen chain in homozygous mice interferes with the normal function of type XI collagen in regulating the size of cartilage collagen fibrils. This would account for the thick fibrils, but how abnormal fibrillogenesis disturbs cartilage development and growth is not well understood. The *cho* mouse is potentially a model for Stickler dysplasia type III.

Pathogenesis

In most instances, mutations of *COL2A1* cause disease in the heterozygous state. This presumably reflects the vulnerability of the homotrimeric type II collagen molecule to the dominant negative effects of mutant α chains from a single mutant allele [55,56]. Of the eight possible ways chains from mutant and wild-type alleles can combine, seven contain at least one mutant chain. It is not known how many mutant chains are required to produce a dysfunctional molecule, but it could theoretically be as few as one, although this probably varies according to mutation. Current dogma holds that the assembly of type II collagen molecules begins with the association of α chain carboxyl propeptides and is

completed after the α chains have wound around each other in a carboxyl to amino direction to form the triple helix. Accordingly, alterations in mutant α chains, such as deletions or insertions of amino acids or substitutions of bulky for compact residues or charged for uncharged residues, or vice versa, may disturb and delay or prevent proper folding of the molecule. Delayed folding may allow for excessive post-translational modification, i.e., overmodification, of certain lysyl and hydroxylysyl residues in the unfolded chains. Abnormally folded molecules may be degraded, poorly secreted, and/or accumulate in the rough endoplasmic reticulum. Alternatively, they may be secreted but not incorporated into fibrils, or their incorporation may produce dysfunctional fibrils. Collagen molecules containing mutant α chains may also be abnormally susceptible to degradation by enzymes that reside in the matrix outside cells. The molecular consequences of abnormal collagen biosynthesis is discussed further elsewhere in this volume (Chapters 8, 9, and 15). The net effect in many, if not most, instances is to reduce the number of collagen fibrils in cartilage matrix. In fact, there appears to be a correlation between the extent of fibril reduction and severity of phenotype, regardless of the specific mutation.

Most mutations associated with Kniest dysplasia disrupt mRNA splicing and lead to skipping of exons and short in-frame deletions in the type II collagen molecule [57]. It is not clear why such deletions produce the Kniest dysplasia phenotype, because they predict disruption of helix assembly in the same manner as other dominant negatively acting mutations.

In contrast to the dominant negative effects of mutations that disturb the folding of type II collagen, many mutations associated with Stickler dysplasia type I are believed to act through haploinsufficiency [58]. Most of these mutations create premature translation stop signals, either because an in-frame stop codon is produced or, more commonly, because the translation reading frame is shifted so that an out-of-frame stop codon becomes in-frame. In either case, the predicted collagen chains are shorter than normal and lack all or part of the noncollagenous carboxyl propeptide necessary for them to be incorporated into triple helical molecules. In principle, haploinsufficiency would reduce the number of collagen fibrils by half.

There are many potential mechanisms by which a reduction in type II collagen molecules or, probably more accurately, in cartilage collagen fibrils, could interfere with skeletal development and growth. They are all speculative in nature and not mutually exclusive. A reduction in the number of collagen fibrils would be expected to alter the mechanical properties of cartilage as a template for endochondral ossification. Given their role in providing structural integrity to tissues, cartilage containing fewer than normal fibrils would likely be less resistant to compressive and other forces exerted on the growth plate as a bone elongates. This could be a direct effect of the reduction in the number of fibrils and/or secondary to loss of proteoglycans, which normally occupy space within the fibrillar network. Proteoglycans attract and hold water within the fibrillar network, which gives cartilage its resilience to deformation.

The cellular events that normally occur in the growth plate could be disturbed by alterations of cartilage collagen fibrils. For example, there is evidence that collagens bind to receptors on chondrocyte surfaces. These interactions could be disrupted by changes in number, composition, or structure of collagen fibrils. Similarly, such changes could interfere with the diffusion, sequestration, and presentation of growth factors to chondrocytes in the growth plate and other cartilaginous tissues.

Relatively little is known about why mutations that produce haploinsufficiency for type II collagen, or disturb the biosynthesis of type XI collagen, produce the Stickler dysplasia phenotype. It is of interest, however, that ocular manifestations are not associated with mutations of *COL11A2*. This is consistent with observations that the α2 chain of type XI collagen in tissues such as cartilage and inner ear, is not expressed and is replaced by the α2 chain of type V collagen in the eye [59] (See Chapter 1, Part V, in this volume).

Clinical Phenotypes

Achondrogenesis Type II (MIM 200610) and Hypochondrogenesis (MIM 120140.0002)

Achondrogenesis type II and hypochondrogenesis represent the severe end of the spectrum of SEDs, or the alternatively designated type II collagenopathies. They are discussed together because the phenotypes blend from the more severe achondrogenesis type II to the slightly less severe hypochondrogenesis [7]. Both are frequently associated with prematurity and hydrops fetalis, and affected infants are either stillborn or die relatively soon after birth.

Affected fetuses and infants exhibit a large head, soft cranium, and flat face. The neck is very short, and the trunk is barrel-shaped. Radiographically, the ribs are short, the iliac wings are hypoplastic, and the acetabular roofs flat (Fig. 1). Rib fractures are common. The long bones are typified by the femora, which are short and broad with irregular cupped metaphyses. The vertebral pedicles are ossified, but the extent of ossification of the vertebral bodies varies. Severely affected infants show lack of ossification of the entire spine and sacrum, whereas milder cases (hypochondrogenesis) have defective ossification of only the cervical vertebrae and the sacrum (Fig. 2). The severity of vertebral ossification defects correlates well with that of long bone changes.

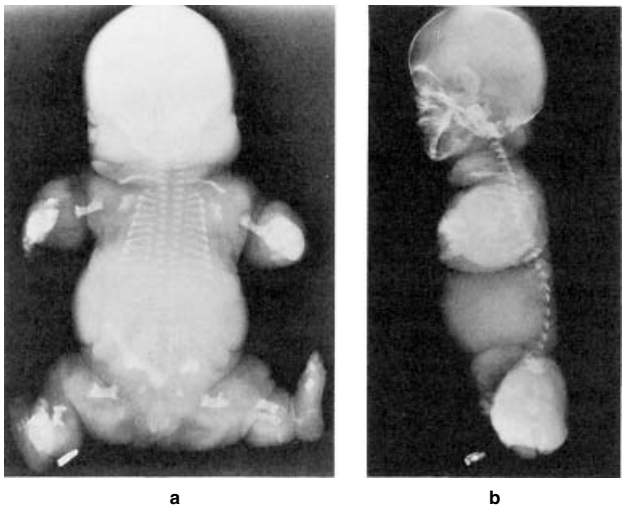
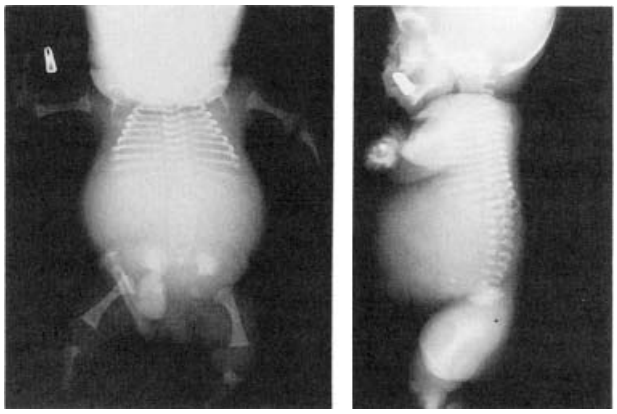


Figure 1. Radiographs of an infant with achondrogenesis type II (**a, b**), showing markedly short ribs, hypoplastic ilia, and flat acetabular roofs. The long bones are extremely short and broad with irregular, cupped metaphyses. The vertebral pedicles are ossified but the bodies are not.



a b

Figure 2. Radiographs of an infant with hypochondrogenesis (**a, b**), showing similar but less severe findings than in achondrogenesis type II. Note ossification of the vertebral bodies.

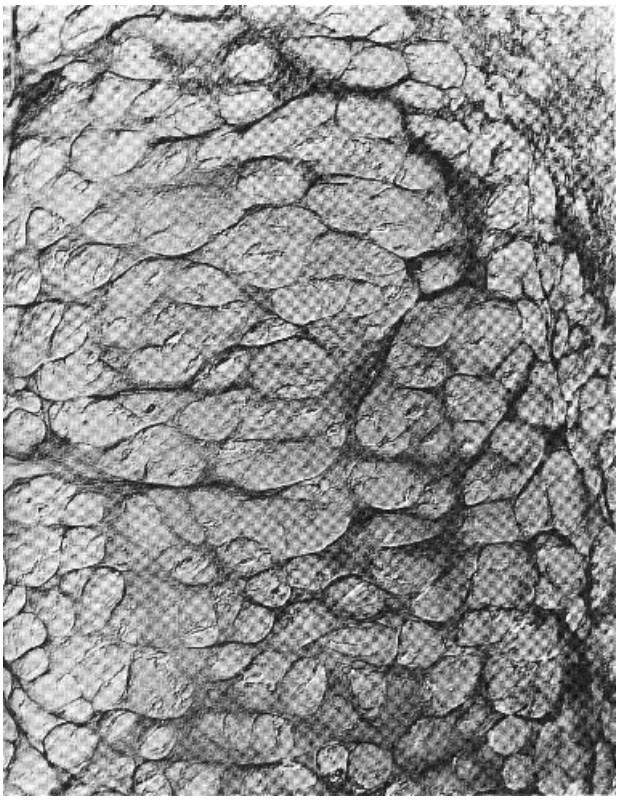


Figure 3. Photomicrograph of epiphyseal cartilage in achondrogenesis type II. Note the large chondrocyte lacunae and scant matrix. Interstitial connective tissue associated with a vascular canal is seen at top right. Toluidine blue stain. ×200.

Histologic examination of epiphyseal cartilage reveals hypercellularity, with reduced matrix and a poorly organized to absent growth plate (Fig. 3) [7]. The relative amount of matrix varies within any one specimen, but that in cartilage from milder cases tends to be more abundant. Cartilage canals are greatly increased in number and in the amount of interstitial connective tissue associated with the structures. The chondrocytes tend to be larger than normal, and electron microscopy reveals many of them to contain dilated rough endoplasmic reticulum, as well as thickening and irregularity of collagen fibrils in the matrix.

Cartilage collagen has been analyzed in several cases of achondrogenesis type II-hypochondrogenesis [8,9,60–65]. The most dramatic finding has been the presence of large amounts of type I collagen in the cartilage, which immunostaining has shown to be associated mainly with the cartilage canals, although small amounts are detectable in the matrix. In some instances, type I collagen has been the only type of collagen detected, while substantial amounts of type II collagen have been identified in other cases. Abnormal type II collagen peptides indicative of overmodification of the type II collagen have been observed [62,65]. The presence of types I and III collagen in cartilage has also been noted in mice null for *Col2a1* [48]. Diminished amounts of type II collagen have further been observed in cartilages from the bulldog calf, which has been considered a bovine model of achondrogenesis type II-hypochondrogenesis [67].

Although achondrogenesis type II was originally considered to be an autosomal recessive disorder [68], it is now clear that it, as well as hypochondrogenesis, results from *de novo*, heterozygous mutations of *COL2A1*. Consequently, the recurrence risk is very low for both conditions. The possibility of germ line mosaicism must be kept in mind during genetic counseling.

SED Congenita (MIM 183900)

SED congenita presents at birth with shortening of the trunk and, to a lesser extent, of the extremities [69–72]. Clubfoot and cleft palate are sometimes present, and the head and facies are usually considered normal. The neck is short, and the chest is barrel-shaped with accompanying kyphosis and exaggerated lumbar lordosis. The limb shortening is most prominent proximally, with the hands and feet appearing relatively normal. These features become more prominent with time. Infants with SED congenita may be hypotonic. Severe axial myopia is common, and retinal detachment may occur. There is considerable variation in severity in SED congenita, and adult patients typically vary in height from 95 to 128 cm [73].

Radiographic changes primarily involve the spine and proximal extremities. The vertebral bodies in the newborn are ovoid in the lateral view, but become markedly flattened with irregular endplates during childhood (Fig. 4). The odontoid process is often hypoplastic and in some cases may be aplastic. The iliac bones are short and square, and the pubic symphysis is poorly ossified at birth. The tubular bones are moderately short with mild metaphyseal irregularity. The epiphyses may be fragmented and show delayed ossification. Epiphyseal changes are most marked in the proximal femora, with a coxa vara deformity occurring commonly.

Patients with SED congenita usually survive infancy and have normal developmental milestones. Typically, a waddling, widebased gait develops in early childhood. Kyphosis and sometimes kyphoscoliosis arises and progresses in childhood; it may compromise respiratory function. Odontoid hypoplasia may predispose to cervico-medullary instability and spinal cord compression, but sudden death is not known to be increased. However, when the diagnosis of SED congenita is made, flexion and extension films of the upper cervical spine should be taken. Delayed ossification in this area can complicate interpretation of

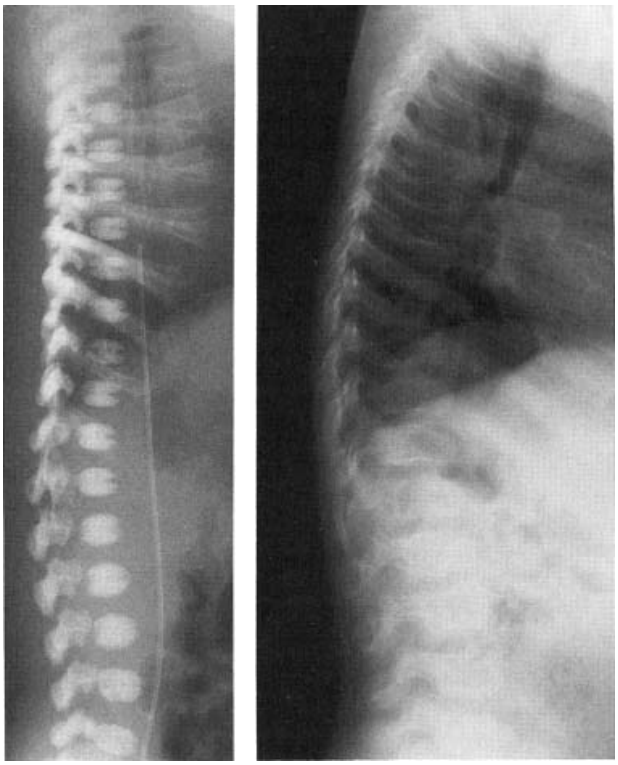


Figure 4. Radiographs of the spine in spondyloepiphyseal dysplasia congenita. **a**: Lateral projection showing ovoid vertebral bodies in a newborn. **b**: Lateral projection showing flattening of vertebral bodies in a 10-year-old child. The vertebral end plates are irregular.

such a study. If cervical instability is detected, other studies such as magnetic resonance imaging or electrophysiologic investigations should be done to search for evidence of spinal cord compression. Careful ophthalmologic evaluation and follow-up are indicated to identify and treat small retinal tears, prior to complete detachment with loss of vision. SED congenita is inherited as an autosomal dominant trait.

Histologically, the growth plate exhibits shorter chondrocyte columns and wider matrix septa than normal [74]. Epiphyseal chondrocytes contain cytoplasmic inclusions by light microscopy. Electron microscopy shows these to be dilated cisternae of rough endoplasmic reticulum, and reveals irregularities of collagen fibrils in the extracellular matrix. Chromatographic and electrophoretic abnormalities of type II collagen have been reported in several cases [32,75–78].

SED Strudwick (MIM 271670)

SED Strudwick is an SED clinical variant [79,80]. Clinically and radiographically indistinguishable from SED congenita during infancy, SED Strudwick type differs in childhood by having greater metaphyseal involvement. It is sometimes referred to as spondylometepiphyseal dysplasia. Skeletal radiographs show a mottled appearance of the metaphyses. Histology shows mild nonspecific changes in organization of the growth plate and dilated rough endoplasmic reticulum of epiphyseal chondrocytes, similar to that seen in SED congenita. A report of affected sibs born to unaffected parents initially suggested autosomal recessive inheritance [80]. However, it is now evident that it is an autosomal dominant condition due to heterozygous mutations of *COL2A1* [81].

Kniest Dysplasia (MIM 156550)

Kniest dysplasia is an autosomal dominant generalized disorder of skeletal tissues that presents at birth with shortening of the trunk and limbs [82–85]. The face is round and flat, the nasal bridge is depressed, and the eyes are prominent. Associated findings may include cleft palate, clubfoot, and inguinal herniae. Joints are often large at birth and continue to enlarge during childhood. The fingers appear long and knobby. Motor milestones are often delayed because of the joint deformities. Axial myopia and hearing loss of both conductive and sensorineural types commonly develop in childhood, and retinal detachment may occur. Intelligence is usually normal.

There is flattening and anterior wedging of the vertebral bodies on radiographs (Fig. 5). Coronal clefts of lumbar vertebral bodies may be seen in infants. The iliac bones are small with irregular acetabular angles. The long bones are short and expanded at the metaphyses, giving rise to a dumbbell appearance. Epiphyses are irregular and fragmented and develop a sclerotic, stippled appearance with age. The femoral necks are short and broad. The capital femoral epiphyses are late to ossify, eventually becoming flat and associated with a coxa vara deformity (Fig. 6). The tubular bones in the hands have flattened and squared-off epiphyses, with narrowing of the joint spaces. There is generalized osteopenia affecting the ends of the long bones.

The major problems result from progressive and painful joint enlargement accompanied by flexion contractures. Muscle atrophy may result from disuse. The combination produces varying degrees of incapacitation and may be complicated by degenerative arthropathy. Symptomatic

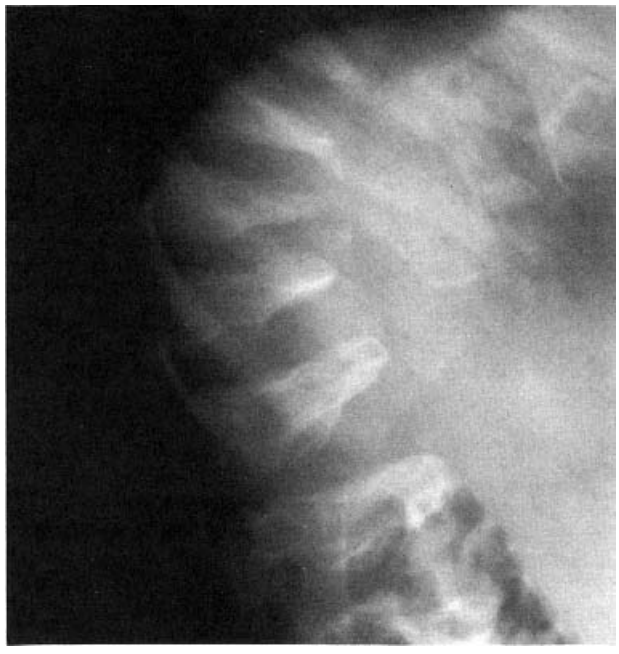


Figure 5. Lateral radiograph of the spine of a 12-year-old child with Kniest dysplasia. Note the flattening and anterior wedging of the vertebral bodies in the lower thoracic and lumbar spine.

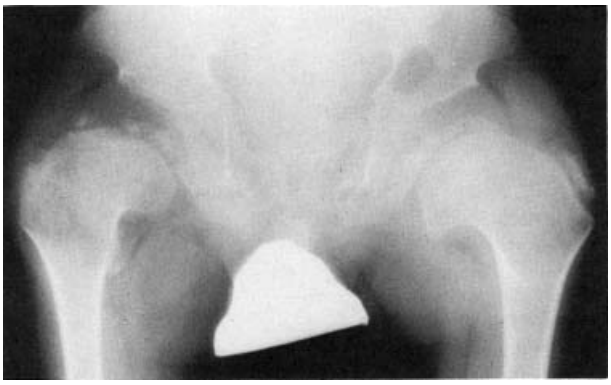


Figure 6. Radiograph of the hips and pelvis of a 12-year-old child with Kniest dysplasia. The capital femoral epiphyses are flat and fragmented. The femoral necks are wide and dysplastic. There is a coxa vara deformity of the hips.

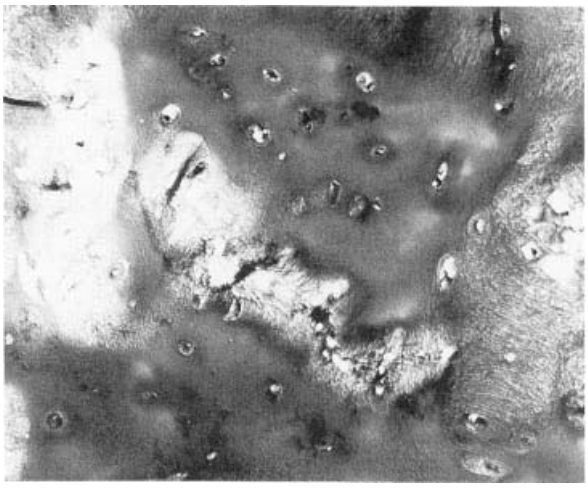


Figure 7. Photomicrograph of epiphyseal cartilage in Kniest dysplasia. Note the extensive fibrous lesions of the matrix, which in some areas form large ''holes.'' Toluidine blue stain. ×200, original magnification.

treatment is indicated for the hearing loss and myopia. Retinal detachment requires ophthalmologic care.

Cartilage is soft, and the most distinctive histologic feature of both epiphyseal and growth plate cartilage is the vacuolar degeneration of both chondrocytes and matrix (Fig. 7). Large cystic lesions are found in paraffin sections, which resemble the holes found in Swiss cheese [74,85]. Cytoplasmic inclusions have been observed in epiphyseal chondrocytes [86]. The inclusions correspond to dilated rough endoplasmic reticulum on electron microscopy, which also shows the collagen fibrils to be abnormally thin and to stain poorly [87,88]. Electrophoretic abnormalities of type II collagen have been observed in at least one instance [89,90]. Most mutations reported to date cause skipping of exons, which leads to small in-frame deletions in the type II collagen triple helix (Table 1).

Stickler Dysplasia (MIM 108300,120290)

Stickler dysplasia type I, which is inherited in an autosomal dominant fashion, classically affects the eyes, the ears, and the skeleton [91–94]. The diagnosis can be made at birth if cleft palate and micrognathia (Pierre-Robin anomaly) are present. Severe axial myopia is typical and retinal detachment sometimes occurs in childhood. Choroidoretinal and vitreous degeneration may occur, as may cataracts. Sensorineural hearing loss may develop in adolescence. Degenerative arthritis, manifested by painful joints and stiffness in the morning and after exercise, usually develops in the second or third decade. Some patients exhibit a Marfanoid habitus and ligamentous laxity, and mitral valve prolapse occurs in around 50% of cases. Short stature is not a feature of this condition.

Spranger et al. reported a child with Kniest dysplasia associated with a heterozygous *COL2A1* mutation. The child's mother, who was mosaic for the mutation, exhibited the clinical phenotype of Stickler dysplasia [95].

Irregular ossification of epiphyses can be seen on radiographs during childhood, and degenerative joint changes are usually evident by adolescence. The tubular bones have narrow shafts with thin cortices. Mild irregularities of the vertebral end plates may be observed. Management depends on the extent of involvement. The cleft palate requires surgical correction, and ophthalmologic care is essential to minimize visual impairment. Surgical replacement of major joints may be required for the arthropathy.

The skeletal and otologic manifestations of Stickler dysplasia type I are found in the nonocular, Stickler dysplasia type II phenotype, but such patients lack the ocular findings [35,36]. OSMED (otospondylo-megaepiphyseal dysplasia), which is recessively inherited, shows similar but more severe changes [18]. Vitreoretinal findings are prominent in Stickler dysplasia type III [96] and Marshall syndrome [19], both of which are due to *COL11A1* mutations.

Disorders of Less Well-Defined Etiology with Similar Clinical Phenotypes

SED Tarda (MIM 313400)

SED Tarda is an X-linked disorder distinct from the other COL2A1-related SEDs. It commonly presents in boys between the ages of 10–14 years with short stature, primarily affecting the trunk [68,97,98]. Vague back pain may occur during adolescence, and precocious osteoarthritis is typical in young adulthood. Average adult height is 145 cm.

Skeletal X-rays show vertebral flattening with a distinctive hump-shaped build-up of bone in the central and posterior portions of the upper and lower end-plates (Fig. 8) [68,99]. This is thought to reflect defective ossification of vertebral ring apophyses. Mild epiphyseal dysplasia is seen in large joints, especially the hips and shoulders, where degenerative changes arise in early adulthood. Eventually, there is degeneration of intervertebral discs with destruction of spinal facet joints [98]. Obligate heterozygote females often show similar, but milder X-ray changes; they often exhibit arthralgia by mid-adulthood.

SED tarda maps to chromosome Xp22.2-22.1 [98,100,101]. Mutations in this region have recently been identified in a gene, *SEDL*, the product of which is proposed to facilitate the secretion of proteins in many cell types [102]. Those identified to date are predominantly frameshifts and premature terminations suggesting that haploinsufficiency for the gene product accounts for the findings.

Metatropic Dysplasia (MIM 250600)

Metatropic dysplasia is an autosomal recessive chondrodysplasia, recognizable at birth by the presence of a long, narrow trunk and mild shortening of the limbs [85,103,104].

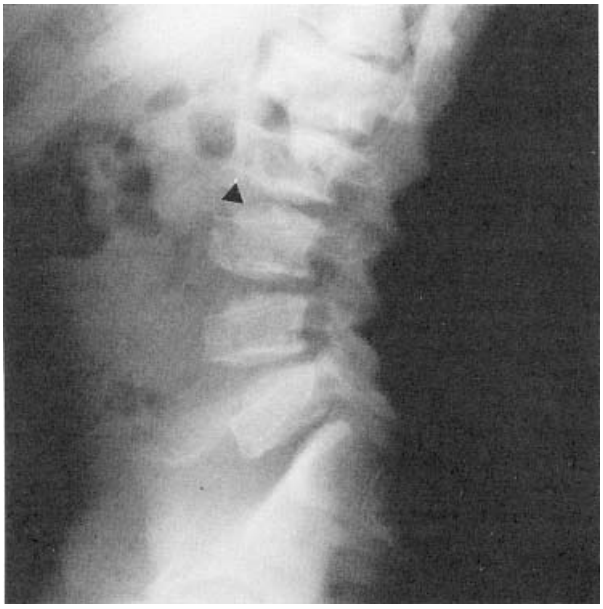


Figure 8. Radiograph of the thoracolumbar spine of an adolescent boy with spondyloepiphyseal dysplasia tarda. A characteristic hump-shaped accumulation of bone (arrowhead) can be seen in the central and posterior portions of the superior and inferior endplates of the vertebral bodies.

A ''tail-like ''appendage is occasionally found at the base of the sacrum. Kyphoscoliosis develops in late infancy and progresses throughout childhood to produce severe short-trunk dwarfism. The name metatropic dysplasia derives from this tendency of the dwarfism to change from the short-limbed to the short-trunk type over time. The joints are enlarged, and their mobility is restricted, except for those of the fingers, which tend to be hyperextensible. Contractures of the hips and knees develop in childhood. Odontoid hypoplasia occurs commonly. The head and face are normal. Developmental milestones are attained at appropriate times, and intelligence is normal. Adult height ranges from 110 to 120 cm [68].

On radiographic examination, the vertebral bodies are thin and either flat or slightly diamond-shaped. The vertebral bodies widen, and the central region of the upper lumbar vertebrae becomes thickened with age. The intervertebral spaces are wide. Lateral projection shows marked platyspondyly (Fig. 9). The iliac bones are short and flared. The combination of a narrow sacrosciatic notch and a lateral notch just above the outer rim of the acetabulum gives the ilium a halberd appearance. The tubular bones are short with markedly expanded, irregular, and deformed metaphyses, producing a dumbbell appearance (Fig. 10).

The major complication relates to the progressive kyphoscoliosis, which is resistant to treatment and which may compromise cardiopulmonary function. Cervical instability from the odontoid hypoplasia may require surgical fusion. The progressive joint contractures may lead to considerable disability.

The growth plate histologic findings are relatively nonspecific [68,74]. The cartilage-bone interface is irregular due to uneven metaphyseal vascular invasion, and foci of dysplastic, unmineralized cartilage may extend into the subchondral bone. Metachromatic inclusions have been observed in epiphyseal chondrocytes, which may be excessively vacuolated.

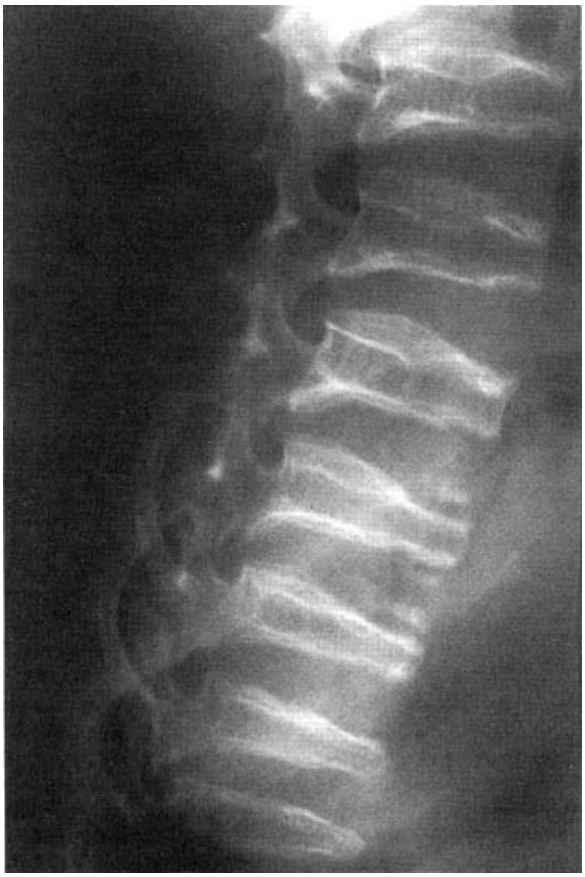


Figure 9. Lateral radiograph of the spine of a 4-year-old child with metatropic dysplasia showing severe platyspondyly.

Dominant Metatropic-like Spondylo-Epi-Metaphyseal Dysplasia

Dominant metatropic-like spondylo-epi-metaphyseal dysplasia (see MIM 250600), described by Beck et al. [105], is clinically and radiologically distinct from metatropic dysplasia. We have observed several children with the disorder. Newborns are generally proportionate at birth. Longitudinal growth begins to fall off between the first and second years. Physical findings include an attractive facies without midface hypoplasia, a short neck, and a barrel-shaped chest. The extremities and the hands are short and the joints wide. A characteristic waddling gait is seen in early childhood, when hip and knee flexion contractures develop, giving rise to a crouched appearance. Other joints exhibit ligamentous laxity. Kyphoscoliosis arises in late childhood and is usually mild. Genu valgum is also a common finding that may require surgical intervention. Ligamentous laxity is exaggerated, particularly in childhood. Other problems include pain in the major joints of the lower extremities that contributes to the decreased mobility and atlantoaxial instability from odontoid hypoplasia, which may require surgical intervention. Adult heights vary from 116 to 123 cm.

Radiographic findings are present at birth, but become more pronounced with time. The spine shows marked

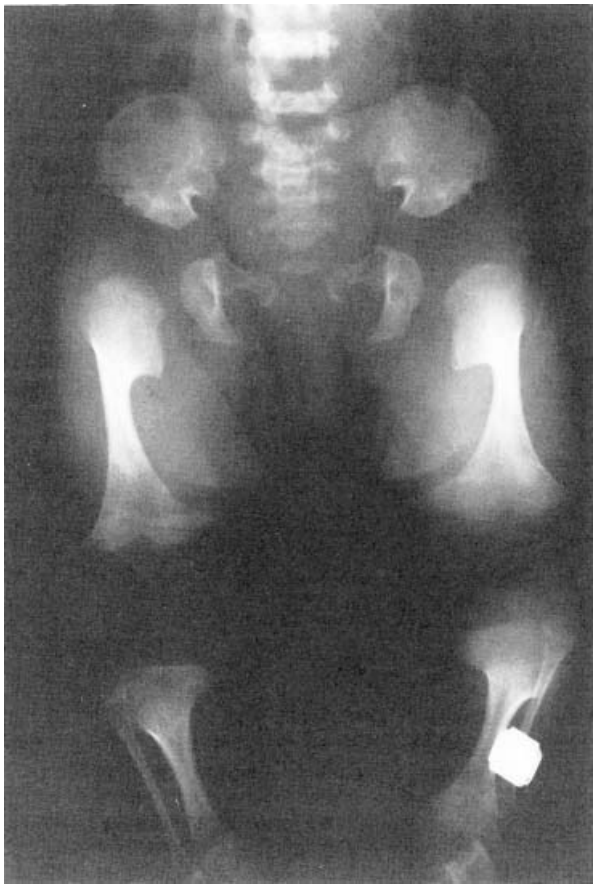


Figure 10. Radiograph of the lower lumbar spine, pelvis, and lower extremities of a newborn with metatropic dysplasia. The vertebral bodies are thin and flat. The iliac bones are short and flared, and the sacrosciatic notches are narrow. The tubular bones are short with markedly expanded, irregular, and deformed metaphyses producing a characteristic dumbbell appearance.

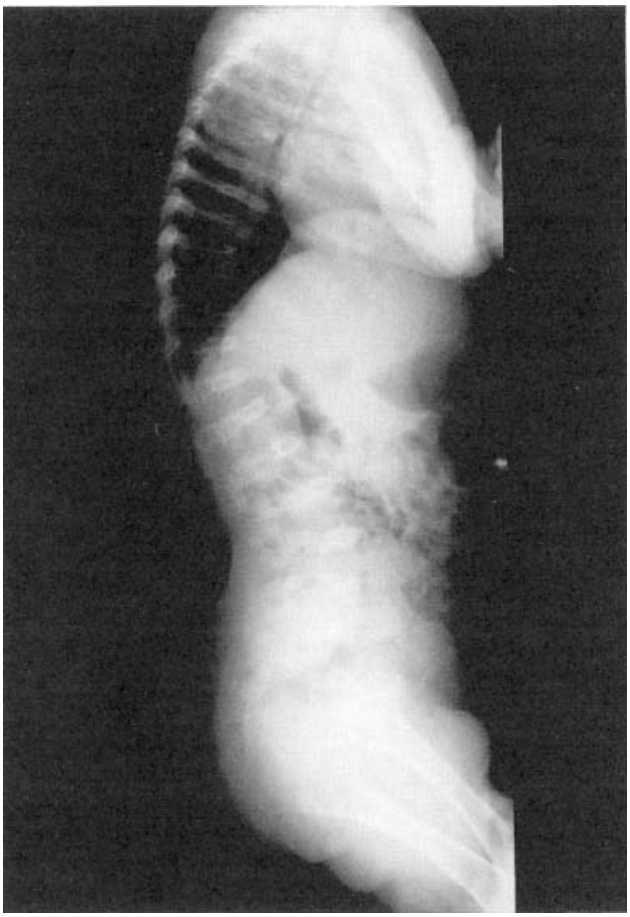


Figure 11. Radiograph of a newborn with dominant metatropic-like spondylo-epi-metaphyseal dysplasia showing marked platyspondyly.

platyspondyly, and mild to severe odontoid hypoplasia is a constant finding. The metaphyses are flared and irregular, while the epiphyses are small and fragmented. The capital femoral epiphyses are dysplastic and have a coxa vara deformity (Fig. 11). Extra ossification centers seen around the knee in late childhood give a bulbous appearance to the knees.

Autosomal dominant inheritance has been observed, and gonadal mosaicism was suspected in one family (unpublished observation). The growth plate has not been examined, and the basic defect is not known.

Brachyolmia

Brachyolmia refers to at least three conditions that present in early to mid-childhood with mild short stature that mainly affects the trunk [106–108]. The Hobaek type (MIM 271530) is an autosomal recessive form. Poorly defined back and hip pain arise typically during adolescence, and back stiffness and pain commonly occur in adulthood. Some patients may exhibit scoliosis.

Skeletal radiographs show generalized platyspondyly, which is most marked in the thoracic region. The lateral margins of the vertebral bodies extend well beyond the vertebral pedicles on the anteroposterior projection, and there is mild irregularity of the vertebral end plates. The iliac bones are short. The femoral necks are short and broad, and linear densities may extend from the physeal region into the femoral neck.

Microscopic examination shows that the epiphyseal chondrocytes are unevenly distributed, often in groups, and many are large, exhibiting a degenerative appearance [107]. They occupy large lacunae that are encircled by a broad band of interconnected dense fibrils that immunostain as type II collagen [109]. The band area stains poorly for proteoglycans. The growth plate contains fewer proliferative and hypertrophic cells than normal, and these tend to be organized into round clusters.

Two other forms of brachyolmia have been described. The Maroteaux type is inherited in an autosomal recessive manner, whereas the other (MIM 113500) is an autosomal dominant trait. The latter is sometimes called brachyrachia. Both have similar clinical features to the Hobaek type. In the Maroteaux type, radiographs are similar to those of the Hobaek type, but show rounding of the anterior and posterior vertebral borders with less extension of the lateral borders [108]. The vertebral changes are more severe in general and patients have scoliosis in the autosomal dominant type [108].

Dyssegmental Dysplasia

Two autosomal recessive disorders have been designated as dyssegmental dysplasias because of segmentation defects of the spine [110–114]. Apparent at birth, both are characterized clinically by very short extremities, an unusual facies, a short neck, and a narrow thorax. There may be a cleft palate, and joint mobility may be restricted. Infants with the milder form, the Rolland-Desbuquois type (MIM 224400), may survive beyond the neonatal period, whereas infants with the severe Silverman-Handmaker type (MIM 224410) usually die at or before birth. Other anomalies may occur, especially encephaloceles.

The two types can be distinguished radiographically [114]. The changes in the Rolland-Desbuquois type resemble those seen in Kniest dysplasia. The limb bones are short and thick. The vertebral bodies have coronal clefts and vary in size and shape on lateral view. The pelvis and scapulae are unremarkable. In the Silverman-Handmaker type, the long bone changes are greater, and there may be midshaft angulation. Segmentation defects of the vertebral bodies are severe and widespread. The ilia and scapulae are hypoplastic and exhibit a dense, amorphous pattern of ossification.

Histologic examination of the mild type shows disorganization of the growth plate and patches of thickened collagen fibrils and excessively vacuolated chondrocytes in epiphyseal cartilage. Areas of mucoid degeneration can be seen in epiphyseal cartilage in the Silverman-Handmaker type. In addition, this type shows abnormal mineralization in the lower growth plate and subchondral bone; large, unfused calcospherites have been observed by scanning electron microscopy [114].

TABLE 3. Reported Mutations of *COL10A1*

Phenotype	Type	Mutation	Reference
Schmid metaphyseal chondrodysplasia	Missense	Gly18Glu	[262]
	Missense	Cys591Arg	[116]
	Missense	Gly595Glu	[124]
	Missense	Tyr597His	[124]
	Missense	Tyr597Cys	[263]
	Missense	Tyr598Asp	[119]
	Missense	Ser600Pro	[117]
	Missense	Leu614Pro	[119]
	Missense	Asp617Lys	[124]
	Missense	Gly618Val	[125]
	Nonsense	Tyr628Ter	[118]
	Nonsense	Tyr632Ter	[127]
	Missense	Leu644Arg	[124]
	Missense	Asp648Gly	[124]
	Nonsense	Trp651Ter	[118]
	Missense	Trp651Arg	[264]
	Missense	Ser671Pro	[265]
	Deletion	Del 13 bp nt 1748	[115]
	Deletion	Del 1 bp nt 1856	[116]
	Deletion	Del 2 bp nt 1856	[118]
	Deletion	Del 4 bp nt 1864	[117]
	Deletion	Del 1 bp nt 1908	[124]
	Deletion	Del 1 bp nt 1956	[117]
	Deletion	Del 2 bp nt 1992,3	[116]
	Deletion	Del 2 bp nt 2029	[117]
	Deletion	Del 10 bp	[266]

DISORDERS DUE TO MUTATIONS OF THE GENE FOR TYPE X COLLAGEN

Mutations of type X collagen are found in Schmid metaphyseal chondrodysplasia. The condition was originally named Schmid metaphyseal dysostosis. This was a misnomer because it is not a dysostosis (a defect in bone formation) nor is the primary defect in the metaphysis. It was grouped with other disorders whose radiographic pictures were dominated by metaphyseal changes, i.e., Jansen metaphyseal chondrodysplasia (MIM 156400) and McKusick metaphyseal chondrodysplasia (cartilage-hair hypoplasia), until the mid-1990s, when mutations in the gene encoding type X collagen were found exclusively in Schmid metaphyseal chondrodysplasia [115–117].

Mutations

Over 25 mutations of *COL10A1* have been reported to date (Table 3). They are all heterozygous and cluster in the carboxyl terminal nonhelical NC1 domain of the molecule. About half lead to truncation of translated products because a premature stop codon is created by a missense mutation or encountered because a deletion shifts the reading frame. Most of the others are missense mutations that change conserved residues in the NC1 domain [115–119]. Mutations have occurred more than once at two codons, i.e., Tyr597 and Trp651.

Animal Models

Potential mouse models have been generated using both transgenic and gene targeting technologies. Jacenko et al. produced transgenic mice harboring an in-frame deletion in the helical domain of type X collagen [120]. The mice exhibited skeletal deformities, most notably kyphoscoliosis, shortening of long bones and craniofacial changes associated with structural abnormalities of the growth plate, especially compression of the hypertrophic zone [120,121]. It was suggested that the mutant type X collagen chains disrupted the assembly of type X collagen molecules in a dominant negative manner. These transgenic mice also displayed evidence of dysfunction of bone marrow, spleen, and thymus. Jacenko et al. proposed that they model spondylometaphyseal and metaphyseal chondrodysplasias.

The *Col10a1* gene in mice has been knocked out by two groups. Those knockout mice generated by Rosati et al. had essentially no abnormal phenotype [122], whereas those reported by Kwan et al. displayed coxa vara, reduced thickness of growth plate and articular cartilage, and an abnormal distribution of matrix proteins in the growth plate [123]. The latter observations suggest that type X collagen influences the distribution of matrix vesicles and proteoglycans in the growth plate and helps to provide support for endochondral ossification.

Pathogenesis

Three mechanisms have been proposed to explain how *COL10A1* mutations associated with Schmid metaphyseal chondrodysplasia act. In the first case, haploinsufficiency may result from an inability of type X collagen chains harboring mutations of essential residues or truncations in the NC1 domain to associate with normal type X collagen chains and be incorporated into triple helical molecules [116,118,124]. *In vitro* studies showing that type X collagen chains containing several Schmid mutations neither assemble into collagen molecules nor associate with normal type X collagen chains support the likelihood of this mechanism [125,126]. The second potential mechanism involves haploinsufficiency through premature decay of mutant *COL10A1* mRNA

transcripts [127]. Nonsense-mediated mRNA decay occurs commonly in transcripts harboring premature termination mutations. There is also evidence that some mutations act in a dominant negative fashion to interfere with assembly of the triple helix [120,128].

Clinical Phenotype

Schmid Metaphyseal Chondrodysplasia (MIM 156500)

This autosomal dominant disorder usually presents after the age of 2–3 years with mild short stature, bowing of the lower extremities, and a waddling gait. Hip pain may be present. Enlargement of the wrists, inability to extend completely the fingers, and flaring of the lower rib cage are associated findings [129–134].

Radiographically, the metaphyseal changes are relatively mild. The tubular bones are moderately short and metaphyses are flared, cupped, and irregularly mineralized (Fig. 12). The physeal space is widened. Changes are most marked at the hips, shoulders, knees, ankles, and wrist. The capital femoral epiphysis is enlarged in early childhood, and severe coxa vara is usually present. The distal metaphyses of the skeleton are only mildly affected. The spine is usually normal, but may show mild irregularities. Histologic changes in the growth plate are not specific.

Disorders of Unknown Etiology with Similar Clinical Phenotypes

Metaphyseal Chondrodysplasia, McKusick Type (MIM 250250)

Metaphyseal chondrodysplasia, McKusick type, is also called cartilage-hair hypoplasia [135–141]. Although growth deficiency usually does not become evident before the second to third year, dwarfism is marked in adults. Children have short extremities, bowed legs, and flaring of the lower rib cage with a prominent sternum. Hands and feet are short and broad. The fingers are short and stubby and exhibit extreme ligamentous laxity. The hair tends to be blond and thin, and the skin is lightly pigmented. Cellular immune deficiency, anemia, Hirschsprung disease, and gastrointestinal malabsorption have been observed. The disorder was first recognized and described in the Amish population, but has since been noted in other ethnic groups.

Short tubular bones with flared, irregularly mineralized, and cupped metaphyses can be seen on radiographs (Fig. 13). The knees are most severely affected. The proximal femora exhibit relatively mild involvement without coxa vara. The distal fibula is disproportionately longer than the tibia. The metacarpals and phalanges are short and broad with metaphyseal changes, and cone-shaped epiphyses may be present. Vertebral end plates may show mild irregularities.

This disorder is associated with several nonskeletal complications. Although not fully defined, affected children may have a variable immune deficiency, with particular problems with varicella infections and possibly vaccinia [142,143]. The problem with varicella infection may be limited to the Amish population, but immune function should be evaluated in all children with this disorder. Chickenpox exposure should be avoided, and the use of attenuated live-virus vaccines may pose risks of serious disease for

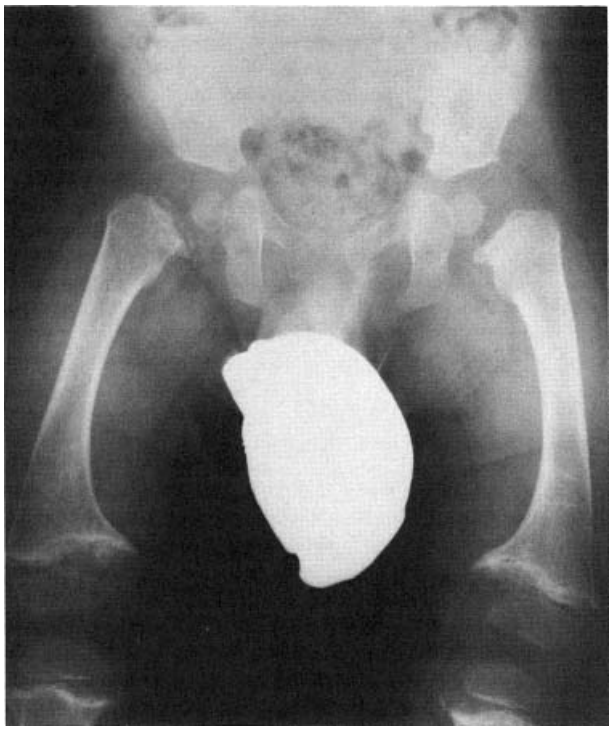


Figure 12. Radiograph of the pelvis and lower extremities of a 5-year-old child with metaphyseal chondrodysplasia, Schmid type. The tubular bones are moderately short and the metaphyses are flared, cupped, and irregularly mineralized.

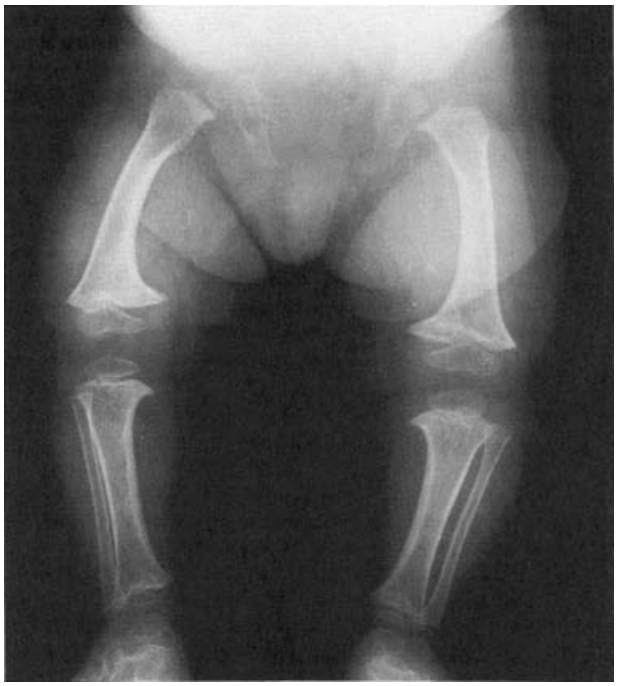


Figure 13. Radiograph of the pelvis and lower extremities of a 3-year-old child with metaphyseal chondrodysplasia, McKusick type. The tubular bones are short with flared, irregular metaphyses. The knees are most severely affected. The proximal femora exhibit relatively mild involvement without coxa vara. The distal fibula is disproportionately longer than the tibia.

children with the disorder. Malignancies of the skin and lymphoid tissue have been observed in adults in the Amish and Finnish populations [144,145]. In a large cohort of Finnish patients, the greatest cancer risk was for non-Hodgkins lymphoma and to a lesser extent for basal cell carcinoma [145].

Metaphyseal chondrodysplasia, McKusick type, is an autosomal recessive disorder. It is especially common in the Amish population, where the founder effect has increased the incidence. Uniparental disomy has been observed [146]. The disease locus has been mapped to chromosome 9p13 [147–149], but its identity is still unknown.

Other Metaphyseal Dysplasias

Growth deficiency associated with metaphyseal irregularities on radiographic examination and immune deficiency is found in three other autosomal recessive conditions [68,150–152]. The skeletal changes occur with pancreatic exocrine insufficiency, cyclic neutropenia, and other hematologic abnormalities in the Shwachman syndrome (MIM 260400) [153–156]. The second disorder is so-called metaphyseal chondrodysplasia with thymolymphopenia, and the third is adenosine deaminase deficiency [157] (see MIM 102700). The biological connection between immune deficiency and metaphyseal chondrodysplasia is not known: however, it has been suggested that cellular proliferation and differentiation, which are common to both, are defective. Moreover, transgenic mice expressing a dominant negative *COL10A1* mutation display immune-related defects [120].

Spondylometaphyseal Dysplasia

There are several disorders in which the radiographic abnormalities are primarily of the spine and the metaphyses of long bones. The best delineated is the Kozlowski type of spondylometaphyseal dysplasia (MIM 271660), which is inherited as an autosomal dominant trait [68,158,159]. It usually presents during early childhood, with a waddling gait and mild short stature mostly affecting the trunk. The hands and feet are short and stubby, and hip mobility may be mildly limited. Scoliosis may develop during adolescence. The head and face are normal. Adult heights range from 130 to 150 cm [68].

Generalized flattening of the vertebral bodies is seen on skeletal radiographs (Fig. 14). They extend laterally beyond the vertebral pedicles as seen on anteroposterior view. The iliac bones are short and flared, and the acetabular roofs are horizontal. Tubular bones exhibit flaring and irregular mineralization of the metaphyses. The latter is greatest in the femora where coxa vara is common.

Histologic abnormalities have been detected in the growth plate, but they are not specific. They include poor organization, irregular vascular invasion, and fewer proliferative chondrocytes than normal [74]. Granular inclusions have been detected in epiphyseal chondrocytes by electron microscopy [87]. Gel electrophoresis of proteoglycan extracts revealed a rapidly moving band not seen in controls, although its significance has not yet been determined [87]. Analysis of cyanogen bromide-derived peptides of type II collagen has been normal [87,160].

Ikegawa et al. have reported a mutation in *COL10A1* in a family with spondylometaphyseal dysplasia [161]. They raise the possibility that mutations in this gene may account for some cases of this disorder. However, Lachman suggests the radiographic features are more consistent with Schmid metaphyseal chondrodysplasia with mild changes of the spine [162].

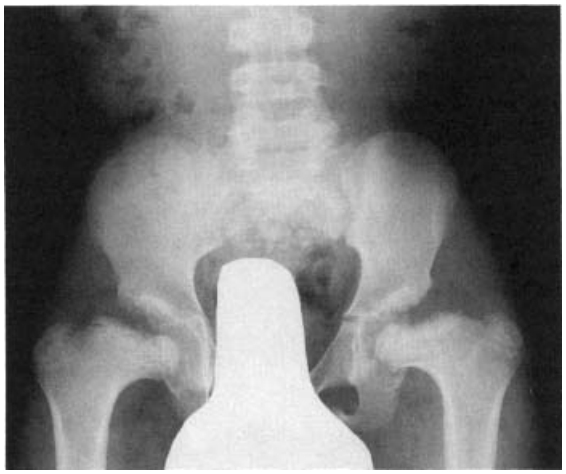


Figure 14. Radiograph of the pelvis of an 11-year-old with spondylometaphyseal dysplasia, Kozlowski type, showing a severe coxa vara deformity of the proximal femora. Note the platyspondyly.

DISORDERS DUE TO MUTATIONS OF GENES FOR CARTILAGE OLIGOMERIC MATRIX PROTEIN (COMP) AND TYPE IX COLLAGEN

The disorders in this group include pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED). MED is genetically heterogeneous (EDM1, EDM2, EDM3), but EDM1 is allelic with PSACH.

Nomenclature and History

Maroteaux and Lamy first described PSACH in 1959 and many clinical reports delineating the phenotype followed [163]. Fairbanks and Ribbing independently described the clinical and radiographic findings of a condition that involved a generalized epiphyseal dysplasia with skeletal findings similar to, but much milder than PSACH [164,165]. The term multiple epiphyseal dysplasia (MED) was coined to describe this condition. Mutation analysis studies have shown that all cases of PSACH and many of MED, specifically those of EDM1, result from mutations in cartilage oligomeric matrix protein (COMP). Case reports suggesting a recessive form of PSACH [166] are now attributed to germline mosaicism for mutant *COMP* alleles [167,168]. Mutations in *COMP* cause a range of phenotypes from normal to mild short stature with or without hip and knee pain (MED) to severe disproportionate short stature with joint symptoms (PSACH). In contrast, mutations in *COL9A2* (*EDM2*) and *COL9A3* (*EDM3*) genes cause a mild epiphyseal dysplasia [169–171].

COMP Protein and Gene

COMP is a large pentameric glycoprotein found in the extracellular territorial matrix of chondrocytes; it is also abundantly present in tendon and ligament [172–175] (see also Chapter 4, this volume). It is the fifth member of the thrombospondin gene family and has homology to the other family members, particularly thrombospondin-3 and thrombospondin-4 [176]. Although its function has not yet been defined, it may exhibit adhesive properties similar to those of other thrombospondins [177]. It has been demonstrated that both collagen types I and II interact with COMP through their C-terminal globular domains and that

this interaction is promoted by the presence of Zn^{2+} but not other cations [178].

The 2.4 kb COMP cDNA is encoded by 19 exons [176]. COMP has a bouquet appearance upon rotary shadowing, with five identical subunits or chains [179]. Each subunit is composed of a pentamer formation domain (exons 1–3), four epidermal growth factor (EGF)-like domains (exons 4–7), seven calcium-binding domains (exons 8–14), and a globular domain (exons 15–19). An alternative numbering scheme for the exons is based on the thrombospondin nomenclature (Table 4) [177]. All but three of the mutations reported to date have been identified in the calcium-binding domains, which are highly conserved between thrombospondin family members in all species [176], suggesting that this region has an important role in maintaining protein structure (Figure 15).

Mutations

COMP

Fifty-one *COMP* mutations have been reported to date, with the majority resulting in PSACH [167,168,175, 180–189]. Only eleven result in the MED (EDM1) phenotype (Table 4). The most common PSACH mutation, a 3 bp deletion involving amino acids 469–473 (exon 13, exon 17B in the alternative scheme), accounts for more than 30% of the total mutations [180]. Moreover, exon 13 appears to be a hotspot for mutations and should be targeted for mutational analysis (Fig. 15). Twenty-eight of the mutations involve aspartic acid residues that are highly conserved and make up the calcium-binding motif, DXDXDXXXDXXD, that is found in all thrombospondins [177]. Three mutations found outside the calcium-binding domains occur in exon 16 that encodes part of the globular domain. No genotype/phenotype correlations can be made, as mutations affecting the same codon have been found for both PSACH and MED [182] (Fig. 15).

Hall and Dorst [166] first suggested germline/somatic mosaicism in PSACH. Molecular analysis of *COMP* alleles in families with more than one sib affected with PSACH born to parents of average stature, confirmed that mosaicism occurs [167,168]. This suggests that all cases of PSACH are secondary to heterozygous mutations in *COMP*, and that germline mosaicism should be considered when counseling all isolated cases. A recurrence risk of 1–2% is suggested (Hecht, unpublished results) and prenatal diagnosis is possible after the mutation is identified in the affected parent. Targeted ultrasound is not a useful prenatal diagnostic procedure as the skeletal findings are not obvious at birth.

COL9A2 and COL9A3

Mutations in two of the type IX collagen genes produce a phenotype that is similar to mild MED (EDM1) [169–171]. The clinical reports suggest that mutations in these genes specifically affect the articular cartilages of the knee and, to a lesser extent, those of the hip. Overall, adult height does not appear to be reduced. Three mutations have been identified in *COL9A2* [169,171] and one in *COL9A3* [170] and each involves the intron 3 splice site and affects the processing of exon 3. The small number of reported mutations makes it difficult to predict what the spectrum of the mutations will be or to correlate genotype with phenotype.

Animal Models

There are no precise animal models for chondrodysplasias due to *COMP* or for *COL9A2* or *COL9A3* mutations. However, type IX collagen biosynthesis has been disturbed in mice by mutations introduced into *Col9a1* in two instances. In the first, mice expressing a transgene encoding a truncated form of the α1(IX) chain displayed osteoarthritis and a mild chondrodysplasia phenotype [190]. Mice null for *Col9a1* also developed osteoarthritis [191]. Hagg et al. showed that the α1(IX) deficiency prevented assembly of type IX collagen molecules and suggested that absence of these molecules predisposes to osteoarthritis [192].

Pathogenesis

Given the pentameric nature of COMP, mutations in PSACH and MED presumably exert a dominant negative effect on COMP function as 97% of all COMP molecules are predicted to have from one to five mutant subunits or chains under such circumstances [180]. However, it is not known if dysfunction requires a certain number of mutant subunits per molecule, i.e., if there is a threshold, or if mutant molecules behave differently depending on how many mutant chains they contain. Studies of chondrocytes in cartilage from PSACH and MED patients show large lamellar inclusions in rough endoplasmic reticulum that contain COMP, aggrecan and type IX collagen [87,185,188,193,194]. *In vitro* studies of redifferentiated PSACH chondrocytes indicate that the massive retention of these extracellular matrix proteins compromises chondrocyte function and causes chondrocyte death (Fig. 16) [194], suggesting that the decrease in linear growth may be related to the loss of growth plate chondrocytes. Moreover, compared with control chondrocytes, PSACH chondrocytes appear to produce smaller amounts of a matrix that contains diminished amounts of COMP [185,194]. Thus, the growth deficiency associated with COMP mutations may reflect the dysfunction and/or premature loss of chondrocytes that contribute to endochondral bone growth, whereas the precocious osteoarthritis may reflect a structurally abnormal and diminished cartilage matrix that erodes over time. Disturbed secretion of type IX collagen may contribute to the articular manifestations given the observations in mice.

Clinical Phenotypes

Pseudoachondroplasia (MIM 177150)

PSACH is an autosomal dominant disorder characterized by disproportionate short stature, short extremities, and ligamentous laxity [68,163,195]. It is distinguished from achondroplasia by a normal appearance and size at birth, a normal head circumference, and a normal to attractive facial appearance. The diagnosis is usually made between the first and second years of life, when skeletal growth decelerates and a waddling gait appears. Limb shortening becomes accentuated with age. The hands are short and broad and usually show ulnar deviation. The forearms may be bowed. Ligamentous laxity is generalized, but especially marked in the hands and fingers. However, mobility at the elbows may be restricted and flexion contractures may develop at the hips. Genu varum, genu valgum, and "windswept" deformities of the knees, in which both knees deviate in the same direction, develop in childhood. They respond to surgical correction but tend to recur. Odontoid hypoplasia occurs occasionally and, with ligamentous laxity, can cause cervical instability [196,197]. Precocious osteoarthritis of the hips and knees is typical, and surgical replacement is often required. In a natural history study, 33% of patients greater than age 19 years reported having hip replacement [198]. Developmental milestones are attained

TABLE 4. Reported Mutations of *COMP*

Phenotype	Type	Exon (Alt[1])	Mutation	Families	Reference
PSACH	Missense	8 (13C)	Asp271His	1	[168]
PSACH	Missense	8 (13C)	Leu272Pro	1	[168]
PSACH	Missense	9 (14)	Asp290Asn	1	[187]
PSACH	Missense	9 (14)	Cys292Trp	1	[168]
PSACH	Missense	9 (14)	Gly299Arg	1	[187]
MED	Missense	9 (14)	Asp302Val	1	[168]
PSACH	Missense	9 (14)	Gly309Arg	2	[188]
PSACH	Missense	10 (15)	Cys328Arg	1	[181]
MED	Missense	10 (15)	Asp342Tyr	1	[181]
PSACH	Missense	10 (15)	Asp349Gly	1	[187]
MED	Missense	10 (15)	Asp361Tyr	1	[186]
MED	Missense	10 (15)	Asp361Val	1	[187]
MED	Deletion	10 (15)	367delArgGly	1	[186]
MED	Missense	10 (15)	Cys371Ser	1	[184]
PSACH	Deletion	10 (15)	372delAsp	1	[181]
PSACH	Deletion	10 (15)	372delAsp	1	[182]
PSACH	Missense	11 (16)	Cys387Gly	1	[187]
PSACH	Deletion	11 (16)	391delProAsn SerAsp/insVal	1	[186]
PSACH	Deletion	11 (16)	395delGlnLysAsp	1	[168]
MED	Missense	11 (16)	Asp408Tyr	1	[186]
PSACH	Missense	12 (17A)	Gly427Glu	1	[189]
PSACH	Missense	13 (17B)	Asp437Gly	1	[168]
PSACH	Missense	13 (17B)	Gly440Arg	1	[186]
PSACH	Missense	13 (17B)	Gly440Glu	1	[182]
PSACH	Missense	13 (17B)	Asp446Gln	1	[185]
PSACH	Missense	13 (17B)	Asp446Gln	1	[188]
PSACH	Missense	13 (17B)	Pro449Thr	1	[189]
MED	Missense	13 (17B)	Asn453Ser	1	[182]
PSACH	Deletion	13 (17B)	457delGlu	1	[167]
PSACH	Deletion	13 (17B)	459delSer	1	[180]
PSACH	Missense	13 (17B)	Cys468Tyr	1	[180]
PSACH	Deletion	13 (17B)	469delAsp	5	[180]
PSACH	Deletion	13 (17B)	469delAsp	1	[175]
PSACH	Deletion	13 (17B)	469delAsp	2	[189]
PSACH	Deletion	13 (17B)	469delAsp	7	[182]
PSACH	Deletion	13 (17B)	469delAsp	3	[187]
MED	Insertion	13 (17B)	469insAsp	1	[267]
PSACH	Insertion	13 (17B)	469insAspAsp	1	[168]
PSACH	Insertion	13 (17B)	469insAspAsp	1	[267]
PSACH	Missense	13 (17B)	Asp472Tyr	1	[180]
PSACH	Missense	13 (17B)	Asp473Asn	1	[189]
PSACH	Missense	13 (17B)	Asp473Gly	1	[187]
PSACH	Missense	13 (17B)	Asp475Asn	1	[189]
PSACH	Missense	13 (17B)	Asp482Gly	1	[268]
PSACH	Missense	14 (18A)	Asp507Gly	1	[189]
PSACH	Missense	14 (18A)	Asp509Ala	1	[189]
PSACH	Missense	14 (18A)	Asp509Gly	1	[189]
PSACH	Missense	14 (18A)	Asp511His	1	[189]
PSACH	Deletion	14 (18A)	512delValValAspLys	1	[184]
PSACH	Missense	14 (18A)	Asp518Asn	1	[189]
PSACH	Missense	14 (18A)	Asp518His	1	[168]
PSACH	Missense	14 (18A)	Asp518His	1	[187]
MED	Missense	14 (18A)	Asn523Lys	1	[183]
PSACH	Missense	14 (18A)	Thr527Ala	1	[175]
PSACH	Missense	16 (19)	Glu583Lys	1	[168]
PSACH	Missense	16 (19)	Thr585Met	1	[182]
MED	Missense	16 (19)	Thr585Arg	1	[182]
PSACH	Missense	16 (19)	His587Arg	1	[189]

[1]Alternative exon naming scheme used by some authors. PSACH refers to pseudoachondroplasia, MED to multiple epiphyseal dysplasia.

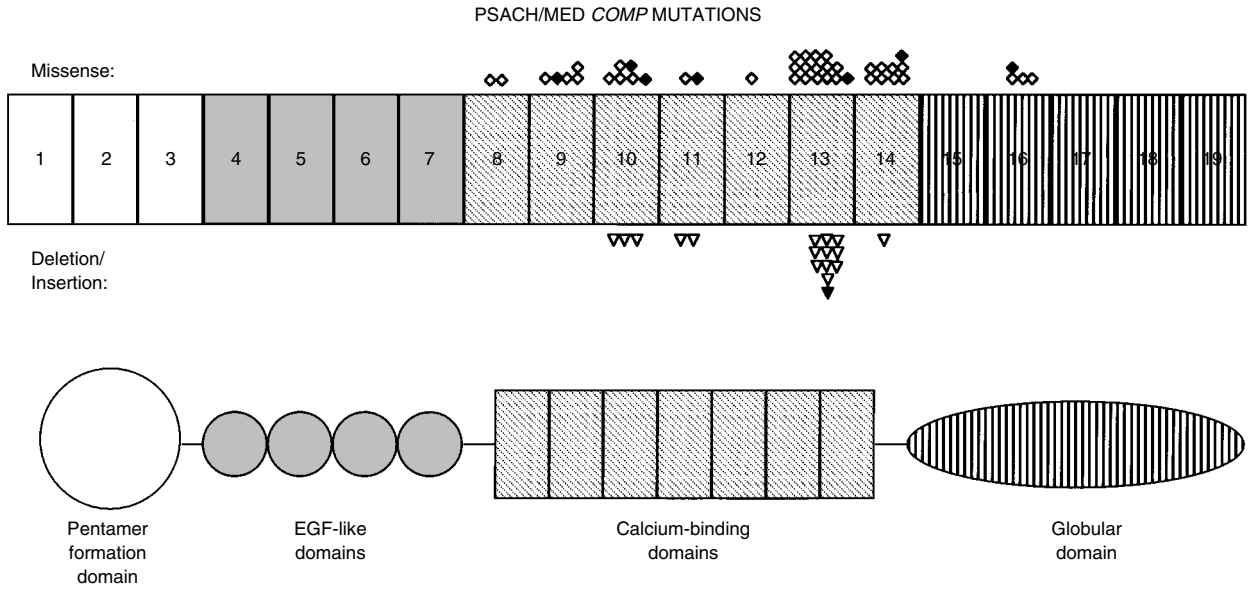


Figure 15. Genomic and protein structures of cartilage oligomeric matrix protein (COMP) are shown, corresponding exons and protein domains have similar patterns. Missense mutations are denoted by diamonds (♦) and deletion and insertion mutations by triangles (▼). The figures are open for pseudoachondroplasia (PSACH) mutations and closed for multiple epiphyseal dysplasia (MED) mutations.

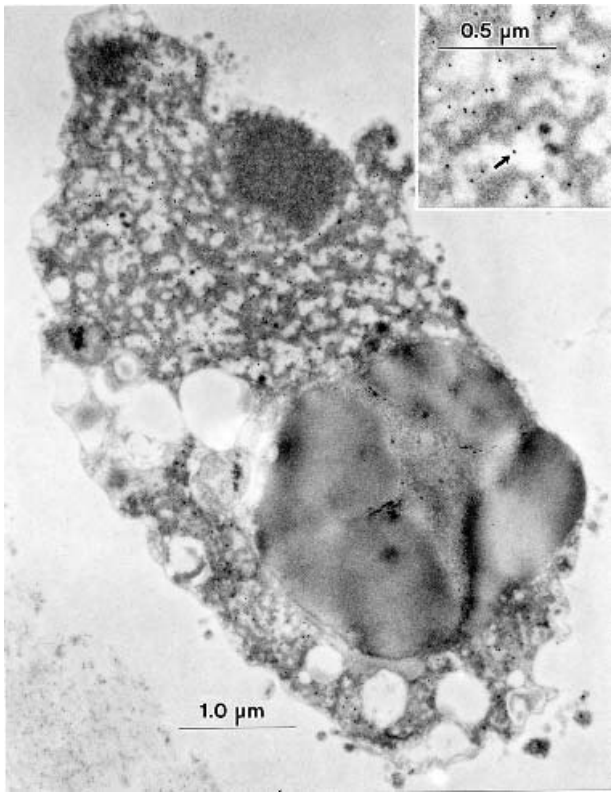


Figure 16. Transmission electron micrograph demonstrating a dead pseudoachondroplasia (PSACH) chondrocyte with large cisternae of rough endoplasmic reticulum. The presence of relatively large amounts of cartilage oligomeric matrix protein (COMP) within the cisternae is visualized by immunogold staining with anti-COMP (inset; arrow).

normally, and intelligence is normal. Adult height ranges from 105 to 128 cm [73,198].

Radiographs show involvement of epiphyses, metaphyses, and the spine [163,199]. The diagnosis can be made at birth by the presence of platyspondyly, even though affected infants are proportionate and have a normal birth length. In early childhood, the vertebral bodies display an ovoid shape and anterior tongue-like projections on the lateral view (Fig. 17). The anterior projection diminishes with age, but irregularities of the end plates persist. The tubular bones are short, with markedly irregular and flared metaphyses (Fig. 18) and the epiphyses are small and poorly formed.

Multiple Epiphyseal Dysplasia (MIM 132400)

Individuals with MED (EDM1) present in mid-childhood with an abnormal gait, hip pain, and Legg-Perthes disease (avascular necrosis of the capital femoral epiphysis) [164,165]. Stature may be slightly reduced but is most often within the normal range. Fingers are short, and genu varum and valgum may occur in childhood. The diagnosis of MED is often made in a child presenting with bilateral Legg-Perthes disease. The treatment for this complication is similar to that provided to normal children with isolated Legg-Perthes disease. Joint erosion occurs in early adulthood and hip replacement becomes necessary because of debilitating hip pain. Restriction of activities that stress the joints is strongly recommended for both PSACH and MED.

Radiographic findings in MED (EDM1) are similar to, but much milder than those seen in PSACH [199] (Fig. 19). All of the epiphyseal centers are small and underossified, especially in the hips. There is less metaphyseal and vertebral involvement compared to PSACH, although platyspondyly has been reported in MED.

In three families with mutations in *COL9A2* (*EDM2*) and in one family with a *COL9A3* (*EDM3*) mutation (Table 5), the presenting complaint was waddling gait, mild short stature, genu valgum and hip pain [169–171]. Epiphyseal

TABLE 5. Reported Mutations of *COL9A2* and *COL9A3*

Locus	Phenotype	Type	Mutation	Reference
COL9A2	MED (EDM2)	Splice	IVS3 +2 T>C skip exon 3	[169]
		Splice	EXN3 −1 G>A skip exon 3	[171]
		Splice	IVS3 +5 G>C skip exon 3	[171]
COL9A3	MED (EDM3)	Splice	IVS2 −2 A>T skip exon 3	[170]

MED refers to multiple epiphyseal dysplasia, EDM2 and EDM3 to multiple epiphyseal loci 2 and 3.

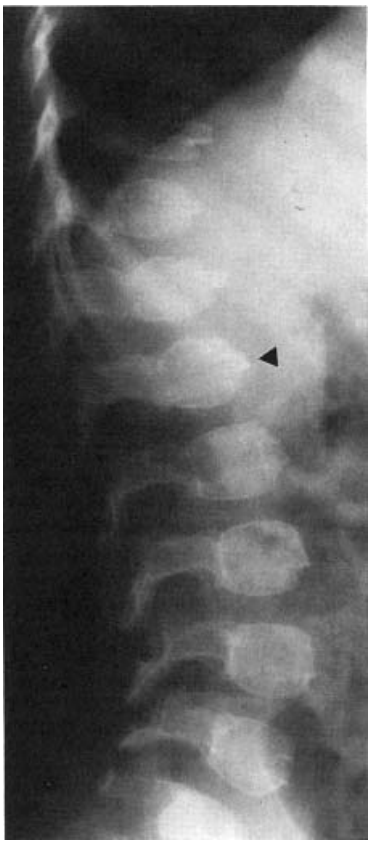


Figure 17. Lateral radiograph of the spine of a 3-year-old with pseudoachondroplasia (PSACH), showing the ovoid shape of the vertebral bodies with anterior tongue-like projections (arrowhead).

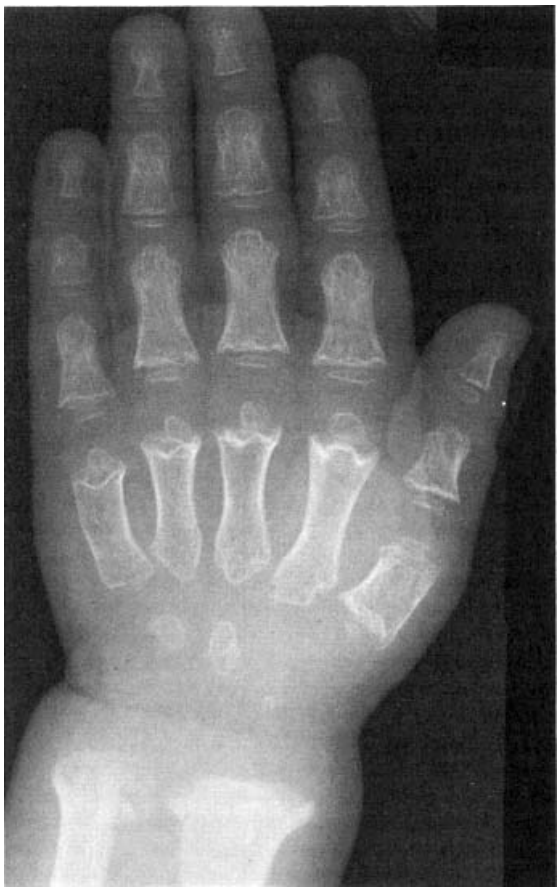


Figure 18. Radiograph of the hand of a 3-year-old with pseudoachondroplasia (PSACH). All tubular bones are short and show markedly irregular and flared metaphyses.

dysplasia appears to be limited to the large joints of the lower limbs. Hip replacement occurring after the sixth decade has been observed in a few individuals.

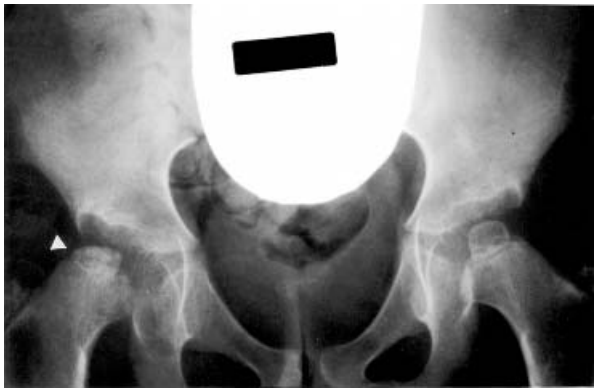
MISCELLANEOUS DISORDERS

Fibrochondrogenesis (MIM 228520)

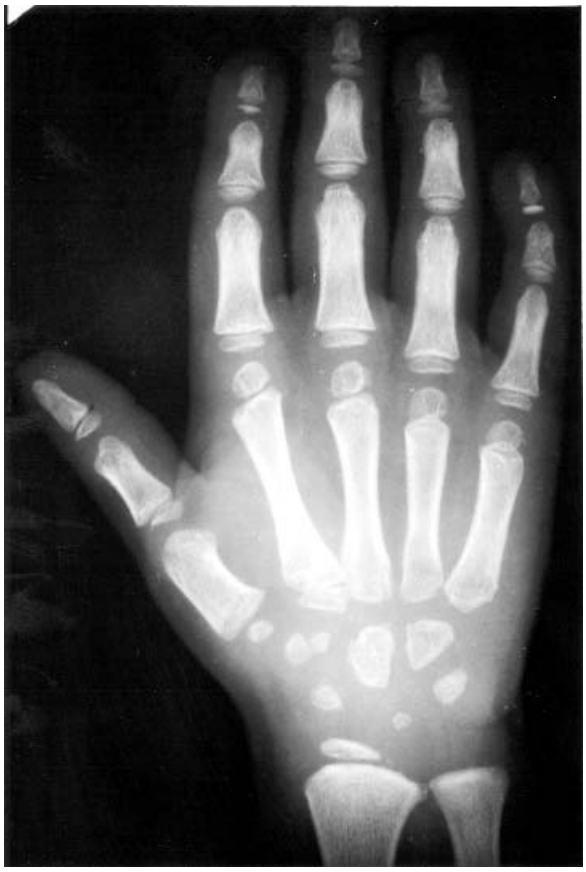
Fibrochondrogenesis is an autosomal recessive chondrodysplasia characterized by moderate shortening of the limbs, enlargement of the joints, a proportionate head and trunk, a narrow chest, and a round flat face with prominent eyes. Cleft palate is a frequent associated finding. Death occurs soon after birth from respiratory insufficiency [200–204].

The vertebral bodies are flattened and project a pyriform silhouette on lateral radiographic examination. The iliac and pubic bones are short and broad. The tubular bones are also short and broad, with irregular metaphyses. The bones of the skull, hands, and feet are normal.

The epiphyseal cartilage is hypercellular, and the cells are elongated on microscopic examination (Fig. 20). The matrix is very fibrous, forming interwoven septa through the cartilage [87]. These septa extend into and disrupt the normal architecture of the growth plate, where relatively few proliferative and hypertrophic chondrocytes are seen. Although the epiphyseal cartilage resembles fibrocartilage morphologically, cyanogen bromide-derived peptide mapping of the cartilage collagen shows only type II collagen, and the absence of type I collagen has been confirmed by immunostaining [87]. The significance of these morphological changes is unknown.



a



b

Figure 19. Radiographs of pelvis (**a**) and hand (**b**) from a patient with multiple epiphyseal dysplasia (MED) with a cartilage oligomeric matrix protein (COMP) mutation. The capital femoral epiphyses are small and the one on the patient's right shows early changes of Legg-Perthes disease (arrowhead).

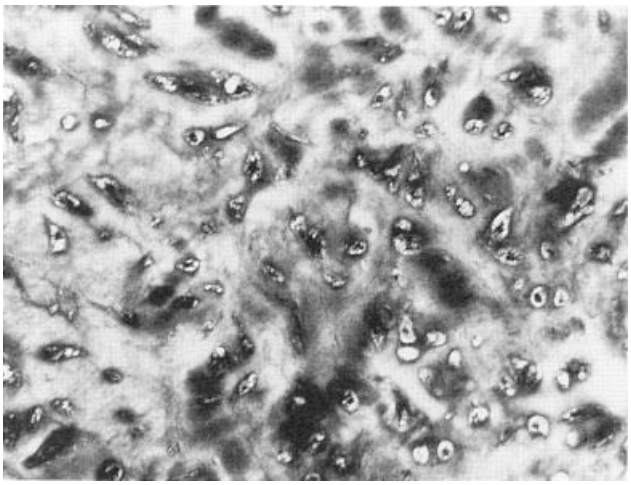


Figure 20. Photomicrograph of epiphyseal cartilage in fibrochondrogenesis. The cartilage is hypercellular and many of the cells display an elongated shape. The matrix stains unevenly and appears more fibrous than normal. Toluidine blue stain. ×200, original magnification.

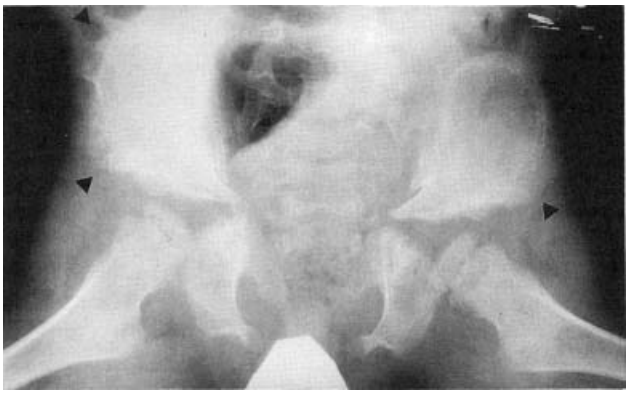


Figure 21. Radiograph of the pelvis of a 7-year-old child with Dyggve-Melchior-Clausen dysplasia. The short iliac bones have a distinctive lace-like mineralization along the iliac crests (arrowheads).

Dyggve-Melchior-Clausen Dysplasia

Dyggve-Melchior-Clausen dysplasia is a rare disorder, characterized by short-trunk dwarfism [205–208]. Growth deficiency usually becomes evident in late infancy or early childhood. The sternum becomes prominent and the lumbar lordosis exaggerated. The hands and feet are small, and the fingers have a clawed appearance. Mental retardation is a common but inconstant feature. The condition exhibits autosomal recessive inheritance in most families (MIM 223800); however, an X-linked pattern (MIM 304950) was observed in one large family [209].

Skeletal radiographs in Dyggve-Melchior-Clausen dysplasia show generalized flattening of the vertebral bodies, with irregular notches of the end plates. The iliac bones are short and broad, and there is a distinctive lace-like mineralization of the iliac crests (Fig. 21). A spur can be seen in childhood projecting medially from the femoral neck, which tends to be horizontally oriented. The capital femoral epiphysis is late to ossify. The tubular bones are short, with irregular ossification of the epiphyses and metaphyses.

The epiphyseal and growth plate cartilages exhibit heterogeneity in their morphologic appearance [210]. The matrix is extremely fibrous in some areas. Dense bundles

of fibers traverse both cartilages, disrupting the normal architecture and giving an appearance similar to that of fibrocartilage. Immunostaining, however, reveals that the fibers are comprised of type II collagen, rather than type I collagen as in fibrocartilage. In other areas of both epiphyseal and growth plate cartilage, the matrix is much less fibrous. Epiphyseal chondrocytes in these areas are found in clusters containing as many as 10 cells. The clusters stain intensely for proteoglycan, and many of the cells contain cytoplasmic inclusions. The clusters may also be found in the growth plate; those near the cartilage-bone interface are associated with deposits of mineral, which are much larger than normal.

Patients with features of Dyggve-Melchior-Clausen dysplasia who lack mental retardation are sometimes referred to as having Smith-McCort dysplasia [211]. Nakamura et al. observed dilated cisternae of the rough endoplasmic reticulum in iliac crest chondrocytes from two patients with this condition [212].

Craniometaphyseal Dysplasia

This disorder is characterized by craniofacial hyperostosis accompanied by metaphyseal widening and diaphyseal sclerosis of tubular bones [213–216]. It is usually evident during infancy from the excess of bone over the bridge of the nose, the prominent forehead, and ocular hypertelorism. Hyperostosis of bones at the base of the skull may encroach upon cranial foramina leading to hearing loss, optic atrophy and facial paralysis. Intelligence is normal. Diaphyseal sclerosis dominates long bone radiographs of affected infants, while club-shaped metaphyseal widening of these bones is typical in children and adults. Life expectancy and adult height are usually normal.

Craniometaphyseal dysplasia must be distinguished from Pyle disease, which has less skull and more metaphyseal involvement radiographically, and craniodiaphyseal dysplasia, which exhibits more severe sclerosis of the skull and long bone abnormalities that involve diaphyses more than metaphyses [99]. Both autosomal dominant and recessive forms of craniometaphyseal dysplasia have been delineated. The recessive form is more severe. The dominant form has been mapped to chromosome 5p15.2–p14.1 [217].

Acromelic Dysplasias

The acromelic dysplasias are a heterogeneous group of disorders in which the skeletal abnormalities mainly affect the distal extremities [68,218]. They all show some degree of growth deficiency, indicating involvement of bones that contribute to height as well. In autosomal dominant peripheral dysostosis (MIM 170700), affected persons usually present during adolescence with swelling and reduced mobility of finger joints [219–221]. The hands and feet are short. Radiographs show shortening of metacarpals, metatarsals, and phalanges, with mild metaphyseal abnormalities and cone-shaped epiphyses of these bones.

Acrodysostosis

Acrodysostosis (MIM 101800) is a sporadic disorder, characterized by extremely short hands and feet and an abnormal facies, consisting of a low nasal bridge, short nose, and anteverted nostrils [222,223]. Patients are usually short from birth and often exhibit obesity and mental retardation later. Radiographs show short and wide metacarpals, metatarsals, and phalanges, with cone-shaped epiphyses. Brachycephaly and nasal hypoplasia are seen on skull films, and there may be mild irregularity of the vertebral end plates.

Tricho-Rhino-Phalangeal Syndrome

The tricho-rhino-phalangeal syndrome type I (TRPS I) (MIM 109350) is an autosonal dominant condition [224]. Patients exhibit thin, sparse hair, a pear-shaped nose, mild short stature, and hands and feet that are short and broad. Short metacarpals, metatarsals, and phalanges with cone-shaped epiphyses are observed on radiographs. These features are combined with prominent ears, microcephaly, micrognathia, mental retardation, and multiple cartilaginous exostoses in TRPS type II or the Langer-Giedion syndrome (MIM 150230). TRPS type II occurs sporadically and is associated with deletions of chromosome 8q24.1 [225]. TRPS II is considered a contiguous gene deletion syndrome as the *TRPS I* and *EXT1* genes are deleted. Mutations in the *EXT1* gene give rise to hereditary multiple exostosis syndrome (MIM 133700), an autosomal dominant condition associated with inappropriate bone growth (exostoses) at the ends of the long bones [226]. Deletion of one copy of the *EXT1* gene in TRPS II is also associated with unregulated bone growth that is identical to that observed in hereditary multiple exostosis syndrome. The *TRPS 1* gene is a novel zinc finger gene with significant homology to GATA-binding transcription factors. Four mutations in TRPS I patients have been identified [227].

Larsen Syndrome

Larsen syndrome is characterized by multiple congenital joint dislocations, an unusually flat face with a prominent forehead, depressed nasal bridge and widely spaced eyes, and abnormal hands with relatively short metacarpals and cylindrical fingers [99,228–230]. Around 50% of affected infants have a cleft palate or uvula. The dislocations usually involve the hips, knees, and elbows, and laxity of the costochondral cartilage has been described. Other manifestations include short fingernails, equinovarus deformity of the foot, delayed dental development, and other dental abnormalities. Cardiovascular lesions, including congenital septal defects and acquired lesions similar to those found in the Marfan syndrome, i.e., mitral prolapse, aortic dilatation, have been reported [231]. Motor developmental delay is common due to the orthopedic deformities, and spinal cord compression may result from excessive mobility of vertebral bodies. Osteoarthritis secondary to the joint deformities is a common complication in adulthood.

The dislocations are evident radiographically and secondary epiphyseal deformities may be detected. Abnormal curvature of the spine may be seen, especially in the cervical region [232]. Radiographs of the hands often show extra carpal bones, as well as irregularities of the metacarpals and phalanges. An extra calcaneal ossification center is characteristic of Larsen syndrome. It usually appears during late infancy and fuses with the main calcaneal ossification center during childhood.

Most cases of Larsen syndrome are sporadic. However, dominant inheritance has been seen (MIM 150250), as has the occurrence of the condition in sibs of unaffected parents suggesting autosomal recessive inheritance (MIM 245600). However, in at least one instance, evidence of germ line mosaicism has been detected in a family in whom recessive inheritance was suspected [233]. A Larsen syndrome locus, *LAR1*, has been mapped to chromosome 3p in the region of, but distinct from, the *COL7A1* locus [234].

RECENT DEVELOPMENTS

X-Linked Spondyloepiphyseal Dysplasia Tarda

The spectrum of *SEDL* mutations associated with X-linked SED tarda has been defined in different ethnic populations. Gedeon et al. found 21 different mutations in 30 unrelated cases [269]. These included missense and nonsense mutations, and insertions, deletions, and putative splicing mutations, all predicted to change the reading frame, leading to a premature stop codon. Deletions accounted for more than half of the mutations. Several recurrent mutations were detected. In one case, RNA analysis indicated that *SEDL* had escaped X-inactivation [270]. A comparison of *SEDL* with homologs in other species suggests that the gene product, sedlin, normally promotes vesicular transport from the endoplasmic reticulum to the Golgi apparatus, leading to speculation that the secretion of cartilage matrix proteins is disturbed in SED tarda [270].

Dyssegmental Dysplasia and Schwartz-Jampel Syndrome

Mutations of *HSPG2*, the gene encoding perlecan, have been discovered in both dyssegmental dysplasia [271] and Schwartz-Jampel syndrome [272]. Perlecan is a large proteoglycan typically found in basement membranes and in other tissues such as cartilage. Similarities in growth plate morphology and ultrastructure between mice null for *Hspg2* and humans with dyssegmental dysplasia led to the identification of mutations in the latter condition. Cultured fibroblasts from a patient with dyssegmental dysplasia displayed cytoplasmic inclusion bodies that stained for perlecan, and they failed to secrete perlecan into the medium [271].

Cartilage-Hair Hypoplasia

Mutations of a gene coding for a large untranslated RNA component of an enzyme complex involved in processing mitochondrial RNA have been found in cartilage-hair hypoplasia (CHH; metaphyseal chondrodysplasia, McKusick type) [273]. The gene is designated *RMRP*. The enzyme, mitochondrial RNA-processing endoribonuclease (RNAase MRP) can cleave the "primer" RNA needed to copy mitochondrial DNA [274]. In yeast, however, most RNAase MRP resides in the nucleolus, where it is involved in processing 5.8 S ribosomal RNA which has been implicated in the translation of proteins and the control of mitosis. Ridanpää et al. attempted to measure RNAase-MRP activity in CHH cells and found no abnormality. However, it is not known if the assay, which was developed for yeast, was sensitive enough for human cells. Thus, it is not clear if the dysfunctions in CHH cells reflect disturbances in mitochondrial DNA replication or in other functions, including the processing of ribosomal 5.8 S RNA and as yet undiscovered functions of RNAase-MRP.

Two classes of *RMRP* mutations were found [273]. The first class includes insertions or deletions in the promoter region. The second class consists of single nucleotide substitutions or other changes involving no more than two nucleotides in regions of the gene that are transcribed. The "ancestral" Finnish mutation (A to G substitution at position 70) falls into the second class. Among 70 CHH patients studied, mutations were detected in both alleles of 55 cases.

Multiple Epiphyseal Dysplasia

Three loci in addition to *COMP* (*EDM1*), *COL9A2* (*EDM2*) and *COL9A3* (*EDM3*) have been implicated in multiple epiphyseal dysplasia (MED). *EDM4* corresponds to the *DTDST* or alternatively named *SLC26A2* (solute carrier family 26, member 2) locus. Patients with recessively inherited MED (rMED) accompanied by club foot and double-layered patellae have been reported to be homozygous for an Arg279Trp mutation at this locus [275]. Of note, is that homozygosity for this mutation was also detected in two sibships with apparently isolated club foot [276].

EDM5 corresponds to the *MATN3* (matrilin-3) locus. Matrilin-3 is an oligomeric protein present in cartilage matrix. Chapman et al. have identified two different missense mutations in the exon encoding the von Willebrand factor A (vWFA) domain of matrilin-3 in two unrelated families with MED [277]. The sixth MED locus, which may end up designated *EDM6*, is the *COL9A1* locus. A splicing mutation in *COL9A1* has been detected in one family with dominant MED [278].

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Chapter 23, Part III

Skeletal Dysplasias Related to Defects in Sulfate Metabolism

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SUMMARY

- **Sulfated proteoglycans are abundant components of skeletal and connective tissues and particularly of cartilage. Genetic defects in the metabolism of sulfate may affect the biosynthesis or the degradation of sulfated proteoglycans and thus perturb the homeostasis of the extracellular matrix.**
- **Impaired degradation of sulfated proteoglycans, caused by deficiency of one of various lysosomal sulfatases, is found in the lysosomal storage disorders (see Chapter 22, this volume).**
- **Defective synthesis of sulfated proteoglycans has been observed more recently: mutations in two genes involved in sulfate metabolism, *DTDST* (for diastrophic dysplasia sulfate transporter, a sulfate/chloride exchanger of the cell membrane) and *PAPSS2* (phosphoadenosine-phosphosulfate synthase 2, an isoform of a bifunctional enzyme responsible for the metabolic activation of inorganic sulfate to the high-energy sulfate donor, PAPS) may impair the sulfation of newly synthesized proteoglycans and produce a clinical phenotype of skeletal dysplasia.**
- **Mutations in the *DTDST* gene may produce a spectrum of disorders that ranges from lethal, generalized skeletal hypodysplasia (achondrogenesis 1B, atelosteogenesis 2) through a non lethal, short-stature dysplasia (diastrophic dysplasia), to a relatively mild dysplasia with normal stature (recessive multiple epiphyseal dysplasia). The pathogenesis involves sulfate depletion in cells with high sulfate requirement, such as chondrocytes, leading to insufficient sulfation of cartilage chondroitin sulfate proteoglycans, the severity of the phenotype being determined by the nature of the *DTDST* mutations and the residual activity they allow for.**
- **Homozygosity for a stop codon mutation in the *PAPSS2* gene has been observed in members of a family affected by moderately severe spondylo-epi-metaphyseal dysplasia and short stature.**
- **The consequences of phosphoadenosine-phosphosulfate synthase deficiency have been extensively studied in the mouse model *brachymorphic*, which has a phenotype of skeletal dysplasia with short limbs and is caused by a mutation in the orthologous gene *PAPSS2*.**

SULFATE AND THE EXTRACELLULAR MATRIX

Proteoglycans comprise a broad category of macromolecules consisting of core proteins to which from one to several hundred glycosaminoglycan side chains are attached [1,2]. The glycosaminoglycan side chains are composed of repeated disaccharide-sulfate units. Proteoglycans are present in the cell nucleus, the cytoplasm, and the cell membrane, but they are particularly abundant in the extracellular matrix of connective tissues [1,2] (see also Chapter 4, this volume). Proteoglycans are highly sulfated. While sulfation of certain amino acid residues of the core protein has been observed, the bulk of proteoglycan sulfation is directed toward the amino group of N-acetyl-glucosamine and N-acetyl-galactosamine sugars in the glycosaminoglycan side chains of the proteoglycans. Here, up to 80–90% of susceptible sugar residues may be sulfated. Because sulfate groups are dissociated at physiological pH values, sulfated proteoglycans exist as polyanions. There is little direct experimental evidence highlighting the role of sulfate groups in proteoglycans, but some insight has been gained by the observation of the phenotypic consequences of disturbances in proteoglycan sulfation, both in the human and the experimental animal.

THE CELLULAR METABOLISM OF SULFATE

Biological sulfation involves such diverse molecules as proteoglycans, membrane lipids, plasma proteins, and xenobiotics detoxified in the liver [3]. Early studies on the sulfation of phenol in liver extracts showed that ATP could significantly accelerate the sulfation reaction (reviewed in [4]). This observation led to the hypothesis that, by analogy to the phosphate donor, ATP, there be a''universal'' sulfate donor that could serve for different substrate-specific sulfotransferases [4]. Studies in yeast, liver tissue, and cartilage tissue have confirmed this hypothesis by showing that the postulated universal high-energy sulfate donor for sulfotransferase reactions exists in the form of phosphoadenosine phospho-sulfate (PAPS). Investigations into the biosynthesis of the high-energy sulfate donor, PAPS, led to the

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recognition of the metabolic pathway called the''metabolic activation of sulfate'' [3,4](Fig. 1). Inorganic sulfate anions in the cytoplasm may either be derived from extracellular fluid, or formed in the cytoplasm by catabolism of sulfur-containing amino acids and other thiol compounds with the production of sulfite which is oxidized to sulfate. The transit of sulfate ions across cell membranes and epithelial tissues is mediated by carrier proteins. Sulfate transport mechanisms have been identified in the intestinal epithelium [5], renal tubular epithelium (NaSi) [6,7], the basolateral and apical membranes of liver cells [8–10], endothelial cells (SUT-1) [11], fibroblasts [12], smooth muscle cells, and Chinese hamster ovary (CHO) cells [13]. Sulfate transporters function either as sulfate-chloride exchangers (or more generally anion exchangers, as other anions may be transported by some of them) or as sodium-sulfate cotransporters. A coordinated model of the role and importance of each of these transport systems in whole-body sulfate homeostasis in man has not yet emerged [14]. Plasma sulfate concentrations in humans span a broad range (approximately 200–450 μM; higher values may be seen during pregnancy [15]), and the mechanisms by which plasma sulfate is regulated are still not clear.

Cytoplasmic Sulfate and Proteoglycan Synthesis

Studies on the synthesis and sulfation of proteoglycans in various types of cultured cells have shown that sulfate groups on proteoglycans may be derived both from the intracellular catabolism of cysteine and other thiols, and from the extracellular fluid. The relative contribution of the two pathways to the pool of intracellular sulfate apparently varies from one cell type to another. Ion uptake and inhibition studies have indicated that in fibroblasts and chondrocytes, sulfate ions are taken up by an anion exchange mechanism driven by the chloride gradient: intracellular chloride ions are released while sulfate ions are taken up by the cell [12,16]. The molecule responsible for this exchange appears to be the chloride-sulfate exchanger designated diastrophic dysplasia sulfate transporter (DTDST; see below). The availability of extracellular sulfate to cultured cells can be curtailed by reducing its concentration in the culture medium, by competing with chlorate, or by inhibiting its uptake with 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid [16,17]. Under these conditions, some cell types synthesize proteoglycans that are undersulfated [16–23]. Chondrocytes appear to be mostly dependent on extracellular sulfate for proteoglycan sulfation [17,20], whereas CHO cells appear to make more extensive use of cysteine-derived sulfate [13], and in fibroblasts both pathways are utilized [13,16,23]. Thus, a high rate of synthesis of proteoglycans seems to correlate with dependence on the uptake of extracellular sulfate for their sulfation [13]. These indirect conclusions have been confirmed by studies in cells from patients with *DTDST* mutations [24] (see below).

The Metabolic Activation of Sulfate

Cytoplasmic sulfate is activated in two steps; these are catalyzed by two distinct enzymes in lower organisms and plants [3,25] (Fig. 1). ATP sulfurylase (sulfate adenylyltransferase; EC 2.7.7.4) catalyzes the synthesis of adenosine-phosphosulfate (APS) from sulfate and ATP, and APS kinase (adenylylsulfate kinase; EC 2.7.1.25) catalyzes the synthesis of PAPS from APS and ATP [3]. In the marine worm *Urechis caupo* [26], *Drosophila* [27], mouse, rat, and man, a single bifunctional enzyme exists with both activities, ATP sulfurylase and APS kinase (formerly ''ATP sulfurylase-kinase'',

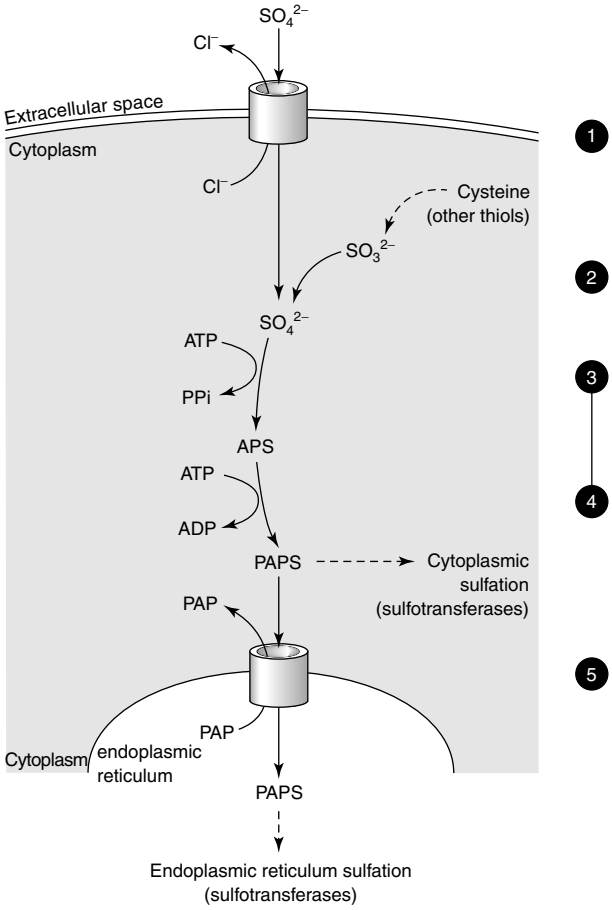

Figure 1. Simplified representation of the cellular metabolism of sulfate in higher organisms. The numbers on the right refer to the following steps: (1) Sulfate-chloride exchange by DTDST. The stoichiometry has not been determined directly, but since the transporter is not ATP-dependent or electrogenic it is assumed to be two chloride ions for one sulfate ion. (2) Sulfite oxidase, catalyzing final oxidation of thiol-derived sulfite to sulfate. (3 and 4) Two-step metabolic activation of inorganic sulfate by the bifunctional enzyme PAPS synthase, of which two genetic isoforms are known, PAPSS1 and PAPSS2. There is evidence to suggest that in some cell types, PAPSS1 (but not PAPSS2) may be localized to the cell nucleus rather than the cytoplasm [41]. (5) Translocation of PAPS into the endoplasmic reticulum by a putative PAPS-PAP antiporter. DTDST, diastrophic dysplasia sulfate transporter; PAPS, phosphoadenosine-phosphosulfate; PAP, phosphoadenosine-phosphate.

ATPSK [28–30]; now ''PAPS synthetase'', PAPSS [31]), contained in the C-terminal and N-terminal segments of the protein, respectively [32,33] (see below). Studies on the bifunctional enzyme of rat chondrosarcoma and mouse cartilage have shown that the substrate of the second reaction is much more likely to come directly from the first active site of the enzyme than from the surrounding fluid, i.e., there is ''channeling'' of substrate from one active site to the other [34,35]. This is consistent with the observation that the equilibrium for the first reaction is shifted toward inorganic sulfate: ''channeling'' of APS toward the second reaction removes product and facilitates the first reaction. Once formed, PAPS is relatively stable. It is the sulfate

donor for sulfation in the cytoplasm. For the sulfation of most membrane-bound and secreted molecules (such as most proteoglycans), PAPS must be transferred to the endoplasmic reticulum and/or the Golgi [36]. Earlier suggestions of a nucleoside sulfate/nucleoside exchange system in Golgi membranes [37] have been partially modified by newer studies that have suggested a PAP/PAPS antiporter activity residing in a 230 kDa protein [38,39]. The role of a further PAPS translocase activity from rat liver Golgi associated with a 70 kDa protein present as a dimer is unclear [40]. Studies on the sulfation of xenobiotics have shown that PAPS availability is the rate-limiting factor in many sulfotransferase reactions [36].

Sulfate activation may not necessarily occur in the cytoplasm in all cell types. Recent evidence suggests a different localization of two PAPSS isoforms (see below): while PAPSS2 is cytoplasmic, PAPSS1 may be associated with the nucleus [41]. The significance of the nuclear localization of PAPSS1 is not yet clear, but may have to do with the abundance of sulfated proteoglycans in the nuclei of certain cell types [42,43].

Deficiencies in specific sulfohydrolases responsible for the lysosomal degradation of sulfated proteoglycans and lipids result in lysosomal storage disorders classified under the mucopolysaccharidoses (see Chapter 22, this volume) or leukodystrophies. Mutations in a neutral arylsulfatase, ARSE, have been implicated in the pathogenesis of one form of chondrodysplasia punctata (see Part II, this chapter), while mutations in, or deletion of, a steroid sulfatase gene are responsible for X-linked ichthyosis [44] (see also Chapter 24, this volume). Defects involving the cellular uptake and the metabolic activation of sulfate, caused by mutations in the *DTDST* and *PAPSS2* genes, have been identified in patients with skeletal dysplasias and are described below.

The Diastrophic Dysplasia Sulfate Transporter (*DTDST*) Gene

The isolation of a human sulfate/chloride exchanger gene was the result of a long-term project aimed at elucidating the molecular basis of diastrophic dysplasia (DTD), a skeletal dysplasia present in most human populations, but particularly frequent in the Finnish population because of a founder effect [45]. The locus responsible for this disorder was initially mapped to chromosome 5 [46]. A more precise mapping to 5q32–q33.1 was obtained using linkage disequilibrium with polymorphic markers on chromosome 5 in the Finnish population [47], as well as with data obtained during the cloning of the Treacher-Collins syndrome gene (MIM 154500). A candidate cDNA encoded by a gene from that region [48] was noted to have homology with *Sat-1*, a rat hepatocyte sulfate transporter [10]. The hypothesis that diastrophic dysplasia was associated with impaired sulfate transport was confirmed by the demonstration of reduced sulfate uptake in cultured fibroblasts from patients with DTD and by the demonstration of mutations in the candidate gene in such individuals [48]. Recognition that the gene responsible for DTD encoded a sulfate transporter was a surprise because there was no prior experimental evidence of abnormal sulfate or proteoglycan metabolism in this disorder.

The coding sequence of *DTDST* (GenBank accession number U14528) is organized in two exons separated by an intron of approximately 1.6 kb, and predicts a protein of 739 amino acids that, by hydrophobicity analysis and analogy with *Sat-1*, was predicted to have 12 transmembrane domains as well as a carboxyl terminal, moderately hydrophobic domain [48]. A further *DTDST* exon has been identified in 5′-position to the two coding exons. This 5′ exon does not contain coding sequence [49].

The DTDST protein (Swiss Prot accession number P50443) belongs to the family of sulfate permeases and has been assigned number 2.A.53.2.1 in the transporter classification system (http://www-biology.ucsd.edu/~msaier/transport/titlepage2.html). Its overall structure, with 12 membrane-spanning domains (Fig. 2), is shared with two other human anion exchangers, PDS (a chloride-iodide transporter involved in Pendred syndrome; MIM 274600 [50]) and CLD (a chloride-bicarbonate exchanger, mutations in which are responsible for congenital chloride diarrhoea, MIM 214700 [51]; CLD was previously known as DRA, for "down-regulated in adenoma"), and there is significant amino acid homology between these three evolutionarily related anion exchangers (see the review by Everett and Green [52]). The function of the carboxyl terminal hydrophobic domain of DTDST is not known, but it has been observed that this domain shows homology to bacterial proteins known as antisigma-factor antagonists, which are kinase antagonists and themselves kinase substrates [53]. A similar cytoplasmic domain is present in CLS and PDS, and the name STAS domain (for sulfate transporter and anti-sigma factor antagonist) has been proposed [53]. If homology between the bacterial anti-sigma antagonists and the carboxyl terminal domain of these three transporters has functional relevance, it may indicate that phosphorylation is involved in regulation of anion exchange.

Northern blot analysis showed that DTDST is expressed in many tissues, but human cartilage was not studied (compare with rat DTDST expression, below). The size of the predominant mRNA species is >8 kb, indicating that there are significant untranslated sequences [48]. Some additional data on DTDST expression in the intestine have been obtained by *in situ* hybridization [54].

Following the characterization of human *DTDST*, rat and mouse homologues have been cloned. The rat gene has five exons with the coding sequence distributed in two exons separated by an intron positioned at the same position as

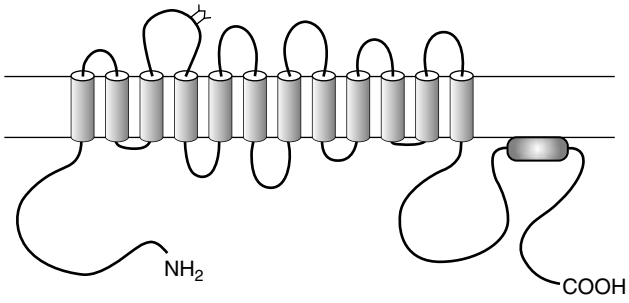

Figure 2. Schematic representation of the DTDST protein as deduced from the primary amino acid sequence (redrawn from [48]). The transporter is predicted to have 12 transmembrane domains and a moderately hydrophobic cytoplasmic domain near the carboxyl-terminus, which has been shown to be homologous to bacterial proteins known as antisigma-factor antagonists, and tentatively called the STAS domain [53] (and see text). This overall structure is shared by the two related anion exchangers, PDS and CLD [52].

in human *DTDST* [55]; the rat protein is composed of 739 amino acids like the human protein. Expression of *Dtdst* in rat is widespread, but highest in cartilage and intestine. Ion uptake and inhibition studies using *Xenopus* oocytes transfected with rat *Dtdst* mRNA confirmed its function as a sulfate/chloride exchanger [55]. A mouse cDNA orthologous to human *DTDST* was cloned during an effort to identify genes induced by bone morphogenetic protein (BMP)-2 and thus associated with osteoblastic differentiation [56,57]. The mouse protein also has 739 amino acids with 81% identity to human DTDST.

The Phosphoadenosine-Phosphosulfate Synthase Genes: *PAPSS1* and *PAPSS2*

Murine and human cDNA and genes coding for phosphoadenosine-phosphosulfate synthase (PAPSS; formerly, ATP sulfurylase-APS kinase) have been characterized by several groups [31–33,58]. Studies on the *Brachymorphic* mouse and on human spondylo-epi-metaphyseal dysplasia (SEMD; see below) have revealed that there are at least two PAPSS genes, *PAPSS1* (MIM 603262; GenBank accession number Y10387) and *PAPSS2* (MIM 603005; GenBank accession number AF091242), present in both mouse and man. *PAPSS1* is on human chromosome 4, while *PAPSS2* is on human chromosome 10 and mouse chromosome 19 [59,60] (to which the brachymorphic locus had earlier been mapped [61]). There are preliminary indications of different expression patterns of the two genes [59,60] (see below). The PAPSS1 protein (Swiss-Prot accession number O43252) and the PAPSS2 protein (Swiss-Prot accession number O95340) are highly homologous; they have two distinct domains, an amino-terminal domain containing the kinase activity, and a carboxyl terminal domain with sulfurylase activity. The mouse proteins (and genes) have a similar arrangement, while each domain has homologs in lower organisms, but the two activities reside on distinct peptides in those species (a detailed list of homologues can be retrieved at http://protein.toulouse.inra.fr/prodom.html). The human PAPSS2 protein has not been extensively characterized so far, unlike the mouse enzyme that has been studied in detail to elucidate the biochemical basis of the *Brachymorphic* mouse mutant (see below).

CLINICAL PHENOTYPES ASSOCIATED WITH DEFECTIVE SULFATION

The Skeletal Dysplasia Family Associated with *DTDST* Mutations

There are currently four distinct phenotypes associated with *DTDST* mutations: achondrogenesis type 1B (ACG1B), atelosteogenesis type 2 (AO2), diastrophic dysplasia (DTD), and recessively inherited multiple epiphyseal dysplasia (rMED). Division into these four categories is useful for differential diagnosis and prognosis but has no biological basis, as the spectrum of disease as judged by both clinical (Fig. 3) and radiological criteria (Fig. 4) is continuous. This phenotypic spectrum ranges from a severe disturbance in embryonic morphogenesis, as seen in achondrogenesis 1B, to such a relatively benign condition as multiple epiphyseal dysplasia. Genotype-phenotype correlations can be drawn, but it appears that the nature of the *DTDST* mutation is not the only factor in determining phenotypic severity. Phenotypic differences have been observed between unrelated and even first degree relatives with the same *DTDST* mutation [62].

Achondrogenesis Type 1B (ACG1B; MIM 600972)

Achondrogenesis is one of the most severe forms of chondrodysplasia in humans, invariably lethal before or shortly after birth. Exact figures of the incidence of achondrogenesis are not available, but it is not exceedingly rare. Reviews with large patient numbers have been published [63,64]. The name *achondrogenesis* (Greek for "not producing cartilage") was given in 1952 by Fraccaro to the condition he observed in a stillborn female with severe micromelia and marked histologic abnormalities of cartilage [65]. The condition described shortly thereafter and also designated achondrogenesis by Grebe [66] is different, although superficially similar to Fraccaro's achondrogenesis because of limb shortening, and has since become known as Grebe chondrodysplasia or Grebe syndrome (MIM 200700). In the 1970s, the heterogeneity of achondrogenesis was recognized. Through a combination of radiological and histologic criteria, achondrogenesis type 1 (ACG1) (then also called Fraccaro-Houston-Harris type or Parenti-Fraccaro type) and type II (ACG2) (called Langer-Saldino type) were distinguished [67,68]. In 1976, it was found that chondrocytes of some (though not all) ACG1 patients contained cytoplasmic inclusions [69]. In the late 1980s, ACG2 was shown to be caused by structural mutations in collagen II and thus represented the severe end of the spectrum of the collagen II chondrodysplasias [70–73] (see also Part II, this chapter). Borochowitz and colleagues [74] and van der Harten and colleagues [75] provided histologic criteria for the subdivision of ACG1 into 1A (with apparently normal cartilage matrix but inclusions in chondrocytes) and 1B (with abnormal cartilage matrix). Using these criteria, some cases from the earlier literature can be unequivocally diagnosed as type 1B, others as type 1A [76]. In 1994, before the *DTDST* gene had been cloned, biochemical evidence of a defect in sulfate metabolism in ACG1B was obtained. Cartilage extracts derived from a newborn with ACG1B were found to stain poorly with toluidine blue after gel electrophoresis, and cultured fibroblasts synthesized proteoglycans that could be marked with radiolabeled glycine or methionine but not with sulfate [77,78]. When pulsed with $^{35}SO_4^{2-}$, fibroblasts from the same patient failed to synthesize both APS and PAPS. The original interpretation as a sulfate activation defect [77,78] analogous to that seen in the *Brachymorphic* mouse (see below) proved to be wrong, as cocultivation studies with DTD fibroblasts, sulfate uptake studies, and, finally, *DTDST* mutation analysis showed that the primary defect in ACG1B was a sulfate uptake defect caused by *DTDST* mutations and, thus, that ACG1B and DTD were allelic disorders [76].

A fetus with ACG1B is frequently in breech position and is immediately perceived as abnormal at birth (Fig. 3A). There is abundant soft tissue relative to the short skeleton giving the infant a fat or hydropic appearance and there is disproportion between the head, which is of normal or near-normal size, and length, which is much shorter than normal. The face is flat, the neck short, and the soft tissue of the neck thickened. The thorax is narrow and the abdomen protuberant. Umbilical or inguinal herniae are frequent. The limbs are severely shortened. The fingers and toes are similarly short and stubby (an important difference from ACG2). The feet and toes are rotated inwardly in a fashion reminiscent of diastrophic dysplasia (see below). ACG1B infants may die before birth, for reasons which are not understood. Even when heart action is present at birth, respiratory insufficiency and death follow shortly.

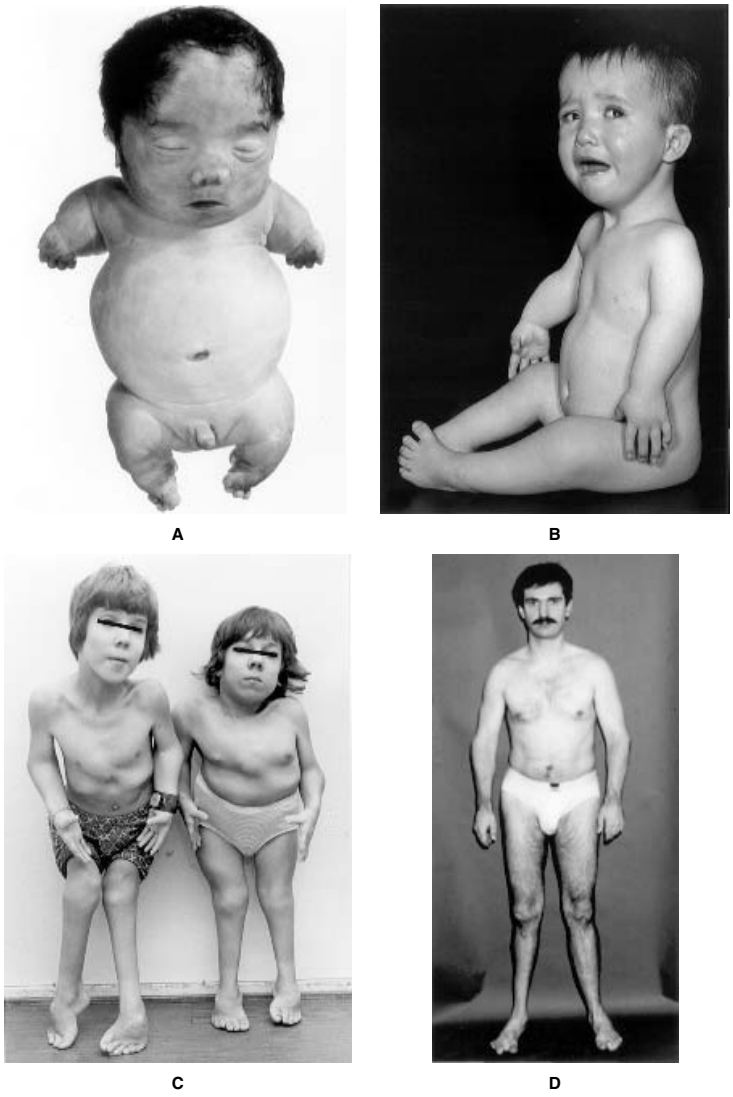

Figure 3. Clinical manifestations of DTDST skeletal dysplasias. **Panel A** shows a newborn with achondrogenesis type 1B, born at week 34, and deceased 25 minutes after birth. Note the flat face, narrow thorax with protuberant abdomen, and severe micromelia with short, stubby fingers and toes. This patient was homozygous for the DTDST *del*V340 mutation (reprinted from [78]) **Panel B** shows a two-and-a-half-year-old girl with diastrophic dysplasia; note the relatively short limbs, the contractures at the knees, and the adducted forefoot. **Panel C** shows a later stage of diastrophic dysplasia in a 14-year-old girl and her 11-year-old brother. Note the short stature and the following characteristic changes: equinovarus-adductus deformity of the feet, muscular hypoplasia of the legs and thighs, flexion contractures of the hips leading to anterior tilting of the pelvis and pronounced lumbar lordosis, limitation of elbow extension, stiff fingers, and the so-called ''hitchhiker'' thumbs. The patients, now adults, are physically handicapped but professionally active at academic level (reprinted from [147]; courtesy of Dr. I. Kaitila, Helsinki, with kind permission of the patients). **Panel D** shows an adult man with recessive MED; he has a tall-normal stature. Foot deformity and muscular hypoplasia are much less pronounced; mild finger deformities are also present (not evident in this photograph) (reprinted from [119]).

Differential diagnosis from other forms of achondrogenesis and other lethal chondrodysplasias requires analysis of radiographic features, histologic study of chondro-osseous tissue, and biochemical and molecular studies (reviewed in ref. [76]; and see below).

Atelosteogenesis Type 2 (AO2; MIM 256050[1]*)*

Sillence et al. [79] first applied this name in 1987 to a group of fetuses or newborns who had previously been considered to have severe DTD, but who had some skeletal features (e.g., a peculiar tapering of the distal humeri) that they considered similar to those seen in another, unrelated disorder, atelosteogenesis 1 (MIM 108720). Nine years later, biochemical studies and mutation analysis showed clearly that AO2 is indeed a severe form of diastrophic dysplasia [80], while AO1 is unrelated to DTDST mutations [81], and, thus, the name AO2 is misleading. It is true, though, that the gap between ACG1B and diastrophic dysplasia is so wide as to justify its occupation by an intermediate entity such as AO2. Newborns or fetuses with AO2 present with short limbs, the adducted feet

[1]The MIM entry is still entitled ''neonatal osseous dysplasia 1'', a confusing and obsolete title, which hopefully will be replaced soon.

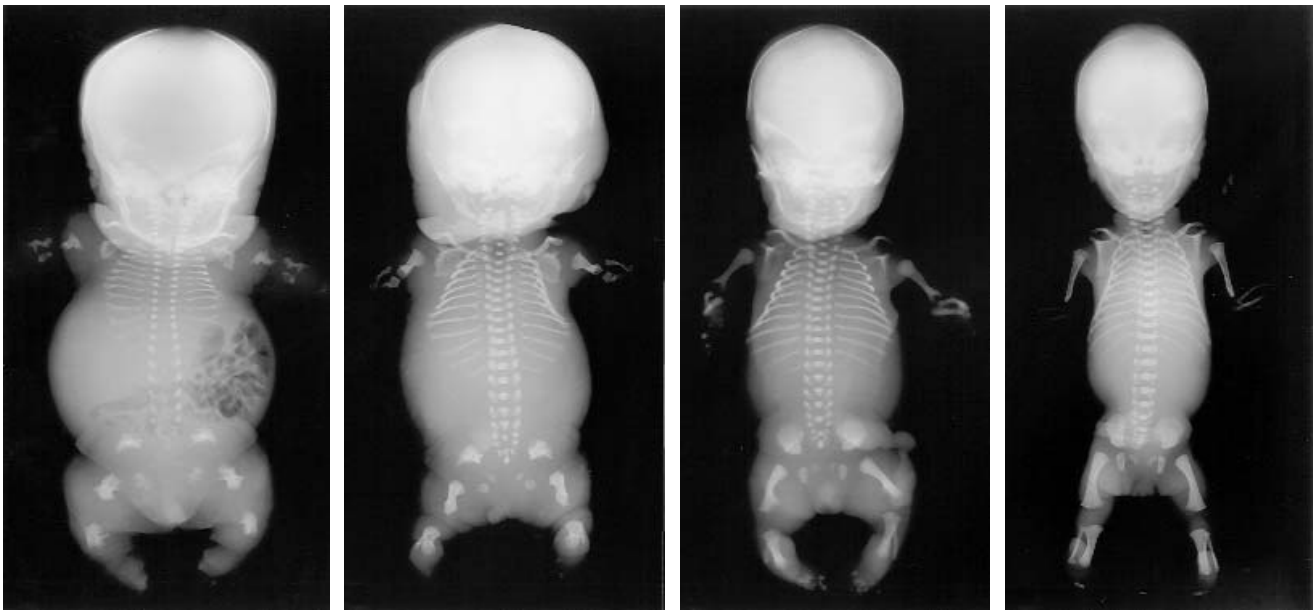

Figure 4. Skeletal morphogenesis is disturbed to different degrees in the DTDST skeletal dysplasia spectrum. The four radiographs of affected fetuses (gestational ages between 20 and 34 weeks) correspond to: achondrogenesis 1B (**panel A**); mild achondrogenesis 1B/severe atelosteogenesis 2 (**panel B**); mild AO2/severe diastrophic dysplasia (**panel C**); and mild diastrophic dysplasia (**panel D**). The gradient in severity can readily be appreciated by comparing the shapes of the femurs, as well as by comparing the lengths of the ribs to that of the spine or the clavicle (which is relatively unaffected because it undergoes desmal, rather than endochondral ossification). The forearms and hands cannot be recognized because of the relative overexposure. Note also narrowing of the interpeduncular space in the lumbar region.

with wide spacing between the hallux and second toe characteristic of ACG1B, and the"hitchhiker thumb" of DTD (see below). The disorder is usually lethal, although survival for several months has been reported. Several cases classified as "severe" or "lethal" DTD prior to 1987 would be diagnosed as AO2 today [82–84]. Radiologic features are similar to those of DTD, with tapering of the distal humerus, as observed with an AP projection radiograph, being more pronounced in AO2 (see Figs. 4B and 4C). Cartilage histology usually shows changes similar to those in DTD [85–87]. A familial, severe skeletal dysplasia known as "de la Chapelle dysplasia", or "neonatal osseous dysplasia 1," may be related to DTD and AO2 but formal proof is still lacking. It seems likely that some patients reported as having de la Chapelle dysplasia had AO2 [85], but the original patients may have had a similar but different disorder [88]. Yet another lethal chondrodysplasia, called McAlister dysplasia [89], appears to be identical to AO2 [90]. These uncertainties show how unreliable radiologic nosology may be without supporting histologic, biochemical and molecular data.

Diastrophic Dysplasia (DTD; MIM 222600)

This condition was one of the first skeletal dysplasias to be delineated clearly. It was described by Lamy and Maroteaux in 1960 as "bone anomalies that simulate achondroplasia during the first years of life but show a quite different evolution" [91]. The name diastrophic dwarfism refers to the "twisted" body shape of affected individuals [91]. DTD is a severe, though usually nonlethal, form of skeletal dysplasia with marked short stature (both the trunk and the limbs are shortened, but the limbs more severely so), bilateral clubfoot, cleft palate, characteristic hand deformities, changes of the ear pinnae, progressive kyphoscoliosis of the spine, and joint stiffness (see Figs. 3 B and C and 5).

The clinical features of DTD have been extensively reviewed [92,93]. The newborn with DTD has a normal-sized head, a slightly shortened trunk, and more markedly shortened extremities. There may be contractures at the hip, elbow, and knee joints. The fingers are short and the thumb is both proximally placed and deviated to the radial side (the so-called hitchhiker thumb). The metatarsals are usually adducted (the great toe stands out as a hitchhiker toe) and there is equinovarus deformity of the feet not dissimilar to that produced by primary neurologic disorders. The hitchhiker thumbs and toes have been observed as early as in the 15th and 16th weeks of pregnancy [94], (and unpublished observations), indicating an early developmental anomaly rather than a deformation. The face may be slightly flattened and the forehead may be broad, but these changes are not consistent in the newborn. Flat hemangiomas on the forehead are common.[2] In more than half of DTD newborns, a peculiar process affects the ear pinnae. Within the first weeks of life, the pinnae develop acute inflammation and swelling with fluctuation or cystic changes on palpation. The inflammation subsides within weeks, leaving thickened and sometimes deformed pinnae, which may eventually calcify [91,93]. When present, these changes are a valuable diagnostic

[2] The reason for this puzzling phenomenon is unknown; it suggests a link between sulfation and angiogenesis.

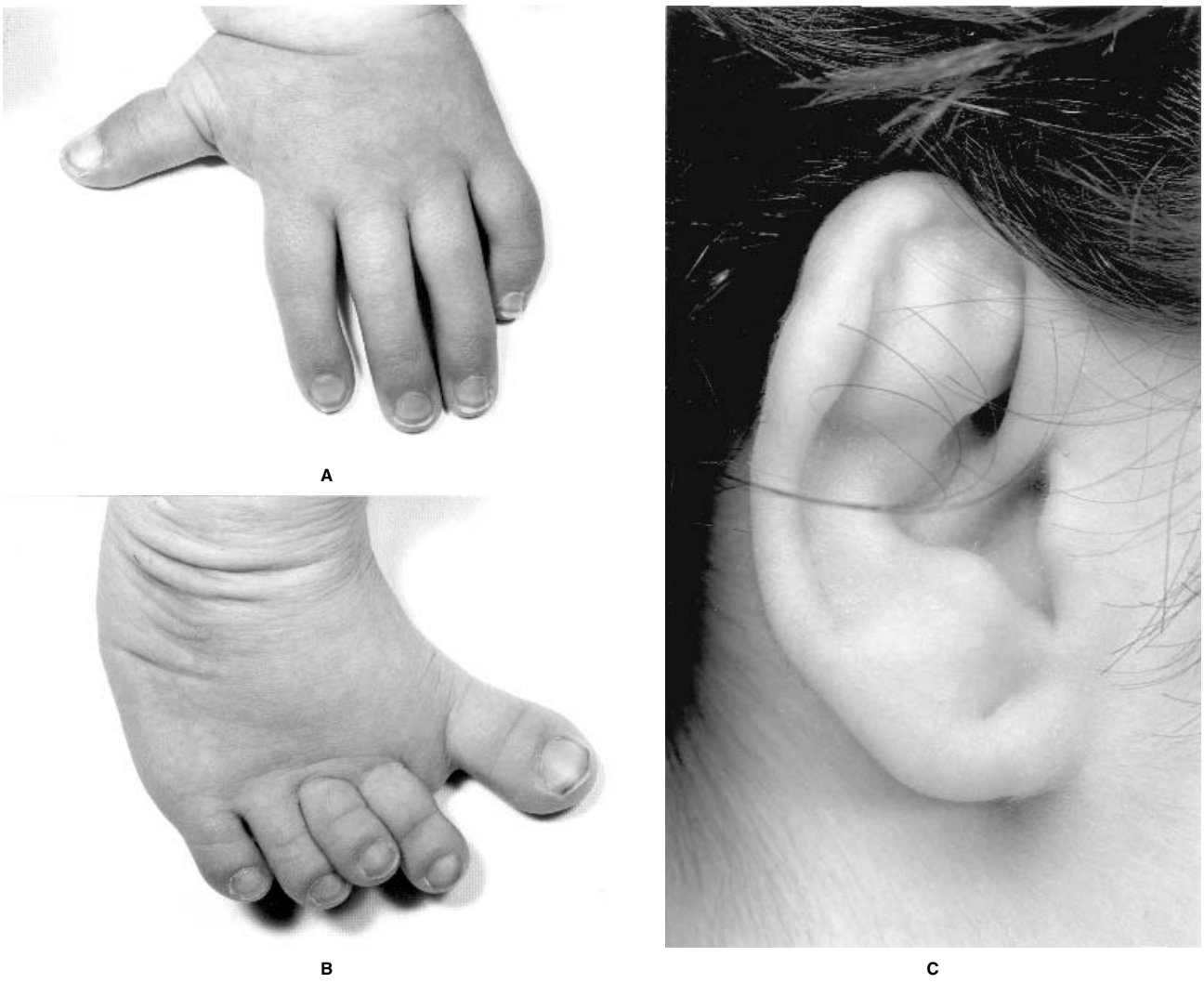

Figure 5. Accessory clinical signs in diastrophic dysplasia. Panels A and B show the hand and foot of the two-and-a-half year-old girl with DTD shown in Figure 3. The hand shows the so-called ''hitchhiker '' thumb deformity, which comprises both proximal insertion and radial deviation of the thumb. The foot shows adduction of the forefoot and wide spacing between the first and second toes; surgical release of the Achilles tendon had already been performed. The pathogenesis of these hand and foot changes, which can be seen as early as the 14th week of pregnancy, is not well understood. Their appearance suggests an increased traction of ligaments and tendons on the ventral aspect of the developing limbs; how this increased traction arises is not clear, but it may have to do with impaired growth (elongation) of tendons or with cell adhesion or proliferation. A pronounced ''hitchhiker'' thumb deformity is not pathognomonic (it can sometimes be seen in the Rubinstein-Taybi syndrome (MIM 180849)), but is highly suggestive of a DTDST skeletal dysplasia. The foot deformity often includes an abnormally short Achilles tendon leading to an *equinus* malposition (see the adolescent patients in Figure 3). The right panel shows the external ear of a 10 year-old girl with DTD. Following acute swelling and tenderness in the neonatal period, indurated nodules have developed. The pathogenesis of this phenomenon, which is pathognomonic for DTD, is not understood; among the possible triggers are changes between pre- and postnatal conditions such as plasma sulfate levels, temperature, and hydration.

clue; their pathogenesis is obscure, but may be related to the absence of an anticalcifying effect of some sulfated proteoglycans; precocious ossification of carpal bones, a double-layered patella, and duplication of sternal ossification centers (see below) may have a similar pathogenesis. Posterior palatal clefting occurs in approximately one-half of cases. Neonatal adaptation may be troubled by respiratory insufficiency because of the small rib cage and tracheal instability and collapsibility, and mechanical ventilation is required in a significant proportion of cases. Mortality in the first months of life is increased, mainly because of respiratory complications such as pneumonia, sometimes aspiration pneumonia [92,95].

The facial appearance of children and young adults with DTD, though not really dysmorphic, has some typical features (Fig. 3B). The forehead is high and broad; the palpebral fissures are relatively small and may be downwardly slanting; the nose is not shortened or stubby as in other chondrodysplasias, but, rather, long and thin because of hypoplastic *alae nasi*; the nares are not anteverted; the facial tissues are tight; the mouth is small, and the mandible normally developed.

Already in the newborn period, and throughout life, the disorder appears to involve not only the skeleton, but also the tendons, ligaments, and joint capsules. These are tighter and shorter than normal, causing restricted joint mobility. The pathogenesis of this ligamentous involvement is not understood; it looks as if growth of the tendons and joint capsules might be impaired[3]. Pretibial dimples may be present, possibly a consequence of reduced intrauterine movements. Because of foot deformities and shortened tendons, many adult individuals with DTD are unable to place their heels on the ground and thus stand solely on their metatarsals and toes. The appearance of an adult individual with classical DTD, who stands on his toes because of severe clubfoot and has marked lumbar lordosis and thoracic kyphoscoliosis (Fig. 3C), is quite typical; this appearance originally prompted the name "diastrophic" for twisted. Based on the characteristic body posture and hand changes, DTD has been diagnosed retrospectively in historical patients [96].

The skeletal changes of DTD are progressive. Degenerative arthrosis of the hip is common in young adults (pain and anterior tilting of the pelvis are the consequences), and the spine may develop excessive lumbar lordosis, thoracolumbar kyphosis, and scoliosis. In the lumbar spine, a decrease of the vertebral interpedicular distance is frequently observed, although it is not as marked as in achondroplasia; related neurological symptoms are only rarely observed (see below). The knee may be unstable in childhood but flexion contractures develop with progressive valgus deformity and lateral positioning of the patella [97]. The patella may be fragmented [97] (see also under recessive MED, below). In addition to brachydactyly, there may be ulnar deviation, phalangeal synostosis, and ankylosis of the finger, with significant disability. In older children and adults, ulnar deviation of the second finger frequently occurs together with radial deviation of the fifth finger (clinodactyly), giving a characteristic "bracket" appearance. Precocious calcification of carpal bones, simulating increased skeletal maturation, is quite common in childhood [98]. A characteristic metacarpophalangeal length pattern has been described [99]. Currarino has pointed to the peculiar double-layered ossification pattern of the sternum in DTD [100], which is reminiscent of the double-layered patella seen in recessive MED (see below). For details of the radiographic features of DTD, see [84,93,101].

In addition to the skeletal abnormalities, a mild degree of muscular hypoplasia of the thighs and legs is common. Neurologic complications may occur, particularly in the cervical region, probably owing to the vulnerability of the cervical spine in DTD and related disorders. Broadening of the cervical spine, with a characteristic "cobra-like" appearance, can often be seen on antero-posterior radiographs, and cervical kyphosis may be seen in lateral radiographs in most newborns; in most cases it ameliorates with age, but in some cases, severe cervical kyphosis may lead to spinal cord compression spontaneously and during intubation procedures [102–106]. Prenatally acquired neuronal degeneration and gliosis of the cervical spinal cord has been observed in a DTD infant with severe cervical kyphosis who died immediately after birth of respiratory insufficiency [82]. Cervical spina bifida occulta has been reported to be very frequent in DTD [102,107]. Newer MRI findings have confirmed that in DTD, the foramen magnum is of normal size but the cervical spinal canal is narrowed; the typical kyphosis may be the consequence of abnormal intervertebral disks [108].

Hearing loss is unusual, and vision defects are seldom observed, although a tendency towards myopia has been reported [109]. The absence of overt corneal changes is remarkable, considering the importance of keratan sulfate in maintaining corneal transparency; a higher affinity of PAPS for keratan-sulfotransferases rather than chondroitin-sulfotransferases may be the explanation. Mental development and intelligence are usually not affected.

Adult stature was found to range between 100 and 140 cm in an early review of American and European patients [93]. A 1982 study reported a mean adult height of 118 cm [110], while a more recent study of Finnish DTD patients (who are genetically homogeneous at the DTDST locus) gave a mean adult height of 136 cm for males and 129 cm for females [111]. Growth curves have been produced that can be used for monitoring of DTD patients [110,111]. The discrepancy in mean height between the older studies and the more recent Finnish study may be due to mutation heterogeneity but may also reflect bias towards more severely affected patients in the older studies.

Based on their experience with Finnish DTD individuals, the Helsinki group has reviewed selected clinical features of DTD such as cleft palate [112], anomalies of dentition [113], and changes in the spine [114], hip joint [115], knee [97], patello-femoral joint [116], and foot [117].

It has long been appreciated that the DTD phenotype may be more variable than in the patients originally described by Lamy and Maroteaux [91]. Affected infants dying in the perinatal period were previously diagnosed as having "severe DTD" but would now be assigned to the AO2 category (see above under AO2). Individuals with milder expression of DTD, particularly those having only mild statural deficit, were previously designated as "DTD variant," but subsequent studies have shown that the differences between classic DTD and so-called diastrophic variant are only quantitative [93] and the "death" of the DTD variant has been decreed [118]. Molecular studies have confirmed this pronouncement. In addition to interfamilial variability, several authors have observed marked differences between affected sibs [62,93,118]. These observations suggest that other genetic or epigenetic factors influence the phenotype.

Recessively Inherited Multiple Epiphyseal Dysplasia (rMED; MIM 226900)

This entity is a recent addition to the DTDST osteochondrodysplasia family. The first reported patient (Fig. 3D) had had clubfoot and hip dysplasia at birth, but no palatal clefting, hitchhiker thumb, or ear swelling, and had grown normally (adult height, 180 cm). He developed hip pain and digital deformations around puberty and was diagnosed as having MED with double-layered patellae [119]. He was shown to be homozygous (and each parent heterozygous) for the Arg279Trp allele (see below), and his fibroblasts exhibited a sulfate incorporation defect similar to that of other DTD patients [119]. The radiologic changes in the hips and the presence of clubfoot are reminiscent of DTD, but the absence of palatal clefting, ear deformity, and hitchhiker thumbs, and — most importantly — the tall-normal stature make the diagnosis of DTD (*nanisme diastrophique*) untenable and justify a separate entity. Patients with a similar form

[3]Perhaps the defective proteoglycan sulfation leads to abnormal packing of collagen fibers or to abnormal adhesiveness of fibroblasts to the extracellular matrix.

of recessively inherited multiple epiphyseal dysplasia have previously been reported [119], and grouped in the McKusick catalog under entry 226900. Following the identification of the patient mentioned above, several other patients who are homozygous or compound heterozygous for the Arg279Trp mutation and who have epiphyseal dysplasia and normal or only mildly short stature have been recognized [120]. In 12 individuals homozygous for DTDST mutation Arg279Trp, a diagnosis of skeletal dysplasia had been made at birth in 2 cases, and in childhood or adolescence in 10 cases. Abnormal findings at birth were present in four cases only (clubfoot in three, cleft palate in one); ear cysts and hitchhiker thumbs were not observed. Leading symptoms and signs were joint pain (8/12 cases), hand and/or foot deformities (4/12), scoliosis or joint contractures (each 1/12). Ten of the 12 cases suffered from chronic joint pain. Stature was below −2 SD for age in 0/2 prepubertal and 4/10 postpubertal patients. A double-layered patella had been observed in four of six patients for whom lateral knee films were available [120].

Diagnosis and Differential Diagnosis of DTDST Skeletal Dysplasias

The differential diagnosis of DTDST-associated chondrodysplasias is usually made within the context of other chondrodysplasias of similar severity and radiographic appearance. ACG1B should be differentiated from other types of achondrogenesis, fibrochondrogenesis, dyssegmental dysplasia, and even thanatophoric dysplasia, lethal osteogenesis imperfecta, and several other less well-defined conditions [76,121]. There are subtle radiographical signs suggesting the correct diagnosis, but cartilage histology and histochemistry can more reliably identify ACG1B, and the final diagnosis should rest on biochemical studies in fibroblast or chondrocyte cultures and on the identification of *DTDST* mutations. As prenatal recognition and pregnancy termination of fetuses affected by skeletal dysplasias (including ACG1B) is increasingly common, a protocol leading to precise diagnosis and thus allowing the provision of accurate genetic counseling is of great importance [76,122]. AO2 should be differentiated from other severe skeletal dysplasias as mentioned for achondrogenesis, and from other forms of atelosteogenesis [85–87,121]; as the disorder is usually lethal, the same considerations noted above for ACG1B apply. Most newborns with AO2 show foot deformities and "hitchhiker" thumbs, as seen in DTD. DTD is usually diagnosed through radiographic abnormalities and the presence of clinical abnormalities including cleft palate, clubfoot,"hitchhiker" thumb and first toes, and the development of painful swelling of the external ear cartilage days to weeks after birth. The latter signs are, however, not always present and their absence does not rule out DTD. Cartilage tissue is not routinely available from DTD patients; diagnostic confirmation can be obtained by analysis of sulfate uptake and incorporation in cultured fibroblasts or by mutation analysis (see below). Mutation analysis proved diagnostic in a DTD patient with unusual radiographic features (severe platyspondyly and broadening of the long bones) suggesting a different disorder [123].

In milder cases, the diagnosis may be difficult [62,119]. The association of clubfoot with skeletal changes suggestive of bone dysplasia, even in the absence of typical hand or ear changes or cleft palate, should alert the physician to the possibility of a DTDST disorder. The rare and still ill-defined condition known as"pseudodiastrophic dysplasia" (MIM 264180) [124] is distinguishable from true DTD by the absence of thumb and ear deformities and by differences in the radiographic changes. There is no DTDST defect in this disorder [125] (and unpublished results); pseudodiastrophic dysplasia may be related to the Desbuquois syndrome (MIM 251450), in which proximal placement and radial deviation of the thumb can be seen; interestingly, advanced bone age can also be seen in this disorder, as in DTD. A distinctive criterion is the presence of joint laxity in Desbuquois syndrome.

The biochemical diagnosis of a DTDST skeletal dysplasia can be made in fibroblast or chondrocyte cultures. When cells from ACG1B, AO2, DTD, or MED patients are incubated in culture medium containing $Na_2{}^{35}SO_4$, they exhibit a characteristic defect in the incorporation of $^{35}SO_4$ into macromolecules. ^{3}H-labeled glycine or glucosamine can be used to provide reference values [78,80,126]. Direct assay of sulfate uptake is technically difficult and thus less suitable for diagnostic purposes [24]. Mutation detection has been efficient, the *DTDST* gene being relatively small [127]. This provides an alternative to biochemical studies in those cases where no cell cultures are available. When the clinical and radiologic features are typical, mutations on both alleles can be found in over 90% of cases (see below); however, the occasional observation of patients with skeletal changes compatible with a DTDST disorder whose fibroblasts show a sulfate uptake defect, but in whom no mutation can be identified, points to the occurrence of pathogenic mutations in the noncoding parts of the *DTDST* gene that have not been explored so far [127].

Prenatal diagnosis of ACG1B, AO2, and DTD is frequently requested by families in which a previous child (or pregnancy) has been affected. When molecular studies are not available, ultrasonography may be used. Achondrogenesis can be recognized as early as weeks 13 to 15 [122,128], and AO2 and DTD a few weeks later [94,129,130]. DTD has been diagnosed (or excluded) by analysis of chromosome 5 markers linked to the disorder [131]. Identification of the mutations segregating in a given family has allowed direct prenatal diagnosis in several families at risk for ACG1B, AO2, and DTD [127]. The possibility of biochemical diagnosis by sulfate incorporation and/or uptake studies in chorionic villus biopsies or cells cultured from such biopsies has not been explored, but the need for such a diagnostic modality is limited when the molecular defects are known.

Approach to Management

The management of DTDST skeletal dysplasias depends on the time of diagnosis and on the severity of the condition itself. A frequent clinical setting is that of unexpected ultrasonographic recognition of severe fetal skeletal dysplasia *in utero*. When quite specific signs are present, such as a clearly recognizable 'hitchhiker' thumb and adducted feet, a mutation screening can be considered but will be time-consuming. Counseling of the parents regarding the probability of survival should be given on the basis of empirical evidence such as measurement of the chest and limbs. If a pregnancy is terminated or if the newborn is unable to survive, it is imperative to obtain materials for proper diagnosis—good X-rays, a source of DNA, a fibroblast culture, and cartilage for histology are minimum requirements.

Intubation may be required for respiratory distress in vital newborns affected by severe skeletal dysplasias such as AO2 and DTD. In some cases, intubation is transitory and may allow survival with reasonably good quality of life. In other cases, it may be impossible to wean the baby from the respirator and troublesome ethical concerns may arise [132].

Therefore, early decisions must be taken cautiously, and consultation with a skeletal dysplasia expert as soon as possible after birth (if not before) is recommended.

No medical treatment being available, the management of individuals with DTD or rMED must take into account orthopedic aspects on the one hand, and psychosocial, educational and professional issues on the other. Cleft palate is similar to that seen in collagen II disorders and can be treated by standard procedures [112], but great care must be taken in intubation procedures because of the potential instability of the cervical spine (see below). Treatment of the orthopedic complications of DTD is a major challenge. Casting for foot deformities is efficacious but burdened by a very high rate of recurrence (''recalcitrant'' clubfoot deformity [101]), so that surgical lengthening of the Achilles tendon and/or arthrodesis is the treatment of choice, to be performed at an appropriate age; casting may help to maintain the surgical results. Contractures at the knees and hips are difficult to treat even surgically; at those sites, physiotherapy may be the only option until painful arthrosis of the hips (caused by both the malposition of the femoral head because of the contractures and the intrinsic cartilage defect) and movement restriction will make hip joint replacement necessary (most commonly in the third or fourth decade [115,133]). Soft tissue release and tenotomies can then be combined with hip joint replacement.

The tendency to contractures makes the treatment of spinal deformity as difficult as that of lower limb deformity. Low-grade cervical kyphosis in newborns and infants is frequent, and in the majority of cases lessens with age without specific treatment [134], although in some cases, myelopathy may occur (see above). Severe thoracic kyphosis requiring surgical treatment is less frequent; combined anterior/posterior vertebral arthrodesis has been successful in one series of patients [135]. Thoracolumbar scoliosis is frequent, affecting at least 50% of DTD individuals. The spine is particularly rigid in DTD, perhaps because of the combination of stiff ligaments with abnormal vertebral disks [108]. The Helsinki team has been conservative, with only two patients operated on because of scoliosis in a series of over 100 DTD individuals [114]. These authors also cite the rare occurrence of lumbar spinal stenosis requiring laminectomy (narrowing of the spinal canal is a frequent finding in DTD; see above).

The psychological and social implications of musculoskeletal dysplasia in DTD are not unlike those seen in other skeletal conditions with short stature. These have been approached by the use of lifestyle questionnaires and health-related quality of life scores [136–143]. Areas where individuals with skeletal dysplasia have significantly lower scores include mobility, usual activities, sexual activity, marital adjustment, and physical discomfort and pain [136–143]. In addition, there are age-specific difficulties, mostly during the time of puberty, which include school and hobbies, friends, and physical appearance [142]. An open and direct approach to these problematic aspects may help the affected individual to develop coping strategies. Interestingly, it appears that unaffected individuals perceive these complications as more severe than affected individuals do; the capacity for adjustment in affected individuals is high.

The Spectrum of DTDST Mutations

Table 1 lists the *DTDST* mutations identified so far in patients and families with DTDST chondrodysplasias, including those reported previously [24,48,49,126,127]; Figure 6 shows their positions along the DTDST protein. Proof of pathogenicity has not been obtained, except for IVS1 + 2T > C and 418delT, which have been shown to cause reduced mRNA levels [49,144], and 1045–1047delGTT, which has been shown to abolish DTDST activity in a *Xenopus* oocyte model [145]. However, assumption of pathogenicity is justified in that some predict a truncated polypeptide chain, and others affect amino acids that are either in transmembrane

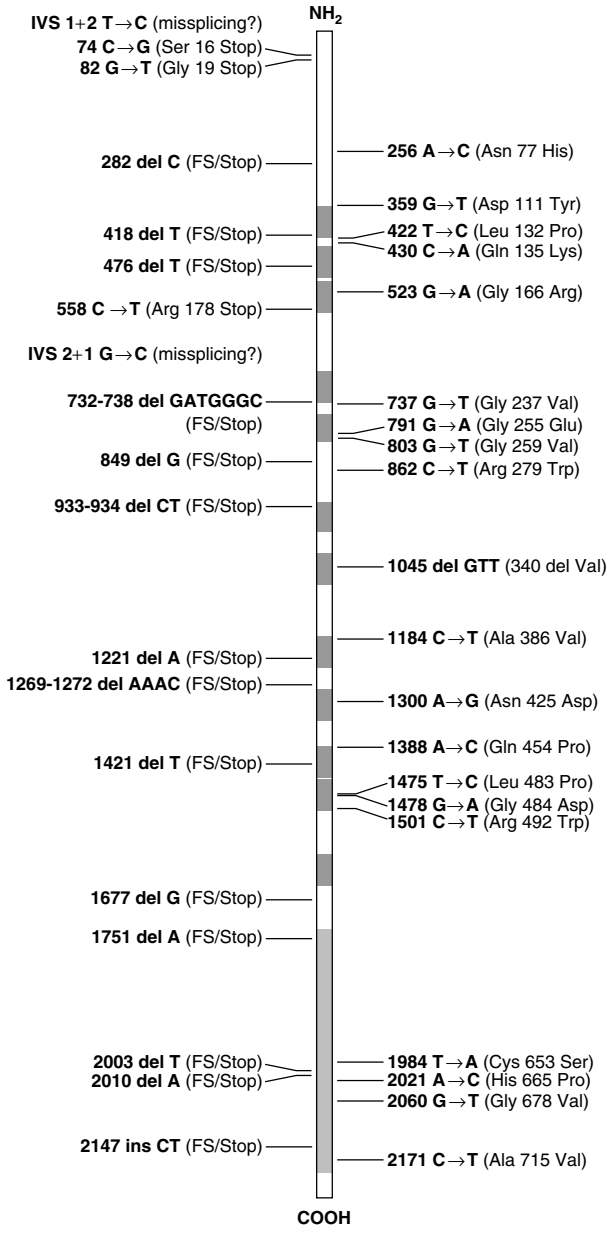

Figure 6. Overview of mutations observed in DTDST skeletal dysplasia patients. The DTDST protein is represented as a vertical bar; the 12 putative transmembrane domains are shaded dark gray; the 3′-cytoplasmic, hydrophobic, putative STAS domain is shaded light gray. Mutations inducing premature stop codons and splice-site mutations are shown on the **left**; on the **right** are mutations leading to amino acid substitutions or deletions of a single amino acid. Of the 20 mutations on the right side, 15 are located within transmembrane domains or the STAS domain (see text).

TABLE 1. Overview of Mutations in the *DTDST* gene

Nucleotide Change	Predicted Change at Protein Level	Associated Phenotype	Remarks	References
IVS1 + 2T > C	Interference with splicing?	DTD, AO2	"Finnish" mutation, giving reduced mRNA levels with intact coding sequence; severity dependent on compounding mutation	[49]
74C > G	S16X	DTD		[127]
82G > T	G19X	DTD		[127]
256A > C	N77H	DTD	Observed in a mild DTD variant; mouse and rat genes have serine at this position	[127]
282delC	FS/stop	DTD		[127]
359G > T	D111Y	DTD		[127]
418delT	FS/stop	AO2		[144]
422T > C	L132P	ACG1B	ACG1B "variant" with proximally pointed femurs	[127]
430C > A	Q135K	DTD		[127]
476delT	FS/stop	ACG1B		[126]
523G > A	G166R	DTD		[127]
558C > T	R178X	ACG1B, DTD	Multiple patients	[126]
IVS2 + 1G > C	Interference with splicing?	DTD	Multiple patients	[48]
732–738delGATGGGC	FS/stop	DTD		[127]
737G > T	G237V	rMED	Compounded with R279W in a rMED patient; not in transmembrane domain, possibly "mild" mutation	Freisinger, Superti-Furga and Bonafé, unpublished
791G > A	G255E	AO2, DTD	Multiple patients	[80]
803G > T	G259V	AO2	Multiple patients; in one case severe AO2, borderline to ACG1B	[127]
849delG	FS/stop	DTD	Compounded with C653S in a DTD patient	[157]
862C > T	R279W	AO2, DTD, rMED	Most frequent mutation outside Finland; probably recurrent; when homozygous, results in rMED	[80,119,144]
933–934del CT	FS/stop	DTD		[127]
1045–1047delGTT	V340del	ACG1B, DTD	Recurrent mutation (see text)	[49,126,145]
1184C > T	A386V	DTD	Homozygous in a patient with classic DTD	[127]
1221delA	FS/stop	ACG1B		[126]
1269–1272delAAAC	FS/stop	DTD		[127]

(*Continued Overleaf*)

TABLE 1. (*Continued*)

Nucleotide Change	Predicted Change at Protein Level	Associated Phenotype	Remarks	References
1300A > G	N425D	ACG1B, AO2	Recurrent mutation	[126]
1388A > C	Q454P	DTD	Nonlethal, severe variant DTD with broad bones	[123]
1421delT	FS/stop	DTD	Severe variant	[127]
1475T > C	L483P	ACG1B		[155]
1478G > A	G484D	DTD		[127]
1501C > T	R492W	DTD	Multiple patients	[127]
1677delG	FS/stop	DTD		[127]
1748C > T	T574I	DTD (?)	Observed as the only mutation in one patient, and in another patient who had two *other* mutations, never in controls –could be rare polymorphism	[127]
1751delA	FS/stop	ACG1B, AO2, DTD	Multiple patients	[48]
1984T > A	C653S	DTD, rMED	Multiple patients; observed in a rMED case, so probably relatively mild mutation	[127]
2003delT	FS/stop	DTD		[127]
2010delA	FS/stop	DTD		[48]
2021A > C	H665P	AO2		[127]
2060G > T	G678V	ACG1B	Rare	[126]
2092A > T	T689S	none	Neutral polymorphism (see text)	[48,145]
2147–2148 ins CT	FS/stop	DTD		[127]
2171C > T	A715V	AO2	Rare	[80]

Note: numbering of nucleotides and amino acids follows reference [48], while the numbering of exons has been revised after the discovery of a 5′ untranslated exon [49].

domains or are conserved in man, mouse, and rat (with the exception of Asn77His, where both mouse and rat have serine).

The 862*C* > T (Arg279Trp) mutation is the most common mutation in Caucasians, accounting for roughly one-third of alleles [127]; it is the second most frequent mutation in Finland, where approximately 90% of DTD alleles carry the so-called Finnish mutation (see below) [49]. The ethnic distribution observed so far for 862*C* > T would be compatible with a Central European origin, but its high frequency could also be explained by independent recurrences as it involves a CpG dinucleotide; indeed, arginine-to-tryptophan substitutions (caused by transition of the cytosine in the first position of the codon to thymine; CGG to TGG) are among the commonest mutations in human genes.

IVS1 + 2T > C, the so-called "Finnish" mutation which may be carried by as many as 1–2% of Finnish individuals, was identified only recently [49]. When it became clear that the Finnish mutation causes reduced mRNA levels [80] but does not reside within the coding sequence of *DTDST*, attention was directed at the flanking sequences. A previously unrecognized 5′ untranslated exon was recognized, and a point mutation at position +2 of the splice donor site was identified. Individuals homozygous for the IVS1 + 2T > C mutation have reduced (though not abolished) levels of mRNA with intact coding sequence [49,80]. Thus, the mutation presumably interferes with splicing and/or with further mRNA processing and transport, although no direct evidence for this has been obtained so far. In accordance with the probable "leakiness" of this mutation, homozygous individuals have diastrophic dysplasia.

The 1045-1047delGTT (del Val340) mutation has been observed in patients of Turkish [126], Hispanic [126], Japanese [145], British, French, and even Finnish [49] origin. This observation, and the occurrence of the mutation at a short direct triplet repeat, suggests it may be a recurrent mutation. This mutation has been shown to abolish DTDST activity in a *Xenopus* oocyte model [145]. Its observation in patients with ACG1B supports the notion that it abolishes transporter activity [126].

Mutation 558*C* > T (Arg178X) has been identified in patients from different ethnic origins and appears to be the fourth most common mutation [126]. Its occurrence at a CpG dinucleotide (again within an arginine codon) would be compatible with independent recurrence. It predicts a truncated protein, and its identification in a patient with ACG1B is again consistent with there being no residual activity of DTDST.

A mutant allele leading to a serine-threonine substitution, 2092*A* > T (Ser689Thr), has been observed [48]. The conservative nature of this substitution, the presence of a serine codon in the corresponding position of the mouse [56] and rat [55] genes, and the observation of the mutation in heterozygous and homozygous states in healthy individuals [49,145] (and J. Hästbacka and A. Superti-Furga, unpublished observations) all suggest it is a neutral polymorphism — the only common one observed so far in this gene.

The results of mutation analysis of affected individuals obtained so far [127] suggest that (i) most mutations are in the coding region and cause structural changes in the transporter, (ii) there is extensive allelic heterogeneity, with only a few common mutations, (iii) there is a paucity of polymorphisms at both genomic and protein levels, implying high selective pressure, and (iv) the high rate of mutation detection implies that there is no second locus producing skeletal dysplasias similar to the DTDST chondrodysplasias; the clinical and radiographic pattern is rather specific for *DTDST* mutations. Correlation of the clinical phenotypes with the underlying mutations suggests the following [119,120,127,146,147]: (i) homozygosity or compound heterozygosity for mutations predicting premature truncation of the protein or amino acid substitutions within transmembrane domains usually results in the more severe phenotype, ACG1B; (ii) amino acid substitutions within extracellular loops or within the N-terminal or C-terminal cytoplasmic domains may have less severe consequences for the transporter protein, and when compounded with a severe mutation can rescue the phenotype from ACG1B to AO2 or DTD; (iii) although originally identified in a patient with AO2 [80], the Arg279Trp allele is associated with non-lethal dysplasias, and produces mild DTD or MED when homozygous [119,120] (and unpublished observations).

Pathogenesis of DTDST Skeletal Dysplasias

Investigations into the pathology and pathogenesis of DTDST chondrodysplasias have focused almost exclusively on the skeletal system. The clinical phenotype points to an involvement of the tendons and joint capsules, but, unfortunately, no data are available yet for these tissues. There are at least two ways by which impairment of DTDST function might result in skeletal dysplasia. The first involves changes in the composition, architecture and mechanical properties of the extracellular matrix in cartilage; the second, still hypothetical, may involve secondary changes in the fibroblast growth factor (FGF) signaling pathway in chondrocytes, which is partly dependent on heparan sulfate proteoglycans.

Chemistry and Pathology of Cartilage. On dissection, ACG1B cartilage is brownish, translucent, and friable, rather than having the white, firm, and elastic nature of normal cartilage. Its consistency is similar to that of a cooked apple, and it can be easily cut through [76]. In this respect, it resembles cartilage from severe collagen II disorders such as ACG2 or Kniest dysplasia (see Part II, this chapter). Light microscopy of cartilage of patients with ACG1B, AO2, and DTD reveals marked changes. In the patient described by Fraccaro, the cartilage looked so different from normal cartilage tissue that the name "achondrogenesis," meant to signify failure of cartilage to differentiate properly, was coined ([65] and M. Fraccaro, personal communication). The changes are generalized in ACG1B, while in AO2 and DTD they may be less pronounced and — more importantly — highly abnormal areas, usually in the interterritorial matrix, may alternate with less severely disturbed areas around clusters of chondrocytes. There is a reduction in the volume of the extracellular matrix with a corresponding increase in cell density. There is a dearth of ground substance in the matrix, corresponding to the deficiency in sulfated proteoglycans, which leads to the"unmasking" of collagen fibers. These become abnormally coarse, and thus visible under the light microscope. In ACG1B, condensation of collagen fibrils around chondrocytes gives a "collagen ring" appearance [74,76,78] (Fig. 7). In DTD, the pericellular area may be spared and the changes confined to the matrix of the interterritorial area [82,148]. Secondarily, there is a disorganization of the growth-plate, the organized transition zone between cartilage and bone tissue where cartilage growth and bone calcification takes place. The coarse

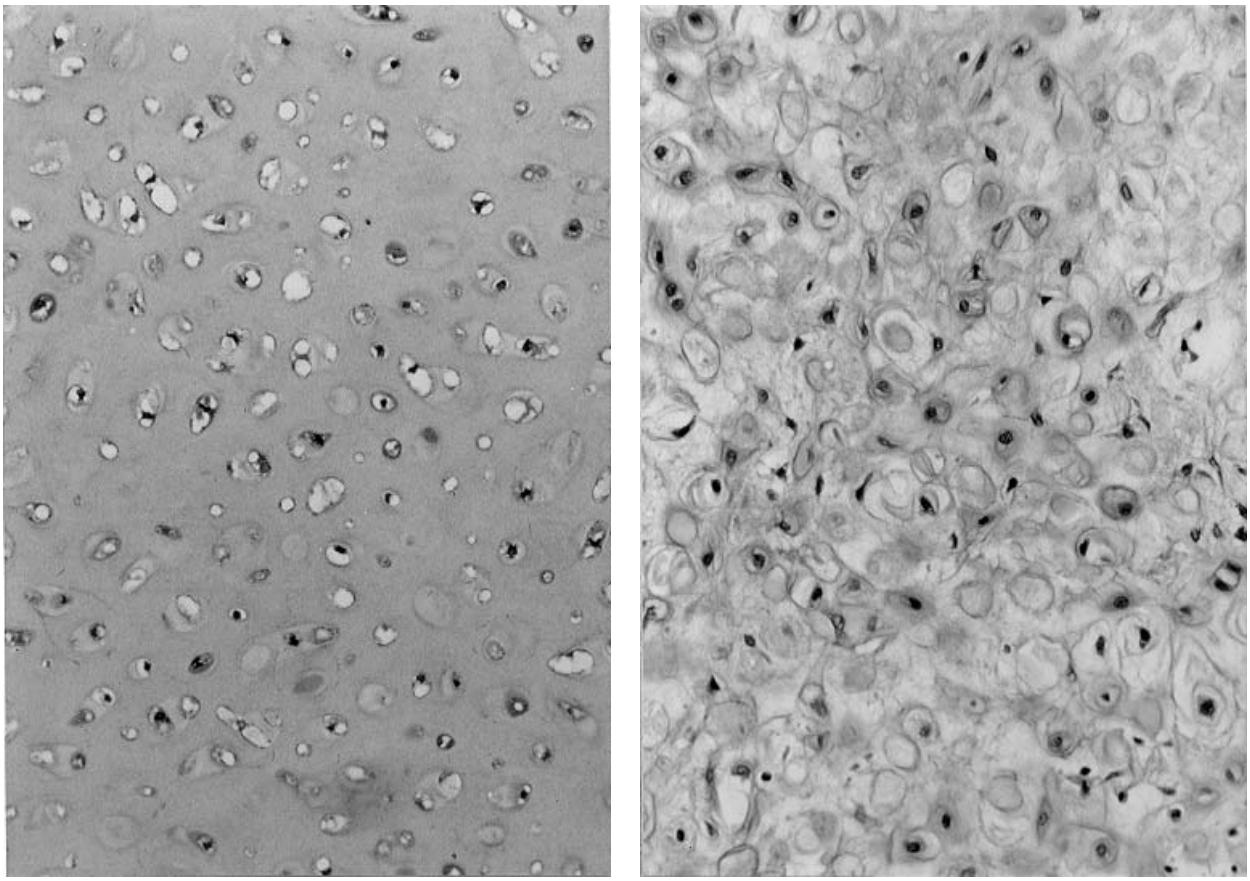

Figure 7. Microscopic appearance of cartilage from a control (**left**) and a newborn with ACG1B (**right**). In the control, the cartilage matrix is homogeneous and individual structures can barely be recognized, corresponding to a ground-glass appearance. In the patient's cartilage, the number of cells is increased. There is a lack of ground substance in the matrix, which leads to unmasking of the collagen fibres which can be accessed directly by the histological stain (visible at higher magnification only); the fibres are also larger than normal and tend to condense particularly around the chondrocytes (collagen ''rings''). [Haematoxylin and eosin staining, magnification approximately 150x] (Reprinted from [76]).

collagen fibers have led investigators on the false track of a collagen defect. An early report on a presumed structural abnormality of collagen II in DTD [149] was disproved by more accurate studies [150] and by exclusion of linkage to the *COL2A1* locus [151]. The observation of a structural anomaly of collagen IX in a DTD patient [152] might be explained by the fact that collagen IX carries a chondroitin sulfate chain and could thus be affected by undersulfation (see below).

Weak or absent metachromasia of cartilage tissue stained with cationic blue dyes such as toluidine or alcian blue is a consequence of the deficiency of sulfated proteoglycans [78]. Rimoin and colleagues, in 1974, studied DTD cartilage histology and suggested an''enzymatic deficiency in chondrocyte mucopolysaccharide metabolism'' [153]; Scheck et al. had reached similar conclusions [154]. Van der Harten studied ACG1B cartilage matrix by semiquantitative histochemical techniques, including pH-dependent staining with alcian blue, and postulated a ''specific defect in the synthesis of sulfated proteoglycans'' [75]. Proteoglycans extracted from ACG1B cartilage migrate more slowly on electrophoresis because of reduced negative charges and are poorly stained by cationic dyes [78,155]. The sulfate content of cartilage hydrolysate is markedly reduced in ACG1B [126] and moderately reduced in AO2 and DTD (A. Superti-Furga and I. Kaitila, unpublished results), while the glycosaminoglycan (GAG) content is only slightly reduced.

The disaccharide composition of cartilage proteoglycans in patients with ACG1B, AO2, and DTD has been studied: while nonsulfated disaccharides account for 2–10 percent of total disaccharides in controls, they range from 11 to 77% in patients (being lowest in DTD and highest in ACG1B) [24]. Chondrocytes from a patient with ACG1B synthesized GAG chains that were of normal length but markedly sulfate-deficient [155]. Thus, it has been proven that functional impairment of DTDST results in deficient sulfation of proteoglycans in cartilage. The mechanism by which the characteristic coarsening of collagen fibers is produced probably involves defective regulation of collagen fibril diameter by undersulfated proteoglycans on the surface of the collagen fibrils themselves [156].

The observation of significantly undersulfated proteoglycans in cartilage of patients with *DTDST* mutations implies that DTDST is a major sulfate transporter in chondrocytes.

The observation of high DTDST mRNA expression in rat cartilage [55] is compatible with this notion. It is not clear yet why cartilage is the only tissue showing overt changes in the presence of *DTDST* mutations, while other proteoglycan-rich tissues (e.g., cornea) do not show overt changes. One possibility is that other sulfate transporters may mediate sulfate uptake in other tissues and cell types; a second possibility is that each of the many cytosolic and endoplasmic sulfotransferases known has a different affinity for PAPS, whereby PAPS deficiency might affect some proteoglycans more than others; a third possibility is outlined below.

A further modulating factor in the phenotypic expression of *DTDST* mutations is sulfate production from the oxidation of cysteine (and perhaps other thiols). We have found that in spite of their defect in the incorporation of exogenous sulfate, patients' fibroblasts synthesize proteoglycans that are sulfated almost to the extent of those from control fibroblasts [24]. In some cell types, such as CHO cells [13] and mouse fibroblasts [23], intracellular sulfate may be derived from cysteine rather than taken up from the extracellular medium. Indeed, cysteine-derived sulfate is incorporated at highly increased rates in DTDST-mutant fibroblasts and chondrocytes *in vitro* [90,157]. Alternative sulfate-producing pathways probably explain the ability of patients' fibroblasts to achieve reasonably good sulfation in spite of the defect in sulfate uptake. It has not yet been shown that this occurs *in vivo* and thus may modulate the tissue-specificity of the phenotype. Chondrocytes, on the other hand, appear not to be able to rescue sulfation through this alternative pathway, perhaps because of a higher requirement, or because of the reduced availability of thiols in cartilage tissue which is largely avascular.

Proteoglycans and Fibroblast Growth Factor (FGF) Signalling. The notions that FGFs require heparan sulfate for efficient signal transmission [158], and that mutations in FGF receptors lead to abnormal skeletal development (Part IV, this chapter) have led to the hypothesis that malfunction of the FGF signaling pathways in chondrocytes may contribute to the development of skeletal dysplasia in the presence of *DTDST* mutations [48,159]. It has not yet been shown that heparan sulfate proteoglycans, which are less abundant than chondroitin sulfate proteoglycans and largely cell-associated rather than in the extracellular matrix, are undersulfated in DTDST dysplasias: the sulfotransferase for heparan has a lower K_M for the sulfate donor, PAPS, than does that for chondroitin, and therefore heparan sulfation may remain normal even when chondroitin sulfation is impaired [21,22]. Moreover, the functions of the various FGF/FGF receptor (FGFR) systems with respect to chondrocyte proliferation and differentiation are still incompletely understood. A mouse model in which the *FGFR3* gene had been disrupted was longer than control mice, rather than dwarfed [160]. Considering these uncertainties, the clinical significance of the recent observation of subnormal proliferative response to FGF stimulation by chondrocytes in which sulfation had been inhibited by treatment with chlorate [55] is uncertain.

Human Spondyloepimetaphyseal Dysplasia Associated with a Mutation in *PAPSS2 (ATPSK2)*

Ahmad et al. have described a large family from Pakistan in which a form of spondyloepimetaphyseal dysplasia (SEMD) segregates as a recessive trait [161]. The clinical phenotype includes short stature, short and bowed lower limbs with enlarged knee joints, kyphoscoliosis, and mild brachydactyly. Radiographs showed platyspondyly, delayed ossification of epiphyses, and osteoarthritic changes. Corneal changes were not reported (although slit-lamp examination was not performed), and intelligence was normal. Ahmad et al. found cosegregation of the disorder with chromosome 10q, in a region which is syntenic with the *Brachymorphic* locus on mouse chromosome 19 [60]. Two pairs of genes coding for ATP sulfurylase/APS kinase (now called PAPS synthetase; PAPSS) were isolated from homologous chromosomal regions in man and mouse, respectively; *ATPSK1 (PAPSS1)* was excluded as the locus for human SEMD and *Brachymorphic*, while a stop codon mutation in human *ATPSK2 (PAPSS2)* cosegregated with human SEMD [60], and a missense mutation in mouse *Atpsk2 (Papss2)*—the same mutation identified shortly earlier by Schwartz and her group [59](see below)—with *brachymorphic*. Why the clinical phenotype of ATPSK2 (PAPSS2) deficiency differs from that of the DTDST disorders is unknown at present.

Spondylar Dysplasia/Brachyolmia: Another Possible Defect in Proteoglycan Sulfation

In 1973, Mourao et al. described sibs with isolated, generalized platyspondyly (a condition also named "brachyolmia," short trunk) and reported that they excreted undersulfated chondroitin sulfate C in their urine [162]. In a later report on the same family, peripheral corneal opacities were noted [163], and in 1981, experiments with patients' serum were reported which suggested a defect in PAPS-chondroitin sulfotransferase [164]. Sewell et al. reported another patient with short stature and brachyolmia. When her urinary glycosaminoglycans were digested with chondroitinase, the resulting disaccharides showed an abnormal mobility on electrophoresis consistent with reduced sulfation [165]. A similar observation was reported (but not shown) in yet another patient [166]. Horton et al. reported three sibs with a recessive condition they designated as"brachyolmia, Hobaek type" which might be the same condition: no biochemical studies were done, but cartilage histology showed glycosaminoglycan deficiency and condensation of collagen fibers, findins that might well fit with a proteoglycan defect [167]. Shohat et al. reported patients with similar abnormalities, again without biochemical studies [168]. In summary, it is possible that one form of generalized platyspondyly exists that is associated with undersulfation of cartilage proteoglycans [169], but its biochemical and molecular basis remains to be characterized.

THE *BRACHYMORPHIC* MOUSE

Brachymorphic (*bm*) is a spontaneously occurring, recessively inherited mouse mutation described by Lane and Dickie in 1968 [170]. The phenotype of homozygous *bm/bm* mice becomes apparent a few days after birth and consists of a dome-shaped skull, and an abnormally short and thick tail. Their growth rate is retarded but adult weight is close to normal. The limb bones are shorter than in the normal mouse. The *bm* mouse has considerably stimulated research into proteoglycan and sulfate metabolism, and for a long time was the only model for abnormal PG synthesis. Orkin and coworkers first observed ultrastructural changes in cartilage suggestive of defective proteoglycan synthesis [171] and showed, by DEAE-cellulose chromatography and cellulose acetate electrophoresis of cartilage glycosaminoglycans, that chondroitin sulfate was undersulfated [172]. Incubating *bm* cartilage with different radiolabeled sulfate compounds, Schwartz and colleagues found that sulfate incorporation was

reduced when inorganic sulfate was provided, but normal when PAPS was used, and suggested that the defect might be impaired PAPS synthesis [173]. Sugahara and Schwartz went on to show that the activities of ATP sulfurylase (EC 2.7.7.4) and APS kinase (EC 2.7.1.25) were moderately and markedly reduced, respectively, in *brachymorphic* cartilage [174]. It was then shown that the biochemical defect was expressed in cartilage and liver but not in skin or brain [175–177]. Copurification of the two enzymatic activities from rat chondrosarcoma and mouse cartilage indicated that they resided in a single protein molecule, and it could be shown that the bifunctional enzyme from *bm* cartilage was defective in "channeling" the product of the first reaction (APS) to the second reaction [35]. The quest for the ATP sulfurylase/APS kinase gene yielded two closely related genes, termed *Atpsk1* and *Atpsk2*: their expression is tissue-specific and a mutation in the kinase domain of *Atpsk2* causes the *brachymorphic* phenotype [59]. Expression of the mutated ATPSK2 in liver (and possibly other tissues) probably explains the abnormalities in hepatic sulfate conjugation and blood clotting seen in *brachymorphic* mice [61,178]. It should be noted that the denominations "ATP sulfurylase" and "APS kinase" used in this paragraph for reasons of historical consistency, have recently been replaced by "PAPS synthase." This makes good sense for those organisms in which the two activities reside in a single polypeptide.

PERSPECTIVES

Insight into the biochemical and molecular basis of skeletal dysplasias associated with impaired sulfation of cartilage proteoglycans has highlighted the importance of proteoglycans in cartilage homeostasis and skeletal growth and development and, at the same time, revealed new aspects of sulfate metabolism. A comprehensive model of whole-body sulfate metabolism and the role of the different organs and tissues is still missing. The observation that fibroblasts of patients with *DTDST* mutations make extensive use of cysteine-derived sulfate would indicate a possible therapeutic avenue, but the task of delivering micro- to millimolar concentrations of thiols to the chondrocyte by the oral route appears impossible at present.

Cloning of the gene responsible for Pendred syndrome (MIM 274600) [179], a form of heritable deafness associated with thyroid gland dysfunction, has revealed homology with *DRA1*, a chloride transporter gene (mutations in which cause congenital chloride diarrhea [51], MIM 214700), and indirectly with DTDST, leading to speculation about its possible role as a sulfate transporter, but later evidence [50,52] suggests that pendredin is a chloride-iodide transporter.

Girard and his coworkers have identified a novel sulfate transporter molecule, called SUT-1, which is a sodium/sulfate cotransporter analogous (but not identical) to NaSi-1 of the renal tubule [11]. Both SUT-1 and DTDST are expressed in high endothelial venules [11]. The physiological role of SUT-1 remains to be determined. The same group has recently discovered that PAPS1 may be located in the cell nucleus under certain conditions [41]; this intriguing finding raises questions about sulfation processes in the nucleus and the role of nuclear glycosaminoglycans.

Genetic defects in one of several sulfotransferase activities [180] can be expected to produce clinical phenotypes, according to substrate specificity and tissue specific expression, including skeletal phenotypes. A genetic deficiency of the core protein of aggrecan, the major chondroitin-sulfate proteoglycan in cartilage, causes severe chondrodysplasia in chickens (*nanomelia*) [30] and mice (*cartilage matrix deficient*) [181]: the human counterpart remains to be identified. A further link between sulfate metabolism and skeletal development may be adduced from the role of sulfated proteoglycans in signalling pathways [182,183]: thus, a defect in heparan 2-sulfotransferase activity leads to a complex renal/ocular/skeletal malformation phenotype in mice [184]. A multitude of substrate-specific sulfotransferases exist, deficiencies of which may potentially have clinical consequences. It appears that the elucidation of sulfation processes and their associated biologic mechanisms and related clinical disorders is only just beginning and may yield more biologically and clinically relevant information.

RECENT DEVELOPMENTS

The Cellular Metabolism of Sulfate

Markovich has provided a masterly review on sulfate transport in mammals [185].

Recessively Inherited Multiple Epiphyseal Dysplasia and Clubfoot

Huber et al. [186] have described four individuals who were diagnosed as having "apparently isolated" clubfoot in infancy or childhood, with clinical and radiographic signs of multiple epiphyseal dysplasia becoming apparent only later. All were found to be homozygous for the *DTDST* Arg279Trp mutation. We had already reported a series of Arg279Trp homozygotes and emphasized that none had received a diagnosis of a skeletal dysplasia until late childhood or later [120]. Bonafé et al. [187] have tested a large population of families with idiopathic clubfoot, mainly of Hispanic origin, for *DTDST* mutations and found just one instance of heterozygosity for Arg279Trp. In summary, it appears that *DTDST* mutations can cause apparently isolated clubfoot but are probably a rare cause of it. Consequently, newborns and children with idiopathic clubfoot should be evaluated or followed up closely for the possibility that the clubfoot may be the presenting sign of recessive multiple epiphyseal dysplasia.

The Spectrum of *DTDST* Mutations and their Consequences

Karniski [188] has established a reporter system in *Xenopus* oocytes to assay the residual sulfate transport activity allowed for by a number of *DTDST* mutations. Though potentially useful to understand genotype-phenotype correlations, the results obtained with this system appear to be at odds with extensive observations with affected human subjects; e.g., the delVal340 mutation results in a high residual sulfate transport activity in the *Xenopus* system but is invariably associated with achondrogenesis 1B when homozygous in humans; four other mutations appeared to have no significant effect on sulfate transport in the *Xenopus* system in spite of their association with skeletal dysplasia in humans. One of the factors responsible for the discrepancy between the observations in the *Xenopus* system and the experience with affected individuals may be the fact that the *in vitro* experiments are performed at temperatures between 16°C and room temperature rather than at 37°C.

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Chapter 23, Part IV

Craniosynostosis Syndromes and Skeletal Dysplasias Caused by Mutations in Fibroblast Growth Factor Receptor Genes

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SUMMARY

- **The human skeletal dysplasias and craniosynostosis syndromes are a phenotypically diverse and genetically heterogeneous group of conditions associated with disordered bone growth. Mutations in three of the four known fibroblast growth factor receptor (FGFR) genes cause human disorders involving skeletal dysplasia and/or craniosynostosis.**
- **In this chapter, we consider the craniosynostosis syndromes and skeletal dysplasias caused by FGFR mutations. First, the biology of the FGFRs is presented. Subsequently, the clinical features and molecular etiology of each condition are described. Finally, we discuss the general principles that emerge from an overview of FGFR mutations and the conditions they cause.**
- **Craniosynostosis syndromes caused by FGFR mutations include Pfeiffer syndrome, Crouzon syndrome, Apert syndrome, Jackson-Weiss syndrome, Beare-Stevenson syndrome, Muenke syndrome, and Crouzon syndrome with acanthosis nigricans. The human skeletal dysplasias caused by FGFR mutations are members of the achondroplasia family of skeletal dysplasias. These include achondroplasia, hypochondroplasia, thanatophoric dysplasia, and severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN).**
- **Pfeiffer syndrome is an autosomal dominant condition caused by mutations in *FGFR1* and *FGFR2*. Affected persons exhibit craniosynostosis, and broad thumbs and great toes. Type 1 (classical) has the most favorable prognosis and intelligence is normal. There is a higher risk of neurodevelopmental problems and a decreased life expectancy in types 2 and 3. A cloverleaf skull is seen in type 2, but not type 3.**
- **Crouzon syndrome is an autosomal dominant condition caused by mutations in *FGFR2*. It is characterized by coronal or multiple suture synostoses, shallow orbits, ocular proptosis and a beaked nose.**
- **Apert syndrome is characterized by brachycephaly, midface hypoplasia, broad thumbs and great toes, and symmetric syndactyly of the hands and feet. Two specific *FGFR2* mutations account for 99% of all cases.**
- **Achondroplasia is the most common of the human skeletal dysplasias. Primary skeletal features include rhizomelic short stature, megalencephaly, and spinal stenosis. More than 95% of cases are caused by one of two mutations at *FGFR3* nucleotide G1138, both resulting in Gly380Arg substitutions.**
- **The phenotypic and radiographic features of hypochondroplasia are similar to, but milder than, those of achondroplasia. Two common *FGFR3* mutations result in hypochondroplasia, but these account for less than 90% of cases. Several studies have suggested that hypochondroplasia may exhibit genetic heterogeneity.**
- **Thanatophoric dysplasia is the most common neonatally lethal form of human skeletal dysplasia. Type I is characterized by curved femurs and may or may not include a cloverleaf skull. Type II exhibits cloverleaf skull and straight femurs. A number of different *FGFR3* mutations have been found in type I, but only a single mutation, Lys650Glu, has been seen in type II.**
- **There is overlap between the skeletal dysplasia and craniosynostosis phenotypes. Some craniosynostosis syndromes show involvement of the appendicular skeleton, while some skeletal dysplasias show craniosynostosis. Acanthosis nigricans, a dermatologic condition characterized by hyperpigmentation and thickening of the skin, is consistently seen in SADDAN and Crouzon syndrome with acanthosis nigricans, and occasionally in the other members of the achondroplasia family of skeletal dysplasias.**
- **The pathophysiologic mechanism of action of all known FGFR mutations studied to date is that of gain of function. The mutations render the receptors constitutively active and relatively ligand-independent.**

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GENERAL BIOLOGY OF THE FIBROBLAST GROWTH FACTOR RECEPTORS

In humans, the fibroblast growth factor receptors (FGFRs) comprise a family of four tyrosine kinase receptors (FGFR1–4) that bind fibroblast growth factors (FGFs) with variable affinity [1]. The FGF family of proteins consists of at least nine structurally related, heparin-binding polypeptides that play a key role in the growth and differentiation of various cells and tissues of mesenchymal and neuroectodermal origin [2–4]. FGFs are also implicated in chemotaxis, angiogenesis, apoptosis, and spatial patterning [5,6]. The prototypical FGF, FGF-2 (also called basic FGF), is a potent angiogenic protein, and stimulates smooth muscle cell growth, wound healing, and tissue repair. FGF-2 is thought to play a role in hematopoiesis and is also implicated in the differentiation and function of the skeleton, eye and nervous system [1,4].

The FGFRs have a highly conserved protein structure. The mature FGFR proteins are membrane-spanning tyrosine kinase receptors with an extracellular ligand-binding domain consisting of three immunoglobulin (Ig)-like domains, a transmembrane domain, and a split intracellular tyrosine kinase domain [1,7]. Between the first and second Ig domains is a stretch of 4 to 8 acidic amino acids, termed the "acid box." Alternative splicing in the FGFR genes results in at least 12 distinct isoforms for each gene [8–10]. Variants include those that lack one or more Ig-like domains, the acid box or the transmembrane domain. Some isoforms have a truncated carboxyl terminus, while others have regions of alternative sequence.

Ligand binding requires dimerization of two monomeric FGFRs and includes a heparin-binding step [5]. Promiscuous dimerization is observed; besides dimerizing with itself, FGFR1 may dimerize with FGFR2, 3, or 4 [7]. Similar dimerization combinations of other FGFR monomers are also possible. Differing combinations of dimers are observed in different tissues and stages of development, and this diversity of dimers probably plays an important role in skeletal differentiation.

The process of FGF binding involves contacts with both the IgII and IgIII domains. It is known that the relative importance of these contacts varies with different FGFs. Autophosphorylation of intracellular tyrosine residues results from binding of ligand to the receptor [11,12] and initiates the intracellular signalling cascade by activating intrinsic protein tyrosine kinase activity. This, in turn, leads to binding by the intracellular domain of proteins containing Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains [13] and subsequent activation of the RAS/mitogen-activated protein kinase (MAPK) pathway and phosphatidylinositol hydrolysis [14,15].

There is a high degree of homology among the four human genes encoding the FGFR monomers, each of which contains approximately 20 exons [16,17]. Among the human FGFRs, amino acid identity for the extracellular domain is 64–74% and for the kinase domain it is 72–85%. Sequence comparisons demonstrate that FGFR4 is the most highly diverged member of the family.

The *FGFR3* cDNA [18] is 4.4 kb in length and contains an open reading frame (ORF) of 2520 nucleotides encoding an 840 amino acid protein. A 3′-untranslated region of 1800 nucleotides, a consensus polyadenylation signal sequence, and a poly (A) tail follow the ORF. Both human and mouse FGFR3 genes have been characterized [19–21] and span 16.5 kb and 15 kb, respectively. Both genes consist of 19 exons and 18 introns and have translation initiation and termination sites located in exons 2 and 19, respectively. The 5′ flanking regions lack typical TATA and CAAT boxes. However, several Sp1, AP2, Zeste, Krox 24 and IgHC.4 putative *cis*-acting elements are present in the promoter region, which is contained within a CpG island [22]. The promoter regions of the human and mouse *FGFR3* genes are similar, with conservation of several putative transcription factor binding sites, suggesting an important role for these elements and their corresponding transcription factors in the regulation of *FGFR3* expression [21]. It has been shown that the 100 bp of sequence 5′ to the *FGFR3* initiation site is sufficient to confer a 20 to 40-fold increase in transcriptional activity [22]. There is an Sp1 binding enhancer element in intron 1 containing two purine rich elements, between nucleotides +340 and +395. *FGFR3* sequences between −220 and +609 are sufficient to promote tissue-specific expression [22].

At least three polymorphisms have been found within the *FGFR3* gene, in close proximity to the achondroplasia mutation site. These include a G to C transition toward the 5′ end of intron 9 that creates a *Pfl*MI site; a C to T transversion in intron 10 that creates a *Pml*I site, and a single G deletion in a stretch of 11 consecutive guanosine residues in intron 9. In one analysis of the frequency of the *Pfl*MI and *Pml*I polymorphisms in the general population, 10 of 224 chromosomes (4.5%) carried the *Pfl*MI polymorphism and 30 of 288 (10.4%) carried the *Pml*I polymorphism [23].

The FGFR genes are characterized by a complex array of alternative splicing. Alternative patterns of splicing include forms that lack the first Ig-like domain, soluble forms comprising the first one or two Ig-like domains only, and intracellular forms that lack the transmembrane domain [24,25]. One specific FGFR isoform, seen in FGFR1, FGFR2, and FGFR3, but not FGFR4 [9,16] is due to the presence of an alternative splice site found in the third Ig-like domain (closest to the membrane) and typically contains an alternative exon for this domain. The Ig-like domain 3 is encoded by two separate exons: exon IIIa encodes the amino-terminal part of the domain, while the carboxyl-terminal half is encoded by either exon IIIb or IIIc [8,9]. This latter form is of particular importance to the pathophysiology of the craniosynostosis syndromes.

The splice forms differ in their ligand affinity and preferential ligand binding, as well as in their tissue-specific expression. FGFR3 with exon IIIb has a high ligand specificity for acidic FGF/FGF-1 [9], and is expressed in mouse embryo, skin and epidermal keratinocytes [26]. The splice form of FGFR3 containing exon IIIc is detected in developing mouse brain and spinal cord, as well as in developing bone [27].

The developmental expression patterns of the various FGFR splice forms are highly complex [28,29]. In several instances, the expression of different FGFRs or alternative FGFR splice forms appears to be mutually exclusive, suggesting that switching of FGFR expression may play an important role in differentiation. The formation of heterodimers when different FGFRs are expressed simultaneously [7,11,30], as well as the availability of multiple alternative splice forms, compounds the complexity of the signaling pathways. The FGFR signal transduction pathway plays an important role in many different cellular processes, including differentiation, migration, mitogenesis, apoptosis, and pattern formation. Mice homozygous for null mutations of either *Fgfr1* or *Fgfr2* die very early in development, indicating that both FGFR1

and FGFR2 play crucial roles in early embryogenesis. Disruption of fetal development in the *Fgfr1* and *Fgfr2* null mice is attributable, respectively, to defects in mesodermal patterning [31,32] and trophectoderm function [33].

Developmental expression studies of Fgfr3 suggest that this protein plays a significant role in skeletal development. Outside the nervous system, the highest levels of *Fgfr3* expression are observed in cartilage rudiments of developing bone. During endochondral ossification, *Fgfr3* is expressed exclusively in resting cartilage [26]. In the mouse, *Fgfr3* has a unique pattern of expression during organogenesis. It is expressed in the germinal epithelium of the neural tube, and at 1 day post-partum and in the adult mouse and rat brain, is expressed diffusely [26,34].

Homozygosity for *Fgfr3* null mutations is compatible with life, but results in increased length of the long bones and vertebral column, as well as deafness [35,36]. Mice homozygous for *Fgfr4* null mutations are normal [33]. Mice heterozygous for any of the *Fgfr* null mutations are normal.

Analysis of chimeras and targeted insertion of specific mutant alleles by homologous recombination have been used to investigate the functions of *Fgfr1* and *Fgfr2* in later development of the mouse. Chimera analysis has shown that *Fgfr1* plays a role in migration through the primitive streak, with mutant cells demonstrating autonomous behavior [37,38]. Other mutations have revealed an important role for Fgfr1 in limb patterning [38,39] and for Fgfr2 in limb induction [40].

CRANIOSYNOSTOSIS SYNDROMES

Craniosynostosis is the premature fusion of one or several sutures of the calvaria leading to an abnormal shape of the skull. The major sutures of the calvaria include the coronal, lambdoid, and squamosal, all of which are paired (Fig. 1). The sagittal and metopic sutures are single

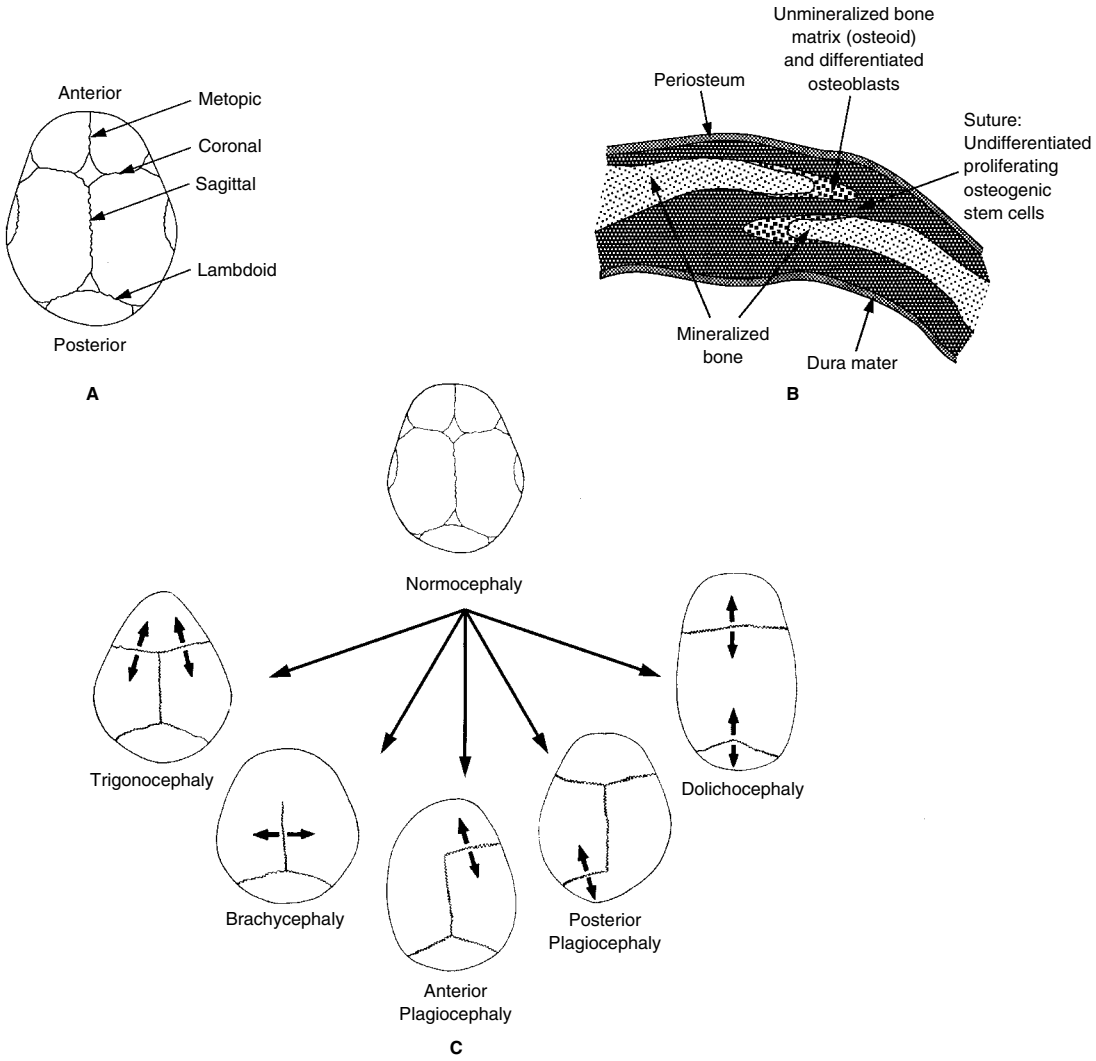

Figure 1. Diagram of the sutures of the skull **A:** Superior view of the normal infant skull. Note that the anterior fontanelle is bordered by the metopic, coronal, and sagittal sutures, the posterior fontanelle by the sagittal and lambdoid sutures. **B:** Cross-section through the coronal suture. The skull bones overlap slightly. In craniosynostosis, the narrow space separating the bones is obliterated. **C:** Premature fusion of the sutures leads to an abnormal head shape. Compensatory expansion (arrows) occurs, particularly at neighboring unfused sutures. (Reprinted from [235], by permission of McGraw-Hill Companies Inc.)

(Fig. 1A). At birth, these sutures are open and the nonmidline sutures overlap slightly (Fig. 1B). Depending on which of the sutures are fused prematurely, the skull can develop a characteristic shape. Simple synostosis of the coronal sutures leads to brachycephaly or short-headedness; variant head shapes include acrocephaly and oxycephaly (pointed head), or turricephaly (tower-shaped head). Unilateral coronal synostosis gives rise to anterior plagiocephaly which is associated with asymmetry of the face and skull (Fig. 1C). Dolichocephaly, or long-headedness, is seen in premature fusion of the sagittal suture. A variant is scaphocephaly (keel-shaped head). Trigonocephaly, or triangular-shaped head, results from premature closure of the metopic suture. Posterior plagiocephaly is due to unilateral fusion of the lambdoid suture. Compound synostosis, i.e., in which two or more sutures are involved, can lead to complex head shapes including cloverleaf (''Kleeblattschädel'') malformation [41].

Classification

Various classifications of craniosynostosis have been applied over the years. One system uses anatomic nomenclature dependent on which suture is involved. This descriptive classification is used in cases of isolated craniosynostosis which do not fit into any of the known craniosynostosis syndromes. A clinical classification based on the craniofacial findings, anomalies in other organ systems, and pedigree information has been used to describe well-known (''classical'') and rare monogenic craniosynostosis syndromes. Over 100 such syndromes are known [42]. The recent identification of mutations, especially in the FGFR genes, has provided new insight into the clinical classification.

Frequency

The most accurate surveys of the frequency of craniosynostosis among live-born infants suggest a prevalence of 1 in 2,100 to 1 in 3,000 [43–47]. The most common craniosynostosis syndromes include Apert (4.5%, of all cases with craniosynostosis), Saethre-Chotzen (4.5%), Crouzon (4.5%), and Muenke (8%) syndromes. Autosomal recessive craniosynostosis syndromes are rare. Craniofrontonasal syndrome is the most common X-linked craniosynostosis syndrome.

CRANIOSYNOSTOSIS SYNDROMES CAUSED BY FGFR MUTATIONS

Pfeiffer Syndrome

Pfeiffer syndrome (MIM 101600) was first described in 1964 [48] in a three-generation family demonstrating autosomal dominant inheritance. It is characterized by craniosynostosis, broad thumbs, and broad great toes. Numerous familial cases have been described since the original report [49,50]. Penetrance is complete; expressivity is variable. In addition to familial occurrence, sporadic cases due to new mutations are well known. These sporadic cases commonly have a more severe phenotype. The birth prevalence of Pfeiffer syndrome is not known; however, it is clearly less common than Apert, Crouzon, Muenke, or Saethre-Chotzen syndromes.

Clinical subtypes of Pfeiffer syndrome were proposed before the underlying etiology was known [49]. Classic Pfeiffer syndrome, or type 1, has the best prognosis and is compatible with a normal life span and normal intelligence in most cases. In contrast, Pfeiffer syndrome types 2 and 3 have a higher risk of neurodevelopmental problems and reduced life expectancy [51–53]. Both types 2 and 3 are almost always sporadic and the clinical findings are more severe, including severe ocular proptosis and elbow ankylosis. Type 2, but not type 3, has a cloverleaf skull. Exceptions with a more favorable outcome have been reported [54].

Linkage studies in families with Pfeiffer syndrome have demonstrated genetic heterogeneity. Loci on chromosomes 8p11.2 [55,56] and 10q25–q26 [57] correspond to mutations in the genes coding for fibroblast growth factor receptors 1 and 2 (*FGFR1*, *FGFR2*), respectively. Pfeiffer syndrome type 1 has been reported with mutations in *FGFR1* and *FGFR2*, whereas patients with types 2 and 3 have mutations in *FGFR2* only. Several large families initially considered to have findings consistent with Pfeiffer

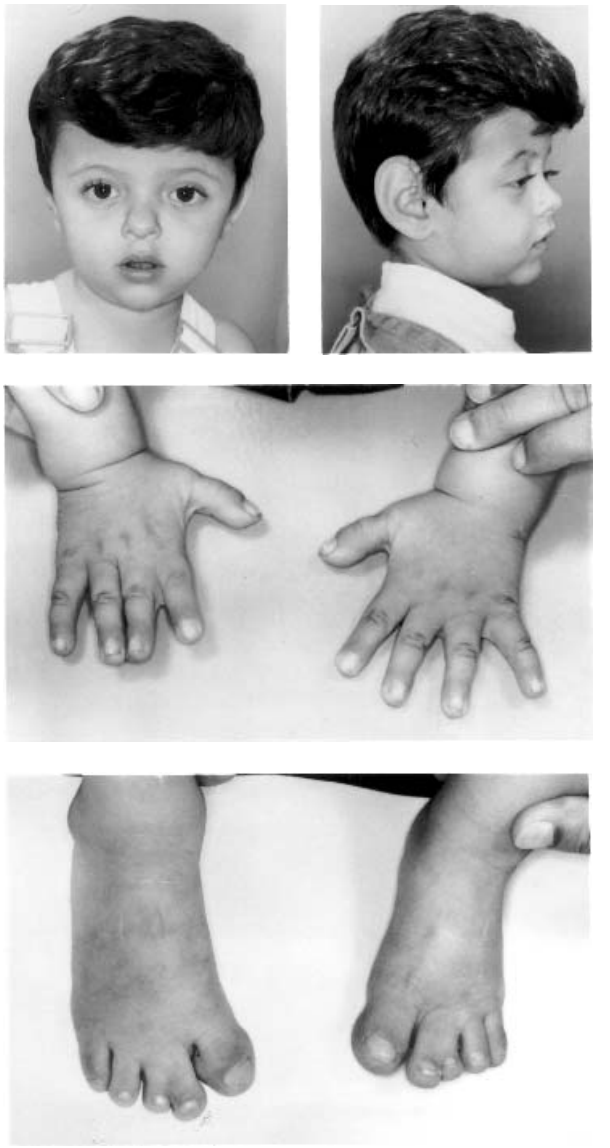

Figure 2. Mild craniofacial and limb anomalies in Pfeiffer syndrome due to *FGFR1* mutation. Brachycephaly with hypertelorism. Partial cutaneous syndactyly of fingers and toes. Note broad, medially deviated great toes. (From Schell et al. [75]. Reprinted by permission of Brazilian Journal of Dysmorphology and Speech-Hearing Disorders.)

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Figure 3. Craniofacial and limb anomalies in Pfeiffer syndrome due to *FGFR2* mutation. Cloverleaf skull with ocular proptosis and severe midface hypoplasia in sporadic Pfeiffer syndrome (**upper panels**). Brachycephaly with hypertelorism, downslanting palpebral fissures and midface hypoplasia in familial Pfeiffer syndrome (**middle panels**). Broad thumbs and symphalangism of all fingers (**lower panel**). From Schell et al. [57]. Reprinted by permission of Human Molecular Genetics.)

syndrome were excluded from either of these two regions but mapped to chromosome 4p16 and were later demonstrated to have Muenke syndrome [58,59].

Craniofacial features in Pfeiffer syndrome are variable and secondary to synostosis mostly involving the coronal sutures [60,61]. Findings include a turribrachycephalic skull, midface hypoplasia with relative mandibular prognathism, a beaked nose, a low nasal bridge, hypertelorism, downslanting palpebral fissures, ocular proptosis, strabismus, a highly arched palate, and crowded teeth (Figs. 2 and 3).

Intelligence is usually normal, although mental deficiency has been observed, particularly in sporadic cases. Rare central nervous system anomalies include large ventricles, hydrocephalus, Arnold-Chiari malformation, and seizures [62].

Anomalies of the hands and feet in Pfeiffer syndrome are distinctive and unique among the craniosynostosis syndromes [63–65]. Characteristically, the thumbs and great toes are broad and deviated away from the other digits (Figs. 2 and 3). However, the thumbs may be of normal width. Brachydactyly is often present, as is partial soft tissue syndactyly of the fingers and toes. Radiographic findings of the hands include malformed and fused phalanges of the thumbs, short middle phalanges (brachymesophalangy) of the second and fifth fingers with complete absence in severe cases, symphalangism (complete osseous fusion of proximal, middle, and distal phalanges which occurs over several years), and occasional fusion of the proximal ends of metacarpals 4 and 5. The distal phalanx of the great toe is broad, and the proximal phalanx is malformed. Broad and short first metatarsals and fusion of carpal and tarsal bones have been described (Figs. 4 and 5).

Additional manifestations may include cloverleaf skull (type 2 Pfeiffer syndrome), fusions of cervical and lumbar

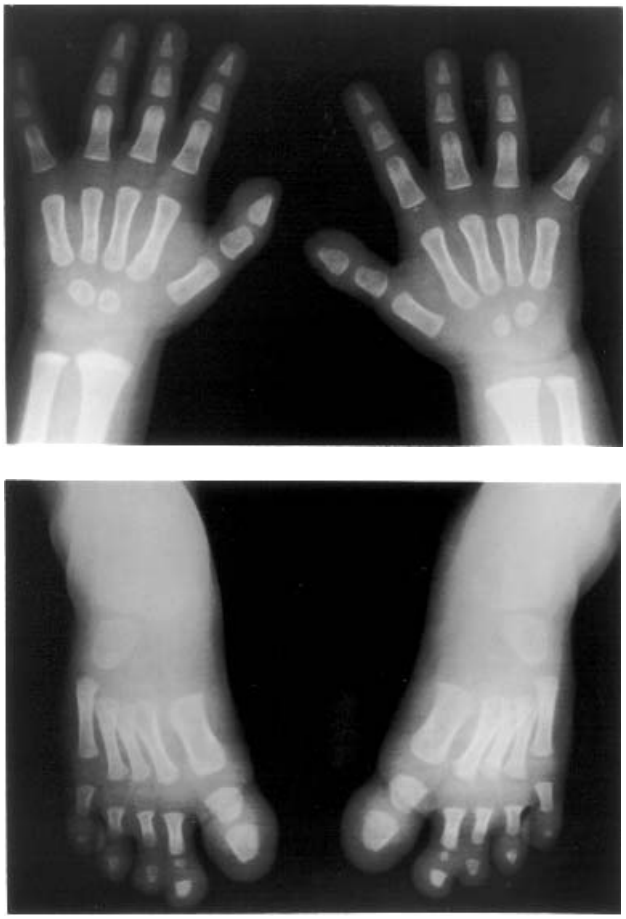

Figure 4. Radiographs of hands and feet in Pfeiffer syndrome with *FGFR1* mutation. Mild brachydactyly and mildly broad thumbs and broad medially deviated great toes. (From Muenke and Wilkie [235]. Reprinted by permission of McGraw-Hill Companies Inc.)

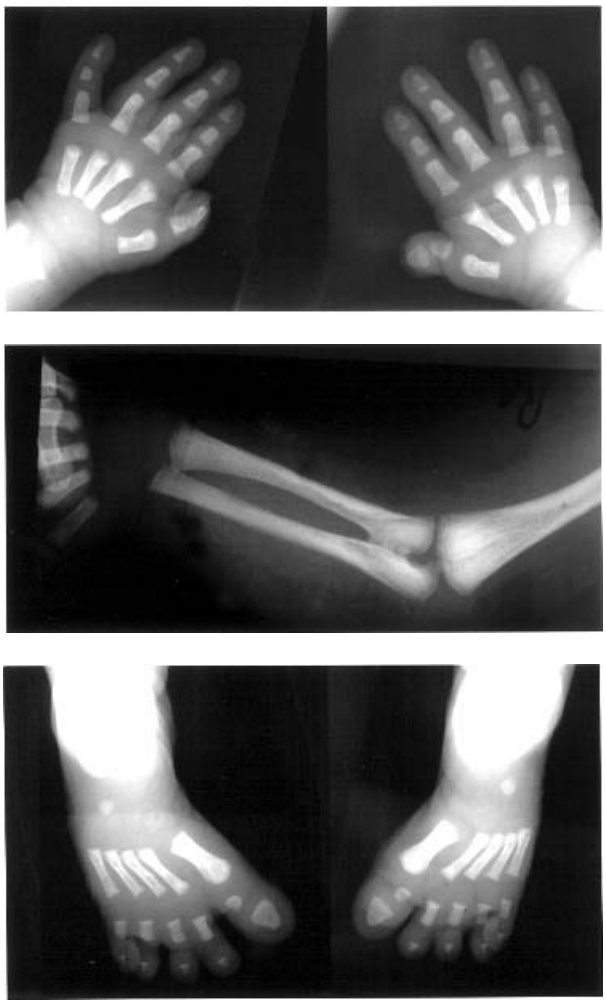

Figure 5. Radiographs of hands, elbow, and feet in sporadic Pfeiffer syndrome with *FGFR2* mutation. Brachydactyly of hands and feet. Abnormally broad proximal phalanges of thumb and great toe. Radioulnar and early humeroulnar fusion of elbow. (From Muenke and Wilkie [235]. Reprinted by permission of McGraw-Hill Companies Inc.)

vertebrae [61,66], cubitus valgus, synostosis of the radio-humeral and ulnar-humeral joints, a shortened humerus, abnormalities of the pelvis, coxa valga, and talipes equinovarus. Abnormalities affecting other organ systems are of low frequency and include hearing loss [67], optic nerve hypoplasia, iris coloboma [68], choanal stenosis/atresia, bifid uvula, tracheal stenosis [69], supernumerary teeth, gingival hypertrophy, widely spaced nipples, intestinal malrotation [70], anal atresia or malpositioned anus [71], pyloric stenosis, umbilical hernia, cryptorchidism, and bifid scrotum [72].

Pfeiffer Syndrome Caused by FGFR1 or FGFR2 Mutations

Prior to the finding that Pfeiffer syndrome is a genetically heterogeneous disorder linked to *FGFR1* on chromosome 8p11.2 and *FGFR2* on chromosome 10q25–q26 [56,57], it was considered one clinical entity. The majority of large families with Pfeiffer syndrome studied by linkage and mutation analysis could be assigned to either *FGFR1* or *FGFR2*. An *FGFR1* Pro252Arg mutation was identified in multiple affected individuals from unrelated families and in sporadic cases [55,57,73–76]. The phenotype in individuals with the *FGFR1* Pro252Arg mutation (Figs. 2 and 4), although somewhat variable even among affected members of the same family, is consistently milder than that of Pfeiffer syndrome due to *FGFR2* mutations.

Clinical findings in Pfeiffer syndrome due to *FGFR2* mutations are consistently more severe (Figs. 3 and 5). The original family [48] and a two-generation family with seven affected members [57] were linked to chromosome 10q25–q26 markers but no mutation was detected when *FGFR2* exons IIIa and IIIc (M. Muenke, unpublished results) were sequenced. These families presumably carry an *FGFR2* mutation outside the IIIa and IIIc exons. Pfeiffer syndrome families with known *FGFR2* exon IIIa and IIIc mutations have been reported [57,75,77–79]. The clinical findings in these families consist of the characteristic craniofacial and limb anomalies in "classic" Pfeiffer syndrome, which are more severe than those resulting from the *FGFR1* Pro252Arg mutation. All sporadic cases of Pfeiffer syndrome reported with severe clinical findings including cloverleaf skull [51,53,57,77,78,80–85] have had *FGFR2* mutations.

Apert Syndrome

Apert syndrome (MIM 101200) was first described in 1906 [86], since when there have been numerous clinical reports (for reviews see [87–92]). It is characterized by brachycephaly, midface hypoplasia, broad thumbs and great toes, and symmetric syndactyly of the hands and feet. The inheritance pattern is autosomal dominant in the few families with parent-to-child transmission. Germline mosaicism was proposed in an unaffected couple who had two children with Apert syndrome [93], although this has not been confirmed at the molecular level. The great majority (~98%) of Apert syndrome cases are sporadic. *De novo* occurrence has been clinically correlated with increased paternal age [94], and the exclusively paternal origin of new mutations has been demonstrated at a molecular level [95]. The birth prevalence of Apert syndrome, estimated as between 1 in 65,000 to 1 in 80,000 newborns, is high among the craniosynostosis syndromes (4.5% of all cases with craniosynostosis) [96,97].

Clinical findings in Apert syndrome tend to be more severe and to affect more organ systems than in many of the other common craniosynostosis syndromes. The skull malformations include turribrachycephaly with a disproportionately high cranium with frontal bossing and occasionally asymmetry. The anterior fontanelle is extremely wide open at birth, extending from the root of the nose to the posterior fontanelle. This midline calvarial defect does not close completely until the third year of life. Other anomalies of the skull include a malformed and often asymmetric cranial base and a very short anterior fossa.

Facial anomalies consist of hypertelorism with downslanting palpebral fissures and ocular proptosis. Strabismus is common, as are myopia, hyperopia, astigmatism, and dissociated eye movements [98]. The midface is hypoplastic with relative mandibular prognathism. The nasal bridge is depressed, the shape of the nose beaked. The palate can be highly arched, often with a median furrow or cleft of the soft palate, and the uvula may be bifid. Dental anomalies (malocclusion, delayed or ectopic eruption of teeth) are common [99]. Significant hearing loss is seen in some.

Central nervous system findings include anomalies of the corpus callosum, septum pellucidum, and limbic structures; hypoplasia of the cerebral white matter and heterotopic gray matter; megalencephaly; and/or ventriculomegaly [87]. Progressive hydrocephalus is present in only a minority of patients with ventriculomegaly [100]. Intelligence is variable and ranges from normal (40–50%) to mild or moderate mental retardation [101].

Hands and feet are syndactylous, characteristic for Apert syndrome, and the pattern differs from that seen in most of the other craniosynostosis syndromes [102–104]. In the hand, the central three digits are always syndactylous, with a broad thumb and part of the fifth finger separate (type 1 according to the classification of Upton [102]). In the "mitten-hand" deformity, the fifth finger is part of the syndactylous mass (type 2), and fusion of all digits ("rosebud") is seen in type 3. Syndactyly of the feet may involve the three lateral digits (type 1), digits 2–5 (type 2), or all digits (type 3). Fusion of digits is associated with fusion of the corresponding nails. Thumbs and great toes are broad and deviate away from the other digits. Incomplete postaxial polydactyly of the hands and preaxial polydactyly of the feet may be present [105,106]. Other limb anomalies include limited mobility at the glenohumeral joint which worsens with growth, moderate shortening of the humerus, and limitation of elbow extension [90]. Progressive synostosis of the bones in the hands, feet, and cervical spine is common. Fusions of the cervical vertebrae are present in 68% of patients with Apert syndrome, C5-C6 fusion being the most common [107].

Skin findings include acneiform eruption in more than 70% of patients. Other cutaneous manifestations are common and include hyperhidrosis, hyperkeratosis, hypopigmentation, nail infections, and skin dimples at the shoulders, elbows, and knuckles [91]. Low-frequency anomalies in the cardiovascular, respiratory, gastrointestinal, and genitourinary systems are reviewed elsewhere [89].

Apert Syndrome Shows Clinical and Allelic Heterogeneity

Two mutations in *FGFR2*, Ser252Trp and Pro253Arg, account for ~99% of Apert syndrome mutations elucidated to date, in a ratio of approximately 2:1 [108,109]. In one study, comparison of patients with the two different mutations showed a higher frequency of cleft palate and a tendency toward more severe craniofacial malformations with the Ser252Trp mutation, but more severe syndactyly with the Pro253Arg mutation [104]. This suggests that distinct molecular mechanisms are responsible for the craniofacial and limb abnormalities of Apert syndrome. Although a different study did not report significant correlations [110], this was based on a smaller sample size and there appeared to be a systematic bias in the classification of syndactyly [104].

Crouzon Syndrome

Crouzon syndrome (MIM 123500) was first described in 1912 [111] and there are numerous reports in the literature (for reviews see [112,113]). It is characterized by coronal or multiple suture synostosis with maxillary hypoplasia, a prominent beaked nose, shallow orbits, and ocular proptosis [100,114]. Crouzon syndrome has an autosomal dominant mode of inheritance, with high penetrance and moderate variability in clinical findings. Approximately half of all cases are familial, while the other half are new mutations with evidence of advanced paternal age. Crouzon syndrome is one of the most common craniosynostosis syndromes, with a birth prevalence of 1 in 65,000 newborns (approximately 4.5% of all craniosynostosis cases) [115].

Clinical findings include brachycephaly, scaphocephaly and trigonocephaly [116]. In one large family, the proband had a cloverleaf skull, several sibs had classic Crouzon syndrome, and several relatives had proptosis and midface hypoplasia without craniosynostosis [117]. Intelligence is usually normal. Seizures are infrequent. Brain anomalies (agenesis of the corpus callosum, for example) are rare, whereas progressive hydrocephalus is not uncommon and may be associated with herniation of the cerebellar tonsils [100,118,119]. Syringomyelia is a rare but important association [100].

Hypertelorism and external strabismus are common. Other ophthalmologic findings may include poor vision, optic atrophy, nystagmus, and exposure conjunctivitis and keratitis [120]. Mild to moderate hearing deficit is present in half of all cases. A hypoplastic maxilla and relative prognathism are common. Cervical spine fusions are also common (30%), mostly involving C2-C3 [121,122]. Calcification of the stylohyoid ligament occurs in 50% of patients [123]. Hands and feet are clinically normal, although minor differences are noted in metacarpophalangeal profile patterns [124–126]. Airway obstruction and visceral anomalies are rare compared with Apert and Pfeiffer syndromes [123,127].

Linkage studies have mapped Crouzon syndrome to chromosome 10q24–q25 [128], and numerous heterozygous mutations have been identified in *FGFR2*.

Crouzon Syndrome with Acanthosis Nigricans

Although Crouzon syndrome was first reported in 1912, Crouzon syndrome with acanthosis nigricans was not mentioned until 1948 [129]. Acanthosis nigricans is a dermatologic condition resulting in thickening of the skin with hyperpigmentation in the flexion creases of the thorax and limbs. With the identification of a consistent heterozygous mutation (Ala391Glu) in *FGFR3* [130–132], it has been recognized as a distinct clinical and molecular entity (MIM 134934.0011). Crouzon syndrome associated with acanthosis nigricans is rare. It is usually reported in females, with an early onset of skin findings including hyperpigmentation, hyperkeratosis, and melanocytic nevi. Verrucous hyperplasia develops predominantly in flexural areas such as the axillae, but also in the neck, face (perioral, periorbital, and nasolabial areas), chest, and abdomen. Craniofacial anomalies are similar to those in Crouzon syndrome itself, and include brachycephaly due to coronal synostosis, hypertelorism, downslanting of the palpebral fissures, and ocular proptosis. Additional anomalies have been described in some patients: choanal atresia, cemental dysplasia of both jaws (cementomas), hydrocephalus, hypoplasia of the foramen magnum, scoliosis, and spinal stenosis with paresthesia of the lower limbs.

Muenke Syndrome

Muenke syndrome (MIM 134934.0014 and 602849; also called Muenke craniosynostosis or *FGFR3*-associated coronal synostosis) was first described in 1996 in sporadic cases and autosomal dominant families with a unique point mutation in *FGFR3* (Pro250Arg) [58,59]. Penetrance is incomplete. The phenotypic spectrum is so variable that patients with this specific mutation had previously been diagnosed variously as having Pfeiffer, Saethre-Chotzen, or Crouzon

Figure 6. Craniofacial and limb anomalies in Muenke syndrome. Note symmetric facial appearance due to bicoronal synostosis (**upper panels**) and plagiocephaly due to unilateral coronal synostosis (**middle panels**). Mild brachydactyly of hands and feet (**lower panels**). (Reprinted from Muenke et al. [59], by permission of American Journal of Human Genetics.)

syndrome [58,59,133–136], as well as Jackson-Weiss syndrome [137] (below), Adelaide-type craniosynostosis (MIM 600593) [138], Ventruto syndrome (MIM 134934) [139], nonsyndromic craniosynostosis [58,59] or brachydactyly-associated craniosynostosis [140,141].

The birth prevalence of Muenke syndrome has not been accurately determined, but a figure of 1 in 30,000 is suggested by one study [141]. The mutation associated with this syndrome is common in sporadic or familial patients with uni- or bicoronal synostosis whose findings do not fit into any of the classic craniosynostosis syndromes. The mutation rate is estimated to be one of the highest in the human genome and comparable to that of achondroplasia (1138G>A in *FGFR3*, see below) and Apert syndrome (755C > G in *FGFR2*) [141].

Clinical findings observed in over 60 patients from more than 20 unrelated families include bi- or unicoronal synostosis (67% of cases), midface hypoplasia (59%),

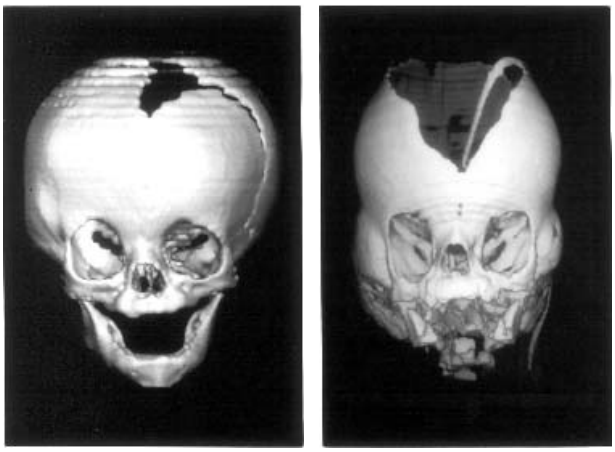

Figure 7. Three-dimensional CT scans in craniosynostosis syndromes. Plagiocephaly with facial asymmetry in a newborn with Muenke syndrome due to right unicoronal and metopic suture fusion (**left**). Severe synostosis of multiple sutures leading to cloverleaf skull and an extremely wide open fontanelle in a newborn with sporadic Pfeiffer syndrome and an *FGFR2* (Ser351Cys) mutation (**right**). (From Muenke and Wilkie [235]. Reprinted by permission of McGraw-Hill Companies Inc.)

downslanting palpebral fissures (50%), and ptosis (27%) (Fig. 6) [59]. In cases with bicoronal synostosis, bulging of the temporal fossae may give a wide-faced appearance [142]. However, it is of note that some mutation carriers do not show any signs of craniosynostosis, having only macrocephaly (6%) or even a normal head size [59,143,144]. In a prospective study, the Pro250Arg *FGFR3* mutation was identified in 4 of 37 individuals with unicoronal synostosis only, some cases of which had previously been attributed to intrauterine constraint [143]. Anterior plagiocephaly has also been described [134,141] (Fig. 7).

Sensorineural hearing loss is seen in 37% of cases, as is developmental delay. Mental retardation was noted in 4 of 9 patients with the Pro250Arg *FGFR3* mutation [134]. The extreme phenotypic variability is emphasized by a five-generation family in which the Pro250Arg mutation in *FGFR3* segregates with moderate congenital bilateral sensorineural deafness and low penetrance of craniosynostosis [145]. A greater severity of phenotype in females than males was suggested [142].

Hand anomalies are found in some, but not all, affected individuals [59] and include brachydactyly (30% of cases) and clinodactyly (42%) with characteristic radiographic findings, such as thimble-like middle phalanges (60%), coned epiphyses (75%), and carpal fusions (13%) (Fig. 8). Some patients (26%) have broad halluces that are not deviated. Radiological findings in the feet include short, broad middle phalanges (13%); coned epiphyses (86%); and calcaneocuboid fusions (35%). Height is normal, in contrast to the FGFR3-associated dwarfism syndromes.

Jackson-Weiss Syndrome

Jackson-Weiss syndrome (MIM 123150) was first reported in 1976 [146] in a large Amish kindred, in which 88 affected individuals were directly examined and another 50 reliably reported to be affected. Inheritance was autosomal dominant with high penetrance and variable expression. The syndrome is characterized by craniosynostosis with midface hypoplasia,

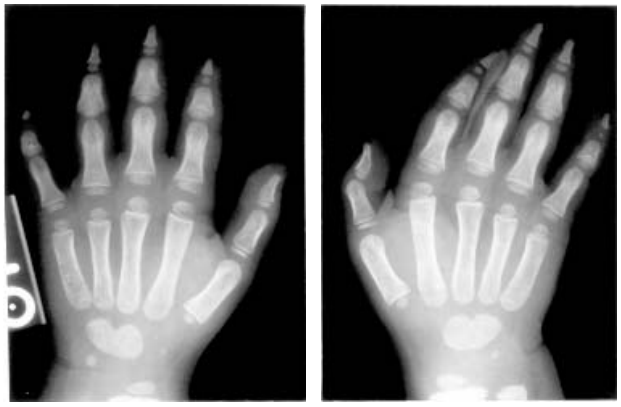

Figure 8. Radiographs of hands in Muenke syndrome. Brachydactyly with short thimble-like middle phalanges of the second through fifth fingers and fusion of the capitate and hamate bones of the wrists. (From Muenke and Wilkie [235]. Reprinted by permission of McGraw-Hill Companies Inc.)

frontal prominence, and foot anomalies with various cutaneous syndactylies and varying degrees of medially deviated and enlarged great toes. Thumbs were normal in all cases except one with fusion of the phalanges, and only one individual was reported with webbing of the fingers. Some affected individuals had no craniofacial anomalies on clinical and radiographic examination. Intelligence was normal. The variability of clinical findings was so marked that, with the exception of Apert syndrome, the entire spectrum of acrocephalosyndactylies was seen in the reported family [146]. Because of the lack of thumb anomalies in Jackson-Weiss syndrome, several families with craniosynostosis and foot anomalies were initially assigned this diagnosis [137,140] but were later found to have mutations in *FGFR1* and *FGFR3*, respectively, and not the *FGFR2* Ala344Gly alteration identified in the original family [147]. There is also overlap between the Jackson-Weiss phenotype and phenotypes associated with different mutations of *FGFR2*, leading to further confusion. In the opinion of the present authors, the diagnosis of Jackson-Weiss syndrome in sporadic cases or small families is likely to cause more confusion than enlightenment. We therefore propose that the designation Jackson-Weiss syndrome be reserved for the original large family, until a new nomenclature for the acrocephalosyndactylies, combining clinical and molecular data, has been agreed upon.

Beare-Stevenson Syndrome

Beare-Stevenson syndrome or the cutis gyrata syndrome of Beare and Stevenson (MIM 123790) was first described in 1969 [148]. It is a rare, lethal, sporadic condition for which there is a total of only 9 published cases [148–154]. Characteristic clinical findings include corrugated skin furrows (cutis gyrata) affecting the scalp, forehead, face, neck, trunk, hands and feet, and acanthosis nigricans. Other skin anomalies include skin tags and an enlarged umbilical stump. Craniosynostosis can be severe, and was present in four of six cases, with cloverleaf skull occurring in three of these [42]. Additional findings in some cases include hypertelorism, a broad nasal bridge, ear defects, cleft palate, hypodontia, choanal atresia, bifid scrotum, anogenital anomalies, and coccygeal eversion.

All reported cases have been sporadic, with an increased paternal age in some. Heterozygous mutations of *FGFR2*, Ser372Cys and Tyr375Cys, have been described, whereas some patients did not have mutations in these regions, suggesting further genetic heterogeneity [153,154].

SKELETAL DYSPLASIAS CAUSED BY *FGFR3* MUTATIONS

All four phenotypes in the achondroplasia family of skeletal dysplasias (achondroplasia, hypochondroplasia, thanatophoric dysplasia and SADDAN) are inherited as autosomal dominant conditions caused by mutations in the *FGFR3* gene on human chromosome 4p16.3. In all of these conditions, penetrance is complete. The majority of cases occur as a result of new mutations.

Achondroplasia

The phenotype of achondroplasia (MIM 100800) may be somewhat subtle in the newborn, but the astute clinician will recognize the relatively short limbs and macrocephaly with frontal bossing and mid-face hypoplasia [155]. The limbs are more notably short in the proximal segment, although the entire limb is short; this is called "rhizomelic" dwarfism. Other features of the limbs include limitation of elbow extension and the so-called "trident hand," in which there is a distinct space between the distal phalanges of the third and fourth digits. Hyperextensibility of the knees and hips is common. A thoracic gibbus may be present at birth and usually resolves spontaneously, coincident with the development of improved muscle tone and the commencement of independent sitting. The expected height for an adult with achondroplasia is 45 to 55 inches (Fig. 9). The growth curves for head circumference (Fig. 10) overlap with those for unaffected children, but true megalencephaly is often present. There is an increased risk of intracranial bleeding during vaginal delivery [156].

The affected newborn is often hypotonic, but muscle tone usually improves during the first and second years of life. One consequence of the low muscle tone during infancy is a delay in the development of motor milestones. Typically, intelligence is normal [157].

Radiographic abnormalities include caudad narrowing of the vertebral interpedicular distance and a notch-like sacroiliac groove in the pelvis. The long bones are short and relatively wide, as are the tarsal and metatarsal bones and all of the phalanges [158,159] (Fig. 11).

The age-specific mortality for achondroplasia is relatively high in the first four years of life, approaching 7.5% in the first year and 2.5% in years 1 to 4 [160]. The mortality curves approach those of unaffected children in later childhood, adolescence and early adulthood, but climb again at the end of the fourth decade [160]. The cause of this increase in age-specific mortality in mid-adulthood is unknown.

Relatively small ear canals often lead to poor drainage and result in frequent episodes of otitis media. These may result in compromised hearing if not recognized and treated appropriately in early childhood.

Neurologic complications may arise from a relatively small foramen magnum present in the newborn period in almost all children with achondroplasia. Compression of the cervico-medullary junction by a small foramen magnum may result in generalized hypotonia, hyperreflexia or clonus, and/or central hypopnea or central apnea [161]. The increased mortality rate in young children is attributable to foramen magnum stenosis. Surgical decompression of

a compromised cervico-medullary junction by a surgeon experienced in the procedure has a high probability of improving neurologic function [162]. Hydrocephalus may result from stenosis of the sigmoid sinus secondary to narrowed jugular foramina [163,164]. Conventional treatment for hydrocephalus in affected patients is the placement of a ventriculo-peritoneal shunt, although venous decompression at the jugular foramen has been proposed as an alternative [165].

In older persons, the narrow spinal canal frequently results in symptomatic thoracic and/or lumbar spinal stenosis. Initial symptoms are usually back pain and pain on walking which are relieved by squatting. These may progress to unremitting pain, leg weakness and bladder and bowel incontinence if untreated. This complication is amenable to surgical correction by spinal laminectomy [166].

Pulmonary compromise may result from any or all of three contributing factors. A relatively small chest may lead to restrictive lung disease. Small upper airways may contribute to the development of obstructive apnea requiring treatment by continuous positive airway pressure (CPAP) or tracheostomy [167–169]. Finally, compression of the cervico-medullary junction may cause central hypopnea or central apnea necessitating, in the most severe instances, surgical decompression of the foramen magnum.

In older patients with achondroplasia, obesity is often a significant problem [170,171]. Up to a height of 75 cm, the mean weight:height curves are comparable for average-statured children and children with achondroplasia, but above 75 cm, the weight:height curves for achondroplasia rise significantly above those for the general population [170]. Obesity contributes to the morbidity associated with lumbar stenosis, and also contributes to nonspecific joint problems and may play a role in the early mortality seen in some adults with achondroplasia.

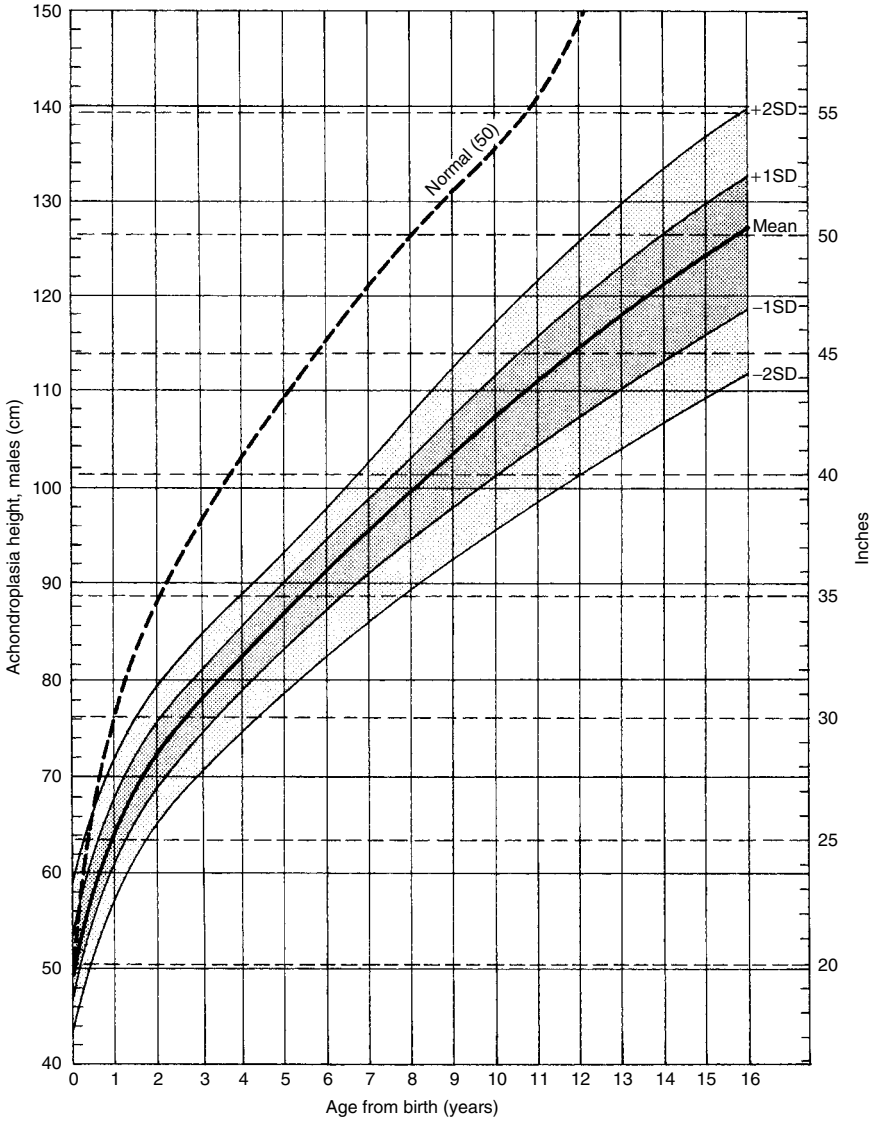

Figure 9. Height curve charts for achondroplasia. Achondroplasia, height, males and females, birth to 16 years. (From Horton et al. 1978 [236]. Reprinted by permission of Journal of Pediatrics.)

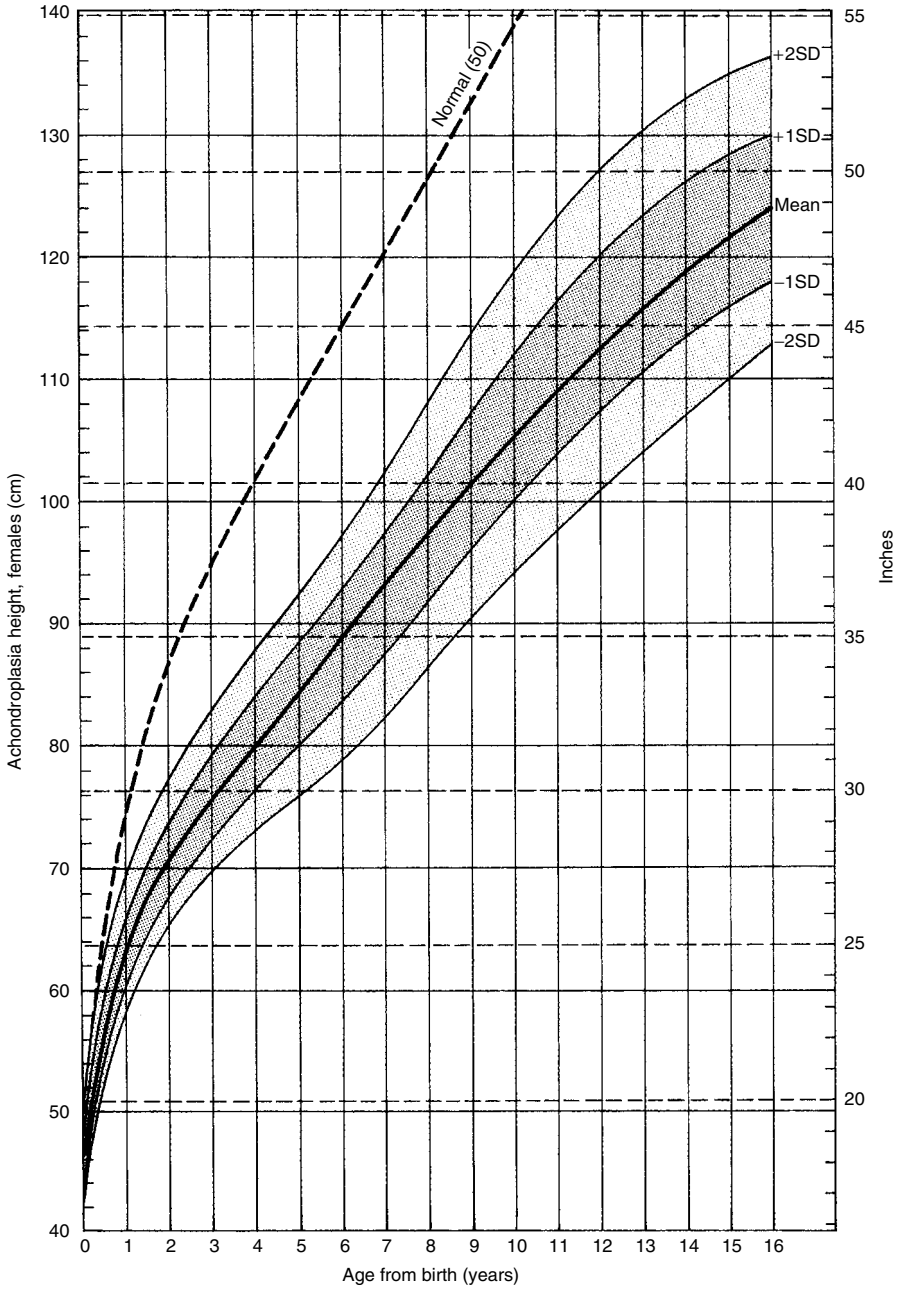

Figure 9. (*Continued*)

Homozygous Achondroplasia

Couples in which both individuals have achondroplasia (Fig. 12) are at 25% risk of having children who inherit two copies of the achondroplasia mutation. These children are said to have homozygous achondroplasia, or, colloquially, "double dominant" achondroplasia [172,173]. This phenotype is almost invariably lethal within the first days to months of life. It may be difficult to distinguish clinically from thanatophoric dysplasia (below), but the family history is the key to this differential diagnosis. Children with homozygous achondroplasia have pronounced rhizomelic dwarfism, marked mid-face hypoplasia, large heads relative to the size of their bodies, and short ribs resulting in a small thorax and restrictive respiratory compromise. A small foramen magnum may also lead to lethal compression at the cervico-medullary junction.

Hypochondroplasia

The phenotype of hypochondroplasia (MIM 146000) (Fig. 13) is very similar to, but generally milder than, that which presents in achondroplasia [174,175]. Affected persons have rhizomelic short-limbed dwarfism, but the short stature is less extreme than in achondroplasia and the growth curve actually overlaps the height ranges of

average children. The craniofacial manifestations are not as pronounced. Head size is usually within the normal range, and there are rarely complications of cervical or lumbar spinal stenosis. A few reports have suggested that learning disabilities may be increased among children affected by hypochondroplasia [176,177].

Thanatophoric Dysplasia

Thanatophoric dysplasia (MIM 187600), a neonatally lethal condition, the name of which means "death bearing," lies at the other end of the severity spectrum from hypochondroplasia. Almost all children with thanatophoric dysplasia die within the neonatal period, although there have been a few reports of survivors into the first decade [178,179]. Children born with this condition have very severe rhizomelic dwarfism, profound midface hypoplasia and extremely short ribs resulting in a small thorax. Death is thought to be due to respiratory failure, as a result of the small thorax and compromised upper airways, or to neurologic impairment, secondary to an extremely small foramen magnum (Fig. 14). Molecular analysis (see below) has enabled the distinction between the two types of thanatophoric dysplasia: thanatophoric dysplasia type I has curved femurs with a cloverleaf skull. Thanatophoric dysplasia type II is associated with straight femurs, with or without cloverleaf skull.

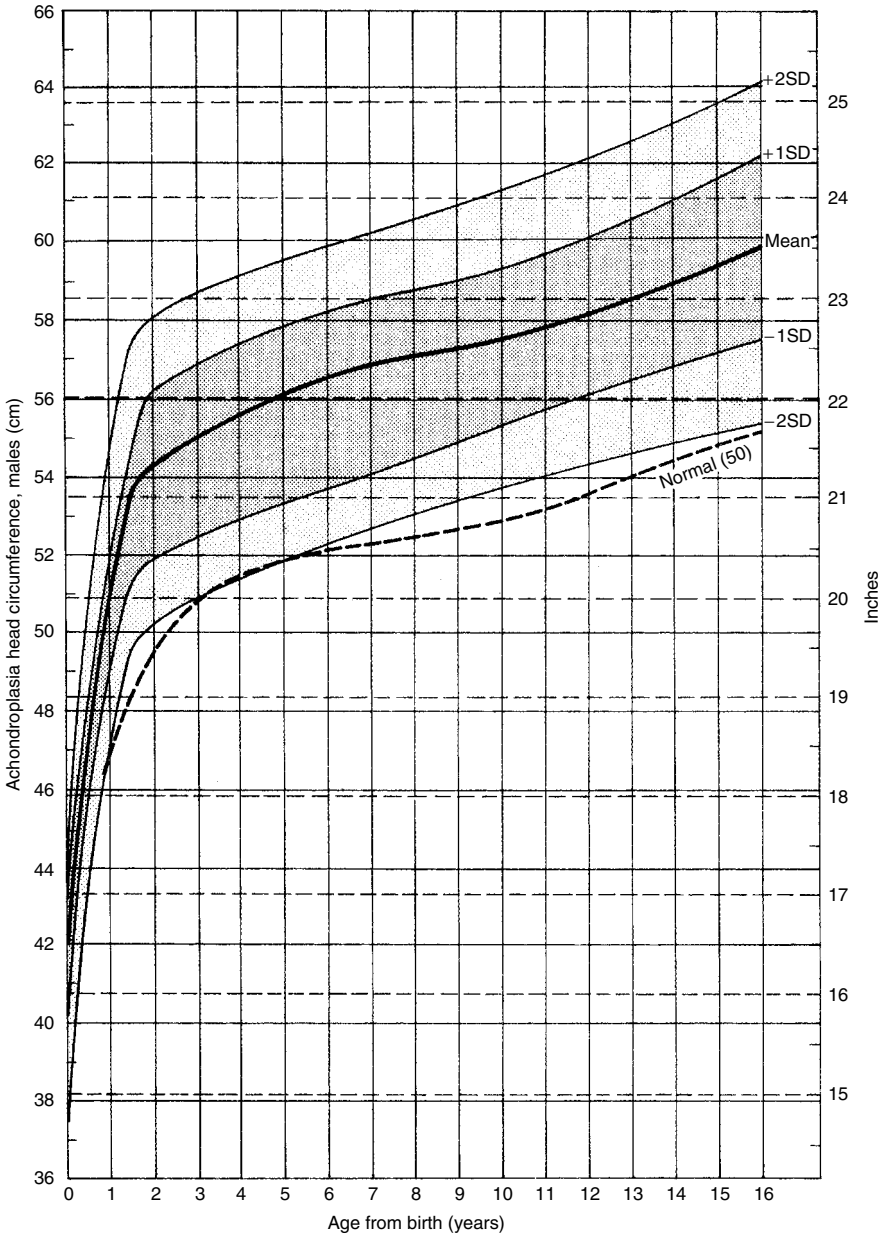

Figure 10. Head circumference charts for achondroplasia. Achondroplasia, head circumference, males and females, birth to 16 years. (From Horton et al. [236]. Reprinted by permission of Journal of Pediatrics.)

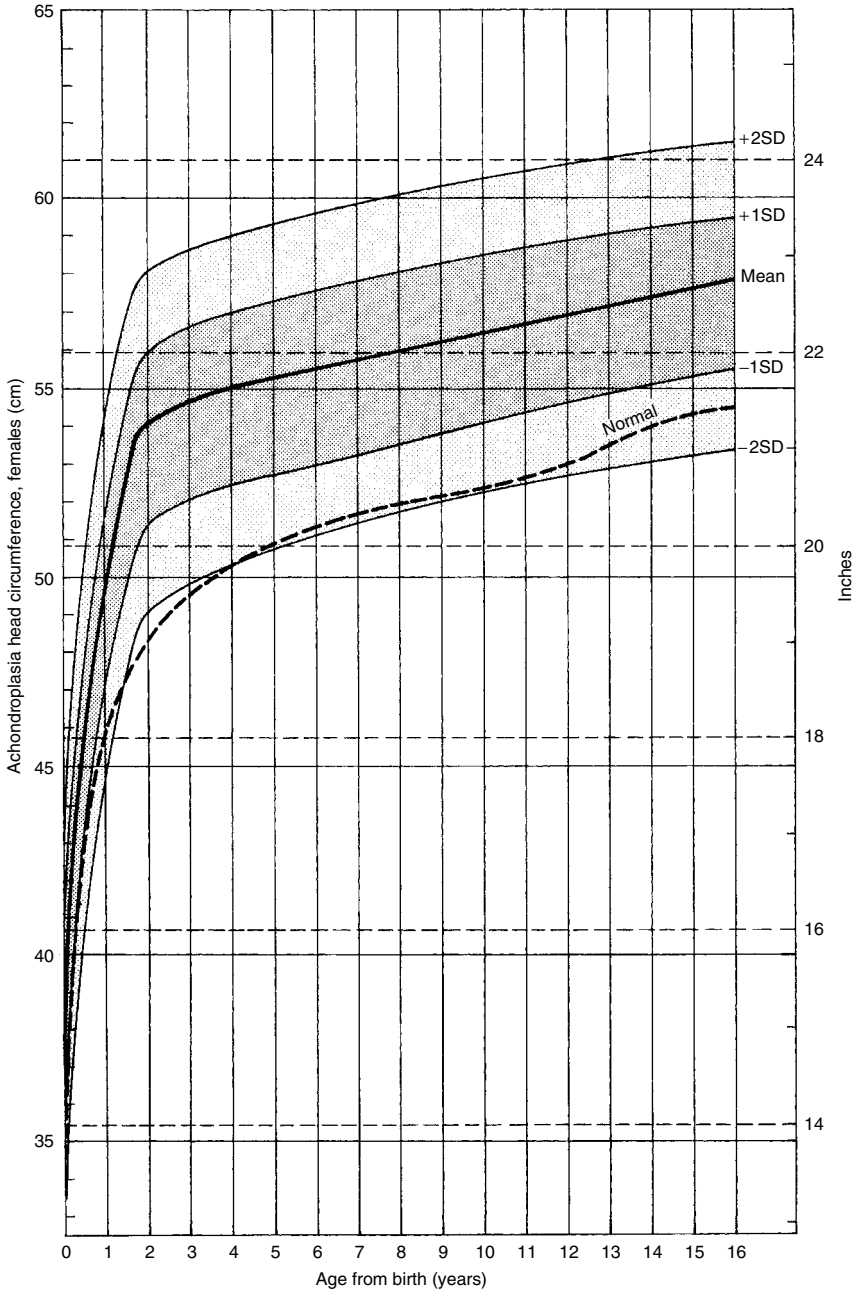

Figure 10. (*Continued*)

Severe Achondroplasia with Developmental Delay and Acanthosis Nigricans (SADDAN)

This phenotype (MIM 134934.0015), first described in 1999, is caused by a single specific mutation in the *FGFR3* gene [180]. Affected persons have profound short stature (one affected 5-year-old child was at the 50th percentile for a 1-year-old with achondroplasia), marked craniofacial characteristics including midface hypoplasia, and severe developmental delay and eventual mental retardation (Fig. 15). They also develop acanthosis nigricans. Medical complications are similar to those seen in achondroplasia, but occur earlier in life and with greater severity. Hydrocephalus and seizures occurred in all three reported children with the SADDAN phenotype [181]. In each case, the disorder resulted from a 1949A>T mutation causing a Lys650Met substitution. This is the same codon in which mutations causing thanatophoric dysplasia type II are found.

COMMON FEATURES OF FGFR DISORDERS

FGFR Mutations in Craniosynostosis Syndromes and Skeletal Dysplasias

Mutations in *FGFR2* were initially identified in Crouzon syndrome in 1994 [147,182]. A candidate gene approach was used after linkage had been established to chromosome 10q

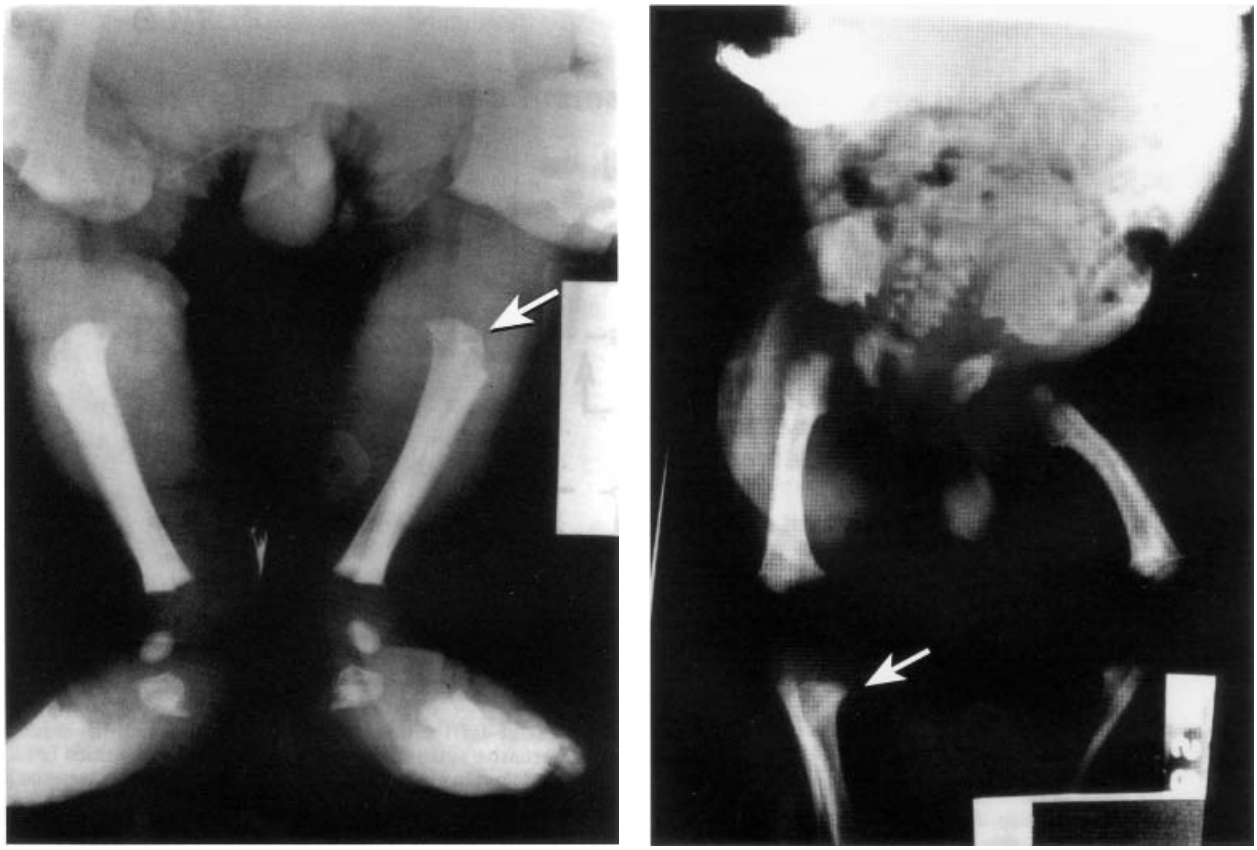

Figure 11. Radiographic features of achondroplasia. Lower limbs in a young child. Note widened metaphyses, ''chevron seat'' epiphyses (arrowed), and short long bones. Radiographically, manifestations can also include lumbar lordosis and mild thoracolumbar kyphosis, with anterior beaking of the first and/or second lumbar vertebrae; small cuboid-shaped vertebral bodies with short pedicles and progressive narrowing of the lumbar interpedicular distance; small iliac wings with narrow greater sciatic notch; short tubular bones; metaphyseal flaring; short trident hand with short proximal midphalanges; and short femoral neck. (Figures courtesy of Dr. Ralph Lachman, Cedars-Sinai Medical Center, Los Angeles.)

in both Crouzon and Jackson-Weiss syndromes [183,184]. Further FGFR mutations were identified in several other craniosynostotic disorders shortly afterwards. To date, heterozygous mutations of *FGFR1*, *−2*, and *−3* have been identified in seven distinct craniosynostosis syndromes. These are: mild Pfeiffer syndrome (*FGFR1*); Apert, Crouzon, mild and severe Pfeiffer, and Beare-Stevenson syndromes (*FGFR2*); Muenke syndrome and Crouzon syndrome with acanthosis nigricans (*FGFR3*). In addition, *FGFR2* mutations have been identified in some other craniosynostosis phenotypes including Jackson-Weiss syndrome.

In 1994, the gene causing achondroplasia was mapped to a region of 2.5 Mb of DNA at the telomeric end of the short arm of chromosome 4, 4p16.3 [185–187]. The candidate region contained the gene encoding FGFR3, which had been cloned in the search for the Huntington disease locus in the same region. Mutations in the *FGFR3* gene were found in the DNA from patients with achondroplasia within 6 months of recognition of the map location [188,189]. Soon thereafter, *FGFR3* mutations causing thanatophoric dysplasia were found [190]. The identification of *FGFR3* mutations that cause hypochondroplasia [191] completed the elucidation of the molecular basis of the achondroplasia family of skeletal dysplasias and confirmed the allelic nature of these disorders.

The paucity of incidences of familial recurrence of achondroplasia speaks against significant levels of germline mosaicism, although there have been a few reports of multiple affected children from families with unaffected parents [192,193]. Germline mosaicism has been demonstrated in families with recurrence of other skeletal dysplasias, including osteogenesis imperfecta type II [194] (see also Chapter 8, this volume) and pseudoachondroplasia [195] (see also this chapter, part II).

Almost all cases of achondroplasia are caused by the same Gly380Arg substitution. Exceptions include three cases with reportedly classical achondroplasia, in which the mutation was 5 codons downstream producing Gly375Cys [196–198]. This mutation also affects the transmembrane domain of FGFR3. One patient with a Gly346Glu mutation has also been reported [199].

Nature of Mutations

The great majority of the mutations seen in the FGFR genes are missense, with a few small insertions, deletions or combined insertion/deletions, all of which remain in-frame.

Figure 12. Typical achondroplasia, in a husband and pregnant wife. Note the disproportionate short stature with rhizomelic (proximal) shortening of the limbs, relative macrocephaly, and midface hypoplasia. Some of the additional manifestations of achondroplasia are lumbar lordosis; mild thoracolumbar kyphosis, with anterior beaking of the first and/or second lumbar vertebrae; short tubular bones; short trident hand; and incomplete elbow extension. The child was found to have heterozygous achondroplasia. (From Vajo et al. [237]. Reproduced by permission of Endocrine Reviews.)

Figure 13. Typical hypochondroplasia. Notice small stature, especially in the bowed lower limbs, and stubby hands and feet. In hypochondroplasia, limbs are usually short, without rhizomelia, mesomelia, or acromelia, but there may be mild metaphyseal flaring. Brachydactyly and mild limitation in elbow extension can be evident. Spinal manifestations may include anteroposterior shortening of lumbar pedicles. The spinal canal may be narrowed or unchanged caudally. Lumbar lordosis may be evident. (From Beals RK [238]. Reproduced with permission of Journal of Bone and Joint Surgery.)

Also found are acceptor and donor splice site mutations of exon IIIc and two *Alu* element insertions.

Mutations in *FGFR1*, *FGFR2*, and *FGFR3*, associated with craniosynostosis are illustrated in Figure 16. Figure 17 illustrates all known *FGFR3* mutations.

The cysteine residues in the IgIII domain of FGFR2 are the sites of highest mutation frequency. The Cys342 residue alone accounts for about 40% of all IgIII mutations. Mutations have been identified which reflect all six amino acid substitutions that can arise by mutating one nucleotide of the TGC codon (Fig. 18). In addition, approximately 35% of mutations at non-cysteine residues within IgIII are substitutions of cysteine. Generation of an odd number of cysteines (1 or 3) in IgIII leads to intermolecular covalent dimerization.

Mutations at the 5′ end of the IIIc exon are thought to result in preferential splicing to the IIIb exon and hence increased expression of the distinct keratinocyte growth factor receptor (KGFR) splice form. This outcome would be expected to confer unique ligand binding characteristics upon the cell and hence represents a potential mechanism for gain-of-function [21] (see below, Biochemical Analysis of Fibroblast Growth Factor Receptor 3 Mutations.)

The first reports of mutations in *FGFR3* causing achondroplasia [188,189] demonstrated that 38 of 39 mutations found were exactly the same, a G to A transition at nucleotide 1138. The remaining mutation was a G to C transversion, also at nucleotide 1138. Both mutations result in the substitution of arginine for the glycine residue at position 380 in the transmembrane domain of the protein (Fig. 19). Most analyses were performed on heterozygous achondroplasia patients but the Gly380Arg mutation was also detected in several cases of homozygous achondroplasia. A larger series reported in 1995 found a Gly380Arg mutation

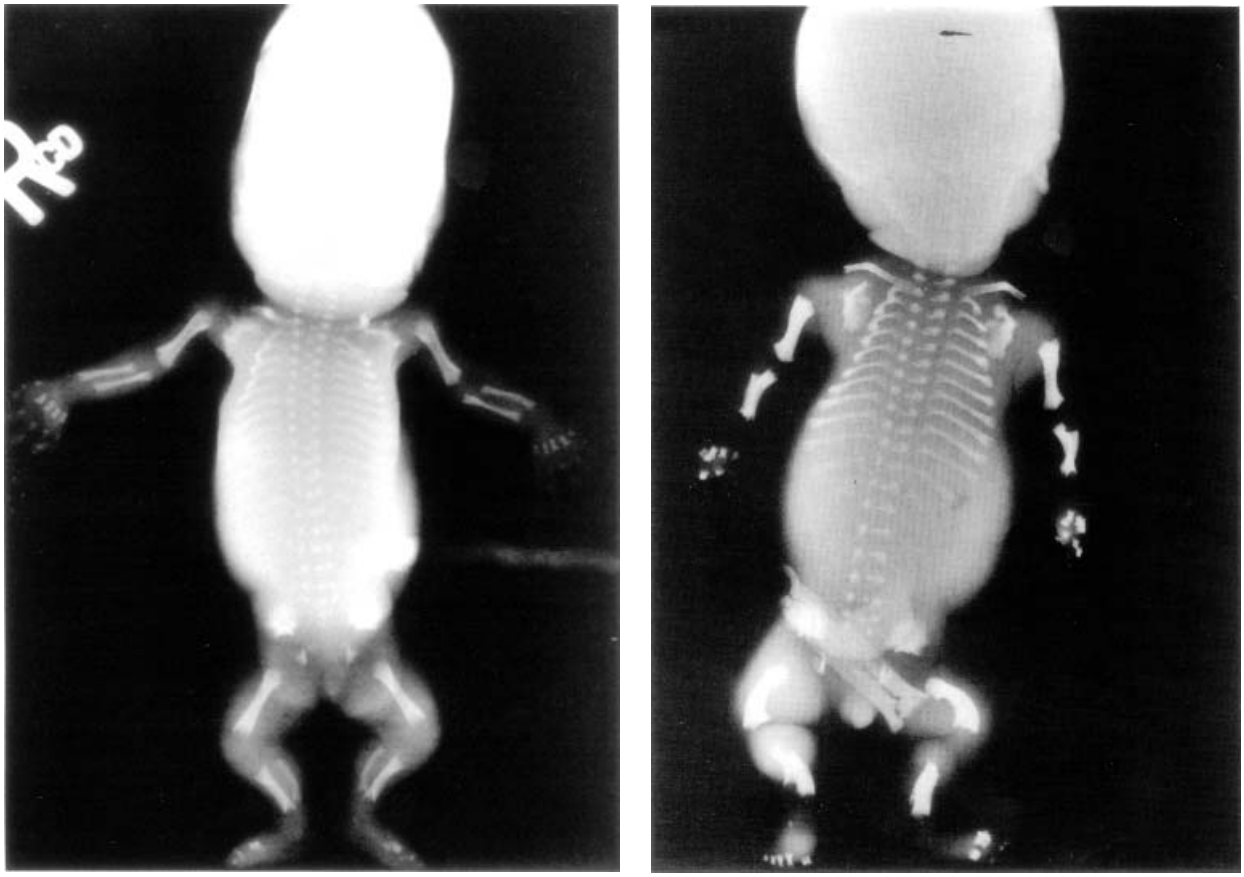

Figure 14. Thanatophoric dysplasia (TD). The features of this condition include micromelic shortening of the limbs, macrocephaly, platyspondyly, and a reduced thoracic cavity with short ribs. **A:** TD type II. Note the straight femurs. Cloverleaf skull may also be a feature of TD II. **B:** TD type I. Note the curved femurs. (Figures courtesy of Dr. Ralph Lachman, Cedars-Sinai Medical Center, Los Angeles.)

on 153 of 154 alleles, confirming the remarkable degree of genetic homogeneity of this disorder [200]. In this later series, the G to A transition accounted for 150 alleles, while the G to C transversion was found in three. (The remaining proband of the 154 patients studied was later recognized as having SADDAN dysplasia.) Stoilov et al. [201] found the Gly380Arg mutations in the DNA from 21 of 23 persons with achondroplasia.

Studies from Sweden, Japan and China have demonstrated Gly380Arg mutations in almost all patients with achondroplasia analyzed [202–204]. A total of 16 Swedish, 10 Chinese, and 20 Japanese patients were studied for the presence of *FGFR3* mutations. All of the Swedish and Chinese patients, and 18/20 of the Japanese patients were heterozygous for the 1138 G>A transition [202–204].

The FGFR mutations vary greatly in frequency, with some being highly recurrent. The C>G nucleotide substitutions encoding the Pro250Arg mutation in *FGFR3* and the Ser252Trp mutation in *FGFR2* represent the most common transversions known in the human genome, with frequencies per haploid genome estimated as 8×10^{-6} [141] and 5×10^{-6} [205] respectively.

Similarly, the estimated mutation rate for *FGFR3* Gly380Arg is between 5.5×10^{-6} and 2.8×10^{-5} per gamete per generation [200]. These rates are based on an estimated frequency of achondroplasia of between 1/15,000 and 1/50,000 live births [206,207].

These observations, taken with the relatively high incidence of achondroplasia, suggest that particular nucleotides in the FGFR genes are among the most mutable nucleotides in the human genome. The altered nucleotide in the common achondroplasia mutation *FGFR3* G1138 occurs in the context of a CpG dinucleotide, which might explain some of the increased incidence at this site. However, the mutation at G1138 is two to three orders of magnitude higher than the rate previously reported for transversions and transitions at CpG dinucleotides [200,208].

The close relationship between specific mutations and specific phenotypes in the FGFR family of genes is unusual. Unlike most human genetic disorders, in which there is a wide range of mutations causing a similar phenotype, in this family of disorders there is a very tight correlation between specific mutations and the phenotypes with which they are associated. In achondroplasia, more than 97% of cases are caused by one of two mutations that substitute arginine for glycine at codon 380 in the transmembrane domain of the protein [202–204]. More than 80% of cases of hypochondroplasia are caused by mutations resulting in the substitution of asparagine for lysine at codon 540 [191].

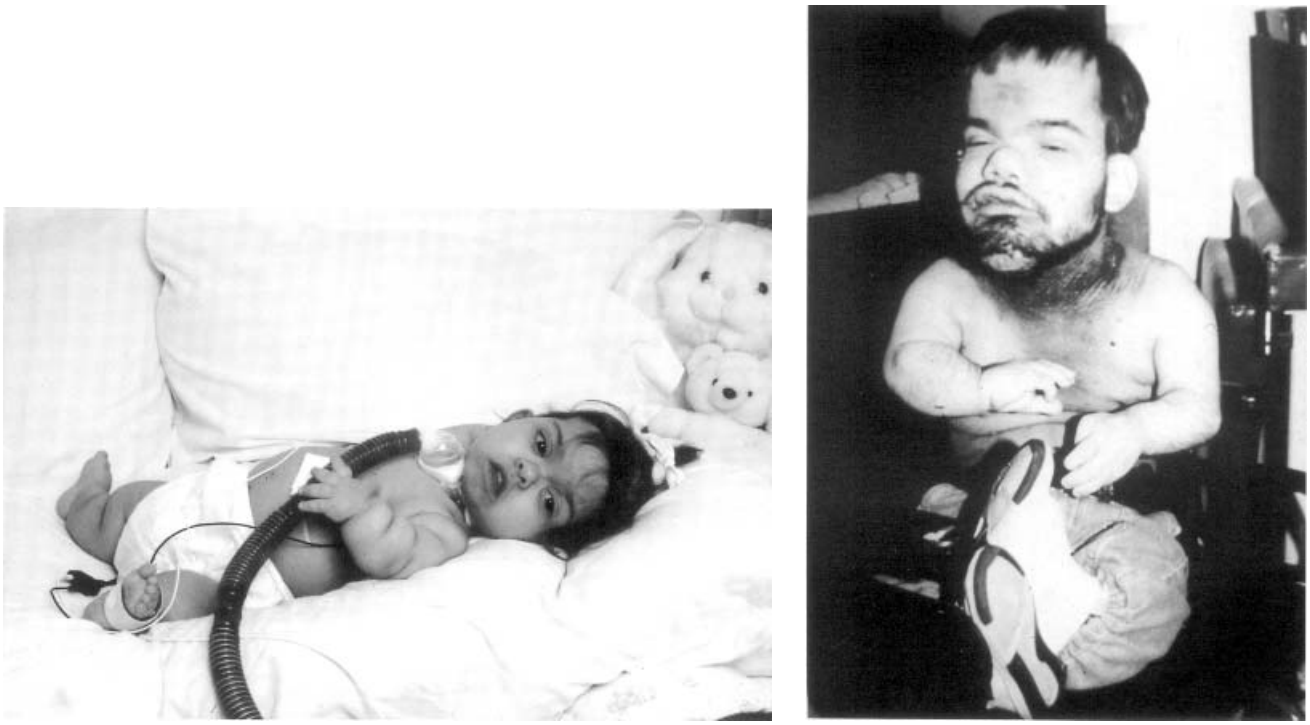

Figure 15. SADDAN dysplasia is characterized by extreme short stature, severe tibial bowing, profound developmental delay, and acanthosis nigricans. **A:** 5-year-old girl. Notice the moderate bowing of the femurs with reverse bowing of the tibia and fibula. **B:** Man in early twenties. Notice the extreme short stature and severe acanthosis nigricans especially around the neck. Individuals with SADDAN dysplasia also have had seizures and hydrocephalus during infancy with severe limitation of motor and intellectual development. (From Bellus et al. [181]. Reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley and Sons, Inc.)

Thanatophoric dysplasia is an interesting case in point; analysis of mutation data allowed clinicians to go back to their patients and sort out two distinct clinical subsets, based on the molecular data. Thanatophoric dysplasia type I is associated with curved femurs and other tubular bones and infrequently a cloverleaf skull, and a variety of mutations in multiple different domains of the FGFR3 molecule. Thanatophoric dysplasia type II, however, presents straight femurs, may or may not show cloverleaf skull, and is invariably caused by mutations resulting in a Lys650Glu substitution in the intracellular tyrosine kinase domain of the FGFR3 molecule [180,209,210].

Mutations at identical positions in FGFR paralogues are observed in several regions of the molecule. The best example of this phenomenon is seen in a Ser-Pro dipeptide in the IgII-IgIII linker, part of a sequence of 16 amino acids conserved between all four human FGFRs. Mutations of proline to arginine in this linker in FGFR1 cause Pfeiffer syndrome [55], in FGFR2, Apert syndrome [108] and in FGFR3, Muenke syndrome [59] (Fig. 20).

The FGFR family of disorders represents several allelic series, i.e., mutations in one gene can cause different phenotypes. Mutations of both *FGFR2* and *FGFR3* have been associated with more than one syndrome. The observation that allelic missense mutations cause distinct dominant phenotypes suggests that different signalling pathways are activated by unique gain-of-function mutations. This point is made particularly apparent by the recognition of distinct *FGFR3* missense mutations in the syndromes of hypochondroplasia, achondroplasia, thanatophoric dysplasia and SADDAN (severe achondroplasia with developmental delay and acanthosis nigricans) syndrome [211]. Craniosynostosis is rare in these latter disorders except in the case of thanatophoric dysplasia.

Paternal Age Effect in Achondroplasia and Apert Syndrome

As has been the case in several different autosomal dominant conditions, studies of families with Apert syndrome have demonstrated that unaffected fathers of affected infants were older, on average, than fathers of unaffected children [97]. Similar observations have been made in families with achondroplasia, and one report demonstrated that the achondroplasia mutation was exclusively present on the paternal allele in a cohort of 40 families [23]. In Apert syndrome, the two common mutations were exclusively paternal in origin in 57/57 cases and exhibited a significant paternal age effect [205]. These observations indicate that these mutations arose during spermatogenesis in the unaffected father.

Pathogenesis of FGFR Disorders

FGF/FGFR Signalling in Cranial Sutures

Several studies have demonstrated expression of FGFs and FGFRs at the mRNA level in mouse cranial sutures [212–214]. Placement of an FGF2-soaked, heparin-coated bead over the coronal suture in mouse embryos

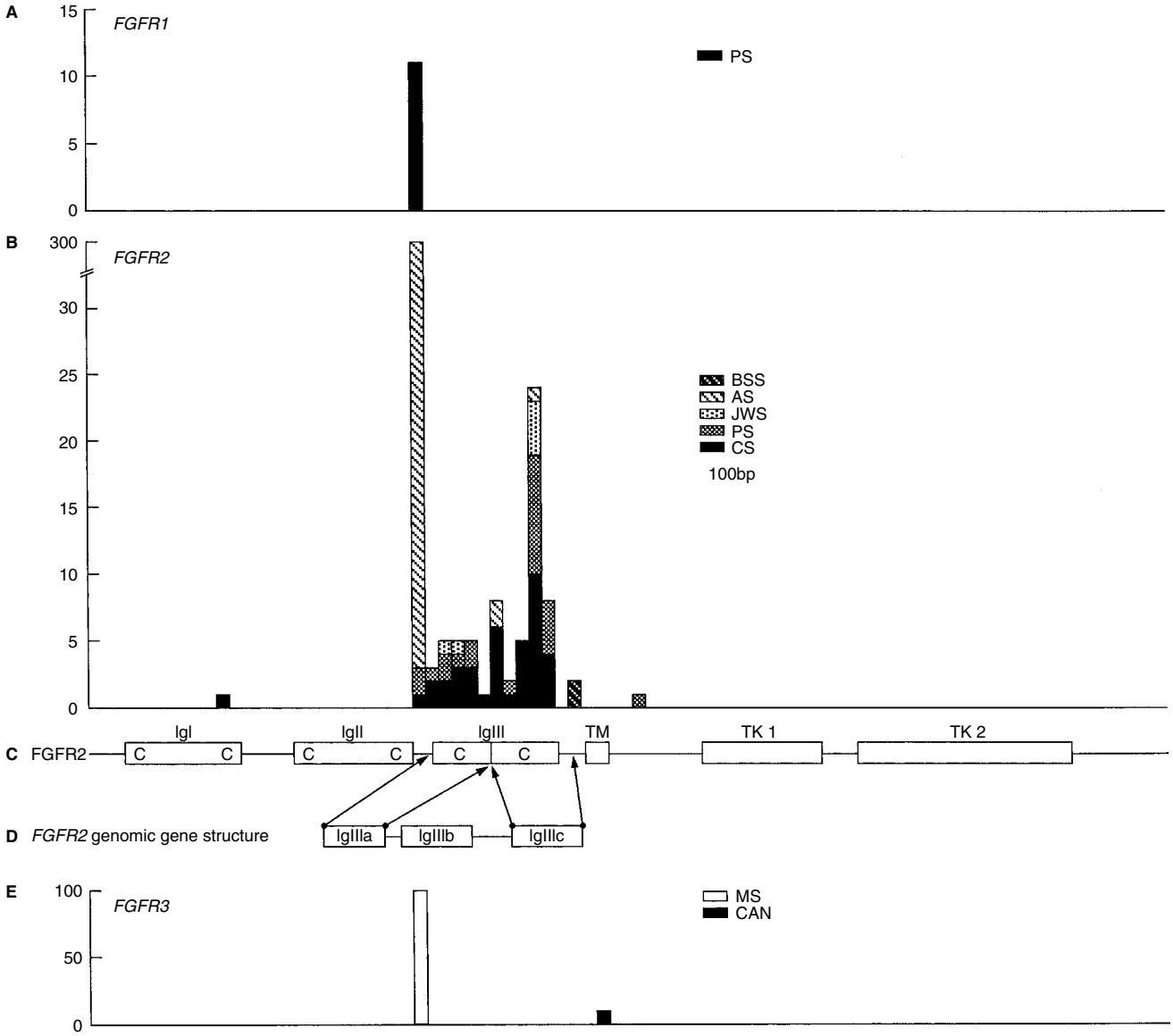

Figure 16. Number and location of the reported mutations in *FGFR1* (**A**), *FGFR2* (**B**), and *FGFR3* (**E**) superimposed on the predicted structure of the *FGFR2* protein with three Ig-like domains in the extracellular region (IgI–IgIII), a transmembrane segment (TM), and a split intracellular tyrosine kinase activity region (TK1 and TK2) (**C**). Genomic structure of the region of *FGFR2* involved in alternative splicing of the IgIII-like domain [108]. (**D**), FGFR mutations have been identified in patients with the different craniosynostosis syndromes: Apert syndrome (AS), Beare-Stevenson syndrome (BSS), Crouzon syndrome (CS), Crouzon syndrome with acanthosis nigricans (CAN), Jackson-Weiss syndrome (JWS), Muenke syndrome (MS), and Pfeiffer syndrome (PS). (From Hehr and Muenke [239]. Reproduced with the permission of Molecular Genetics and Metabolism.)

resulted in differentiation of the sutural tissue underlying the bead, with a new suture-like structure being formed around the bead. Expression of Fgf2 was observed in the rat posterior frontal suture at the time of suture fusion [214]. A mouse model of Crouzon syndrome, Bulgy-eye (Bey), has a retrovirus inserted in the intergenic region between the tandem *Fgf3* and *Fgf4* genes, resulting in significantly higher expression levels of both genes in the cranial sutures of affected mice [215].

Histological examination of 19–28 week human fetuses with Apert syndrome showed increased calcified bone matrix and stronger alkaline phosphatase staining of cells in the subperiosteal area compared to controls, indicating increased maturation of preosteoblast cells. In culture, these cells demonstrated increased bone differentiation markers including alkaline phosphatase and osteocalcin [216].

One seemingly paradoxical result is that immunoassay of FGFR2 protein in the cranial sutures of Apert and Crouzon syndrome patients showed reduced levels [216,217]. However, this is likely to be a consequence of down-regulation of FGFR caused by excessive signalling through the FGF/FGFR pathway, a feedback loop that has been demonstrated in

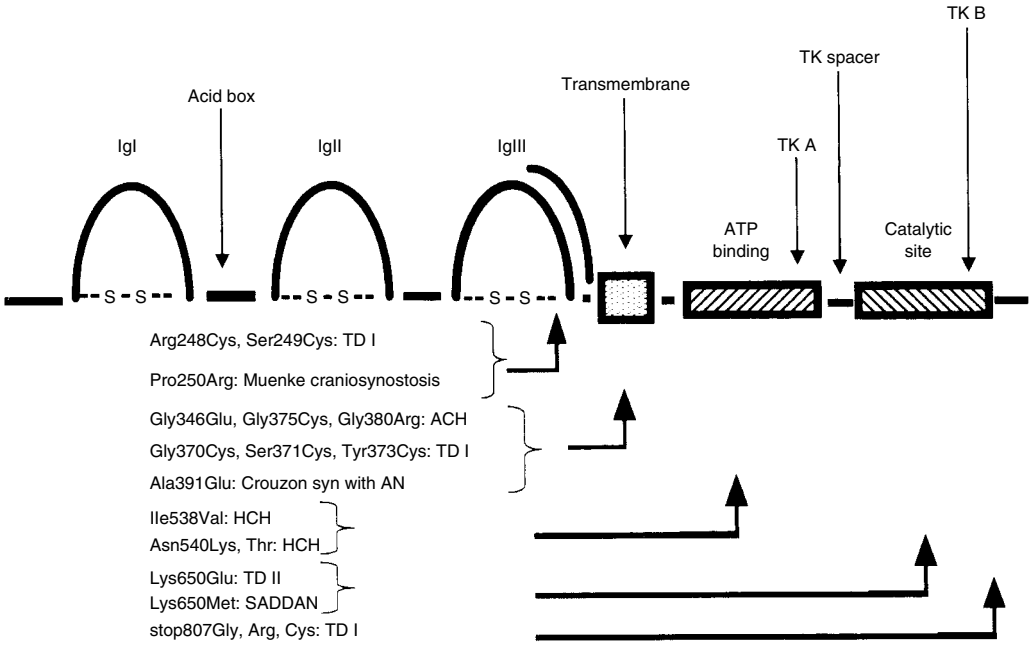

Figure 17. Schematic diagram of a prototypical FGFR protein. Three Ig-like domains (IgI–IgIII) are indicated by loops, closed with disulfide bridges. These Ig-like domains are extracellular and responsible for ligand binding. Alternative splicing in the C-terminal half of the third Ig-like loop is indicated by an extra ''half'' loop. The acid box is a stretch of acidic amino acids found in all FGFRs between IgI and IgII. The tyrosine kinase (TK) domains are found intracellularly. The TK A domain contains the ATP-binding site. The TK B domain contains the catalytic site. Also shown are known *FGFR3* mutations and their approximate corresponding locations within the protein. TD, thanatophoric dysplasia; ACH, achondroplasia; AN, acanthosis nigricans; HCH, hypochondroplasia.

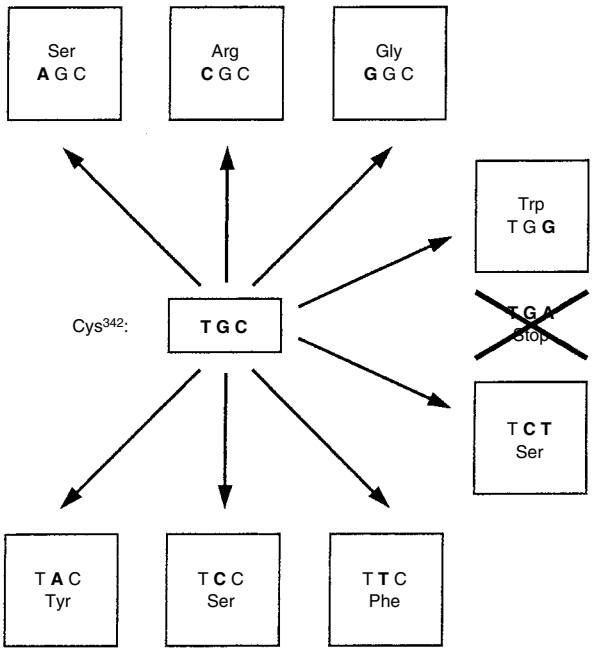

Figure 18. Observed point mutations resulting in the substitution of cysteine at position 342 of *FGFR2*. All possible missense mutations at this position have been identified in patients with craniosynostosis syndromes with one exception, the nonsense mutation TGC > TGA. This further supports the hypothesis of a ''gain-of-function'' effect of the heterozygous FGFR mutations in craniosynostosis. (From Hehr and Muenke [239]. Reproduced by permission of Molecular Genetics and Metabolism.)

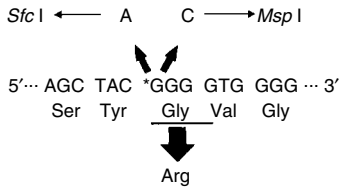

Figure 19. The common *FGFR3* mutations causing achondroplasia both result in Gly380Arg amino acid substitutions. Shown is the *FGFR3* sequence surrounding the site of the common mutation. An 1138 G>A mutation creates a novel *Sfc*I site; an 1138 G>C mutation creates an *Msp*I site. The nucleotide changed in the common mutation (G1138) is depicted by an (*). The regular glycine residue (Gly380) is underlined.

several experimental systems [218,219]. Such studies suggest that signalling through the FGF/FGFR pathway has different consequences at different intensities. Low intensity, tonic signalling stimulates sustained cell proliferation, prevents preosteoblast differentiation, and hence maintains the suture. Higher intensity signalling, consistent with activating mechanisms for FGFR mutations, causes FGFR downregulation and suture differentiation.

Biochemical Analysis of Fibroblast Growth Factor Receptor 3 Mutations

All of the *FGFR3* mutations observed to date result in constitutive activation of the receptor. *In vitro* biochemical studies suggest that the phenotypic differences among the disorders in the achondroplasia family of skeletal dysplasias

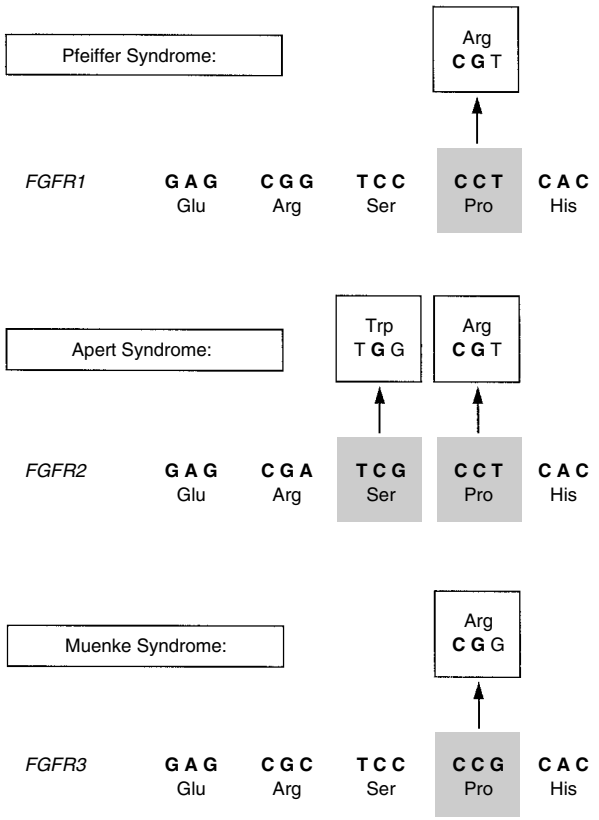

Figure 20. Observed point mutations in patients with Pfeiffer syndrome (PS), Apert syndrome (AS), or Muenke syndrome (MS) at an identical position in all three FGFR paralogues (*FGFR1, FGFR2,* and *FGFR3*) resulting in a substitution of proline to arginine. (From Hehr and Muenke [239]. Reproduced by permission from Molecular Genetics and Metabolism.)

are due, at least in part, to varying degrees of ligand-independent FGFR3 activation.

These considerations predict that loss of *Fgfr3* function will result in a different phenotype. In support of this prediction, mice homozygous for a targeted total disruption of the *Fgfr3* gene exhibit skeletal and inner ear defects. The skeletal manifestations in these mice include kyphosis, scoliosis and kinking of the tail; in addition, the long bones exhibit overgrowth, in direct contradistinction to the shortening of the long bones seen in achondroplasia and related disorders. These observations suggest that the normal function of FGFR3 is the negative regulation of bone growth [35,36]. Thus, *FGFR3* mutations in the achondroplasia family of skeletal dysplasias are thought to be gain-of-function mutations that activate, in a ligand-independent manner, the fundamentally negative growth control exerted by the FGFR3 pathway [35,36,220].

Based on current knowledge of signal transduction by the FGF pathway, activation of FGFRs normally occurs only after ligand binding [221]. Analysis of *Xenopus* receptors has demonstrated that point mutations may activate FGFRs by a number of distinct mechanisms [222].

Additional evidence for the gain-of-function hypothesis was provided by Webster et al. [223]. In an *in vitro* model system, profound constitutive activation of the FGFR3 tyrosine kinase (approximately 100-fold greater than of the wild type) was associated with the Lys650Glu mutation, which causes thanatophoric dysplasia type II (TDII) [223]. Altered kinase activation was dependent on both the position of the altered amino acid, and its charge. The authors speculated that the TD II mutation in the FGFR3 activation loop mimicked the conformational changes that activate the tyrosine kinase domain. Ligand binding and autophosphorylation of the receptor normally initiate this domain. Using immunoprecipitation followed by an *in vitro* kinase assay, Webster and Donoghue [224] also found that the mutation in thanatophoric dysplasia type II increased autophosphorylation of the FGFR3 relative to the wild type or achondroplasia mutation.

Subsequently, similar constitutive FGFR3 activation has been associated with the Gly380Arg mutation causing achondroplasia [224,225]. The downstream events initiated by FGFR3 activation are represented in Figure 21. The thanatophoric dysplasia type II mutation displays a higher level of autophosphorylation than the achondroplasia mutation, suggesting that the severity of the conditions may correlate with the degree of autophosphorylation caused by the receptor mutation [225].

The constitutive tyrosine kinase activity of FGFR3 with the TDII mutation specifically activates the transcription factor Stat1 (signal transducer and activator of transcription) [226]. This mutant receptor also induces nuclear translocation of Stat1 and expression of the cell-cycle inhibitor p21 (WAF/CIP1), resulting in growth arrest of the cell. Stat1 activation and increased p21 (WAF/CIP1) expression were found in chondrocytes from a TDII fetus, but not in those from an unaffected fetus. These observations suggest that in thanatophoric dysplasia, Stat1 may be used as a mediator of bone growth retardation, and that abnormal Stat activation and p21 (WAF/CIP1) expression due to the mutant FGFR3 may be responsible for the phenotype [226].

Thompson et al. [227] constructed a chimeric receptor containing the transmembrane and intracellular domains of FGFR3 with the rare Gly375Cys achondroplasia mutation fused to the extracellular domain of platelet-derived growth factor. PC-12 cells lack this growth factor. Strong neurite outgrowth in cells transfected with these constructs would suggest activation of the receptor. Ligand-dependent autophosphorylation of the chimeric receptor was observed, as well as strong phosphorylation of mitogen-activated protein (MAP) kinase, phospholipase C and Shc. Compared with cells transfected with a chimera with normal *FGFR3* sequences, cells transfected with the *FGFR3*-achondroplasia chimera were more responsive to ligand, with a less sustained MAP kinase activation, indicative of a primed or constitutively "on" condition. This observation is consistent with the hypothesis that these mutations weaken ligand control of the FGFR3 receptor, and may provide a biochemical explanation for the observation that the TD phenotype is more severe than that of achondroplasia [227].

CRANIOSYNOSTOSIS SYNDROMES AND SKELETAL DYSPLASIAS: GENERAL GUIDELINES FOR MANAGEMENT

Genetic Counseling

Almost all of the conditions discussed in this chapter are autosomal dominant disorders. Most cases represent new mutations, in which case the recurrence risk to unaffected parents is low. However, the possibility of

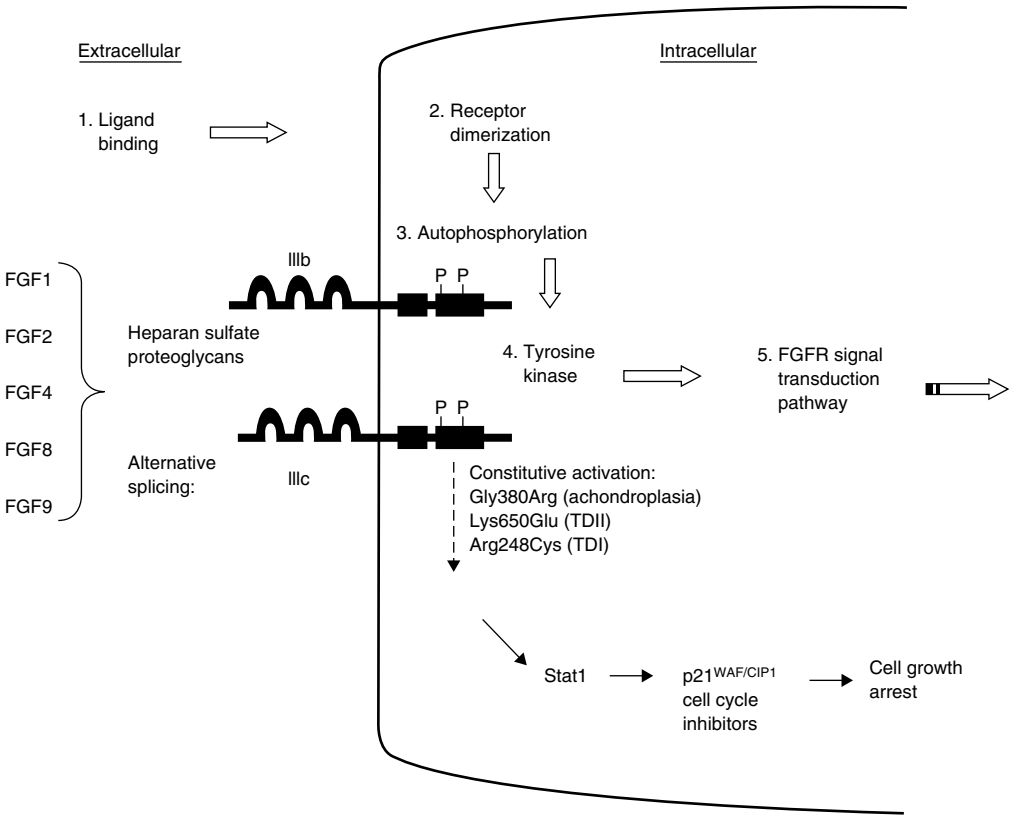

Figure 21. A putative model for FGFR3 signalling. The receptor is shown with both extracellular and intracellular domains. Binding of the ligand (FGF) to the receptor in the presence of heparan sulfate proteoglycans results in receptor dimerization and autophosphorylation of several FGFR3 tyrosine residues in the cytoplasmic domain, which stimulates tyrosine kinase activity. These phosphorylated tyrosine residues provide a means to recruit and phosphorylate other molecules, furthering the FGFR3 signal transduction pathway. It has been shown that mutations in the *FGFR3* gene can allow constitutive, ligand-independent activation of the receptor. For the common achondroplasia and thanatophoric dysplasia mutations, this leads to the activation of Stat1 and cell cycle inhibitors, eventually leading to cell growth arrest.

germline mosaicism in one of the unaffected parents means that the risk is not zero [192,193]. It is recommended that any couple who have had a child with one of these conditions consult a genetic counselor if they are considering further reproductive options.

Prenatal Diagnosis

The relatively small number of mutations and tight correlation between specific mutations and phenotypes makes prenatal diagnosis more accessible for this group of disorders than for many others. However, the high frequency of new mutations makes the application of prenatal testing impractical in most cases. Moreover, known genetic heterogeneity in some conditions, such as hypochondroplasia, may lead to a false negative outcome if not considered.

Therapy

Careful monitoring of the head circumference in infancy is essential in all of these conditions. Deviations from expected growth curves should lead to evaluation for possible hydrocephalus, or neurosurgical intervention for craniosynostosis. Anticipatory monitoring for central and/or obstructive apnea and hearing loss is advised for persons with achondroplasia and many of the craniosynostosis syndromes. Spinal stenosis is common in adults with achondroplasia [166]. The development of paresthesias or weakness in the lower extremities, or loss of bowel or bladder control, should be promptly evaluated.

Growth hormone trials for achondroplasia have not been a resounding success. While growth accelerates during treatment, the ultimate effect on height is still unknown [228,229]. Considering the cost and inconvenience of daily injections, many practitioners and families conclude that the treatment is not worthwhile.

Surgery

Common surgical interventions in achondroplasia include foramen magnum enlargement, placement of a ventriculoperitoneal shunt, placement of pressure-equalizing (PE) tubes in the tympanic membrane, spinal laminectomy, and tibial osteotomy. Limb lengthening is a less commonly performed and controversial approach to increasing height in persons with achondroplasia.

For persons with craniosynostosis syndromes, neurosurgical intervention to release the prematurely closed cranial sutures may be undertaken. In case of increased intracranial pressure, a VP shunt may be placed in these disorders as well. Finally, hand surgery to release the syndactyly associated with the craniosynostosis syndromes, particularly Apert syndrome, is sometimes feasible.

Foramen Magnum Enlargement

Stenosis of the foramen magnum is a common complication of achondroplasia. It is particularly pronounced in infancy, and may compress the cervico-medullary junction, resulting in central apnea and/or severe hypotonia and evidence of neurological compromise. Enlargement of the foramen magnum can be a life-saving procedure for these children [162].

Placement of a Ventriculo-Peritoneal Shunt

Enlarged ventricles are the rule rather than the exception in achondroplasia. Nonetheless, in some children, the head circumference enlarges more rapidly than expected (see growth chart for head circumference). Imaging of the ventricles in these children often reveals massive enlargement of the ventricles, an indication for the placement of a ventriculo-peritoneal shunt for hydrocephalus. In cases where it is not clear whether the intracranial pressure is increased, it is possible to place an intraventricular pressure monitor intraoperatively. The neurosurgeon may then watch the pressure for two or three days in an intensive care setting, before a decision is reached to place a shunt.

Placement of Pressure-Equalizing (PE) Tubes

Otitis media is a common childhood complication of achondroplasia. Frequent episodes of otitis media may lead to scarring of the tympanic membrane and significant hearing loss. To prevent this complication, PE tubes are sometimes placed to prevent the build-up of pressure in the middle ear and facilitate drainage.

Laminectomy

Because of the high frequency of spinal stenosis in adults with achondroplasia, spinal laminectomy is the most commonly performed surgical procedure in adulthood [166]. The anatomy of the spine in achondroplasia results in narrowing of the spinal canal towards the caudal region. Hence, lumbar stenosis is the most common type. However, thoracic and even cervical stenoses do occur and may require surgical correction by laminectomy.

Osteotomy

Genu varus (bowed legs) are a common orthopedic complication of achondroplasia in childhood. The orthopedic correction of genu varus is through a tibial osteotomy, in which a wedge-shaped piece is removed from the tibia to straighten the bone.

Limb Lengthening

Perhaps the most controversial of surgical procedures for achondroplasia is that of limb lengthening [230]. Technically, the procedure has been refined significantly over the past decade. It involves creating a surgical fracture and inserting pins on either side of the fracture site. The pins are mechanically distracted in millimeter increments over a period of 6–12 months. Fracture callous fills in the space between the distracted ends of the fracture, and the bone is lengthened. When both upper and lower segments of the leg are lengthened, as much as a foot (30 cm) in height may be gained. Potential complications include both neurologic and vascular damage to the limb if the lengthening occurs too quickly. The long-term stability of the new bone has yet to be evaluated.

CONCLUSION

Many questions remain to be answered about the pathogenesis of FGFR disorders. The precise molecular mechanisms of downstream signaling due to allelic mutations have yet to be elucidated. Similarly, an explanation for the extraordinarily high mutation rates at specific FGFR nucleotides is still unknown. Nonetheless, the study of FGFR mutations in human craniosynostosis and skeletal dysplasia syndromes has yielded much new information about the FGF/FGFR signalling pathways and their involvement in skeletal development.

RECENT DEVELOPMENTS

All *FGFR2* mutations in **Pfeiffer syndrome** described to date localize to the extracellular IgIII domain. However, the original family described by Pfeiffer in 1964, although shown to be consistent with linkage to FGFR2, does not have causative mutations in *FGFR2* exons 7 to 9 (IgIII). Recently, Kan and colleagues [240] have identified *FGFR2* substitutions of two adjacent nucleotides, 514GT and 515CT, predicting the amino acid substitution Ala172Phe, in all affected family members who were available for testing. This alanine substitution is in a key position in the IgII domain crucial for the formation of the immunoglobulin domain. This is the first mutation in the IgII domain of FGFR2 (or any FGFR), suggesting that this region may harbor mutations in patients with Pfeiffer and other craniosynostosis syndromes.

Bellus et al. [241] have reported three new mutations at Lys650 in *FGFR3* exon 15, each causing the **hypochondroplasia** phenotype. The three mutations include G1950T and G1950C, both of which result in a Lys650Asn substitution, and A1948C, resulting in a Lys650Gln substitution. Both amino acid substitutions result in constitutive activation of the FGFR3 tyrosine kinase activity, but to a lesser degree than observed with mutations at the same codon resulting in the more severe phenotypes of thanatophoric dysplasia type II and SADDAN.

Several new **animal models** of *FGFR3* disorders have been reported. Iwata and her colleagues have described mouse models for the Lys650Glu and Lys650Met mutations causing thanatophoric dysplasia type II and SADDAN, respectively [242, 243]. Observations in the thanatophoric mice suggest that signaling through Fgfr3 both promotes and inhibits chondrocyte proliferation at different times during development. Chondrocyte differentiation, by contrast, was suppressed throughout embryonic development. Most mice carrying the *Fgfr3* Lys644Met mutation (the equivalent of the human SADDAN mutation) survived the neonatal period and exhibited long bone abnormalities milder than those seen in the thanatophoric dysplasia type II model.

Chen and colleagues [244] have constructed a mouse carrying an *Fgfr3* Ser365Cys mutation, the equivalent of one of the mutations causing thanatophoric dysplasia type I in humans. The mutant mice demonstrated shortened limbs with reduced proliferation and differentiation of chondrocytes at the growth plate. The mutation also caused down regulation of expression of Indian hedgehog (*Ihh*) and parathyroid hormone-related protein (*PTHrP*) receptor genes.

Transgenic mice with altered Fgfr1 and Fgfr3 signaling in growth plate chondrocytes have been reported by Wang et al. [245]. *Fgfr1* was activated in the growth plate in cells that normally express FGFR3, and comparison was made with mice expressing a similarly activated *Fgfr3* construct.

APPENDIX

Saethre-Chotzen Syndrome

Although Saethre-Chotzen syndrome is not caused by FGFR mutations, it is included here because it is one of the major human craniosynostosis syndromes. Saethre-Chotzen syndrome (MIM 101400) is characterized by brachycephaly, a low frontal hairline, facial asymmetry, ptosis of the eyelids, a flat midface, and a thin pointed nose. The mode of inheritance is autosomal dominant, with incomplete penetrance and variable expressivity. The birth prevalence is 1 in 65,000.

Most cases of Saethre-Chotzen syndrome have brachycephaly due to coronal synostosis. Frequently, plagiocephaly and facial asymmetry are also found. Hypertelorism, strabismus, and ptosis of the eyelids are usually noted. The nose is long and pointed, and the nasal septum may be deviated. Maxillary hypoplasia and mandibular prognathism are other findings. The palate is highly arched and narrow and may be cleft. The ears are small and posteriorly rotated, often with a prominent crus [231,232].

Limb findings include brachydactyly and partial cutaneous syndactyly between fingers 2 and 3 and occasionally between toes 2 and 3. The great toes can be broad and deviated towards the other toes. Other skeletal anomalies include contractures of the elbows and defects of the cervical and lumbar spine.

Saethre-Chotzen syndrome is associated with mutations in the *TWIST* gene at chromosome 7p21.1. Mutations may involve cytogenetic deletions, translocations, and heterozygous mutations in the *TWIST* gene [232,233,234].

ELECTRONIC DATABASES/WEB-SITES DETAILING FGFR MUTATIONS

Online Mendelian Inheritance in Man (OMIM): www3.ncbi.nlm.nih.gov/Omim/

FGFR1 MIM 136350
FGFR2 MIM 176943
FGFR3 MIM 134934

PATIENT SUPPORT GROUPS

Alliance of Genetic Support Groups: www.geneticalliance.org/Resources/search.html
Craniosynostosis and Parent Support (CAPS): www.cap2000.org
FaceIt: www.faceit.org
Little People of America (LPA): www.lpaonline.org/lpa.html
Human Growth Foundation (HGF): www.hgfound.org

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Chapter 23, Part V

Defects in Skeletal Morphogenesis

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SUMMARY

- **Heritable diseases of the skeleton provide insights into skeletal patterning, organogenesis, and growth. Dysostoses are malformation syndromes that affect selected embryologically defined skeletal elements, the remainder of the skeleton being normal. They are generally caused by mutations in transcription factors or cytokines that regulate cell differentiation.**
- **Dysostoses can be divided into four groups: disorders of the craniofacial skeleton; disorders of the axial skeleton; disorders of the appendicular skeleton; and disorders characterized by patterning defects as well as a generalized disturbance of growth (dysostoplasias).**
- **Disorders of the craniofacial skeleton include disorders of neural crest cell migration/differentiation (Treacher Collins-Franceschetti syndrome, Waardenburg syndrome, DiGeorge syndrome/Shprintzen Velocardiofacial syndrome) and midline defects (holoprosencephaly).**
- **Disorders of the axial skeleton include disorders affecting vertebral segmentation (spondylocostal dysostosis) and disorders that affect caudal structures (sacral agenesis).**
- **Disorders of the appendicular skeleton are disorders affecting the patterning of hands/feet (polydactylies, syndactylies, and hypoplasias), disorders affecting dorsal-ventral patterning (nail-patella syndrome), and disorders of growth and joint formation (brachydactylies, acromesomelic dysplasia, and proximal symphalangism).**
- **Dysostoplasias, characterized by phenotypes that share aspects of dysplasias and dysostosis, are caused by mutations in genes that are involved in the formation of cartilage and bone as an organ. They include disorders (campomelic dysplasia, cleidocranial dysplasia) resulting from mutations in transcription factors that regulate chondrocyte and osteoblast differentiation, chondroectodermal dysplasia and short-rib-polydactyly syndromes.**

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INTRODUCTION

The identification of genes responsible for human inheritable skeletal disorders is increasingly leading to major advances in our understanding of the molecular and cellular basis of skeletal morphogenesis. Mutations in genes that play a major role during early skeletal development and patterning result in malformations that affect selected, embryologically defined skeletal elements, while the remainder of the skeleton is normal. Such malformations, classified as dysostoses, represent a large group of rare disorders. In this chapter we have included conditions that are well defined clinically, and for which the molecular and/or embryological pathogenesis is relatively clear (for detailed clinical information on these and other related syndromes see Jones 1997 [1]). We have divided the conditions into four broad groups as follows: disorders that primarily affect the craniofacial skeleton; disorders that affect the axial skeleton; disorders that include malformations of the appendicular skeleton; and conditions that include patterning defects as well as a generalized disturbance of skeletal growth. Conditions in this last group share aspects of both dysostoses and dysplasias, dysplasias being disorders that affect the skeleton in a generalized manner because both bone and cartilage are affected. We refer to conditions in this last group as dysostoplasias and discuss how these disorders are caused by mutations in genes that play important roles in skeletal organogenesis.

The general architecture of the vertebrate skeleton is determined by a range of early patterning genes. These genes pattern cells from three distinct embryonic lineages that contribute to the development of bone. The neural crest, the sclerotome, and the lateral plate mesoderm contribute to the craniofacial skeleton, the axial skeleton, and the limbs, respectively (Fig. 1). Organogenesis follows patterning and involves the formation of cartilage and bone as an organ (the skeleton). Chondrocytes or osteoblasts differentiate and form the early anlagen, representing the outlines of the future skeletal elements. Bones then develop, either directly from osteoblastic progenitor cells (intramembranous ossification) or within a temporary framework of hyaline cartilage (endochondral ossification) (for review, see [2]; see also Chapter 1, parts II and III, this volume).

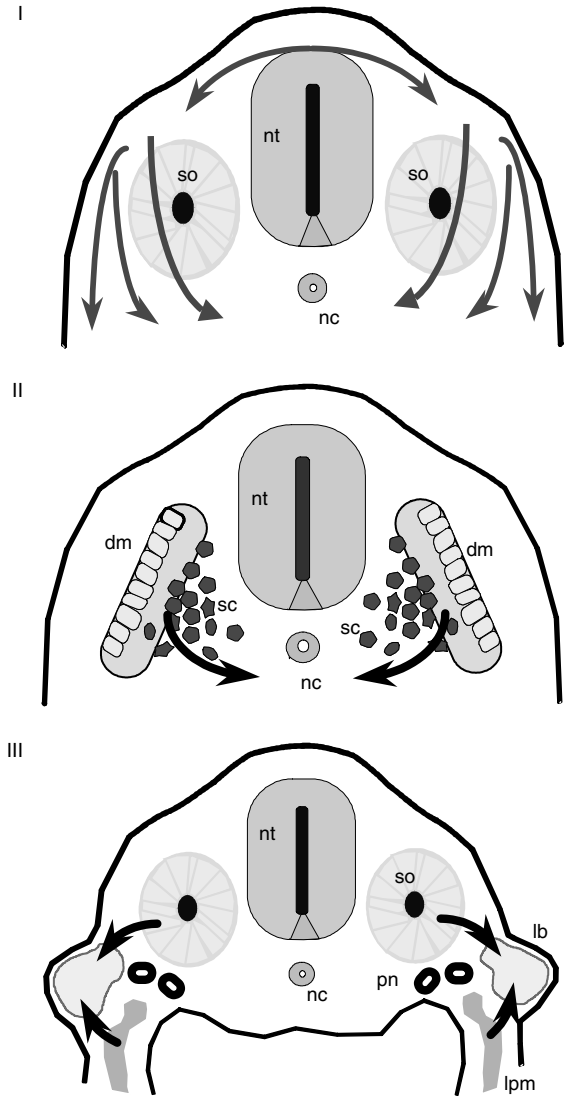


Figure 1. The origin of bone. Patterning of undifferentiated mesenchymal precursor cells involving cell-to-cell signaling and transcriptional regulation leads to migration, determination, and differentiation, processes that will eventually result in the formation of individual skeletal elements. Skeletal precursors originate from three distinct embryonic lineages: the neural crest, the sclerotome, and the lateral plate mesoderm. **I.** Migrational pattern of neural crest cells. Neural crest cells originate from the dorsal part of the neural tube and migrate along specific pathways dorsolaterally and through the somites (arrows). Most cranial bones including the calvaria, the maxilla, the mandible, the teeth and the inner ear are derivatives of the neural crest. **II.** The axial skeleton is derived from the sclerotome. The somites differentiate upon signals from the notochord into the sclerotome and the dermatomyotome. Sclerotome cells migrate towards the notochord where they contribute to the formation of vertebrae and ribs. **III.** Limb development begins with the migration of cells from the lateral plate mesoderm and the somites to form the limb bud and the limb precursor cells. The initial phase of proliferation seems to be under the control of the nearby mesonephros. Limb precursors then differentiate along a genetically determined pattern. Somitic cells contribute to the limb musculature, whereas cells from the lateral plate mesoderm generate the skeletal elements of the limbs. nt, neural tube; so, somite; nc, notochord; sc, sclerotome; dm, dermatomyotome; lb, limb bud; lpm, lateral plate mesoderm; pn, pronephros/mesonephros. Top is dorsal.

Much of what we know about the general mechanisms underlying skeletal morphogenesis is the result of animal studies. Such studies provide a good correlation between specific embryological events and gene function and, consequently, a framework for understanding the fundamental pathways that build and pattern bone. Based on the remarkable conservation of basic developmental mechanisms between animal species, connections to human disorders are frequently possible. The reverse is also true. The identification of genes responsible for human heritable diseases of the skeleton is increasingly leading to major advances in our understanding of the processes that underlie skeletal morphogenesis.

Heritable diseases of the skeleton can be divided into two broad categories. "Dysplasias" are conditions in which bone or cartilage is affected as a tissue and consequently has an abnormal structure and/or function. In contrast, "dysostoses" are malformation syndromes that affect selected, embryologically defined skeletal elements, the remainder of the skeleton being normal. A dysplasia usually affects the skeleton in a generalized manner resulting in disproportionate dwarfism, whereas patients with dysostoses show more localized defects. Genes that are involved in the pathogenesis of dysplasias encode molecules that are important for bone formation and growth, such as extracellular matrix molecules or factors that regulate chondrocyte differentiation/proliferation in growth plates. In contrast, genes that cause a dysostotic phenotype play a major role during early skeletal development and patterning, and their products are frequently transcription factors, for example, *HOX* or *PAX* genes. Dysostoses are consequently disorders of skeletal morphogenesis.

Dysostoses represent a large group of rare disorders, many of which are still ill defined. In the great majority of cases, the molecular pathogenesis is unknown. In this chapter, we will focus on clinically well-defined conditions in which the molecular and/or embryological pathogenesis is relatively clear. Four broad groups can be differentiated. These include disorders that primarily affect the craniofacial skeleton; disorders that affect the axial skeleton; and disorders that include malformations of the appendicular skeleton. Naturally, overlap between these groups is observed. A fourth group is composed of diseases that are characterized by patterning defects as well as a generalized growth disturbance, indicating that the mutated gene is active not only during early embryogenesis but also during fetal and postnatal life. Since these diseases share aspects of both dysplasias and dysostoses, we will refer to them as "dysostoplasias." The results of recent studies indicate that diseases in this group are caused by mutations in genes that are involved in skeletal organogenesis.

DISORDERS OF THE CRANIOFACIAL SKELETON

Development of the craniofacial skeleton involves a complex series of events that are disturbed in many genetic conditions [3–5]. The large number of craniofacial anomalies provide, therefore, an excellent opportunity to study craniofacial development, and a characteristic facial appearance frequently gives a clue to the correct diagnosis in genetic syndromes [6]. The facial bones develop from five primordia — the unpaired frontonasal prominence and the paired maxillary and mandibular prominences. The mandibular and maxillary prominences, derivatives of the first branchial arch, grow toward the midline where they

eventually fuse. The frontonasal prominence gives rise to the frontal bones of the skull, the nose, and the philtrum. In humans, this process begins around 4 weeks of development and by 8 weeks a recognizable face is apparent.

Most of the craniofacial bones are of neural crest origin [7]. Neural crest cells migrate from the dorsal part of the neural tube through the sclerotome into the branchial arches and the frontonasal mass where they contribute to a wide variety of tissues, including bone and cartilage [8,9]. Cell tracing studies in mouse and chick embryos have shown that neural crest cells originating from different rostrocaudal levels migrate into specific areas of the developing face [10]. For example, the first branchial arch is populated by neural crest cells migrating from the caudal midbrain and the first two rhombomeres, while neural crest cells from the fourth rhombomere populate the second branchial arch. Through an as yet unknown series of epithelial–mesenchymal interactions, neural crest cells differentiate and contribute to nerves, muscles, and bones of the head.

Disorders of Neural Crest Cell Migration/Differentiation

Treacher Collins-Franceschetti Syndrome (Mandibulofacial Dysostosis)

Treacher Collins-Franceschetti syndrome (TCOF1 or MFD1; MIM 154500) is a well-characterized craniofacial disorder with autosomal dominant inheritance [11,12]. The clinical features include downward-slanting palpebral fissures with lower lid coloboma or partial to total absence of lower eyelashes, malformations of the auricles, atresia of the external auditory canals, and malformation of the middle ear ossicles resulting in bilateral conductive hearing loss, malar and mandibular hypoplasia, and cleft palate. There is marked phenotypic variation so that some individuals are only partially and mildly affected whereas in others the malformations result in perinatal death due to a compromised airway.

Treacher Collins-Franceschetti syndrome has been mapped to chromosome 5q32–33.1, and a new gene, which the discoverers named *TREACLE*, isolated [13]. *TREACLE* has no strong homology to any other known gene, and the mouse homologue is expressed in a variety of embryonic and adult mouse tissues. The protein is therefore not specific to craniofacial tissues, but it does show high levels of expression at the edges of neural folds and in the branchial arches at the time of critical morphogenetic events [14]. A large number of mutations have been found in *TREACLE*. All of these appear to represent loss-of-function mutations, and Treacher Collins-Franceschetti syndrome is therefore likely to be a consequence of haploinsufficiency for treacle protein [15]. The presence of nuclear and nucleolar localization signals and several phosphorylation sites has led to the hypothesis that treacle is a member of the nucleolar phosphoprotein family, but why mutations result in the characteristic phenotype is not clear [16,17].

Waardenburg Syndrome

The eponym, Waardenburg, covers a group of auditory-pigmentary syndromes that can be separated into several subgroups [18]. Type I Waardenburg syndrome (WS1; MIM 193500) is the type originally described by Waardenburg as a combination of (i) lateral displacement of inner canthi (dystopia canthorum) with short palpebral fissures, a broad and high nasal bridge and hypoplastic alae nasi; (ii) partial albinism manifested by hypopigmented skin lesions, hypochromia iridis (bright blue irises), heterochromia iridis, a hypopigmented ocular fundus and/or a white forelock; and (iii) deafness of the sensorineural type. The hearing loss is congenital and may be unilateral or bilateral and can vary from slight to profound. Dystopia canthorum is the most penetrant of all features, being present in 99% of those affected, the others showing marked interfamilial and intrafamilial variability. WS1 carries a small risk of spina bifida and can be associated with Sprengel's deformity (elevation of the scapula) and cleft lip/palate. Waardenburg syndrome type II (WS2) is similar to WS1 except for the absence of dystopia canthorum. Because this is the most reliable and distinguishing clinical sign of Waardenburg syndrome, the clinical definition of WS2 is somewhat arbitrary, covering any syndrome with deafness/pigmentation defects. WS2 is heterogeneous, with linkage to different loci in different families. Linkage to chromosome 3p13, associated with mutations in the microphthalmia gene (see below), is now classified as Waardenburg syndrome type IIA (WS2A; MIM 193510), while Waardenburg syndrome type IIB (WS2B; MIM 600193) maps to a gene on chromosome 1p21–p13.3. A rare variant of WS1 that includes limb abnormalities with an amyoplasia-like condition affecting the arms has been called type III (WS3; MIM 148820) or Klein-Waardenburg syndrome. A fourth type, characterized by the association of deafness and/or pigmentary disturbances with Hirschsprung's disease (aganglionic megacolon), is called Waardenburg-Shah syndrome or WS4 (MIM 277580).

WS1 and WS3 are caused by mutations in *PAX3* [19–22], whereas WS2 is heterogeneous, being caused by mutations in the microphthalmia (*MITF*) gene in some but not all affected families (see above) [23]. Mutations in endothelin 3 (*EDN3*), or in the gene for its receptor (*EDNRB*), have been identified in patients with Hirschsprung's disease [24,25]. Homozygous individuals can show a WS4 phenotype. In families with dominant inheritance, WS4 is caused by mutations in the *SOX10* gene [26].

DeStefano et al. [27] have attempted to link gene function and phenotype in Waardenburg syndrome. The underlying cause of hearing loss is the requirement for melanocytes in the stria vascularis of the cochlea. The patchy absence of melanocytes leads to the observed pigmentary and auditory defects. Melanocytes are derived from neural crest cells. Considering the involvement of other neural crest derivatives such as the facial bones and the enteric ganglia, it is apparent that the Waardenburg syndromes are neurocristopathies. *PAX3* encodes a transcription factor, one of a family of nine human PAX proteins defined by the presence of a 128 amino acid paired domain [28]. During development, *PAX3* is expressed at the dorsal side of the neural tube, where neural crest cells emerge and start their migration. MITF is a transcription factor known to activate the gene for tyrosinase, a key enzyme in melanogenesis [29,30]. *MITF*, in turn, is directly regulated by PAX3, providing a molecular basis for the similar phenotypes in WS1 and WS2 [31]. *SOX10* is expressed early in development in neural crest cell derivatives [32]. Its role in these cell lineages in unknown.

Animal models. A model for the megacolon associated with WS4 is the dominant spotting mouse (Dom) [33]. The disorder is caused by a premature termination mutation in *Sox10* (MIM 602229), resulting in apoptotic loss of neural crest cells [34]. A defect in neural tube development occurs also in the Splotch mouse, caused by a deletion in the paired

homeodomain of *Pax3* [35]. A mouse model for WS2 is microphthalmia (mi), caused by mutations in *Mitf* [36].

DiGeorge Syndrome/Shprintzen Velocardiofacial Syndrome

The DiGeorge/Shprintzen syndrome (DGS; MIM 188400) comprises with variable expressivity (i) heart defects (mainly of the outflow tract); (ii) hypocalcemia arising from parathyroid hypoplasia; (iii) immune deficiency due to thymic hypoplasia; and (iv) a characteristic facies with telecanthus, a short philtrum, a rather bulbous nose, and submucous or palatal clefting. All phenotypes can be seen within a single family, and most cases have been shown to have deletions of chromosome 22q11. The acronym CATCH22 (cardiac defects, abnormal facies, thymic hypoplasia, cleft palate, and hypocalcemia resulting from 22q11 deletions) has been proposed for this condition [37]. The diagnosis is made by fluorescence *in situ* hybridization using probes corresponding to the deleted segment. Parents should be screened for carrier status. The combination of defects suggests a role for the DiGeorge gene in neural crest cell migration.

Animal models. In the mouse, homozygosity for inactivated alleles of the transcription factor Hoxa3 produces a phenocopy of DiGeorge syndrome [38]. The human *HOXA3* gene is not on chromosome 22q11, but an explanation for why complete loss of *Hoxa3* causes a DiGeorge/Shprintzen–like phenotype in mice, could be that *HOXA3* is an important transcriptional activator of some of the genes that are located within the deletions on 22q11.

Midline Defects

Holoprosencephaly

Holoprosencephaly encompasses an etiologically heterogeneous group of disorders that range from the most severe abnormality with cyclopia (a single eye) and a proboscis above the eye to the occurrence of a single central upper incisor (Fig. 2). Most cases are sporadic, but in some families holoprosencephaly is inherited as an autosomal dominant trait. The condition in such families usually maps to chromosome 7q36 and has been found to be associated with loss-of-function mutations in the sonic hedgehog gene, *SHH* [39]. This form of holoprosencephaly is now designated holoprosencephaly 3 (HPE3; MIM 142945). A second familial form of holoprosencephaly, caused by mutations in the DNA-binding domain of the homeobox protein encoded by *SIX3* on chromosome 2p21, is referred to as holoprosencephaly 2 (HPE2; MIM 157170) [40]. Other forms are associated with alterations in chromosome 21q22.3 (HPE1; MIM 236100) [41] or 18p11.3 (HPE4; MIM 142946) [42].

Animal models. Mice lacking the sonic hedgehog gene die around birth with malformations of the frontal areas of the brain and severe abnormalities of the skeleton. They display cyclopia, and at day 15.5 of gestation, show a proboscis-like extension [43]. These facial features are very similar to those observed in human holoprosencephaly, except that in humans, the phenotype is seen in heterozygous individuals, whereas in mice only homozygous animals are affected.

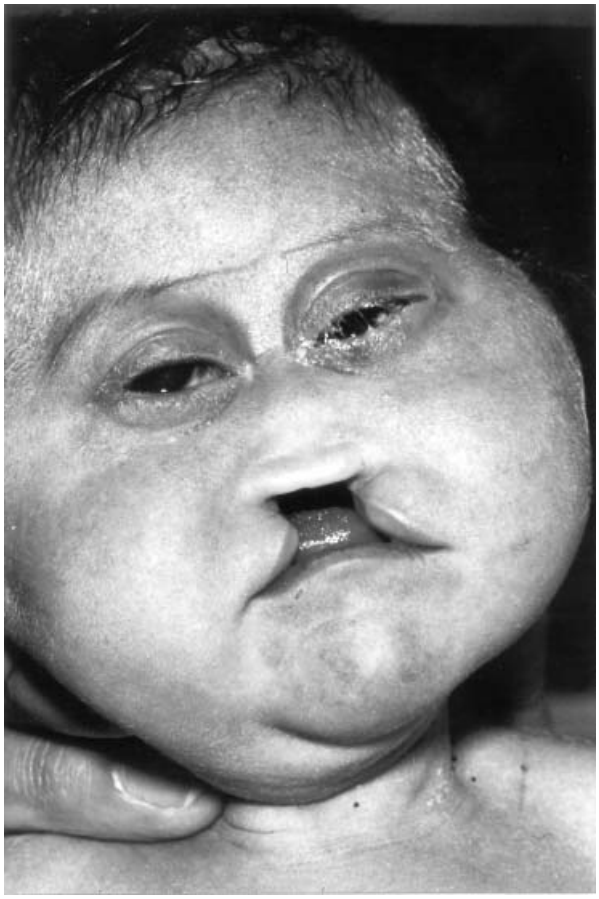


Figure 2. Holoprosencephaly. Patient with holoprosencephaly (newborn) showing midline cleft, hypotelorism, and absence of philtrum and nasal septum.

DISORDERS OF THE AXIAL SKELETON

Malformations of the ribs and/or vertebrae are a relatively common finding. Vertebrae and ribs originate from the sclerotome, a structure formed by the differentiation of somites (Fig. 1). Somites are blocks of epithelial cells with a repetitive, periodic architecture, which originate from the paraxial mesoderm. They determine the migration paths of neural crest cells and give rise to the vertebrae and ribs, the dermis of the dorsal skin, and the skeletal muscles of the body wall and limbs. Somites are laid down sequentially in a head-to-tail order, in a row on each side of the body axis. The budding of somites from the presomitic mesoderm occurs in a precise, regular series with perfect symmetry between the two sides of the body. Recent findings have unraveled some of the basic mechanisms that control somitogenesis [44,45]. The formation of somites involves a molecular segmentation clock that leads to the oscillation of hairy 1 expression in mesodermal cells. The hairy 1 oscillator drives lunatic fringe expression, which in turn drives the decisive pattern of changes in Notch activity and Delta expression in the segmenting mesoderm [46–50]. The Notch-Delta pathway is of crucial importance in somitogenesis as demonstrated by several knock-out mutations — *Mesp2* [51], *Notch1* [52], *Dll1* [53], *lunatic fringe* [54,55], and *Dll3* [56] — and the severe vertebral and rib deformities of homozygous mice with the pudgy mutation (pu), which is caused by a disruption of *Dll3* [56]. Interestingly, these mutations, leading to irregular size and asymmetry of somites, result in similar phenotypes. The skeletal malformations resemble those observed in humans with vertebral malformations.

When the somite is first formed, any of its cells can become any of the somite-derived structures. As the somite

matures, its various regions become committed to form only certain cell types. Patterning of the somite is brought about by the interaction of ventralizing signals from the notochord/floorplate complex and dorsalizing signals from the surface ectoderm and neural tube. Upon these signals, the ventral medial cells of the somite undergo mitosis, become mesenchymal cells again, and migrate towards the notochord. This portion of the somite is called the sclerotome, and will eventually give rise to vertebral and rib chondrocytes. The signalling molecule sonic hedgehog (*Shh*) is the major signal from the notochord/floorplate that initiates and controls sclerotome formation. Mice with inactivated *Shh* alleles develop no sclerotome and are hence devoid of the dorsal parts of ribs and vertebrae [43]. The homeotic (*Hox*) genes are expressed in overlapping domains along the rostrocaudal axis [57,58]. Targeted inactivation or ectopic expression of these genes in the mouse results in the deletion or addition of skeletal elements into shapes resembling other elements [59,60]. Thus, after induction of the sclerotome, *Hox* genes give the positional information necessary for the identity of individual vertebrae.

Spondylocostal Dysostosis

Spondylocostal dysostosis is a genetically heterogeneous group of disorders affecting vertebral segmentation. Clinically, three entities have been delineated. Jarcho and Levin [61] described a severe, recessive form (SCDO1; MIM 277300) that is characterized by severely malformed vertebrae, particularly in the thoracic spine, and a decreased number of ribs that have a crowded origin from the thoracic vertebral bodies and fan out to give a characteristic "crab-like" appearance. Affected individuals frequently die during the neonatal period due to respiratory insufficiency. This type of condition seems to be particularly common in Puerto Rico. A benign form of spondylocostal dysostosis with autosomal dominant inheritance (MIM 122600) has also been described [62]. Its clinical manifestation is extremely variable, ranging from single vertebral defects to multiple affected vertebrae with rib fusions. A third form, also designated spondylothoracic dysostosis (see MIM 277300), is inherited as an autosomal recessive disorder. It is probably the most frequent entity within this group. It is usually more severe than the dominant form but no clinical or radiological feature consistently distinguishes it from the benign dominant form (Fig. 3).

It is likely that these three forms are only the tip of the iceberg. For example, there are many cases of spondylocostal dysostosis with miscellaneous malformations, mostly urogenital. Other types of vertebral malformations affect predominantly the cervical spine with fusion of vertebrae and hemivertebrae. The so-called Klippel-Feil deformity (MIM 148900) is clearly heterogeneous, some forms being genetic in origin, while others have no simple genetic basis, or are due to disruptions. Given the complexity of somitogenesis and the multiple genes involved, it is likely that this phenotypically rather homogeneous group of vertebral segmentation defects is genetically heterogeneous. A locus for autosomal recessive spondylocostal dysostosis has been mapped to chromosome 19q [63] close to the human homologue of the pudgy gene, *Dll3* [56].

Sacral Agenesis/Currarino Triad

Hereditary sacral agenesis (MIM 176450) is an autosomal dominant condition including, with variable expressivity (i) anorectal anomalies such as anal stenosis, anal displacement, or imperforate anus; (ii) a presacral mass with anterior meningocele or presacral teratoma; and (iii) partial sacral agenesis with an intact first sacral vertebra (Fig. 4). The combination of anorectal, sacral and presacral anomalies is known as the Currarino triad. The clinical features of affected individuals can be mild, with constipation being the only complaint, or severe with imperforate anus, anterior meningocele, and the typical sickle-shaped hemisacrum revealed by X-ray of the sacral region, indicative of the Currarino triad.

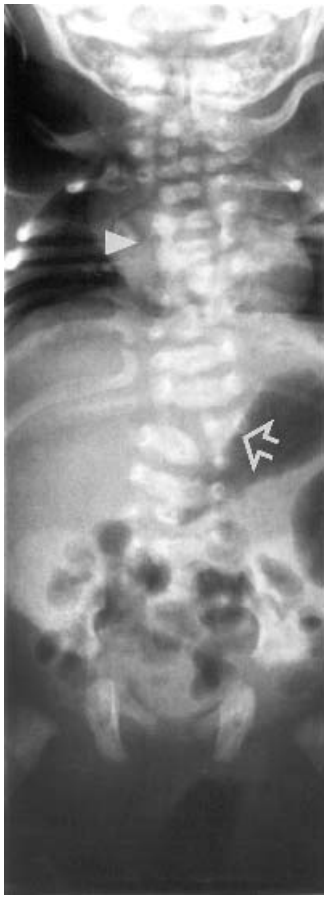


Figure 3. Spondylocostal dysostosis. Radiograph of a patient at age 3 weeks with spondylocostal dysostosis, short trunk dwarfism, hypospadia, and normal limbs. Extensive malsegmentation throughout the vertebral column with vertebral fusions (arrowhead), hemivertebrae (arrow), and rib anomalies (fusion, abnormal position).

Lynch et al. [64] identified the locus for dominant sacral agenesis on chromosome 7q36 close to the gene for sonic hedgehog. Within the critical region, a homeobox gene, *HLXB9*, was identified, and mutations were characterized in several affected families [65]. The mutations were expected to produce severely truncated products that probably reflect loss-of-function alleles. Haploinsufficiency is likely to explain the phenotype since deletions of the 7q35-qter region have frequently been observed in patients with sacral agenesis. Expression of *HLXB9* was observed in lymphoid and pancreatic tissue and in the anterior horn regions of the spinal cord. Clearly, further work is needed to explain the pathogenesis of this syndrome.

Other types of caudal dysgenesis have been described. Complete absence of the sacrum and lower vertebrae together

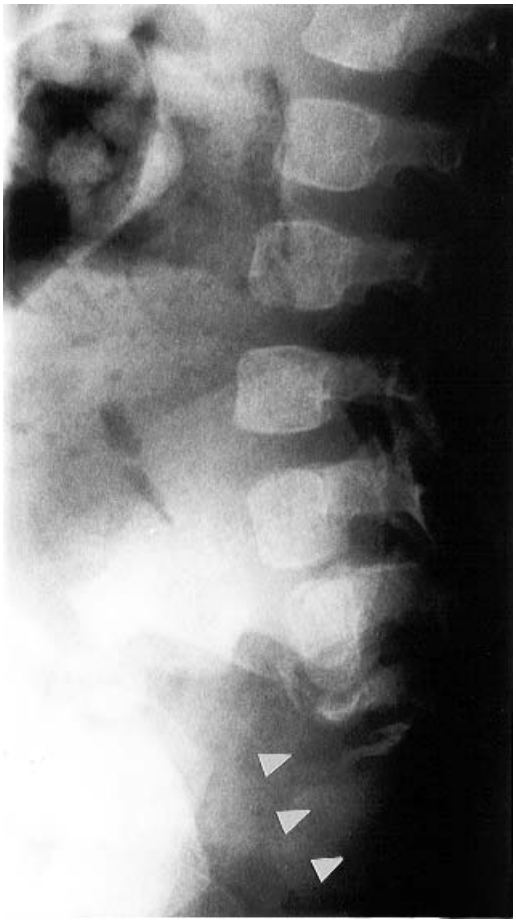


Figure 4. Sacral agenesis/Currarino triad. Lateral view at age 4 years showing normal lumbar vertebrae, but absence of sacrum (arrows).

with multiple other congenital anomalies (urogenital, anal, cardiac) occurs on a sporadic basis and is frequently associated with maternal diabetes [66]. The role of *HLXB9* in this and other types of caudal regression remains to be determined. However, the well-established association between sacral agenesis and maternal insulin-dependent diabetes mellitus and the expression of *HLXB9* in pancreas is intriguing.

DISORDERS OF THE APPENDICULAR SKELETON

Malformations of the hands and/or feet are frequently observed in human malformation syndromes. Some are unspecific and only found in association with other major defects, others are more specific and often diagnostic. The study of model systems and the genetic analysis of human syndromes have elucidated the causes of several heritable limb malformations in humans. In many instances, it is now possible to explain in some detail how malformations occur taking advantage of the recent progress that has been made in understanding the mechanisms of limb morphogenesis.

The limb originates from a dual contribution of the lateral plate and the somitic mesoderm (Fig. 1) [67]. After the initial outgrowth of the limb bud, cells from the lateral edges of nearby somites migrate into the limb and contribute to muscles, nerves, and vasculature. All other limb tissues, including the skeletogenic mesenchyme are derived from the lateral plate mesoderm. Outgrowth of the limb bud is the result of a series of interactions between the mesoderm and the overlying ectoderm (for reviews, see [68,69]). Several fibroblast growth factors (FGFs) can initiate the signalling cascade that results in fully formed limbs when applied to mesoderm of the flank. Cells of the growing limb bud are embedded in a dynamic three-dimensional structure that is controlled by multiple signalling molecules. The outgrowth of the limb along the proximodistal axis is directed by signals emerging from the apical ectodermal ridge (AER), a structure of specialized cells at the outermost edge of the limb bud. Cells from the underlying progress zone are kept in a proliferating nondifferentiated state through signals from the AER such as FGF4 and FGF8 [70,71]. Removal of the AER results in the termination of limb outgrowth. The anteroposterior pattern of the limb bud is established by the zone of polarizing activity (ZPA), a group of cells at the posterior margin of the limb bud. Sonic hedgehog (SHH) mediates the activity of the ZPA [72]. Transplantation of the ZPA or SHH-expressing cells to the anterior margin of a limb bud results in mirror duplications of the digits indicating that the polarity has been maintained, but the polarizing activity is now from both directions, anterior and posterior. Anterior extra digits and an ectopic anterior ZPA are also seen in mice homozygous for inactivated alleles of the Aristaless-like 4 (*Alx4*) gene. This gene, encoding a paired-type homeodomain protein that is expressed in anterior mesenchymal cells in the limb bud, is therefore part of the regulatory mechanism that restricts formation of the ZPA to the posterior mesenchyme in the normal limb [73]. Loss-of-function mutations in *Alx4* cause the mouse mutant phenotype Strong's luxoid (lst) [74]. Dorsoventral patterning requires the expression of the signalling molecule Wnt7a in the dorsal ectoderm and the homeobox-containing transcription factor engrailed 1 (EN1) in the ventral ectoderm [75,76]. *HOX* genes from the 5′ region of the A- and D-clusters on chromosome 7 and chromosome 2, respectively, show characteristic stage-dependent expression patterns that determine the shape and identity of the individual elements [77]. How this works and what the downstream targets of the *HOX* genes are is not clear, but it seems that they work in a dose-dependent fashion regulating proliferation and cell adhesion [78].

Patterning through transcription factors and signaling molecules leads to the formation of cartilaginous templates that are laid down in a proximal to distal sequence, so that the humeral anlage is formed first, followed by the radius and ulna, and lastly by the digits. The humerus and ulna, as well as the metacarpals and phalanges, are first formed as continuous cartilaginous rods that are subsequently segmented into the individual bones [79]. Segmentation leads, through apoptotic cell death, to the formation of joints, a process in which bone morphogenetic proteins (BMPs) or related molecules, such as cartilage-derived morphogenetic proteins (CDMPs, also called growth and differentiation factors, GDFs), play a major role [80,81]. Apoptosis is also involved in the separation of the individual digits, a process that is disturbed in syndactyly. Again, BMPs seem to play a significant role [82,83]. The effects of BMPs on cellular processes depend on their local concentrations. It is not surprising, therefore, that they are subject to both positive and negative regulation at several levels. One important regulator is noggin, a secreted protein that binds and inactivates BMPs. Mice that are homozygous for inactivated noggin (*Nog*)

alleles die at birth with multiple defects, including fusion of bones in the appendicular skeleton [84].

Congenital limb deformities are a genetically and clinically heterogeneous group of disorders. Those affecting the hands/feet (autopodia) can be classified into polydactylies (preaxial, involving digits of the radial side of the limb, or postaxial, involving digits on the ulnar side), syndactylies (fusion of soft tissues and/or bones), or hypoplasias affecting variable parts or segments. The most frequently observed limb malformation is post- or preaxial polydactyly, with a prevalence of 7 to 14 per 10,000 live births. Defects of the stylopodium (humerus or femur) or the zeugopodium (radius/ulna or fibula/tibia) can occur with or without associated hand malformations and can usually be classified as hypoplastic defects. This classification, based on clinical appearance and skeletal radiology, is frequently complicated by a large variation in expressivity and considerable overlap between apparently different malformation syndromes. Detailed molecular analyses should help define the phenotypic spectrum associated with certain mutations.

Polydactyly-Disorders Involving the Hedgehog Pathway

Vertebrate sonic hedgehog (*SHH*) is homologous to the *Drosophila* segment polarity gene hedgehog (*Hh*). Given its central role in development, the mechanisms of SHH signaling and the SHH pathway have been of major interest to the research community. Much of what we know has been delineated in *Drosophila*, but the general pathway and the major players in it are conserved between flies and higher animals and are likely to be similar in humans. Hh is a secreted signaling molecule that undergoes autocatalytic cleavage to generate two fragments [85]. The amino-terminal fragment contains the active part of the molecule. It is modified by the covalent attachment of cholesterol, a process that is essential for proper function [86,87]. The receptor for Hh is patched (Ptc), which forms a receptor complex with smoothened (Smo), a seven-pass membrane protein that transduces the Hh signal [88–92]. In the absence of Hh, Ptc inhibits Smo; this inhibition is released after the binding of Hh to Ptc [93]. As a result of signaling from Smo, the transcription factor cubitus interruptus (Ci) is stabilized, activating downstream targets of Hh.

Ci is a zinc finger transcription factor that acts as a bifunctional modulator of gene expression. In the absence of Hh signal, Ci is proteolytically processed in the cytoplasm into a shortened form causing transcriptional repression of Hh target genes including wingless (*wg*), decapentaplegic (*Dpp*), as well as *Ptc* and *Hh* itself. Hh signaling stabilizes full-length Ci, which, following transport to the nucleus, activates transcription of Hh targets [94,95]. Ci has three vertebrate homologues, GLI1, GLI2, and GLI3. Recent evidence suggests that in vertebrates, as in the fly, hedgehog signaling involves two antagonistic operations, one repressing, the other activating. In contrast to *Drosophila*, however, vertebrates may have solved this by gene duplication with GLI1 functioning as an activator, and GLI3 as a repressor [96].

Greig Cephalopolysyndactyly

Greig syndrome (GCPS; MIM 175700) is a dominantly inherited condition characterized by (i) malformations of the hands (broad thumbs, postaxial polydactyly — frequently pedunculated postminimus, syndactyly primarily of the third and fourth fingers) and feet (preaxial polydactyly/broad toes, occasionally postaxial polydactyly, syndactyly primarily of the first to third toes) (Fig. 5); and (ii) craniofacial anomalies consisting of hypertelorism, broad nasal root, frontal bossing, scaphocephaly, brachycephaly, and macrocephaly. Occasional defects include late-closing cranial sutures, mild mental deficiency, a mild degree of hydrocephalus, and other forms of polydactyly.

Greig syndrome was one of the first dysostoses to be characterized at the molecular level. In 1991, Vortkamp et al. [97] identified *GLI3* as the culprit gene, taking advantage of a 7p translocation segregating with the phenotype in one family. In this and other subsequent families, *GLI3* was shown to be completely deleted or truncated upstream or within the region coding for the zinc finger domain. Thus, Greig syndrome is likely to be caused by haploinsufficiency of the GLI3 gene.

Pallister-Hall Syndrome

Pallister-Hall syndrome (PHS; MIM 146510) consists of (i) malformations of the central nervous system including hypothalamic hamartomas (Fig. 6B), hypopituitarism, and, occasionally, holoprosencephaly with midline cleft-ing; (ii) craniofacial anomalies (microtia, an absent external ear canal, a flat nasal bridge, a short nose, micrognathia); (iii) malformations of the limbs with nail dysplasia, variable degrees of syndactyly and polydactyly (central and postaxial) (Fig. 6A), and distal shortening of the limbs; and (iv) anal defects including an imperforate anus and a variable degree of rectal atresia.

Given the phenotypic overlap between Greig syndrome and the Pallister-Hall syndrome, Kang et al. [98] screened several families for mutations in *GLI3*. They identified frameshift mutations that were located 3′ of the zinc finger-encoding domains. Provided these truncated proteins are stable, they are expected to bind to DNA. What the effects of these truncated proteins are, and how they disturb the hedgehog pathway awaits further investigations.

Postaxial Polydactyly, Type A1

Postaxial polydactyly (a supernumerary digit on the ulnar or fibular side of the hands and feet) is one of the most common malformations in humans. A small extra digit, frequently in the form of a skin tag, has a high prevalence among the African-American population (1:300 to 1:1000 live births). It has been suggested that inheritance is autosomal dominant with variable expression, but autosomal recessive forms have been reported. Many genetic syndromes are associated with postaxial polydactyly such as trisomy 13, the Meckel-Gruber syndrome, or the Bardet-Biedl syndrome. There are, however, families in which postaxial polydactyly is inherited as a dominant trait as the only phenotypic manifestation (PAPA1; MIM 174200). In these cases, the extra digit is well formed, articulates with the fifth or the metacarpal/metatarsal, and is usually functional.

Radhakrishna et al. [99] studied a family with a dominant form of postaxial polydactyly and mapped the phenotype to chromosome 7p, colocalizing it with the *GLI3* zinc finger transcription factor gene defective in the Greig and Pallister-Hall syndromes. A frameshift mutation in *GLI3* was identified 3′ of those identified in patients with the Pallister-Hall syndrome [100]. The truncated protein product would thus contain the zinc finger domain as well as the protease cleavage site.

Animal models. Mouse models for *Gli3* mutations are the mutants extra-toes (xt) [101] and polydactyly Nagoya (Pdn) [102].

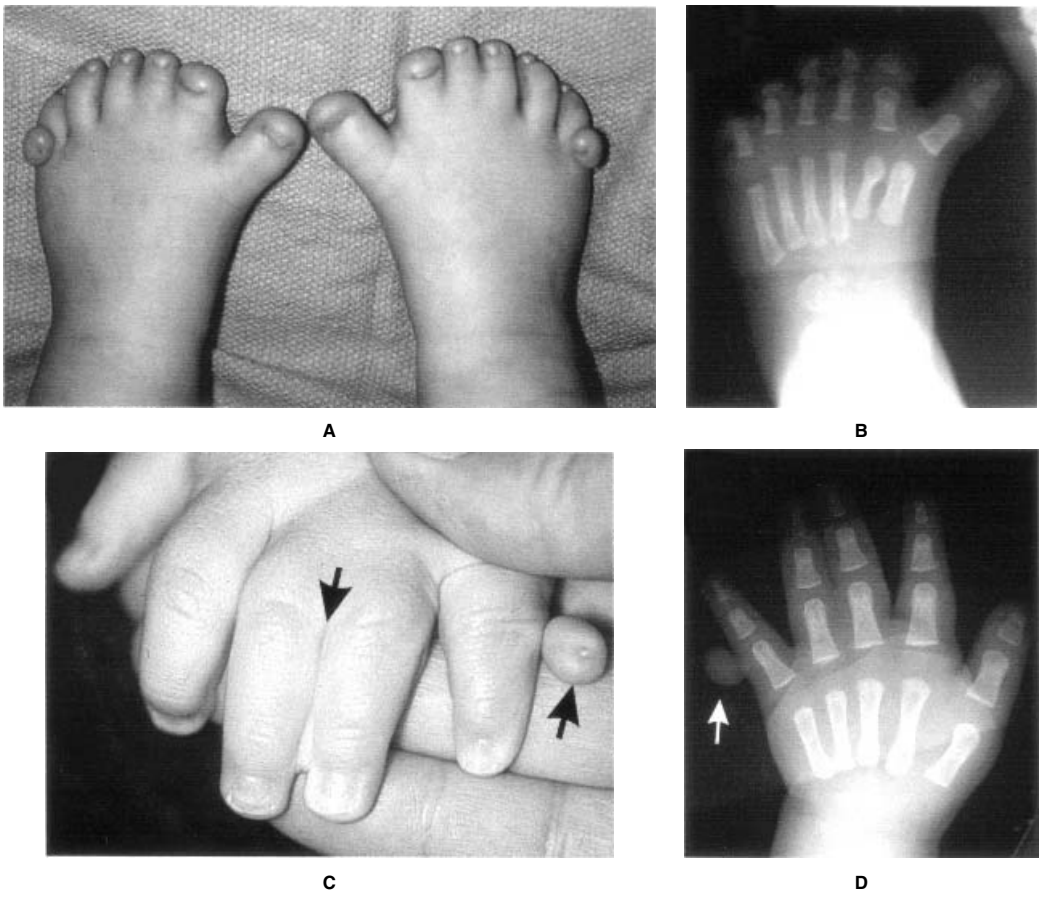


Figure 5. Greig cephalopolysyndactyly. Hands and feet of a female patient with Greig cephalopolysyndactyly syndrome aged 9 months. (**A**) Pre- and postaxial polydactyly of feet with cutaneous syndactyly of toes II to IV, and corresponding radiograph in (**B**) demonstrating complete duplication of first toe. (**C**) Postaxial polydactyly of hands (pedunculated postminimi, arrow) and cutaneous syndactyly of fingers III and IV (arrow). Corresponding radiograph is shown in (**D**). Note postaxial polydactyly without apparent ossification (arrow) and broad thumb.

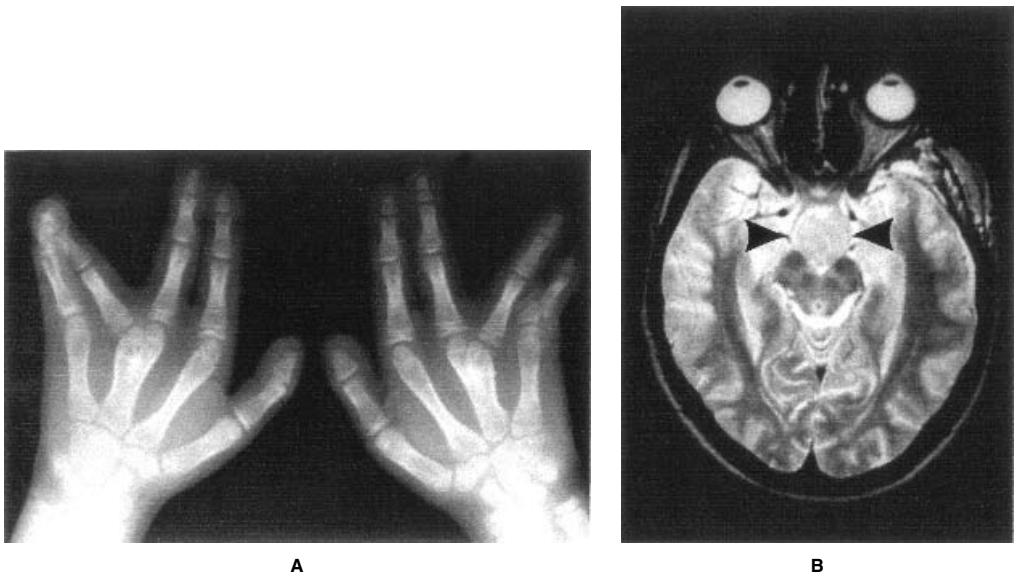


Figure 6. Pallister-Hall syndrome. (**A**) Syndactyly of metacarpals III/IV, surgically corrected postaxial polydactyly. (**B**) Hypothalamic hamartoma (MRI scan, black arrow heads). (With permission from M. Löw et al. [208].)

Townes-Brocks Syndrome

Townes-Brocks syndrome is an autosomal dominant disorder (TBS; MIM 107480) with marked variability in its severity of expression. Clinical features include (i) craniofacial anomalies consisting of external and internal ear malformations (overfolding, large ears, preauricular tags, and conductive and sensorineural deafness) and varying degrees of facial hypoplasia resembling hemifacial microsomia (Goldenhar) syndrome (MIM 141400); (ii) hand anomalies including hypoplastic, broad, or triphalangeal thumbs, preaxial polydactyly, and various other bony defects; (iii) anal defects including imperforate anus, anterior placement, and stenosis; and (iv) different types of renal anomalies (Fig. 7).

Townes-Brocks syndrome is caused by mutations in *SALL1*, the human homologue of the *Drosophila* homeotic gene *spalt* (*Sal*) [103]. SALL1 is a putative C2H2 zinc finger transcription factor similar to GLI3. The mutations described so far probably result in haploinsufficiency, as the predicted truncated proteins are expected to lack all zinc finger domains. In the fly, this transcription factor is required for the specification of posterior head and anterior tail segments; in the developing wing, Sal is activated in response to Hh signaling mediated by Dpp [104,105]. In the fish, *Spalt* has been shown to be a hedgehog target gene and to be expressed in all known hedgehog signalling centers of the embryo [106]. In the mouse, Spalt is expressed in urogenital ridge-derived structures, the notochord, and the limb buds, sites corresponding to the malformations observed in Townes-Brocks syndrome [107].

Smith-Lemli-Opitz Syndrome

Smith-Lemli-Opitz syndrome (SLOS; MIM 270400) is a rather common autosomal recessive malformation syndrome (estimated incidence, 1:20,000 live births) consisting of (i) craniofacial anomalies; (ii) limb malformations such as syndactyly, asymmetrically short fingers, or polydactyly; (iii) genito-urinary abnormalities; and (iv) moderate to severe mental retardation, various defects in brain morphogenesis and seizures. Other occasional defects include cleft palate, abnormal pulmonary lobation, rectal atresia and Hirschsprung disease.

Smith-Lemli-Opitz syndrome is due to a deficiency of 7-dehydrocholesterol-Δ7-reductase, the final enzyme in cholesterol biosynthesis [108]. Many of the features of the syndrome overlap with those found in patients with holoprosencephaly and mutations in SHH. As mentioned above, cholesterol is essential for the proper functioning of SHH [109]. This raises the possibility that the developmental abnormalities associated with defects in cholesterol biosynthesis may be due to impaired sonic hedgehog processing or signaling.

Polydactyly - Other Forms

Synpolydactyly

Syndactyly is the most common congenital malformation of the hand, occurring in approximately 1 in 2,000 live births. Several distinct forms have been delineated, all inherited in an autosomal dominant manner. Synpolydactyly, or syndactyly type II (SPD; MIM 186000), comprises syndactyly between the third and fourth fingers, with digit duplication in the syndactylous web, and syndactyly of the fourth and fifth toes (Fig. 8). Incomplete penetrance and variability both between and within affected families is common. Involvement ranges from partial skin syndactyly without digit duplication to complete duplication extending as far proximally as the metacarpals/metatarsals. Usually, all four limbs are involved, but many individuals have both normal and affected hands/feet indicating the presence of intra-individual variability.

Synpolydactyly is caused by mutations in *HOXD13* [110]. The mutations lead to an in-frame expansion of a 15-residue polyalanine tract, encoded by an imperfect trinucleotide repeat sequence, by 7, 8, 9, 10, or, in one family, 14 additional residues. In an extensive study analyzing a total of 20 families with 99 affected individuals, Goodman et al. [111] found a highly significant increase in the penetrance and severity of phenotype with increasing expansion size. Several nonpenetrant gene carriers were identified, all from families with a 7-alanine expansion. The overall penetrance in the families studied was only 86%, but was close to 100% in families with larger expansions. This has important consequences for genetic counseling. Parents who are clinically unaffected should be tested for carrier status. The authors hypothesized that the polyalanine

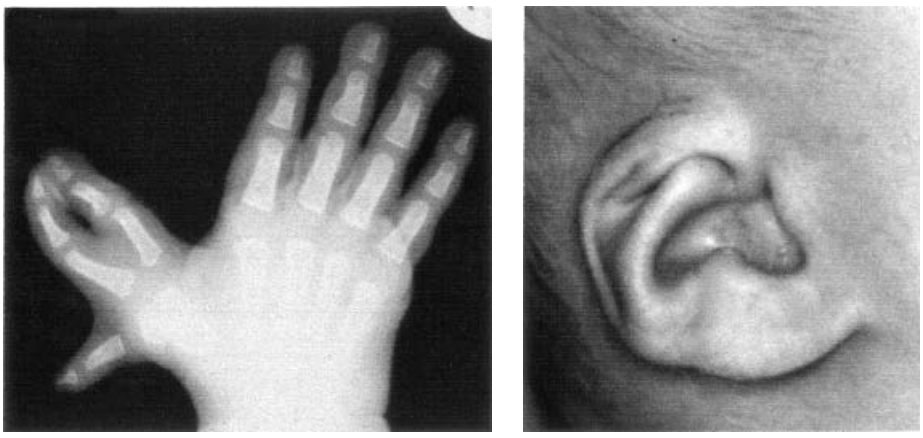


Figure 7. Townes-Brocks syndrome. (**A**) Supernumerary, triphalangeal thumbs. (**B**) Abnormal ear of same patient as in (**A**) with overfolding of the anterior part of the helix and prominent anthelix. (With permission from Kohlhase et al. [103].)

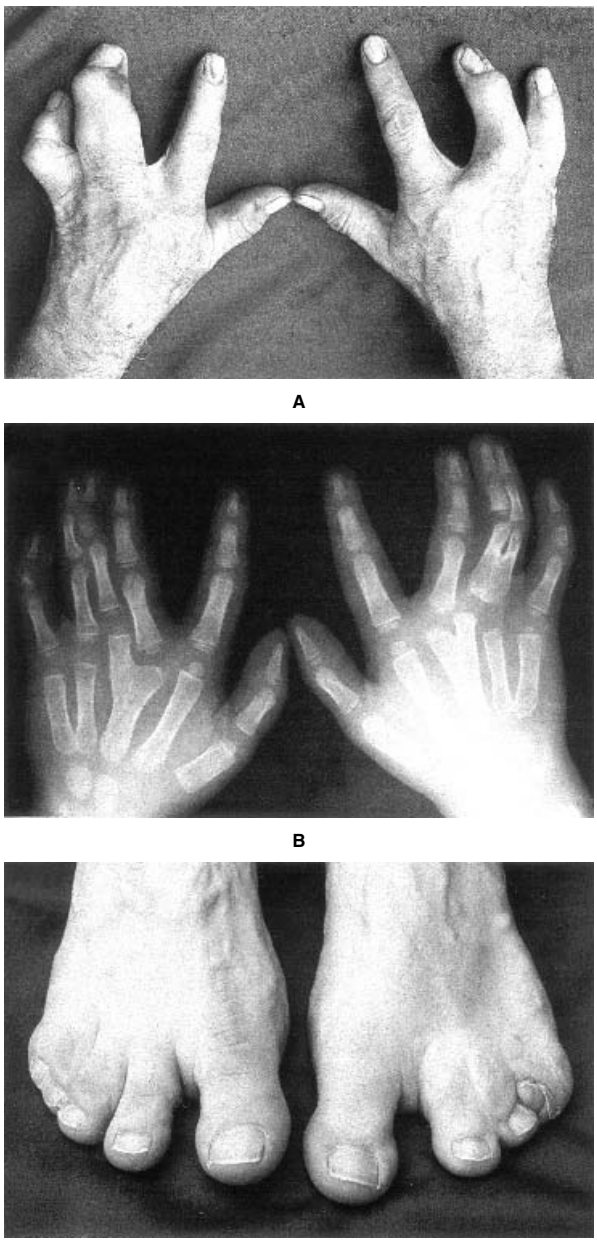


Figure 8. Synpolydactyly. (**A**) Complete membranous and partial osseous fusion of fingers III and IV with an additional finger in the syndactylous web. Frequently, as in this patient, complete cutaneous fusion of finger III, the extra finger and finger IV is observed resulting in a single nail. (**B**) Radiograph of hands of an affected child age 10 showing V-shaped metacarpal III, polydactyly between fingers III and IV, and hypoplastic middle phalanx of finger V. (**C**) Feet of individual in (**A**) showing cutaneous syndactyly of toes V to III/II. The fifth toes are broad and duplicated.

tract expansion leads to a gain of function in direct correlation to the expansion size. Inter- and intra-individual variability, a phenomenon frequently observed in congenital malformations, was more common in families with small expansions indicating that stochastic events are important events in mutations with reduced penetrance.

Recently, two families with frameshift mutations in *HOXD13* have also been described [112]. These mutations are expected to lead to null alleles since the deletions are 5′ of the DNA-binding homeodomain. Interestingly, these patients have the same hand phenotype as those with a polyalanine expansion, but a different foot phenotype consisting of a rudimentary digit between the first and second metatarsal.

The pathogenesis of syndactyly type II has to be considered in the context of the expression of genes in the D-cluster of *HOX* genes in chromosome 2 during development of the limb autopodia (hand/foot). The three most 5′ genes in the cluster, *HOXD11*, *HOXD12*, and *HOXD13*, are expressed in overlapping domains, with *HOXD11* being restricted to the most posterior (ulnar) region, and *HOXD13* being expressed across the entire autopodium. The expression domain of *HOXD12* is intermediate [77]. These expression patterns are controlled by sonic hedgehog, synthesized by the cells of the zone of polarizing activity (ZPA). Thus, the phenotypic abnormalities caused by *HOXD13* mutations are confined to the middle region of its expression domain during development of the distal region of the limb.

Animal models. A murine mutant (spdh) that represents a geno- and phenocopy of synpolydactyly type II has been described [113].

Preaxial/Triphalangeal Thumb

Preaxial polydactyly can occur as an isolated anomaly or as part of a congenital malformation syndrome. Several types have been described. The defect may be unilateral or bilateral affecting hands and/or feet. Isolated unilateral duplication of a biphalangeal thumb is usually sporadic and not associated with thenar abnormalities. Preaxial polydactyly in association with syndactylies and foot anomalies is frequently observed in Greig syndrome. A third type is characterized by triphalangeal thumbs (a long, finger-like thumb with three phalanges instead of two) with variable degrees of preaxial and sometimes postaxial duplication (TPTPS; MIM 190605). This type is inherited as an autosomal dominant trait with new cases most likely representing *de novo* mutations. Zguricas et al. [114] described the variable phenotype and expressivity in three large Dutch families. Severely affected individuals had bilateral triphalangeal index-like digits with additional preaxial digits and hypoplastic thenar muscles. At the mild end of the phenotypic spectrum was an extra delta-shaped phalanx between phalanges I and II leading to an ulnar deviation of the interphalangeal joint of the thumbs.

This type of polydactyly has been mapped by three separate groups to the long arm of chromosome 7, close to the SHH locus (7q36) [115–117]. A mouse mutant with preaxial polydactyly was recently reported as a consequence of a transgene insertion into a site close to the sonic hedgehog locus. In the abnormal limbs, expression of sonic hedgehog (as well as of the transgene reporter) was induced ectopically in the anterior region, suggesting that the transgene had interrupted a regulatory region necessary for the restriction of expression of *sonic hedgehog* to the posterior mesenchyme [118]. It is possible, therefore, that patients with preaxial polydactyly have mutations in such a regulatory region.

Absence/Hypoplastic Defects of Hands

Split-Hand/Foot Malformation

The rather nonspecific descriptive term ectrodactyly, derived from the Greek "ektroma" (abortion) and "daktylos" (finger), is applied to a number of genetic and

nongenetic malformations with absence deformities of the hands. Unilateral transverse defects (terminal aphalangia or aphalangia) can be caused by congenital constriction rings (amniotic bands) and are usually sporadic. Other sporadic atypical unilateral forms have also been described. The split hand malformation, sometimes called lobster claw deformity, is also called ectrodactyly. Phenotypically, two types of split-hand/foot malformation (SHFM) are recognized: (i) deficiency of the central rays with a cleft (the "lobster claw" variety) (Fig. 9); and (ii) monodactyly with absence of the radial rays and no cleft. The fifth finger is usually present. The two types can occur within one family and are thus of no genetic significance. Reduced penetrance and extreme variability in expression has been observed in many large pedigrees with SHFM. A particularly instructive family was described by Spranger and Schapera [119] in which three unaffected sibs with normal parents each produced affected offspring. As pointed out by Emery [120], minor hand anomalies may represent partial expression, and skipping of generations occurs frequently. To further complicate the situation, there seems to be non-Mendelian transmission characterized by the overtransmission of SHFM from affected fathers to sons [121,122].

Several cases of ectrodactyly with interstitial deletions of chromosome 7q have been described, and this locus has thus been designated SHFM1 (MIM 183600). Scherer et al. [123] characterized the translocation or inversion breakpoints in six patients and mapped the critical interval to the region 7q21.3–q22.1. Several candidate genes have been identified within this region including two members of the *DLX* family of homeobox transcription factors that have been implicated in limb development.

In a kindred described by Ahmad et al. [124], exhibiting full expression of the trait, monodactylous or split hand and split foot malformations were present in 33 males and only 3 females. Other females showed a significantly milder phenotype with only partial syndactyly and minor malformations of other bones of the hand indicating X-linked inheritance. This assumption was confirmed by linkage analysis localizing the X-linked form of SHFM (SHFM2; MIM 313350) to Xq26 [125]. In several other multigenerational families, linkage to 7q or Xq was excluded [126,127]. A third locus (SHFM3; MIM 600095) was subsequently localized to chromosome 10q25 [128].

Several other forms of syndromic ectrodactyly exist. One is the tibial aplasia ectrodactyly syndrome characterized by

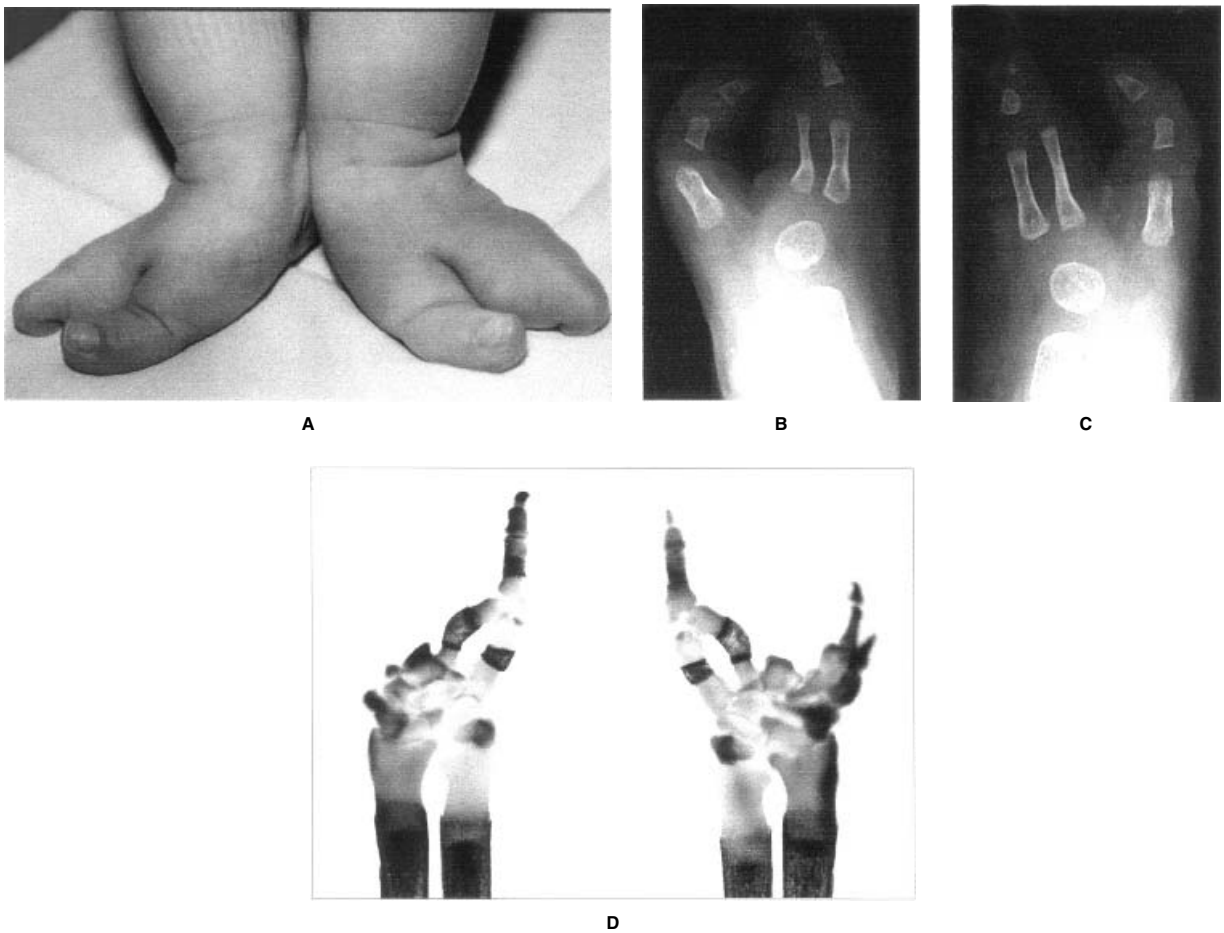


Figure 9. Split-Hand/Foot malformation. (**A**) Split foot in a 6-months-old with corresponding radiographs (**B, C**). This is a typical case with a central defect, absence of digits II and III and fusion of digits IV and V (Courtesy of P. Meinecke, Hamburg). (**D**) Skeletal preparations of limbs from a mouse heterozygous for the dactylaplasia locus. The spectrum of phenotypes frequently observed in one animal includes monodactyly (left) and ectrodactyly (right). The changes resemble those observed in human split-hand/foot malformation. Alizarin red- / alcian blue-stained skeletons.

ectrodactyly of the hands (most commonly split hand with absence of several fingers), variable foot deformities (absence of tarsal, metatarsals and toes), and aplasia/hypoplasia of the tibia and/or fibula. This condition is inherited as a dominant trait with widely variable expression and penetrance. The SHFM phenotype can be associated with ectodermal dysplasia and cleft lip/palate, a combination that has been named EEC syndrome (EEC1, MIM 129900; EEC2, MIM 602077). Other associated anomalies include a characteristic facial morphology with maxillary hypoplasia, a short philtrum and broad nasal tip, lacrimal duct anomalies, choanal atresia, and urogenital malformations. Several cases with anomalies of chromosome 7q have been reported, including a three-generation family with a translocation breakpoint at 7q11.21 [129] suggesting this as an EEC location (EEC1). Another family with EEC syndrome (EEC2) has been mapped to chromosome 19 [130], indicating that this syndrome is genetically heterogeneous. Other defects associated with the SHFM phenotype are split hand with perceptive deafness, split hand with congenital nystagmus, fundal changes and cataract (Karsch-Neugebauer syndrome), and anonychia with ectrodactyly.

Animal models. The mouse mutant "dactylaplasia" (Dac) provides important insights into split hand/foot malformations [131]. This mutation, mapped to the distal portion of mouse chromosome 19, is inherited as an autosomal semidominant trait characterized by absence of the central digital rays. The distal chromosome 19 region is syntenic to the locus for SHFM3 in humans [132]. Interestingly, the loss of central digital rays in Dac limbs appears to be due to cell death in the central portion of the apical ectodermal ridge (AER) [133]. This cell death is associated with a lack of cell proliferation in the mutant AER and this appears to lead to reduced cell proliferation in the mesenchyme below the ridge [134]. Positional cloning has identified a novel member of the *F-box/WD40* gene family as the gene responsible for the Dac phenotype. The product of this gene, called dactylin, may function as an adapter protein that targets specific proteins for degradation by presenting them to the ubiquitination machinery [135]. Intriguing is the finding that the phenotypic manifestation of the Dac mutation depends on alleles in a second locus called mdac. One dominant allele in the mdac locus inhibits manifestation of the phenotype; a second recessive allele allows it. Clearly, further studies of the Dac and mdac mutations will provide exciting insights into the control of AER function and the control of digit outgrowth.

Absence/Hypoplastic Defects of the Radial Ray

Holt-Oram Syndrome

The Holt-Oram syndrome (HOS; MIM 142900) is a dominantly inherited condition characterized by malformations of the heart and upper limbs. The phenotypic spectrum of the hand anomalies ranges from hypoplastic, absent, or triphalangeal thumbs to radial hypoplasia or even phocomelia (Fig. 10). Motion of the shoulder and elbow may be limited due to anomalies of the forearm: hypoplasia or absence of the radius and/or ulna, radio-ulnar synostosis, hypoplasia, or absence of the humerus. Narrow shoulders may be part of the clinical picture caused by anomalies of the humeral head, hypoplasia of the clavicle, rib anomalies, small scapula, and other changes of the shoulder girdle such as deficiency of pectoral and/or other muscles. The vertebrae may also be involved (hemivertebrae, hypoplasia, fusion). Carpal abnormalities, e.g., extra carpal bones, delayed ossification/absence, fusion can be observed as a minimal phenotype and may be more specific of the Holt-Oram syndrome than changes in the thumbs [136,137]. Cardiac involvement is found in the great majority of patients (95%) [138] and may consist of atrial and/or ventricular septal defects, cardiac conduction defects, or other cardiac anomalies such as anomalous pulmonary venous return, or right isomerism suggesting aberrant chamber specification.

The Holt-Oram syndrome was mapped independently by two groups to the long arm of chromosome 12 [137,139]. Positional cloning strategies identified a member of the T-box gene family, *TBX5*, within the interval. Several mutations have been identified that most likely lead to haploinsufficiency [140,141]. *TBX5* is related to the mouse gene *Brachyury* or *T*, encoding a transcription factor involved in the development of the posterior mesoderm during gastrulation. The role of *Tbx5* in limb development has only recently been investigated. The protein is expressed in the atrium and sinus venosus of the developing heart, the forelimb buds, trachea, thoracic wall, and retina [142]. Experiments in the chick demonstrated expression of *Tbx5* in the wing and flank, but not the leg, whereas *Tbx4* was found to be expressed only in the leg [143]. Based on these and other results it is now believed that *Tbx5* and *Tbx4* regulate limb outgrowth and identity [144–147]. The bicoid-related homeodomain transcription factor Pitx1, expressed in developing hindlimbs, the anterior pituitary gland, and first branchial arch, appears to be an upstream regulator of *Tbx4* [148]. Mice that are homozygous for inactivated *Pitx1* alleles show, in addition to changes in the pituitary and cleft palate, abnormalities in the tibia and fibula, patella, and tarsal bones so that the limb more closely resembles a forelimb [149,150]. In addition, expression of *Tbx4* in the hindlimb is reduced. Misexpression of *Pitx1* in the wing-bud of the developing chick embryo, where it is not normally expressed, induces distal expression of *Tbx4* as well as *Hoxc10* and *Hoxc11* genes, and the wings acquire characteristics of hindlimbs. For example, the muscle pattern becomes leg-like and the digits become more toe-like [151].

The Hand-Foot-Genital Syndrome

The hand-foot-genital syndrome (HFG; MIM 140000) is a rare developmental defect with autosomal dominant inheritance consisting of (i) limb anomalies with short thumbs and toes, hypoplasia of the thenar eminence, small feet, clinodactyly V, and fusion or delayed ossification of carpal bones; (ii) urogenital malformations, including duplications of the female genital tract (bifid uterus and cervix, septate vagina), a displaced urethral opening, and malpositioning of ureteral orifices leading to reflux and chronic kidney disease. Hypospadias of varying degrees are observed in affected males.

The gene for the hand-foot-genital syndrome was identified through phenotypic similarities with the mouse mutant hypodactyly (Hd). Hd was shown to be due to a mutation in *Hoxa13* [152], and, based on this observation, *HOXA13* was screened in patients with hand-foot-genital syndrome. A stop codon within the homeodomain was identified in one family indicating that the phenotype is caused by loss of function mutations of the *HOXA13* gene [153].

Absence/Hypoplastic Defects of the Ulnar Ray

The Ulnar-Mammary Syndrome

The ulnar-mammary syndrome (UMS; MIM 181450) is characterized by (i) posterior (ulnar or postaxial) limb

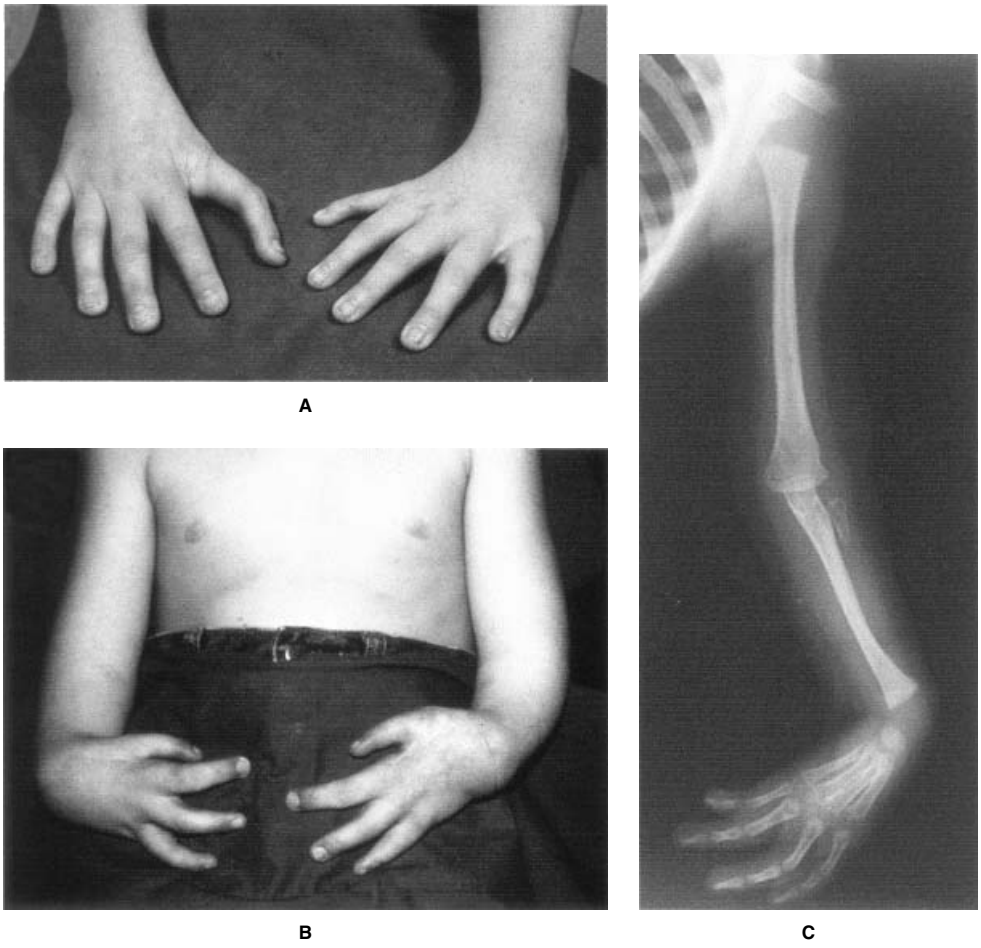


Figure 10. Holt-Oram syndrome. (**A**) 11-year-old patient with Holt-Oram syndrome with triphalangeal thumbs. Note finger-like thumbs and hypoplasia of the patient's left thumb. (**B**) 12-year-old boy with Holt-Oram syndrome showing severe bilateral malformation with hypoplasia of the forearms and left humerus, with limited motion in elbows. Aplasia of both thumbs. (**C**) Radiograph of a similarly affected individual demonstrating hypoplasia of the radius, a hypoplastic humeral head, and absence of the thumb. (Courtesy of S. Spranger, Bremen.)

defects, such as hypoplasia or absence of the most ulnar fingers, including their metacarpals, combined with (ii) apocrine abnormalities that range from diminished perspiration and axillary hair to complete absence of breast tissue, perspiration and axillary hair; (iii) ectopic, hypoplastic, or absent canine teeth; (iv) delayed puberty in males, and (in some affected individuals) micropenis, shawl scrotum, and an imperforate hymen. Known also as Schinzel syndrome and the ulnar-mammary syndrome of Pallister, UMS has been mapped to a region on chromosome 12q that also includes the gene for Holt-Oram syndrome [154]. In fact, the two syndromes are caused by mutations in two closely linked and homologous genes. Holt-Oram syndrome with its anterior (radial) limb abnormalities is the result of mutations in the *TBX5* gene; UMS is caused by loss of function mutations in the *TBX3* gene [155]. UMS is therefore likely to be caused by haploinsufficiency of *TBX3*.

What the downstream targets are for *TBX3* in the different organs that are affected in the UMS syndrome is not known, but further analysis of syndromes displaying phenotypic overlap with UMS may help in identifying such target genes. For example, in a large Dutch family, van Bokhoven et al. [156] have demonstrated linkage to a locus on chromosome 3q27 for a syndrome called limb-mammary syndrome (MIM 603543) with severe hand/foot anomalies combined with hypoplasia/aplasia of mammary glands and nipples, nail dysplasias, hypohidrosis, hypodontia, and cleft palate.

Defects in Dorsal-Ventral Patterning

Nail-Patella Syndrome

The nail-patella syndrome (NPS1; MIM 161200), also referred to as onychoosteodysplasia, is characterized by (i) nail dysplasia (hypoplasia, absence, ridging, flatness); (ii) hypoplastic or absent patella; (iii) characteristic bony projections from the iliac wings (iliac horns); (iv) elbow deformity with an inability to fully extend, pronate, or supinate the forearm due to hypoplasia and/or subluxation/dislocation of the radial head; and (v) nephropathy of various severity and manifestation (glomerulonephritis, nephrotic syndrome) (Fig. 11). Some 10–20% of patients may develop end-stage renal failure. It is caused by mutations in the LIM-homeodomain protein LMX1B [157,158]. This transcription factor is critical for the dorsal/ventral patterning of the limb, and its expression in dorsal limb mesenchymal cells is induced and maintained by Wnt7a secreted

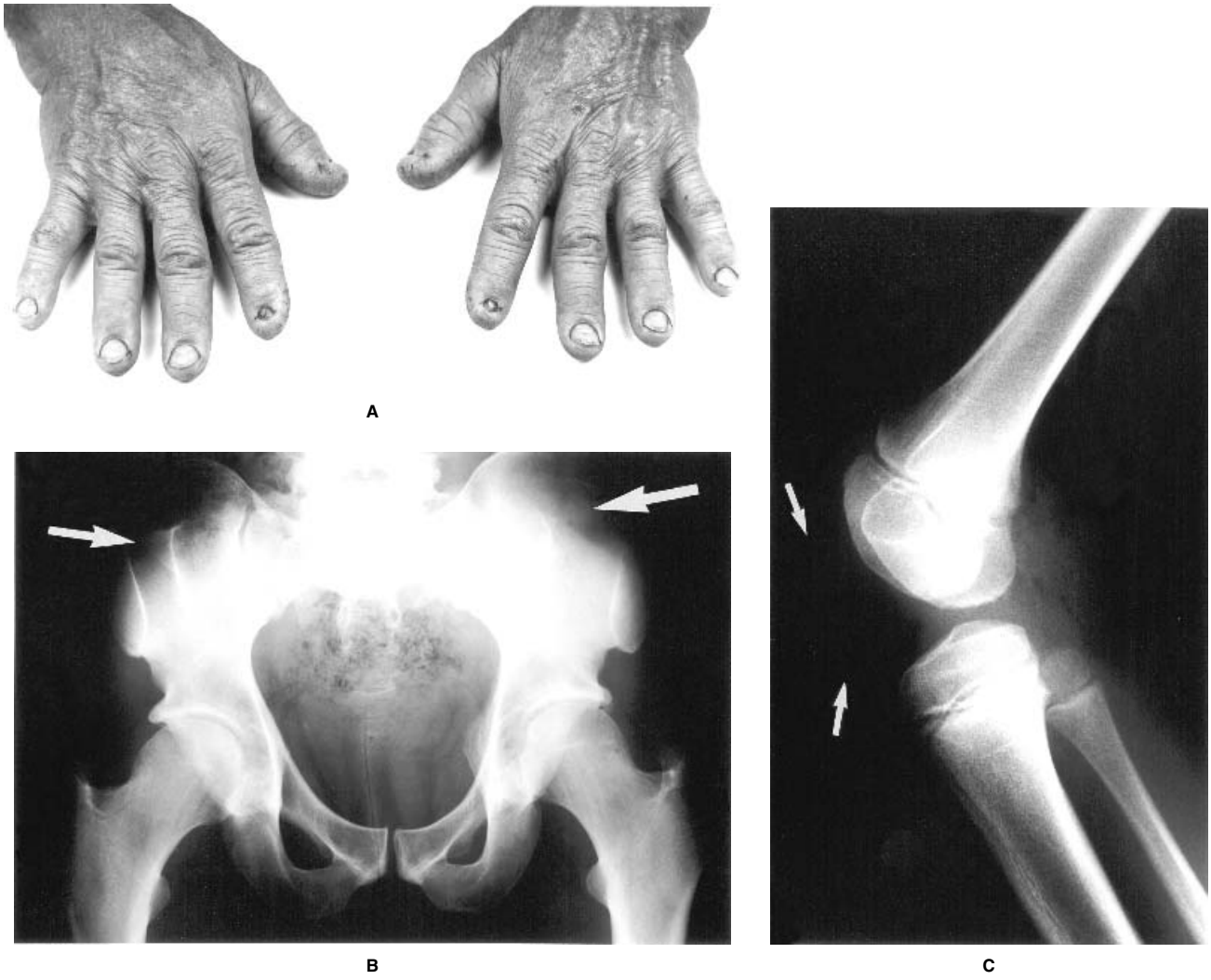


Figure 11. Nail-patella syndrome. (**A**) Hypoplasia of nails in an individual with nail-patella syndrome. (**B**) abnormal pelvis of the patient shown in **A** with flaring of the iliac wings and characteristic iliac horns (arrows). (**C**) Absent patella (arrows) in the affected child.

by dorsal ectodermal cells. Ectopic expression of *LMX1* in ventral mesenchyme generates double-dorsal limbs in chick embryos [159]. *LMX1* is homologous to the *Drosophila* gene *apterous*, a gene that specifies dorsal cell fate and regulates outgrowth of the wing during wing development in the fly [160]. In vertebrates, these two functions are performed by different proteins. While *LMX1* specifies dorsal identity, another *apterous* homologue, *LHX2*, is important for controlling limb outgrowth [160].

Targeted disruption of the *Lmx1b* gene in mice results in a phenotype that is very similar to what is seen in individuals with nail-patella syndrome: skeletal defects that include hypoplastic nails, absence of patellae, and renal dysplasia [161]. The mutations found in patients with the syndrome either affect DNA binding or result in premature termination of translation, consistent with the conclusion that the syndrome is caused by loss-of-function of *LMX1B* [162]. In some families with NPS1, open angle glaucoma has been reported to segregate with the other features of the syndrome [157]. This is also consistent with the mouse knock-out phenotype, in that it includes anterior segment eye abnormalities.

Brachydactyly

Disorders characterized by shortening of fingers and toes, brachydactyly, are divided into a large number of types depending on which bones and/or fingers (toes) are affected. Given what we now know about the specification of digit identity, joint development, and bone growth in hands and feet, this clinical subdivision is useful and appears to reflect the underlying genetic defects.

Brachydactyly, Types A1 and A2

Type A1 brachydactyly (BDA1; MIM 112500) is characterized by (i) rudimentary middle phalanges of all digits, sometimes fused with the terminal phalanges (distal symphalangism); (ii) short proximal phalanges of the thumb and big toe; and (iii) short stature. This form of brachydactyly is also referred to as the Farabee type, in reference to S. W. Farabee who described this disorder in Mendelian terms in his Harvard University Ph.D. thesis in 1903.

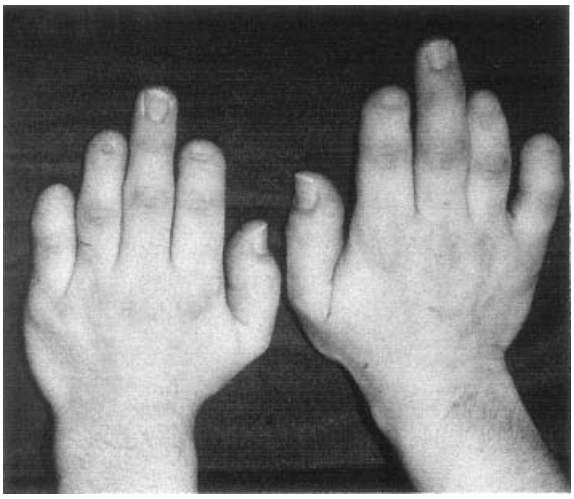
A rare form of brachydactyly type A, with shortening and triangulation of the middle phalanges of only the index finger and the second toe, is classified as brachydactyly, type A2 (BDA2; MIM 112600).

Although several candidate genes have been considered, mutations that result in brachydactyly A1 have not yet been described [163].

Brachydactyly, Type B

Brachydactyly type B (BDB1; MIM 113000) is characterized by rudimentary or absent distal and middle phalanges of fingers two to five with complete or nearly complete absence of the nails. The thumbs are spared (Fig. 12). In some individuals, the thumbs show a variable degree of flattening, splitting, or duplication of the distal phalanges. The feet show similar changes but are generally less affected. Other features are soft tissue syndactyly, symphalangism, carpal and/or tarsal fusions, and shortening of metacarpals and/or metatarsals. There are no other associated malformations.

Brachydactyly type B has been mapped independently by two groups to chromosome 9q22 but there is evidence of genetic heterogeneity in this condition [164,165].



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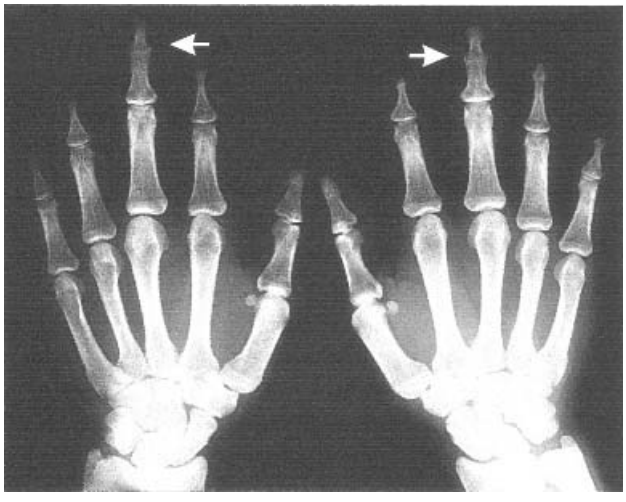


B

Figure 12. Brachydactyly type B. (**A**) Absence or hypoplasia of terminal portions of fingers II to V with mild involvement of finger III and normal thumb in a 31-year-old individual with brachydactyly type B. (**B**) Radiograph of the hands of the individual in (**A**) showing absent middle phalanges of fingers II, IV, and V, hyploplasia of distal phalanges II–V, and distal symphalangism of fingers III (absence of interphalageal joints, arrows).

Brachydactyly, Type C

Type C brachydactyly, also referred to as Haws type brachydactyly (BDC; MIM 113100) is a complex disorder, at least as described by Haws [166] in a large Mormon kindred. The characteristic features include (i) shortening of the middle phalanges of fingers II, III, and V; (ii) a short and frequently oval-shaped metacarpal of finger I; (iii) triangulation of the proximal end of the proximal phalanx of the index finger with prolongation of the radial border, short metacarpals, and symphalangism are occasionally present (Fig. 13).

In a pedigree described by Robin et al. [167], brachydactyly type C mapped to chromosome 20 to an interval previously shown to contain *CDMP1* [168]. *CDMP1*, also known as *GDF5*, is a member of the TGF-β superfamily. Interestingly, the mouse mutant brachypodism, a murine type of brachydactyly, had previously been shown to be caused by homozygosity for functional null alleles in *Cdmp1* [169]. Several mutations in *CDMP1* were identified in patients with brachydactyly type C that are likely to result in functional haploinsufficiency [168].

Acromesomelic Dysplasia, Grebe and Hunter-Thompson Types

Grebe (AMDG; MIM 200700) and Hunter-Thompson (AMDH; MIM 201250) types of acromesomelic dysplasia are autosomal recessive disorders characterized by severe shortening of the four limbs and small digits. The craniofacial and axial skeletons are not affected. The limb abnormalities

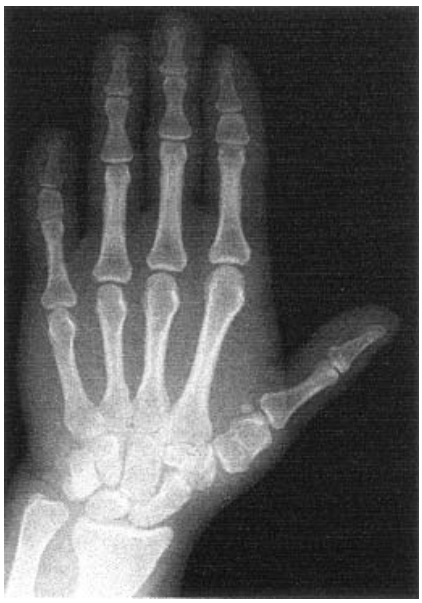


Figure 13. Brachydactyly type C. Radiograph of hand of a mildly affected individual aged 30, showing short first metacarpal and hypoplastic middle phalanges II and V. (Courtesy of B. Zabel, Mainz.)

show a proximal-to-distal gradient of increasing severity. While the humerus/femur is relatively normal, the radius and ulna/tibia and fibula are short, carpal/tarsal bones may be fused and metacarpal/metatarsal bones may be absent. Fingers/toes may show poly- and brachydactyly with phalangeal bones missing. Dislocation of hips, knees, ankles, and elbows is also described (Fig. 14).

In their description of patients with AMDH, Langer et al. [170] pointed to similarities with AMDG, but suggested that the two syndromes were distinct clinical entities based on radiological findings. The similarities between the two syndromes can now be understood based on the fact that they are both caused by mutations in the gene encoding cartilage-derived morphogenetic protein-1, *CDMP1* [171].

In a large Brazilian pedigree with the Grebe syndrome [172], Thomas et al. [173] showed linkage to markers close to *CDMP1*. They further demonstrated that affected individuals were either homozygous for a cysteine to tyrosine substitution in CDMP-1, or compound heterozygous for this mutation in one allele and a single nucleotide deletion leading to a frameshift and premature stop codon in the other allele. Thus, Grebe syndrome seems to be the homozygous form of brachydactyly type C. In the family with AMDH reported by Langer et al. [170], Thomas et al. [174] also found a mutation in *CDMP1*. The mutation, a 22-bp tandem duplication, led to a frame-shift and a mutant protein of almost normal size but with the last 43 amino acid residues totally different from those of the wild-type protein.

The phenotypic differences between AMDG and AMDH may reflect different effects of different mutations in *CDMP1*. In the case of AMDH, affected individuals are homozygous for a frameshift, loss-of-function mutation. In contrast, the Cys to Tyr substitution in CDMP1 in patients with AMDG appears to have a dominant-negative effect in that the mutant protein forms heterodimers with other BMPs and prevents their secretion.

Animal models. Brachypodism (bp), is a recessive disorder caused by mutations in *Gdf5*, the murine homologue of *CDMP1* [169].

Brachydactyly with Hypertension

Brachydactyly combined with hypertension, also referred to as Bilginturan syndrome (MTNB; MIM 112410), is characterized by shortening of both phalanges and

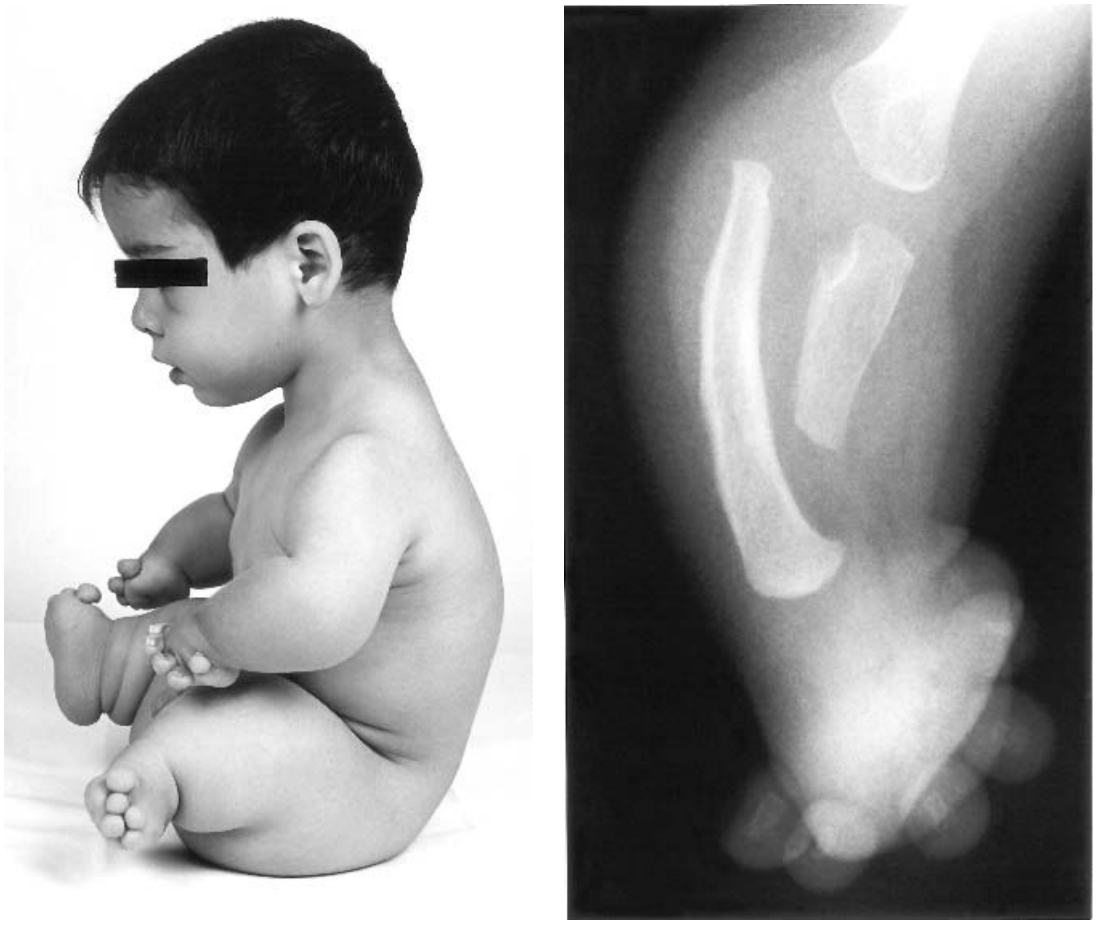


Figure 14. Acromesomelic dysplasia Grebe type. (**A**) 4-year-old boy with Grebe dysplasia showing severe mesomelic limb reduction. Both parents show a mild form of brachydactyly type C (radiograph of father's hand is shown in Fig. 13). Note very short fingers and toes. The legs are shorter than the arms. (**B**) Radiograph of the upper limb of patient in (**A**) showing severely deformed and shortened radius and ulna with rudimentary carpal bones and fingers. (Courtesy of B. Zabel, Mainz.)

metacarpals. The elevation in blood pressure can be severe, with systolic pressures ranging from 110 to 250 mm Hg and diastolic pressures from 100 to 150 mm Hg. Surprisingly, affected individuals are relatively free from end-organ damage, including an absence of hypertensive retinopathy. Linkage analysis of a large Turkish pedigree placed the locus of the affected gene in the chromosome 12p12.2–p11.2 region [175]. Markers in the same region also cosegregate with the phenotype in other families, and in a Japanese family affected individuals were found to be heterozygous for a deletion within this region [176].

Syndactyly

Type III Syndactyly

Among the many clinical forms of syndactyly, autosomal dominant syndactyly type III (SDTY3; MIM 186100) is characterized by usually complete, bilateral syndactyly between digits four and five. The feet are unaffected. The syndactyly typically involves only soft tissue, but fusion of the distal phalanges can occur; the middle phalangeal bone of finger five can be absent or present as a small rudiment.

SDTY3 has been mapped to chromosome 6q22–q24 [177]. This region also contains the locus for oculodentodigital dysplasia (ODDD; MIM 164200). Since affected individuals of one family included in the mapping study had a facial phenotype resembling that of ODDD associated with their syndactyly, it has been proposed that SDTY3 and ODDD are caused by mutations at the same locus [177].

Disorders of Segmentation

Proximal Symphalangism

The autosomal dominant disorder, proximal symphalangism (SYM1; MIM 185800), is an inherited absence of the proximal interphalangeal joints with fusion of carpal and tarsal bones, often associated with early onset conductive deafness in affected individuals (Fig. 15). Ankylosis of the proximal interphalangeal joints also occurs as part of other syndromes, including diastrophic dysplasia (MIM 222600) and type C brachydactyly (MIM 113100).

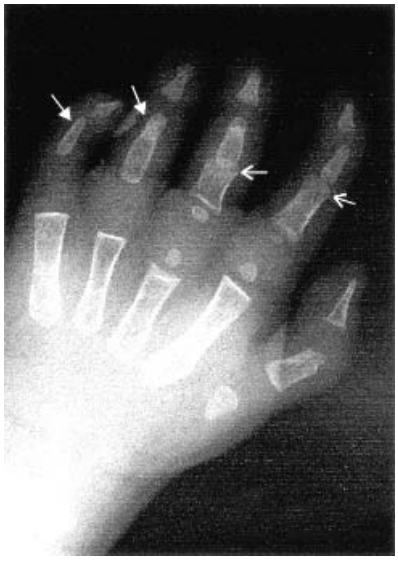


Figure 15. Proximal symphalangism. Radiograph of hand of a 3-months-old patient with conductive hearing loss and decreased mobility of proximal phalangeal joints showing complete bony fusion of the proximal interphalangeal joint of fingers IV and V (arrows), as well as hypoplastic joints and middle phalanges of fingers II and III (arrows). (Courtesy of P. Meinecke, Hamburg.)

SYM1 was mapped to the noggin (*NOG*) locus on chromosome 17q and has been shown to be caused by mutations in *NOG* [178]. A *NOG* mutation also in a family with multiple synostosis syndrome (SYNS1; MIM 186500) suggests that SYM1 and SYNS1 are allelic disorders [178]. The mutations in the human *NOG* gene confirm studies in mice that noggin is essential for the normal formation of joints, and suggest that the protein is particularly important for the development of proximal interphalangeal joints in humans.

THE DYSOSTOPLASIAS

Skeletal organogenesis involves the formation of bone and cartilage as an organ (the skeleton). Condensation of undifferentiated cells follows a preset pattern that outlines the future skeletal elements. The molecular mechanisms that initiate and regulate condensation are largely unknown. The expression of extracellular matrix proteins that promote cell-cell adhesion by providing "sticky" surfaces is likely to be a key event. N-cadherin, N-CAM, and fibronectin are adhesive molecules known to be elevated in sites of precartilaginous condensations [179]. After condensation, mesenchymal precursor cells differentiate into chondrocytes or osteoblasts, and finally these cells produce their specific extracellular matrix (histogenesis). These aspects of early skeletogenesis, the anlage, represent the outlines of the future skeletal elements. Bones then develop either directly from osteoblastic progenitor cells or within a temporary framework of hyaline cartilage. The direct formation of bone is called desmal or intramembranous ossification, and occurs in the flat bones of the skull, the mandible, and part of the clavicle. In contrast, all other bones develop through endochondral bone formation, a process involving the proliferation and differentiation of chondrocytes to form a temporary cartilaginous template which is subsequently replaced by bone (see also Chapter 1, Parts II and III, this volume).

Mutations in genes that are involved in skeletal organogenesis produce phenotypes that share aspects of dysplasias and dysostoses. Among such genes are genes that regulate the differentiation and growth of osteoblasts and chondrocytes. When the genes are expressed only in skeletal tissues, as in the case of that for the transcription factor CBFA1, only bone and cartilage are affected. In contrast, when the gene is important for the differentiation/growth of both skeletal and nonskeletal tissues, as in the case of *SOX9* and the gene for cartilage-hair hypoplasia, characteristic syndromes are produced that combine skeletal abnormalities with disorders of other organ systems.

Cleidocranial Dysplasia

Cleidocranial dysplasia (CCD; MIM 119600) is a dominantly inherited disorder characterized by (i) hypoplasia/aplasia of the clavicles; (ii) delayed ossification of cranial sutures and fontanelles; and (iii) dental anomalies that include delayed eruption of deciduous and permanent teeth and supernumerary teeth of the permanent dentition (Fig. 16A–C) [180]. A large number of other skeletal abnormalities are commonly observed including a narrow, bell-shaped thorax, cervical or missing ribs, and characteristic changes in the pelvis consisting of hypoplastic iliac wings and a wide symphysis pubis. Large femoral epiphyses, and

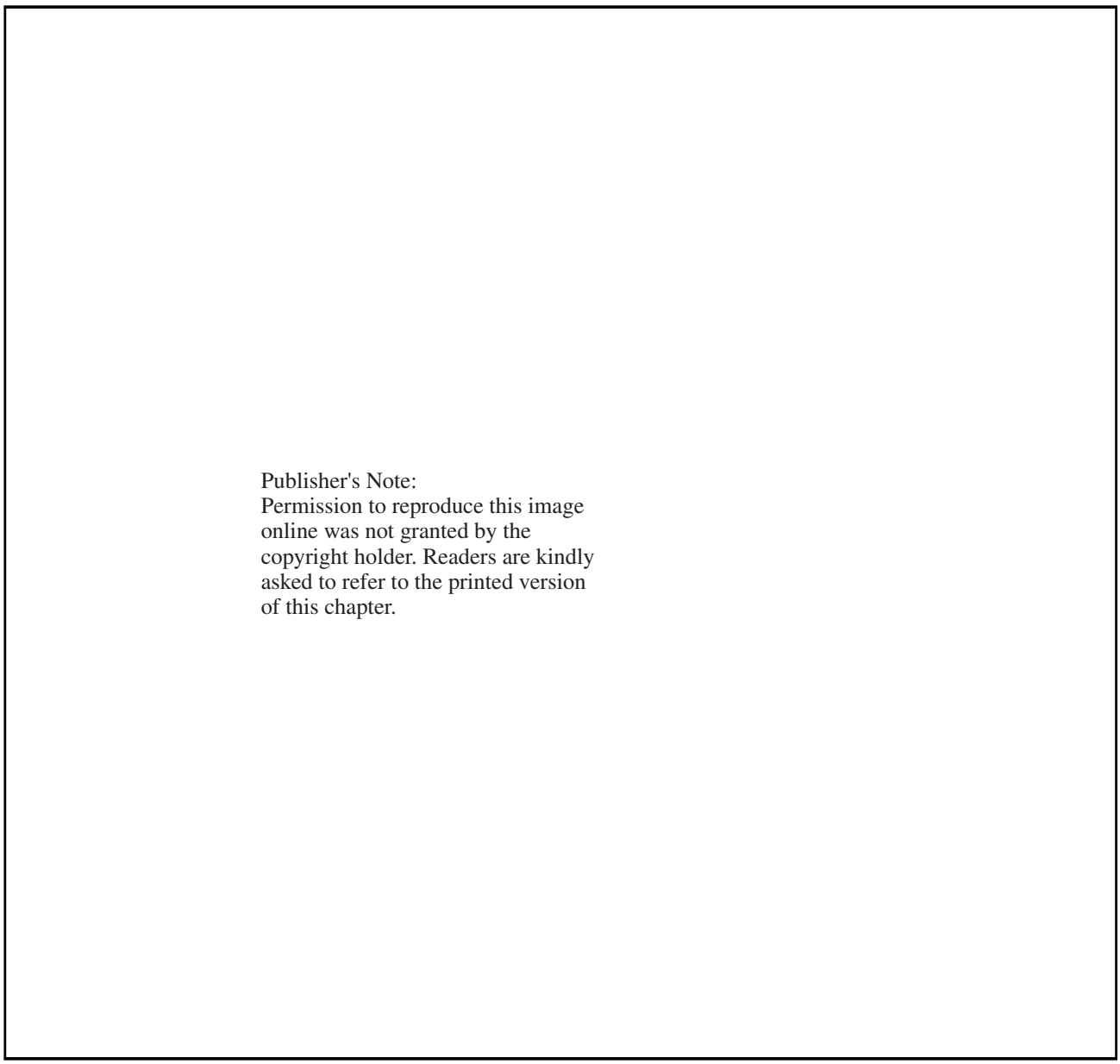


Figure 16. Cleidocranial dysplasia. (**A**) Radiograph of the chest showing rudimentary clavicle (arrow) on the right and straight, distally hypoplastic clavicle on the left. Narrow, cone shaped thorax. (**B**) Skull radiograph demonstrating multiple Wormian bones (arrows) in lambdoid and sagittal sutures. (**C**) Panoramic radiograph of teeth from an affected patient aged 15 years showing multiple impacted supernumerary teeth and partly persistent deciduous dentition. (With permission from Quack et al. [184]). (**E**) Severely hypoplastic clavicles in the Cbfa1+/− mouse compared with (**D**) wild type clavicles (arrows). Alizarin red/alcian blue stained skeletons.

two ossification centers in the second metacarpal and a short middle phalanx of the fifth finger are frequently found. The bone age is generally retarded. The face and skull have a characteristic appearance with a relatively large cranium, frontal and parietal bossing, and a frontal groove originating from ossification defects within the metopic suture. The nose is short and anteverted, and the maxilla is hypoplastic giving the impression of a relative prognathism. The cranial abnormalities are sometimes referred to as the ''Arnold head'' after the founder of a large South African pedigree with the disorder [181]. Growth retardation and slightly retarded skeletal maturation during childhood can lead to short stature in affected individuals. The gene for CCD was mapped to chromosome 6p21 and a small deletion in this region was shown to segregate with the phenotype in one family [182]. This was followed by the demonstration that

CCD is caused by heterozygous loss-of-function mutations in the transcription factor CBFA1 located within the deleted segment [183,184].

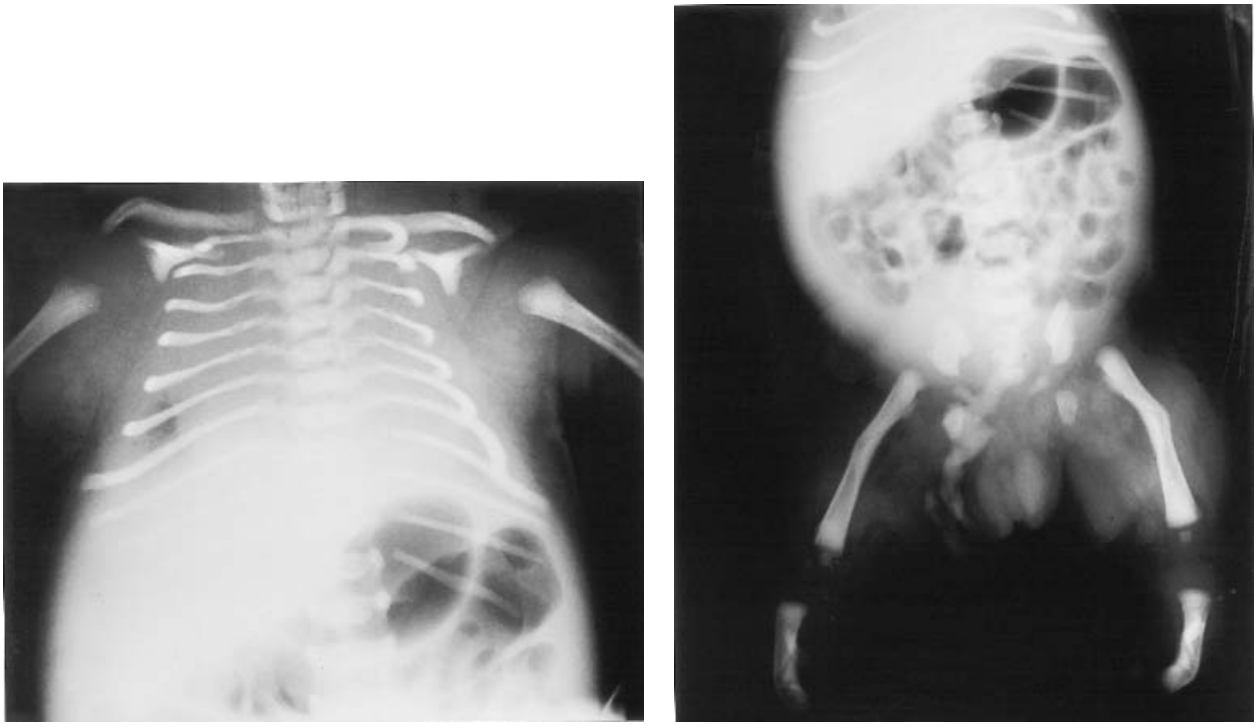
CBFA1 is a member of a small family of transcription factor genes that are homologous to the *Drosophila runt* gene. While *CBFA2* is critical for hematopoietic cell differentiation, *CBFA1* is essential for the differentiation of osteoblasts, during both intramembranous and endochondral bone formation [185]. This seems to be true not only during embryonic development, but also during adulthood since some individuals with severe CCD also have osteoporosis [184]. Mice that are heterozygous for inactivated *Cbfa1* alleles show a CCD phenotype (Fig. 16D and E). Homozygous knockouts die at birth with a skeleton consisting entirely of cartilage and no bone [186,187]. In addition, maturation of chondrocytes to hypertrophic chondrocytes is severely reduced, particularly in proximal limb segments, and invasion of cartilage by blood vessels and hematopoietic cells does not occur. A radiation-induced mouse mutant, Ccd, carries a large deletion on chromosome 17, including the *Cbfa1* gene; heterozygous mutant animals have a phenotype identical to that of heterozygous *Cbfa1* knockouts [186,188].

The major features of CCD can easily be understood on the basis of a reduced differentiation of osteoblasts. The clavicular defect and the deficient maturation of chondrocytes in long bones, however, appear to be the consequences of impaired chondrocyte differentiation and maturation [189,190]. It is likely, therefore, that chondrocytes in some regions of the developing skeleton are dependent on *CBFA1*.

Campomelic Dysplasia

Campomelic dysplasia (CMD1 or CMPD1; MIM 114290) is a dominant disorder caused by haploinsufficiency of the transcription factor SOX9. The phenotypic abnormalities include (i) bowing and angulation of long bones; (ii) hypoplasia of the scapula and pelvis; (iii) abnormalities of the vertebral column with a decreased number of ribs; and (iv) craniofacial abnormalities (cleft palate, micrognathia, hypertelorism, and a flat facies) as a consequence of a small chondrocranium (Fig. 17). Most affected babies die in the neonatal period or early infancy of respiratory distress caused by a small rib cage, narrow airways (defective tracheobronchial cartilage) and hypoplastic lungs. Heart and kidney malformations and the absence of olfactory bulbs and tracts have also been reported.

The major skeletal features of the syndrome suggest a defect in cartilage development. Not only is there a defective formation of cartilage in the airways, but the chondrocranium is much smaller than the neurocranium and many of the craniofacial defects are similar to those associated with mutations in cartilage-specific extracellular matrix molecules such as collagens II and XI. A significant and striking difference between campomelic dysplasia and other chondrodysplasias, however, is that CMD1 is often associated with male-to-female sex reversal [191]. This interesting association can now be understood from the molecular defect in the syndrome. The disease-causing



A **B**

Figure 17. Campomelic dysplasia. Radiographs of a female newborn with dwarfism (39 cm), short limbs, severe hypoplasia of the thorax and neonatal death due to respiratory distress. (**A**) Narrow, bell-shaped thorax, 11 pairs of ribs, lateral clavicular hooks, severely hypoplastic scapulae. (**B**) Hypoplastic iliac wings, poor ossification of os pubis, hip dislocation, long and angulated femurs, short angulated tibiae and hypoplastic/absent fibulae, no ossification in feet. (Courtesy of J. Spranger, Mainz.)

mutations are either heterozygous loss-of-function mutations in the *SOX9* gene or chromosome rearrangements that interrupt important transcriptional regulatory elements scattered over a large region in the 5′ flanking region of *SOX9* [192–194].

SOX9 is a member of a family of transcription factors that contain an SRY-related high-mobility-group (HMG) homology domain [195]. In the mouse, it is expressed in mesenchymal condensations before and during chondrogenesis [196]. One of its regulatory targets is *COL2A1*, the gene encoding the major collagen in cartilage (see Chapter 2, this volume); SOX9 binds specifically to sequences within the first intron of this gene [197]. Loss-of-function mutations in *SOX9* or mutations that decrease transcription of the gene therefore result in decreased expression of collagens II and XI (collagen XI molecules being composed of three different subunits, one of which is the product of *COL2A1*). It has also been demonstrated that the addition of BMP2 to avian embryos results in increased expression of *SOX9* and the induction of ectopic cartilage. Furthermore, ectopic expression of the BMP-binding protein noggin in chick limbs causes loss of *SOX9* expression in digits, suggesting that BMPs may regulate *SOX9* expression [198]. In chimeric mouse embryos containing a mixture of *Sox9* −/− cells and *Sox9* +/+ cells, the *Sox9*-deficient cells are excluded from all cartilages, but are present in perichondrial tissues [199]. This exclusion appears to occur during the condensed mesenchyme stage of chondrogenesis, suggesting that *SOX9* is critical for mesenchymal cell condensation leading to chondrocyte differentiation.

Loss-of-function mutations in *SOX9* are therefore expected to affect chondrogenesis at an early stage as well as the expression of cartilage-specific extracellular matrix molecules by differentiated chondrocytes. *SOX9* is also expressed in differentiating Sertoli cells and it is believed that it plays an essential role in sex determination, thus providing a logical explanation for the sex reversal associated with campomelic dysplasia [200,201].

Ellis-van Creveld and Short-Rib-Polydactyly Syndromes

Ellis-van Creveld syndrome (EVC; MIM 225500) is also referred to as chondroectodermal dysplasia. It is a recessive disorder and, as the designation chondroectodermal implies, the clinical features are a combination of skeletal and ectodermal abnormalities. The major features consist of (i) short stature of prenatal onset that is most pronounced in the distal segments; (ii) skeletal defects including polydactyly, short and broad middle phalanges and hypoplastic distal phalanges, fusion of wrist bones (hamate and capitate), an abnormal pelvis, a narrow thorax with thin ribs, and hypoplasia of the upper lateral tibia (Fig. 18); (iii) hypoplastic fingernails; (iv) cardiac defects, most commonly atrial septal defects and a single atrium; (v) neonatal teeth, or small teeth with delayed eruption; (vi) a frenulum from the alveolar ridge to the upper lip, and defects in the alveolar ridge.

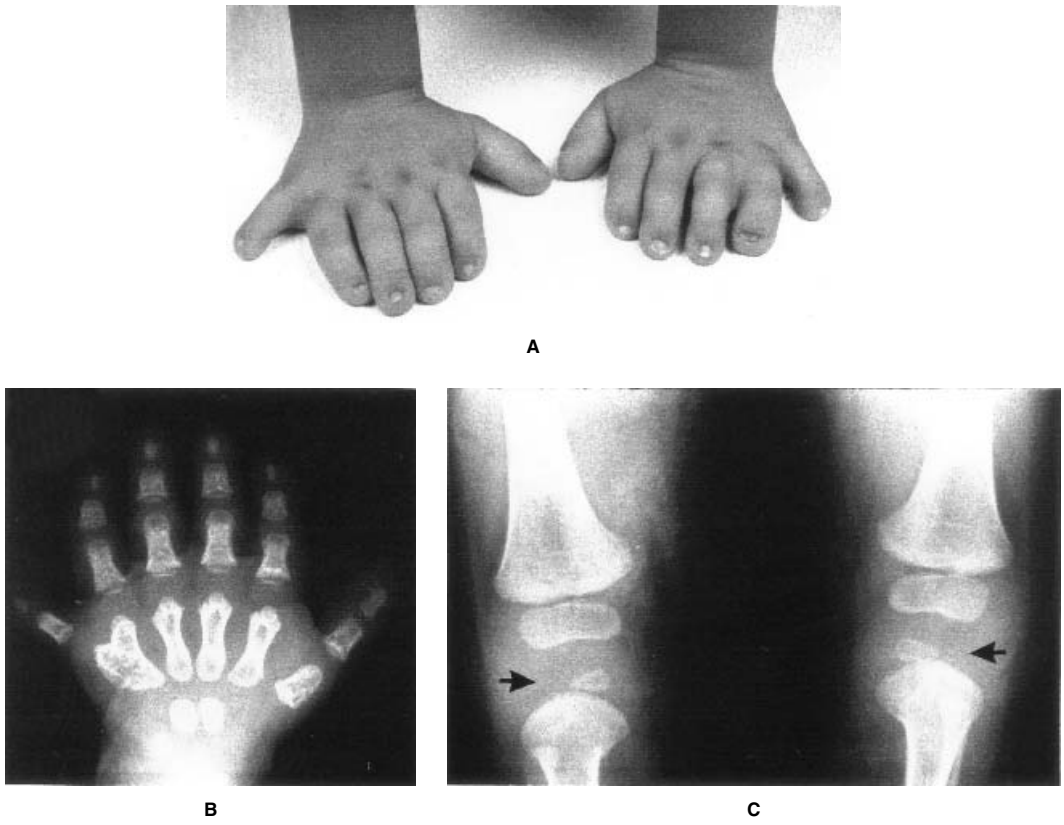


Figure 18. Ellis-van Creveld syndrome. (**A**) Hands of a 2-year-old girl with Ellis-van Creveld syndrome showing broad fingers, hypoplastic nails and postaxial polydactyly. (**B**) Radiograph of individual in (**A**) showing postaxial polydactyly with bony fusion of extra metacarpal and metacarpal V, short and broad middle phalanges with partial duplication of middle and terminal phalanx of finger IV, cone shaped epiphyses and hypoplastic distal phalanges. (**C**) Hypoplasia of lateral part of tibial epiphysis (arrows) is a frequent finding in Ellis-van Creveld syndrome.

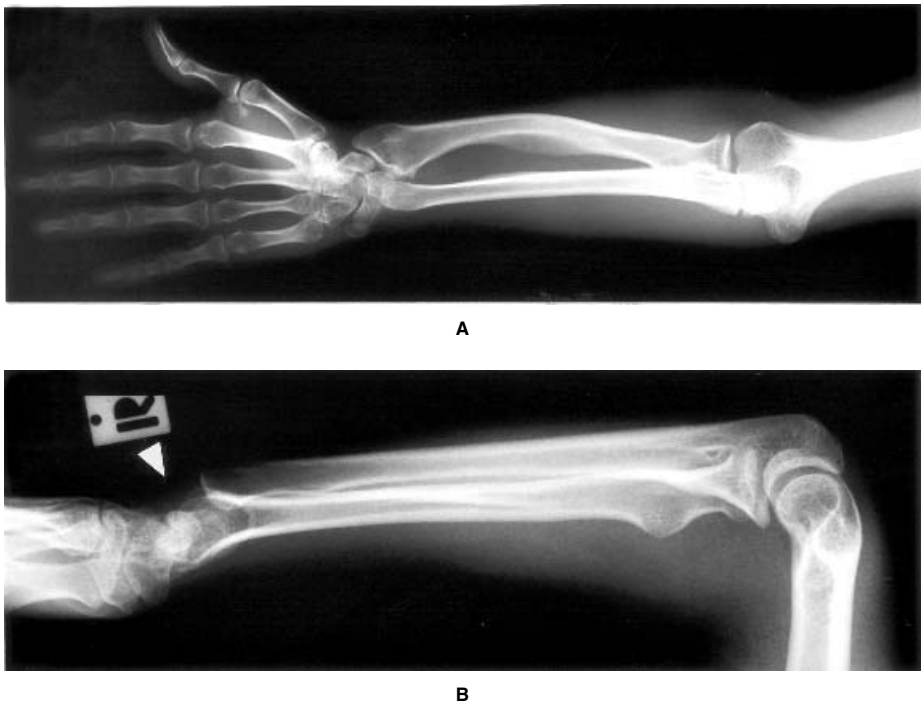


Figure 19. Leri-Weill dyschondrosteosis. (**A**) Radiograph of forearm and hand in a 25-year-old patient with dyschondrosteosis. The radius is bowed and short, the distance between radius and ulna is widened, and there is triangulation of the distal radius. (**B**) Lateral view of same forearm showing Madelung deformity with dorsal subluxation of the distal end of the ulna and volar displacement of the hand (arrowhead).

The syndrome has been mapped to the distal region of chromosome 4p. Two homeobox genes, *MSX1* and *HMX1*, initially proposed as candidate genes for EVC, have both been excluded [202,203].

The short-rib-polydactyly syndromes are a group of at least seven distinct conditions, among them Jeune syndrome, or asphyxiating thoracic dystrophy (ATD; MIM 208500). This group of diseases is characterized by (i) short ribs and subsequent thoracic and pulmonary hypoplasia; (ii) polydactyly, preaxial or postaxial (variable); (iii) short limbs with dysplastic bones, and associated malformations of the heart, the urogenital system and other organs, and clefts. Many of the skeletal changes in the chest, limbs and pelvis are not unlike those seen in Ellis-van Creveld syndrome.

Leri-Weill Dyschondrosteosis

Caused by heterozygous mutations in the pseudoautosomal gene *SHOX*, Leri-Weill dyschondrosteosis (LWD; MIM 127300) is characterized by (i) mild to moderate short stature; (ii) mesomelic shortening of the forearms and lower legs with shortening and bowing of the radius, triangulation of the distal radial epiphysis, distal hypoplasia of the ulna, and widening of the distance between the radius and the ulna. Dorsal dislocation of the distal ulna may lead to elbow dislocation, cubitus valgus. The altered osseous alignment at the wrist leads to the dorsal subluxation of the distal end of the ulna and volar displacement of the hand, an abnormality referred to as Madelung deformity (Fig. 19).

The abnormalities are the consequences of heterozygous loss-of-function mutations (deletions or point mutations) in the *SHOX* gene within the pseudoautosomal region (PAR1) on the X and Y chromosomes [204,205]. Homozygosity for mutations within the gene leads to the Langer type of mesomelic dysplasia (MIM 249700), characterized by severe hypoplasia or aplasia of the ulna and fibula, and a bowed radius and tibia [206]. Two alternatively spliced transcripts of the *SHOX* gene have been found to be expressed in several tissues, including bone marrow fibroblasts [207], but why mutations in the gene should result in short stature and the characteristic abnormalities in forearms and lower legs, is unclear.

RECENT DEVELOPMENTS

Disorders of the Craniofacial Skeleton

Parietal Foramina

Parietal foramina (MIM 168500) are oval defects of the calvaria caused by deficient ossification of the parietal bones. Inheritance is frequently dominant. Mutations in *MSX2*, a transcription factor gene located on chromosome 5q, have been associated with this condition [209]. The presence of a large deletion encompassing the entire gene suggests a loss of function mechanism. Interestingly, *MSX2* has also been found mutated in a family with Boston-type craniosynostosis [210,211]. The *MSX2* mutation in this family is located in the homeodomain and the mutant protein was shown to bind with enhanced affinity to the MSX2 DNA target sequence, implying that the mutation operates through gain of function [212]. These mutations demonstrate that MSX2 dosage is fundamental for human skull development and calvarial osteogenic differentiation.

Another locus for parietal foramina is on chromosome 11p11-12. This region contains *ALX4*, a transcription factor gene previously identified as the cause of the mouse mutant Strong's luxoid [213]. In mice, heterozygous mutations of *Alx4* cause preaxial polydactyly, while homozygotes manifest delayed ossification of the parietal bones and several other

abnormalities. Mavrogiannis et al. [214] were able to identify mutations in *ALX4* in several families with parietal foramina, and concluded that haploinsufficiency of *ALX4*, as in the case of *MSX2*, results in ossification defects of the skull.

Opitz G/BBB Syndrome

Opitz G/BBB syndrome (MIM 300000) is a congenital disorder primarily affecting midline craniofacial development. Manifestations include a typical facial appearance, ocular hypertelorism, cleft lip and palate, mental retardation, and dysplasia of the corpus callosum. Other features of this condition include tracheo-esophageal fistulae, cardiac anomalies, genitourinary malformations, and imperforate anus. The condition is genetically heterogeneous, one locus being on chromosome 22q11.2, another on Xp22.3. The X-linked condition is caused by mutations in *MID1*. *MID1* encodes a microtubule-associated protein that functions as a ubiquitin ligase which targets phosphatase 2A for degradation [215].

Disorders of the Axial Skeleton

Spondylocostal Dysostosis

One form of recessive spondylocostal dysostosis (MIM 277300) has been shown to be due to mutations in *DLL3* [216], the human homologue of the mouse *Dll3* gene, which is the cause of the pudgy mutation in the mouse. The mutations identified predict truncations within the conserved extracellular domains of DLL3 or are missense mutations within other conserved domains.

Robinow Syndrome

Recessive Robinow syndrome (MIM 268310), a skeletal dysplasia with mesomelic short stature, hypogenitalism and heart defects, is frequently associated with malformations of the axial skeleton, mimicking spondylocostal dysostosis. Robinow syndrome has been shown to be due to mutations in the tyrosine kinase receptor ROR2 [217,218]. Inactivation of *Ror2* in the mouse produces a severe lethal skeletal phenotype with vertebral defects, rib fusions, and severe shortening of the extremities [219,220]. The phenotype is comparable to Robinow syndrome, but more severe. The ligand for this receptor and the signalling pathway downstream of the receptor, as well as its function during skeletal patterning and growth are largely unknown. Recessive Robinow syndrome is allelic with brachydactyly type B.

Disorders of the Appendicular Skeleton

Polydactyly Disorders

Oral-facial-digital syndrome type I (MIM 311200) is transmitted as an X-linked condition with lethality in males. It is characterized by a variety of digital anomalies including asymmetric shortening of digits, unilateral polydactyly of feet, clinodactyly, syndactyly and/or brachydactyly. The oral abnormalities consist of frenula, pseudoclefting of the upper lip, and clefts of the palate and tongue. The facies is dysmorphic and hypoplasia of the alar cartilages is frequently found. Other features of this condition include brain malformations and mental deficiency, dry skin, sparse hair, and adult polycystic kidney disease. Mutations have been identified in the *OFD1* gene (formerly *Cxorf5/71-7a*), a gene with unknown function [221].

Absence/Hypoplasia Defects of Hands/Feet

Split-hand split-foot malformation. van Bokhoven and colleagues have mapped one form of split-hand split-foot malformation, which they termed limb mammary syndrome (MIM 603543), to chromosome 3q27. In addition, they were able to show that several families with EEC syndrome, which comprises ectrodactyly, ectodermal dysplasia, and cleft palate (MIM 604292), map to the same region suggesting that these disorders are allelic. Analysis of the p63 gene, a homologue of the tumor suppressor gene p53, revealed heterozygous mutations in several families with EEC syndrome and limb mammary syndrome [222]. Using transactivation studies, the authors provided a molecular explanation for the dominant character of the p63 mutations. A knock out of p63 shows a strikingly similar pattern of involved structures. $p63^{-/-}$ mice have a truncated secondary palate, a hypoplastic maxilla, and a wide variety of abnormalities of the limbs, including syndactyly and central ray defects resembling the split-hand split-foot phenotype [223].

Further analysis of a large number of patients showed that mutations in p63 are the cause of several related disorders, indicating that p63 mutations have highly pleiotropic effects. In a study by van Bokhoven et al. [224], three distinct phenotypes were associated with p63 mutations: limb-mammary syndrome, which is characterized by ectrodactyly, cleft palate, and mammary gland abnormalities, EEC syndrome, and non-syndromic split-hand split-foot malformation. In addition, Hay-Wells syndrome has been shown to be due to p63 mutations [225]. This condition is characterized by ankyloblepharon (congenital partial fusion of the eyelids), ectodermal dysplasia and clefting. Linkage studies and phenotypic overlap suggest that the list for p63 associated conditions is even longer and may include acro-dermato-ungual-lacrimal-tooth (ADULT) syndrome (MIM 103285), recessive cleft lip/palate-ectodermal dysplasia (CLPED1; MIM 225060), and lacrimo-auricular-dental-digital (LADD) syndrome (MIM 149730) [225,226].

Acheiropodia (MIM 200500) is a rare, recessive condition characterized by congenital amputations of the upper and lower extremities. The phenotype consists of a severe reduction deformity resulting in the absence of the radius, ulna, and fibula, and the carpal, metacarpal, tarsal, metatarsal and phalangeal bones. The condition was mapped to chromosome 7q36 in close proximity to the *SHH* gene. Ianakiev et al. [227] were able to identify mutations in the human orthologue of the mouse *Lmbr1* gene. They identified a deletion within the gene in all families tested. Interestingly, expression of the gene is significantly reduced in the mouse mutant hemimelic extra-toes (Hx), which also maps in this region [228]. Hx mice show an ectopic expression of Shh indicating that Lmbr1 may act as a repressor of *Shh*.

Brachydactyly

Brachydactyly type A1 (MIM 112500) has been shown to be due to mutations in the Indian hedgehog (*IHH*) gene [229]. Ihh is one member of the hedgehog family, the more well known being Sonic hedgehog (Shh). Ihh has previously been shown to regulate chondrocyte differentiation via Gli, PTH and the PTH-receptor [230]. The present finding points to the possibility that Ihh also has a function during early condensation/differentiation of phalangeal chondrocytes.

Brachydactyly type B (MIM 113000) has been shown to be caused by mutations in *ROR2*, a gene encoding a receptor tyrosine kinase with unknown function [231,232]. Interestingly, the mutations observed so far cluster in two distinct regions of the gene. The intracellular part of the ROR2 receptor consists of the tyrosine kinase domain and a C-terminal serine-proline-rich domain. Frame shift and premature stop codon mutations are located exactly before or after the tyrosine kinase domain, resulting in truncated proteins that lack either the entire intracellular domain or

the serine-proline-rich domain. Brachydactyly type B is allelic with Robinow syndrome. In contrast to Robinow syndrome mutations, the brachydactyly type B mutations are likely to function as dominant mutations through an as yet unknown mechanism.

Disorders of Segmentation

Mutations in *HOXA11* have been identified in two families with radio-ulnar synostosis and megakaryocytic thrombocytopenia (MIM 605432) [233]. The mutations were single base pair deletions within the homeodomain coding region resulting in a premature stop codon and, presumably, a truncated protein without DNA-binding capacity. The phenotype reflects the developmental expression pattern of Hoxa11, but it remains unclear whether the mutations act as null alleles or exert a dominant-negative effect.

Dysostoplasias

Ellis-van Creveld Syndrome

Ellis-van Creveld syndrome (MIM 225500) is a recessive skeletal dysplasia with short limbs, short ribs, postaxial polydactyly and hypoplastic nails and teeth. In addition, approximately 60% of affected individuals have a heart defect, most commonly a common atrium. The gene for Ellis van-Creveld syndrome has been identified and named *EVC* [234]. It encodes a 992 amino acid protein of unknown function.

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Chapter 24

Disorders of Keratinization

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SUMMARY

- **The disorders of keratinization are a clinically and genetically heterogeneous group, characterized by scaling and hyperkeratosis. The identification of mutations in various components of the keratinization process allows a new classification of these disorders according to their genetic defects.**
- **The keratin disorders are caused by mutations in keratins, leading to a collapse of the intermediate filament network in keratinocytes of the epidermis and the hair shaft. They comprise bullous congenital ichthyosiform erythroderma, ichthyosis bullosa of Siemens, epidermolytic palmoplantar keratoderma, pachyonychia congenita types I and II, and monilethrix. The clinical presentation corresponds to the expression pattern of the mutant keratin.**
- **The disorders of the cornified cell envelope are characterized by impaired formation of the protective barrier of the epidermis. The prototype is lamellar ichthyosis type 1, which is caused by mutations in the *TGM1* gene coding for transglutaminase 1. Mutilating keratoderma with ichthyosis and progressive symmetric erythrokeratoderma are caused by loricrin mutations, leading to impaired incorporation of loricrin into the cornified cell envelope.**
- **The disorders of abnormal lipid metabolism are characterized by an altered lipid composition of cell membranes in various organ systems, accounting for the wide range of symptoms. Defects in several pathways have been identified, including oxidation of fatty alcohol (Sjögren-Larsson syndrome), ceramide metabolism (Gaucher disease), and cholesterol biosynthesis (X-linked dominant chondrodysplasia punctata and CHILD syndrome).**
- **The peroxisomal disorders are characterized by a deficiency of one or more peroxisomal proteins, that normally function in pathways related to lipid metabolism. In rhizomelic chondrodysplasia punctata type 1 several enzymes fail to be imported into the peroxisome, leading to defects in several organ systems. Refsum disease is characterized by ophthalmologic, neurological, and skin manifestations that are the result of inactivating mutations of phytanoyl-CoA-hydroxylase and subsequent accumulation of phytanic acid in various tissues.**
- **The sulfatase disorders comprise X-linked ichthyosis (XRI), X-linked recessive chondrodysplasia punctata (CDPX1), and multiple sulfatase deficiency (MSD). XRI and CDPX1 are monogenic disorders caused by deficiencies of steroid sulfatase and arylsufatase E, respectively. In MSD, several singular sulfatases are catalytically inactive due to a lack of co- or post-translational modification. The clinical presentation can be explained by accumulation of the corresponding substrates in various tissues.**
- **The contiguous gene deletion disorders are caused by deletions of several adjacent genes that are located on the distal short arm of the X chromosome (Xp). The phenotype results from a combination of two or more monogenic disorders and relates clinical findings to corresponding genotypes. The combination of XRI, CDPX1, and Kallmann syndrome has been described as a contiguous gene deletion syndrome due to deletion of the terminal part of Xp.**
- **The proteinase disorders are characterized by defective proteinases or their inhibitors. Netherton disease consists of hair shaft abnormalities and ichthyotic skin changes and is caused by mutations in the serine proteinase inhibitor LEKTI. In Papillon-Lefèvre syndrome, palmoplantar keratoderma is often combined with severe periodontitis. Inactivating mutations of cathepsin C, a lysosomal proteinase, have been demonstrated, which suggests that regulation of proteolysis is critical in epithelial formation and differentiation.**
- **The amino acid metabolism disorder is the Richner-Hanhart syndrome. This is caused by a deficiency of hepatic tyrosine aminotransferase, leading to hypertyrosinemia. It is the only hereditary palmoplantar keratoderma that can be treated efficiently by a dietary regimen through restricting the dietary intake of tyrosine and phenylalanine.**

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- **The disorder of nucleotide excision repair is trichothiodystrophy. This is caused by mutations in the genes *ERCC2* and *ERCC3*, that code for a transcription/repair factor, that normally corrects nucleotide defects at an early stage in DNA excision repair. The clinical presentation consists of generalized ichthyosis, characteristic hair abnormalities, short stature, and mental retardation.**
- **Defects in intercellular communication are caused by connexin mutations or defective calcium-ATPases. In Vohwinkel syndrome, connexin 26 is defective and causes congenital deafness and characteristic skin changes. Connexin 31 mutations are causative in erythrokeratoderma variabilis. Darier-White and Hailey-Hailey diseases are caused by a dysfunction of calcium pumps, which seem to play a role in the differentiation pathway.**

INTRODUCTION

The ichthyoses comprise a large group of disorders characterized by scaling and hyperkeratosis, often associated with other defects and abnormalities. "Ichthyosis" derives from the Greek term *ichthys*, fish, which is descriptive of the scale-like appearance of the skin. Historically, the ichthyoses were divided into subgroups according to distinct clinical features and several classifications have been proposed.

In 1808, Willan first classified the disorders of keratinization and used the term "ichthyosis" for this group of diseases [1]. Brocq separated bullous from nonbullous congenital ichthyoses (congenital bullous erythroderma) and Cockayne and later Siemens introduced the Mendelian thinking of medical genetics into the field. More than 30 years ago, Frost and Van Scott added biological approaches and divided the ichthyoses into two subgroups, the retention and hyperproliferative hyperkeratoses [2], while Schnyder and his collaborators promoted ultrastructural research by electron microscopy [3]. Another major step was the clinical and histopathological differentiation of noncongenital vulgar ichthyoses, that is, the autosomal dominant and X-linked recessive forms, by Wells and Kerr [4,5]. This was rapidly followed by the first identification of a molecular defect in ichthyoses by defining X-linked recessive ichthyosis as a steroid sulfatase deficiency [6,7]. This discovery focused attention on the lipid compartment of the epidermis for nearly two decades and led to the proposal of the "bricks and mortar" model. This model defines the stratum corneum as a two compartment system consisting of corneocytes and intercellular membranes [8]. In 1987, Williams and Elias classified the disorders of cornification into 24 different subgroups (DOC 1-24) [9] and Traupe subsequently distinguished the common ichthyoses of the vulgar type from more severe congenital forms with or without associated multisystemic disease [10]. This practical classification has been adopted to a large extent in recent textbooks [11]. Today, a number of underlying genetic defects and biochemical deficiencies have been identified, necessitating a new and revised classification. In an attempt to classify the disorders of keratinization according to the underlying genetic defect or metabolic deficiency, we first have to understand the mechanisms that regulate keratinization in normal skin.

Keratinocytes represent the most abundant cell type of the epidermis, and keratins are their major gene products. Keratins are members of a multigene family of cytoskeletal proteins, called intermediate filaments (10 nm filaments)(see also Chapter 6, this volume). More than 50 intermediate filament proteins have been identified, of which the keratins constitute the largest group. According to their biochemical properties, keratins are divided into two types, the acidic type I keratins (K9–K20) and the neutral-basic type II keratins (K1–K8) [12]. One of each type is required for the formation of specific pairs of keratin filaments that form a network in the cytoplasm of epithelial cells [13]. The formation of intermediate filaments is a dynamic process, whereby newly synthesized proteins are integrated into the existing network. Filament assembly takes place in several stages and begins with the heterodimerization of one type I (K9–K20) and one type II (K1–K8) keratin in a coiled-coil fashion. Head-to-tail overlaps between heterodimers yield higher order polymers that eventually make up the filament network of the keratinocyte [14]. Keratins consist of a central, α-helical rod domain of approximately 310 amino acids that is divided into four regions, 1A, 1B, 2A, 2B, separated by three linker regions, L1, L12, L2. The rod domain is composed of a seven residue repeating amino acid sequence $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$ termed a "heptad repeat," in which positions "a" and "d" represent hydrophobic residues that are considered crucial for stabilization of the heterodimer [15]. The beginning of the 1A region and the end of the 2B region, also known as helix initiation and termination motifs, are highly conserved among keratins and other intermediate filaments. These regions are critical for the lateral alignment of heterodimers in which the "head" domain of one heterodimer overlaps with the "tail" domain of a second. The expression of keratins is specific for both the tissue and the stage of differentiation. In the normal epidermis, basal cells express keratins K5 and K14; on migration to the suprabasal layers, the expression of the differentiation-specific keratins, K1 and K10, is induced, while K5 and K14 are down-regulated [16] (Fig. 1). K9 is expressed suprabasally in the epidermis of palms and soles, and K2e is expressed in the granular layer of the epidermis [17]. The K6 isoforms, predominantly K6a and K6b, together with K16, have an additional expression pattern in hair, nail, and mucosa. K17 is found in basal cells of complex epithelia.

The normal differentiation process, also known as keratinization or cornification, involves the transformation of a germinative cell in the basal layer to the deceased corneocyte in the corneal layer (Fig. 1). It involves the formation of the cornified cell envelope as a protective barrier against the environment and water loss [18,19]. Cornified cell envelope formation starts in keratinocytes of the upper granular layer with the cross-linking of various molecules that form a rigid structure at the inner side of the plasma membrane [20]. During this process, the plasma membrane is replaced by a lipid layer that is bound to the outer surface of the protein envelope [18]. The initial incorporation of involucrin and cystatin A involves binding of these components to the lipid envelope which is followed by the incorporation of small proline-rich proteins (SPRRs) and loricrin. Whereas involucrin, SPRRs, and loricrin serve as a mechanical barrier [21], proteinase inhibitors, such as cystatin A, elafin and plasminogen activator inhibitor 2 function as a biological barrier against proteinases [22]. Involucrin is expressed in the upper spinous cells as one of the major initial components of the cornified cell envelope. It is thought to act as an initial scaffold that consolidates after fusion with the other components of the cornified cell envelope [23]. SPRRs are synthesized in the upper spinous to granular cells and function as molecular bridges to connect two proteins of the cornified cell envelope [24]. In the

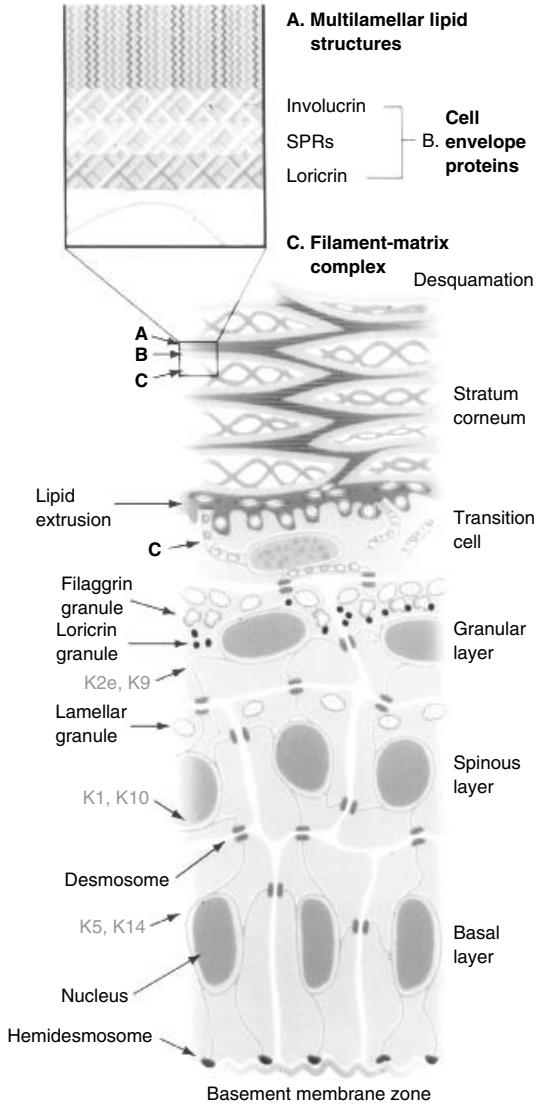

Figure 1. Schematic illustration of the epidermis, showing the normal differentiation process. The epidermis consists of four histologically distinct cellular layers (basal layer, spinous layer, granular layer, and stratum corneum), each with a unique maturation stage of the keratinocyte. The components of the cornified envelope are shown in the inset.

granular layer, keratohyalin granules appear in the cytoplasm and are composed of profilaggrin, which is dephosphorylated and undergoes proteolysis at the zone between the granular and corneal layers, thus yielding filaggrin [25]. Filaggrin is involved in the packing of intermediate filaments into microfibrils through the parallel alignment of already existing intermediate filaments and, to a lesser extent, contributes to the formation of the cornified envelope [26]. S100 family proteins are positioned beneath the plasma membrane in a calcium-dependent manner by annexins, and are covalently linked to the cornified cell envelope by transglutaminase 1 (TGase1) [24]. Loricrin is another protein that accumulates in keratohyalin granules of the superficial granular cells [27]. Loricrin is an insoluble basic protein due to its high content of glycine and other basic amino acids, and represents the major component of the cornified cell envelope [28]. Involucrin, cystatin A, filaggrin, S100 proteins, loricrin and SPRRs are cross-linked by TGase1, one of the four transglutaminases in the epidermis [29]. TGase1 activity is mostly found bound to plasma membranes, whereas TGases 2 and 3 are located in the cytosol. Only TGase1, which is found in the upper spinous and granular layers [30], and TGase3, which is expressed in the granular layer [31], play a role in the formation of the cornified envelope. Moreover, evidence exists that membrane-bound TGase1 is also involved in ceramide lipid attachment to involucrin during protein envelope assembly [32]. Finally, paralleling the formation of the protein envelope, lamellar bodies discharge their contents into the interface between the granular and corneal layers, thereby forming the lipid lamellae. Lamellar bodies are located in the granular layer of the epidermis and contain fatty acids, sphingolipids, cholesterol, and catabolic enzymes such as lipases and proteases [33]. Hydrolases are thought to play an important role in the process of desquamation by dissolving intercellular bridges [34].

In the past few years, the identification of various mutations in components of the keratinization process has revealed the molecular basis of many of the disorders of cornification and provided new insights into the mechanisms that regulate keratinization *in vivo*. Our knowledge today allows a new classification of the disorders of cornification according to their underlying genetic defects (Table 1). However, this classification excludes a group of disorders for which the pathological mechanisms are still unknown. Until the molecular basis of these disorders is identified, we term this group "disorders of keratinization of unknown molecular defect"; the elucidation of the underlying defects behind these disorders will eventually allow us to classify all disorders of keratinization according to their molecular basis.

KERATIN DISORDERS

Bullous Congenital Ichthyosiform Erythroderma (BCIE) (MIM 113800)

Bullous congenital ichthyosiform erythroderma (BCIE) is also known as epidermolytic hyperkeratosis (EHK). The disease was first described by Brocq in 1902 [35]. Subsequently, Lapière termed the disease "granular degeneration," thereby describing his histological findings [36]. The term EHK, which was introduced by Frost and Scott about 10 years later, is often used as a synonym for BCIE and stands for the histopathological picture that is also observed in other keratin disorders such as the two types of pachyonychia congenita, white sponge nevus and Vörners palmoplantar keratoderma [2]. BCIE is an autosomal dominant trait. It has an incidence of 1 in 200,000 to 300,000 newborns, with up to 50% of cases being sporadic. Epidermal nevus of the epidermolytic hyperkeratotic type is a mosaic form of BCIE that is due to postzygotic, spontaneous mutations during embryogenesis. Affected skin alternates with normal skin, and the distribution of the patchy or linear skin lesions is often along the lines of Blaschko. Vertical transmission of the mutation is possible if the germline is involved, causing generalized BCIE in the affected offspring [37].

Clinical and Histological Features

Clinical heterogeneity is high between BCIE families, but rather similar within single families. The onset of the disease phenotype is typically at birth and affected

TABLE 1. Classification of Disorders of Keratinization According to Their Molecular Defects

Disorder	Molecular Defect		Location
	Protein	Gene	
Keratin disorders			
Bullous congenital ichthyosiform erythroderma	K1, K10	*K1, K10*	12q13,17q21–22
Ichthyosis bullosa of Siemens	K2e	*K2e*	12q11–13
Epidermolytic palmoplantar keratoderma	K9	*K9*	17q12–21
Pachyonychia congenita type I	K6a, K16	*K6a, K16*	12q13,17q12–21
Pachyonychia congenita type II	K6b, K17	*K6b, K17*	12q13,17q12–21
Monilethrix	HB1, HB6	*HB1, HB6*	12q13
Disorders of precursor proteins of the cell envelope			
Mutilating keratoderma with ichthyosis	LOR	*LOR*	1q21
Progressive symmetric erythrokeratoderma	LOR	*LOR*	1q21
Autosomal recessive congenital ichthyoses			
Lamellar ichthyosis 1; NCIE 1	TGase1	*TGM1*	14q11.2
Lamellar ichthyosis 2			2q33–35
Lamellar ichthyosis 3; NNCI			19p12–q12; 19p13.2
NCIE 2			3p21
Disorders of abnormal lipid metabolism			
Sjögren-Larsson syndrome	FALDH	*FALDH*	17p11.2
Gaucher disease type II	GCase	*GBA*	1q21
X-linked dominant chondrodysplasia punctata	EBP	*EBP*	Xp11.23–11.22
CHILD syndrome	NSDHL	*NSDHL*	Xq28
Peroxisomal disorders			
Rhizomelic chondrodysplasia punctata type I	PTS2	*PEX7*	6q22-24
Refsum disease	PHYH	*PHYH*	10pter-p11.2
Sulfatase disorders and contiguous gene deletion disorders			
X-linked ichthyosis	STS	*STS*	Xp22.32
X-linked recessive chondrodysplasia punctata	ARSE	*ARSE*	Xp22.3
Multiple sulfatase deficiency	All Sulfatases		Xp22.3
Kallmann syndrome	Anosmin	*KAL1*	Xp22.3
Rud syndrome			X
Proteinase disorders			
Netherton syndrome	LEKTI	*SPINK5*	5q32
Papillon-Lefèvre syndrome	CTSC	*CTSC*	11q14
Amino acid metabolism disorder			
Richner-Hanhart syndrome	TAT	*TAT*	16q22.1-22.3
Disorder of nucleotide excision repair			
Tay syndrome	ERCC2,3	*ERCC2,3*	19q13.2-13.3,2q21
Defects in intercellular communication			
Vohwinkel syndrome	CX 26	*GJB2*	13q11-12
Erythrokeratodermia variabilis	CX 31	*GJB3*	1p35.1
Darier-White disease	SERCA2	*ATP2A2*	12q23-24.1
Hailey-Hailey disease		*ATP2C1*	3q21-24

individuals present with erythroderma, blistering, and peeling (Fig. 2). Erythroderma and blistering diminish during the first year of life and hyperkeratoses develop, predominantly over the flexural areas of the extremities. Maceration of the flexural areas with concomitant bacterial overgrowth often produces a foul odor. A variant of epidermolytic hyperkeratosis with annular and polycyclic erythematous hyperkeratotic plaques on the trunk and proximal extremities has been described [38]. The distinct clinical features with the histopathological finding of epidermolytic hyperkeratosis has led to the term annular epidermolytic ichthyosis (AEI). The same molecular defect that underlies EHK has been identified in two families with AEI [39,40] suggesting that AEI is not a distinct disorder but rather a variant of BCIE. EHK is descriptive of the histopathological findings consisting of perinuclear vacuolar degeneration, lysis of the suprabasal keratinocytes, and a thickened stratum corneum (Fig. 3). Electron microscopy shows clumping of keratin filaments in the suprabasal layers of the epidermis [3,41]: these clumps have been shown to contain keratins K1 and K10 [42].

Molecular Defect

BCIE has been mapped to two keratin gene clusters, the type II keratin gene cluster on chromosome 12q, which contains keratin K1, and the type I keratin gene cluster on chromosome 17q, which contains keratin K10 [43,44]. Several point mutations in the genes for the suprabasal keratins, K1 and K10, have been identified [45–47]. The majority of the mutations in K10 are located in the same

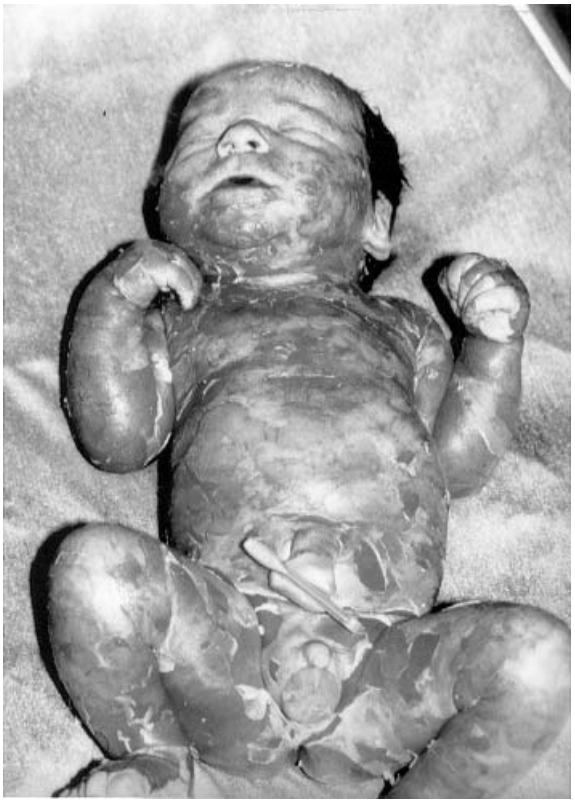

Figure 2. Bullous congenital ichthyosiform erythroderma (BCIE) presenting at birth with generalized erythroderma and blistering. A K1 mutation at the beginning of the rod domain was later identified in this patient. (Courtesy W. Küster, Bad Salzschlirf.)

codon, affecting an evolutionarily highly conserved arginine residue. This site is a "hot spot" due to CpG methylation and deamination [48]. Interestingly, the same arginine residue has been found to be mutated in K14 in the Dowling-Meara form of epidermolysis bullosa simplex (EBS-DM), the most severe form of EBS (see Chapter 15, this volume). An association between the location of the mutation and the severity of the phenotype has been suggested, following the observation that mutations within the initiation and termination motifs lead to more severe phenotypes than mutations located within the rod domain [49]. Clearly, mutations in the highly conserved regions of the rod domain are more disruptive to filament assembly and stability. However, to date a clear genotype-phenotype correlation has not been firmly established for the keratin diseases.

Diagnosis

While a diagnosis can be established clinically in most cases, analysis at the molecular level often facilitates distinction from severe forms of ichthyosis bullosa of Siemens (see below). Prenatal diagnosis by ultrastructural analysis of fetal skin biopsies and amniotic fluid cells has been described [50]. However, both techniques harbor a high number of false-negative results and are thus not considered reliable. Sequence analysis of genomic DNA of chorionic villus samples is a useful tool to identify mutations in the suprabasal keratins, and has been successfully used as early as week 15 [51].

Ichthyosis Bullosa of Siemens (IBS) (MIM 146800)

Ichthyosis bullosa of Siemens is a rare genodermatosis with some clinical features similar to those of epidermolytic hyperkeratosis. Named after Siemens [52], it denotes a milder and more limited form of disease than usually seen with BCIE.

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Figure 3. Light micrograph of a skin biopsy from an 18-month-old patient with bullous congenital ichthyosiform erythroderma (BCIE). The epidermis (E) is broadened (acanthosis) and shows pronounced hyperkeratosis (arrow). Note the cytolysis in the suprabasal layer (arrowhead). D = dermis. (Reprinted with permission from [398].)

Clinical Features

At birth, individuals usually present with blistering, and hyperkeratosis develops shortly thereafter. Erythroderma, however, as seen in most BCIE patients, is absent. The fragility of the epidermis is more superficial with shedding and loss of the uppermost layer, the stratum corneum. This leads to characteristic denuded areas, described as "Mauserung" or a "molting effect" by Siemens in 1937 [52]. These areas are often found on the backs of the hands and feet and on the extremities. Hyperkeratoses are usually found at similar sites of the body as in BCIE, with a lichenified appearance over the flexural areas. The absence of erythroderma and blistering caused by minor mechanical trauma often makes a distinction from BCIE easier [53]. The blistering diminishes with age, and in early adulthood lichenification and hyperkeratoses are predominantly found over the flexural areas of the extremities. The severity of the phenotype differs markedly both between kindreds and among members of the same family.

Histological and Ultrastructural Features

Examination of involved skin shows hyperkeratosis and vacuolization confined to the granular layer. Electron microscopy shows tonofilament clumping in the upper spinous layer of the epidermis, the clumps being localized around the nucleus. The overlying stratum corneum is usually less dyskeratotic than in BCIE and areas in the superficial epidermis may be observed that do not exhibit clumping [54].

Molecular Defect

Initially, IBS was linked to the type II keratin gene cluster on chromosome 12q [55]. Subsequently, "hot spot" mutations affecting the same residue in the helix termination motif of K2e were identified as the underlying genetic defect in several families with IBS [56,57]. Interestingly, several of these IBS families were initially diagnosed clinically as having BCIE. However, when obvious mutations in K1 or K10 could not be found, identification of mutations in K2e suggested that these families had been misdiagnosed [56]. Soon thereafter, several groups identified more K2e mutations in IBS, the majority of which were located at the previously described "hot spot" at the end of the rod domain [58–60]. More recently, mutations have also been identified at the beginning of the rod domain of K2e, but these are not as common as the mutations at its end [61]. The resulting amino acid substitutions have been shown to disrupt filament assembly and stability, similarly to the findings in BCIE. As in other keratins, the ends of the rod domain of K2e are highly conserved and substitutions of these particular residues are deleterious to filament assembly. A genotype-phenotype correlation was suggested in two IBS families in which a nonconservative amino acid substitution was associated with a severe disease phenotype in one family, whereas a conservative substitution at the same site correlated with mild clinical features in the second [62].

Ichthyosis exfoliativa presents with circumscribed areas of blistering and hyperkeratoses, clinical features similar to those in IBS [63]. Until 1994, it was thought that both diseases represented distinct entities, but linkage of both disorders to chromosome 12q indicated the type II keratin genes to be candidate genes for both ichthyosis bullosa of Siemens and ichthyosis exfoliativa [55]. Histologic and ultrastructural examination of skin biopsies indicated that ichthyosis exfoliativa was identical to ichthyosis bullosa of Siemens, and this was confirmed by molecular analysis when mutations in K2e were identified as the underlying molecular defect [58].

Epidermolytic Palmoplantar Keratoderma (EPPK) (MIM 144200)

Vörner described an autosomal dominant keratosis of palms and soles with the characteristic histological finding of epidermolytic hyperkeratosis in 1901 [64]. Years earlier, Thost had examined a large German kindred with palmoplantar keratoderma (PPK) in whom histology did not show epidermolytic hyperkeratosis [65]. Unna reported similar clinical features in another PPK family, although histological findings are not available [66]. Soon thereafter, the Unna-Thost form of PPK was distinguished on the basis of histological findings from the rare Vörner type. More recently, the question arose whether both types are indeed different diseases and the existence of the Unna-Thost type was doubted [67–69]. Re-examination of patients diagnosed with the Unna-Thost type and of descendants of the family originally described by Thost in 1880 consistently revealed the histological finding of epidermolytic hyperkeratosis [67–69]. Moreover, K9 mutations, as discussed below, have been identified in several families with epidermolytic palmoplantar keratoderma, including the family originally described by Thost, indicating that both forms are, in fact, one disease, i.e., epidermolytic palmoplantar keratoderma. Nevertheless, nonepidermolytic diffuse palmoplantar keratoderma of Unna exists and is clinically and histopathologically observed. In one such case, a mutation in the ISIS box of the V1 domain of K1 has been identified [70].

Clinical and Histological Features

Epidermolytic palmoplantar keratoderma is an autosomal dominant disorder in which hyperkeratosis is confined to the palms and soles. The onset of the disease is usually early in life and palms and soles are affected with thick hyperkeratoses. The nature of the hyperkeratoses varies markedly between families and among members of the same family. Additional features frequently seen are knuckle pad-like keratoses over the flexural areas of the finger joints and clubbing of the nails [69] (Fig. 4). The histological finding of epidermolytic hyperkeratosis is reminiscent of the findings in BCIE; however, the abnormalities are restricted to palms and soles in EPPK.

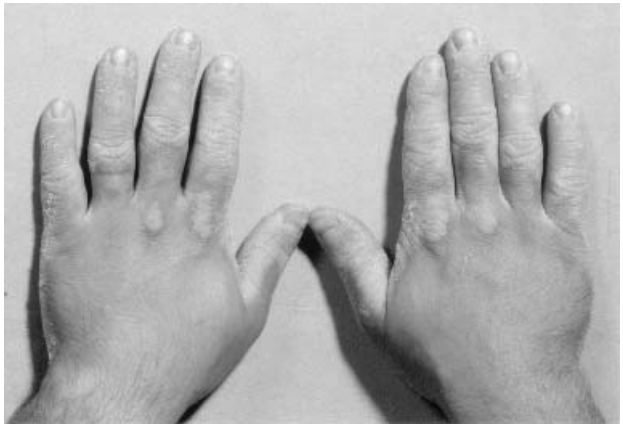

Figure 4. Epidermolytic palmoplantar keratoderma (EPPK) showing hyperkeratosis confined to hands, and pad-like keratoses on the knuckles.

Molecular Defect

The disease has been mapped to the type I keratin gene locus on 17q12-21 [71] and mutations in the suprabasal keratin 9 have been identified in EPPK patients [72–76]. The majority of reported mutations to date lie in the 1A region at the beginning of the central rod domain of K9. Recently, an insertion mutation has been reported in the 2B region of K9, leading to the same EPPK phenotype as previously observed with mutations located in the 1A region [77].

Pachyonychia Congenita (PC); Type I Jadassohn-Lewandowsky Pachyonychia Congenita (MIM 167200); Type II Jackson-Sertoli Pachyonychia Congenita (MIM 167210)

Pachyonychia congenita (PC) is a group of autosomal dominant dysplasias characterized by hypertrophic nail dystrophy accompanied by other features of ectodermal dysplasia. Pachyonychia congenita type I (PC-1) was first described by Jadassohn and Lewandowsky in 1906 [78]. It represents an autosomal dominant ectodermal dysplasia characterized by hypertrophic nail dystrophy, focal nonepidermolytic palmoplantar keratoderma and variable features of oral leukokeratosis and follicular keratosis. In the Jackson-Sertoli form (PC-2, also known as Jackson-Lawler type), pachyonychia and mild focal keratoderma are accompanied by multiple pilosebaceous cysts, natal teeth, and hair abnormalities. It was first reported in 1949 in an Italian family by Sertoli [79] and in six members of a three-generation family by Jackson and Lawler in 1951 [80].

Clinical Features

The clinical features consist of increased thickening and curvature of fingernails and toenails (onychogryphosis), and hyperkeratoses on palms and soles (Fig. 5) and the extensor surfaces of the large joints. In PC-1, leukoplakia of the oral mucosa (Fig. 6) and hyperhidrosis are associated features. When oral candidiasis does not respond to antifungal treatment, oral leukokeratosis associated with PC-1 should be included in the differential diagnosis. In one case, a 2-month-old child presented with oral lesions and was treated for recurrent oral candidiasis. The lesions were unresponsive to antifungals, the development of hypertrophic nail changes was noted, and subsequently the diagnosis of PC-1 was established. At 9 months of age, hyperkeratotic papules, which were not apparent at initial presentation, developed on elbows and knees [81].

The keratoderma in PC-2 is usually milder than that in PC-1 and oral involvement is absent. Multiple epidermoid cysts, pili torti, and teeth abnormalities have been described in PC-2. Moreover, multiple steatocystomas are characteristic and the prominent clinical feature [82]. The presence of epidermoid cysts enables a distinction between the two types. Oligo-symptomatic variants, that is, isolated focal insulated nonepidermolytic keratoderma of the Jadassohn-Lewandowsky type and isolated sebocystoma of the Jackson-Sertoli type, exist, and may be explained by less detrimental mutations in keratins K16 and K17, respectively [83].

Molecular Defect

PC-1 has been associated with mutations in the genes for K6a and K16, located on chromosomes 12 and 17, respectively [84,85]. Most of the mutations reported to date lie in the 1A region of K6a, including deletions and point mutations, suggesting a mutational hot spot [81,86]. Examination of a large kindred affected by PC-2 established linkage of the disease to the type I keratin gene cluster on chromosome 17q and subsequently point mutations in the genes for K6b and K17 were identified [82,85,87].

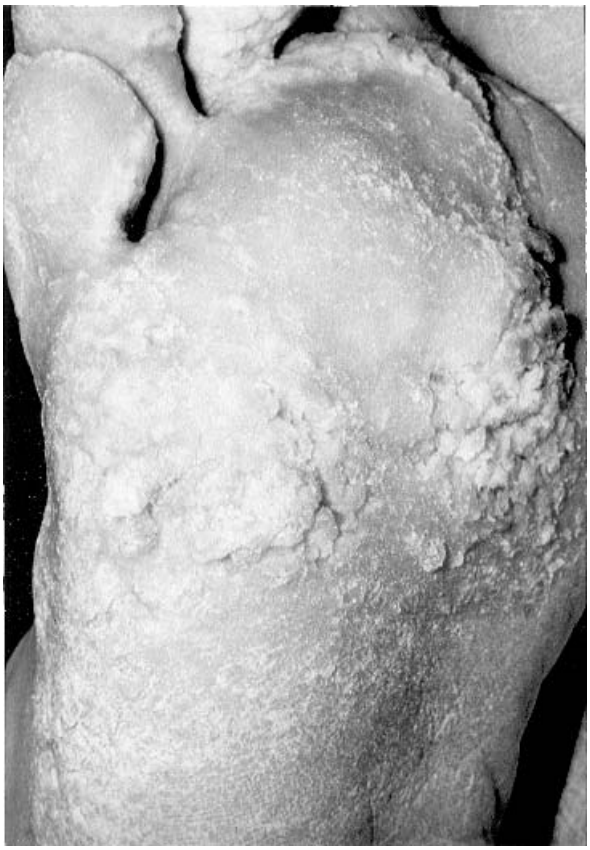

Figure 5. Plantar keratoderma in pachyonychia congenita type 1. (Reprinted from [81], with permission of the publisher.)

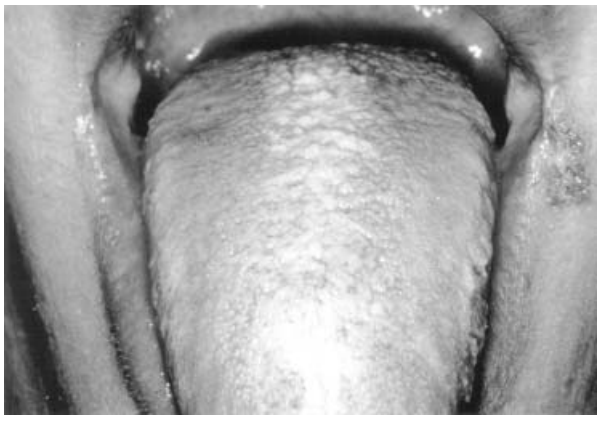

Figure 6. Leukokeratosis of the tongue and oral mucosa in pachyonychia congenita type 1. (Reprinted from [81], with permission of the publisher.)

Monilethrix (MIM 158000)

Monilethrix is a rare hair dystrophy characterized by a variable degree of alopecia due to defects in the structural

proteins of hair and nail, the "hard" keratins. It follows an autosomal dominant inheritance and has been described several times since its first report in 1910 [88].

Clinical and Histological Features

The clinical presentation may be variable, from dystrophic hair confined to a small area to almost total alopecia. Perifollicular hyperkeratosis is a consistent feature and nail defects may be present. Histologically, the hair is beaded as a result of a periodic narrowing of the shaft [89]. Ultrastructural examination of affected hair shows structural defects in the cortex and clumps of the structural proteins of the hair shaft, the hair keratins.

Molecular Defect

Monilethrix maps to the type II keratin gene cluster on chromosome 12q13 [90,91] and has been shown to be caused by mutations in human hair keratins [92]. To date, at least 11 human hair keratins have been identified, of which hair keratins hHB1, 3, and 6 are expressed in the cortex, whereas hHB5 is found primarily in the hair matrix. Heterozygous point mutations in the genes for the hair keratins hHB1 and hHB6 have been identified [92] and one highly conserved glutamic acid residue in the 2B domain of the human hair basic keratin 6 (hHb6) seems to be a mutational hot spot [93].

Treatment of Keratin Diseases

The current treatment of hyperkeratotic skin diseases is symptomatic. Hydration of the skin can be achieved by moistening and lubrication with creams, lotions, oils and ointments. To remove scales, keratolytic and hydrating agents containing urea, ammonium lactate, propylene glycol, and α-hydroxy acids are effective, as well as topical retinoids (e.g., tretinoin) [94]. Topical salicylic acid is nowadays mostly avoided due to its toxicity, particularly when treating large body surface areas in children. It is also increasingly recognized that 12% ammonium lactate in the classical formulation is often not well tolerated due to burning sensations. Thus, it is either used at lower concentrations or combined with other agents. Hyperkeratoses in mild forms of BCIE and IBS respond well to topical treatment with keratolytics and lubricants, which are also effective in minimizing blistering and erosions. In generalized forms of BCIE, and in severe cases of IBS with major skin involvement, systemic retinoids may be considered; however, long-term side effects should be closely monitored. In BCIE patients, good results have been observed with acitretine or, earlier, etretinate, at low doses between 0.2 and 0.5 mg/kg. High doses, however, can cause exacerbation with increased blister formation [95]. Similarly, PC patients benefit from retinoids, and prompt improvement under systemic retinoid therapy has been reported [96,97].

In EPPK, however, retinoid therapy is controversial. Reduction of the palmar and plantar hyperkeratoses has been achieved, but patients do not usually benefit from this treatment because of the development of painful erosions that may impair the normal functioning of hands and feet [95]. Better results have been achieved by removing the hyperkeratoses mechanically. Recently, topical administration of calcipotriol has been proposed [98]. Management of bacterial infections, which frequently occur in BCIE patients, often requires topical and systemic treatment with antibiotics. A decreased frequency of bacterial infections has been observed in BCIE patients under systemic retinoid therapy [94].

DISORDERS OF PRECURSOR PROTEINS OF THE CORNIFIED CELL ENVELOPE

Mutilating Keratoderma with Ichthyosis (MIM 604117)

Mutilating honeycomb keratoderma with associated ichthyosis, also referred to as keratoderma of Camisa, loricrin keratoderma, and variant form of Vohwinkel syndrome, was first described by Wirz in 1930 and Grschebin in 1932 [99,100]. Since then, several cases of palmoplantar keratoderma associated with ichthyosis and constrictions of fingers and toes, leading to auto-amputation have been reported [101–103].

Clinical and Histological Features

The hyperkeratoses and constricting bands on the fingers or toes usually develop during childhood (Fig. 7). The association of three characteristic features has been described by Camisa: diffuse palmoplantar keratoderma with honeycomb appearance, bands constricting the digits (pseudoainhum), and mild ichthyosis [103]. Histologically, the epidermis shows thickening of the granular layer and hyperkeratosis with retention of rounded nuclei in the stratum corneum [104] (Fig. 8).

Molecular Defect

Mutilating keratoderma with ichthyosis is inherited in an autosomal dominant manner. Linkage of this syndrome to markers in proximity to the epidermal differentiation complex on chromosome 1q21 has been demonstrated in a large kindred [105]. Interestingly, the same kindred had been reported previously as three separate families, but it is now possible to connect this large kindred to a common ancestor in the United Kingdom in the mid-1700s. Two different insertion mutations in the loricrin gene (231insG; 209insT) have been identified in unrelated families [102,104,105]. The insertion of an extra guanine or thymidine after codon 231 and at codon 209 produces a mutant protein with a frameshift of its terminal 84 and 107 amino acids, respectively, that is 22 amino acids longer than the wild-type protein. The insertion mutation is considered to be responsible for the disease and is thought to cause accumulation of defective loricrin in the nucleus. The altered protein structure impairs cross-linking by transglutaminases and thus the mutant loricrin is not properly incorporated

Figure 7. Mutilating keratoderma with constrictions of the fingers (pseudoainhum). (Reprinted from [105], with permission of the publisher.)

Figure 8. Histological picture in mutilating keratoderma with retention of nuclei (arrowhead) in the thickened stratum corneum (SC). (Reprinted from [105], with permission of the publisher.)

into the cornified cell envelope. That cornified cell envelope formation is impaired is indicated by the observation that the cornified envelope in mutilating keratoderma with ichthyosis is much thinner than normal [102,103]. It is now clear that loricrin mutations in keratoderma of Camisa are unique to the variant with ichthyosis: in a family with mutilating keratoderma, molecular analysis revealed one of the previously reported insertion mutations in the loricrin gene (231insG), whereas in a second family with sensorineural deafness and localized starfish keratoses rather than generalized ichthyosis (Vohwinkel syndrome), linkage to loricrin was excluded [102]. Therefore, mutilating keratoderma of Camisa with ichthyosis due to loricrin mutations should be delineated from the classic Vohwinkel syndrome, which is now known to be caused by connexin mutations (see below).

Progressive Symmetric Erythrokeratoderma (PSEK) (MIM 602036)

Progressive symmetric erythrokeratoderma was first described by Darier in 1911 [106]. Only about 30 cases have been described since then in the literature [107].

Clinical and Histological Features

At birth, well-demarcated, symmetric, erythematous and hyperkeratotic plaques predominate over extremities, buttocks, and face. These plaques usually remain stable in location and appearance but may progress slightly during childhood. Spontaneous regression of the plaques is also possible [108]. PSEK is usually a localized disorder of keratinization; however, intermittent generalization is frequently reported. The histological findings are similar to those in Camisa keratoderma with hyperkeratosis and parakeratosis.

Molecular Defect

The disease is inherited in an autosomal dominant manner with incomplete penetrance and variable expressivity. A frame-shift insertion mutation of an additional cytosine at nucleotide position 709 in the loricrin gene, only 21 bp upstream of the mutation discovered in Camisa keratoderma, has been found in one family affected by PSEK [107]. The mutation at codon 224 results in a frameshift that changes the terminal 91 amino acids to missense and adds 22 residues to the mutant protein. Clinically, overlaps between PSEK and Camisa keratoderma have been described; moreover, it has been suggested that they are different manifestations of a single condition [109]. The identification of loricrin mutations in both disorders suggests one disease entity; however, it remains to be shown if the site of mutation will correlate consistently with the respective disease phenotype. Interestingly, variants of PSEK with deafness have also been described in the literature [110] that are reminiscent of the true Vohwinkel keratoderma due to connexin 26 mutations (see below).

Treatment of Disorders of Precursor Proteins of the Cornified Cell Envelope

In mutilating keratoderma with ichthyosis, improvement of the condition has been reported with etretinate in a 28-year-old woman and in four members of an affected three-generation family [111]. A similar effect has been reported in an 11-year-old boy [112]. In all cases, the hyperkeratoses diminished and the constricting bands tended to disappear. Likewise, good results have been achieved with etretinate treatment in PSEK patients, and it has been suggested that it should be preferred over isotretinoin [95]. However, hyperkeratoses usually respond better to etretinate treatment than the erythematous component.

ICHTHYOSIS OF THE NEWBORN

Collodion Baby

The collodion presentation at birth can develop into a wide spectrum of ichthyosis phenotypes such as the autosomal recessive congenital ichthyoses, X-linked dominant chondrodysplasia punctata, and Netherton syndrome. Frenk distinguished the self-healing, autosomal recessive collodion baby from the type which develops into lamellar ichthyosis at the ultrastructural level [113]. Larrègue studied 69 collodion babies and found that the majority of cases developed into nonbullous congenital ichthyosiform erythroderma and lamellar ichthyosis [114] (Fig. 9).

Clinical Features

At birth, the newborn is encased in a tight membrane that breaks up during the first weeks after birth, leaving deep fissures and erythroderma. The birth is often premature and impairment of respiration due to the tight membrane, as well as inadequate nutritional and fluid intake, might complicate the infant's condition. Therefore, monitoring of temperature, hydration, and electrolytes is essential.

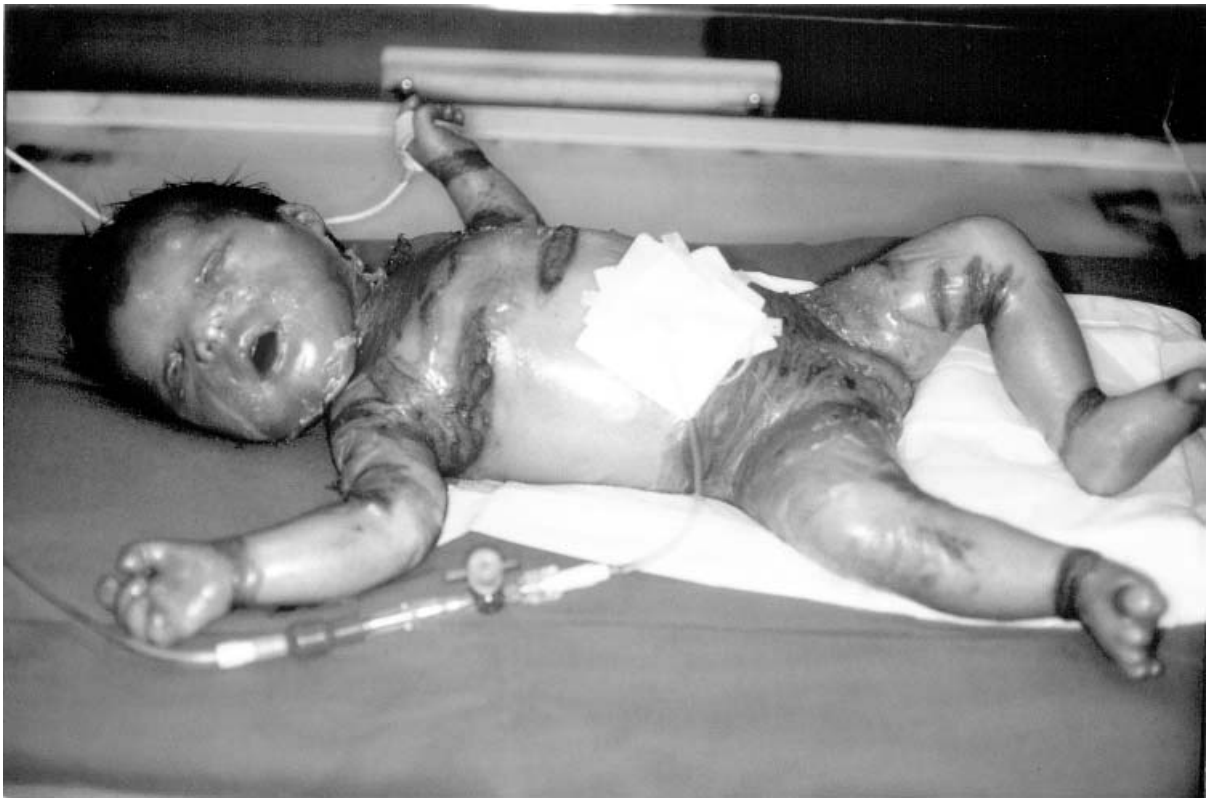

Figure 9. Collodion baby due to nonbullous congenital ichthyosiform erythroderma.

Molecular Defect

The pathogenesis underlying most disorders leading to collodion presentation at birth has been identified, such as transglutaminase deficiency in lamellar ichthyosis, *EBP* mutations in X-linked dominant chondrodysplasia punctata and LEKT1 mutations in Netherton syndrome (see below). A straightforward test to distinguish transglutaminase 1-deficient lamellar ichthyosis from other forms of underlying ichthyosis is an *in situ* transglutaminase assay to determine TGase activity [115] (see below).

Treatment

Collodion babies need to be transferred to a neonatal intensive care unit due to the high risk of death (1976: 33%, 1984: 11%) [114]. Systemic hydration is crucial and potential skin infections need to be recognized early by repeated microbiological swabs. Nevertheless, preventive antibiotic treatment is not recommended, to avoid the development of resistant, uncontrollable strains. Topical treatment should be inert (e.g., cold cream, petrolatum). The desquamation of the collodion membrane takes about 10 days: during that time the newborn should be placed in a humidified incubator and treatment of the skin with wet compresses and lubricants may further hydrate the membrane.

Harlequin Ichthyosis (MIM 242500)

Harlequin ichthyosis is a very severe, usually fatal condition. As a result of improvements in therapy and survival rates, the term harlequin ichthyosis may replace the term harlequin fetus [116]. Less than 100 cases have been described in the literature. The condition is inherited in an autosomal recessive fashion, but dominant mutations may play a role in some sporadic cases [117].

Clinical and Histological Features

Most infants with harlequin ichthyosis are born prematurely. At birth, thick plates of stratum corneum cover the whole body in a diamond-like configuration, reminiscent of the suit of a harlequin clown (Fig. 10). The horny shell restricts vital functions such as respiration and swallowing, and constrictions of limbs and digits may lead to deformations. The nose and ears are deformed and often even completely absent. Water loss and infections are serious complications. The skin of harlequin babies surviving the neonatal period resembles that seen in severe forms of nonbullous congenital ichthyosiform erythroderma (NCIE). Histologically, the stratum corneum is enlarged in most cases [118]. The size of the granular layer is variable and the thickness of the epidermis appears small compared to the broadened stratum corneum. Studies suggest that lamellar bodies, which normally form the lipid lamellae of the stratum corneum, are present in harlequin ichthyosis, but are defective and thus contribute neither to the formation of the lipid lamellae nor to desquamation [119].

Molecular Defect

The underlying genetic defect is unknown. An alteration in the expression pattern of profilaggrin/filaggrin and keratins K6/K16 has been proposed following the observation that filaggrin is absent [117]. Decreased activity of a serine/threonine protein phosphatase has been shown in keratinocytes of affected individuals, suggesting

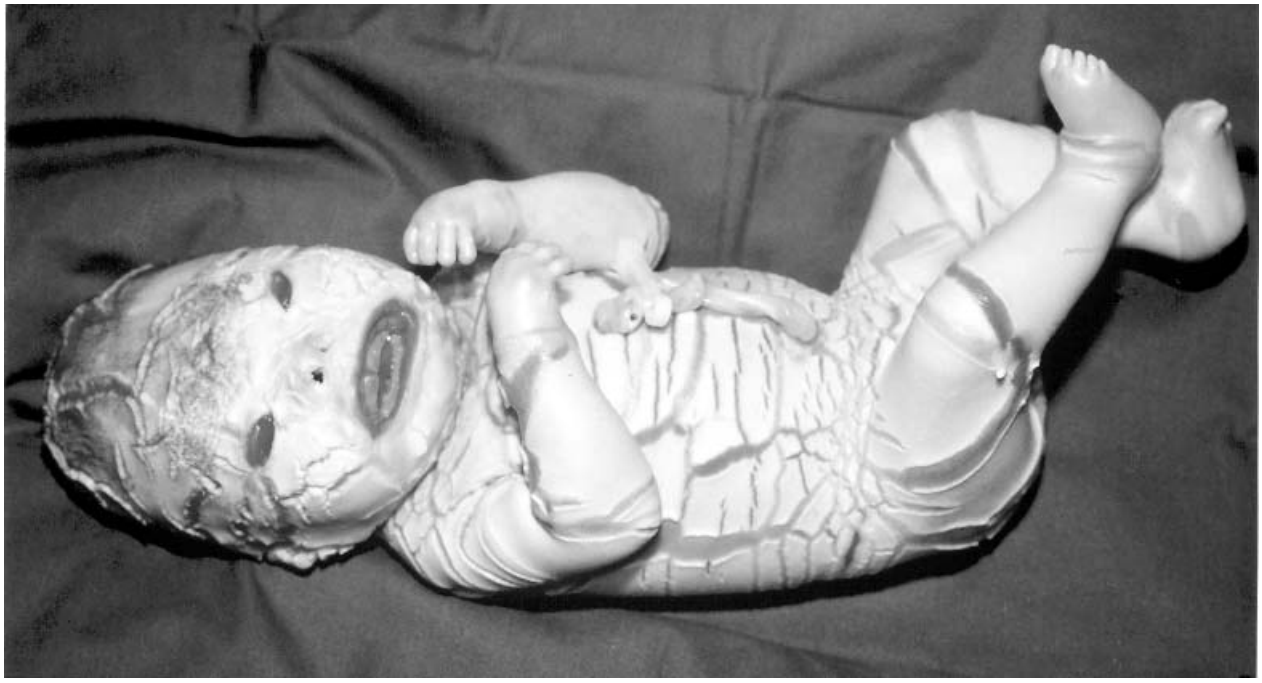

Figure 10. Harlequin fetus that died at birth.

impaired conversion of phosphorylated profilaggrin to the non-phosphorylated form [120]. Calcium-activated neutral proteases are believed to participate in signal transduction in the epidermis; diminished levels in harlequin skin implicate calcium-mediated signaling events in the alteration of differentiation that occurs in harlequin ichthyosis [121]. The corresponding phenotype to harlequin ichthyosis arose spontaneously in a colony of BALB/CJ mice. The mutant gene was mapped to the proximal end of mouse chromosome 19. The disease showed an autosomal recessive mode of inheritance with full penetrance [122].

Management

Management of harlequin ichthyosis remains symptomatic. To date, no generally accepted therapeutic regimen has been established. Systemic retinoid therapy has been shown to improve survival rates [123] and topical administration of paraffin ointment leads to skin improvement allowing movement of the limbs and normal nourishment [124].

AUTOSOMAL RECESSIVE CONGENITAL ICHTHYOSES

Lamellar Ichthyosis Type 1 (LI 1) (MIM 242300), Type 2 (LI 2) (MIM 601277), and Type 3 (LI 3) (MIM 604777); Nonbullous Congenital Ichthyosiform Erythroderma 1 (NCIE 1) (MIM 242100) and 2 (NCIE 2) (MIM 604780), Nonlamellar Nonerythrodermic Congenital Ichthyosis (NNCI) (MIM 604781)

The autosomal recessive congenital ichthyoses (ARCI) are a clinically heterogeneous group that have been distinguished in the past on the basis of clinical presentation and/or ultrastructural findings [125]. Three types of lamellar ichthyosis (LI 1–3), two forms of nonbullous congenital ichthyosiform erythroderma (NCIE 1 and 2), and a nonlamellar, nonerythrodermic ichthyotic type (NNCI) can be distinguished on the basis of chromosomal location. The inheritance pattern of each is recessive, although some cases of dominant inheritance have been described [126]. As more patients are being characterized at the molecular level, it might soon be possible to classify this heterogeneous group still further.

Clinical and Histological Features

The subtypes all have distinct clinical features (Table 2) that range from generalized large brown scales to fine white scales with associated erythroderma [10]. About 60% of collodion babies evolve to the NCIE phenotype or the LI phenotype, the former being slightly more frequent [114].

LI 1 accounts for about 50% of all ARCI. In the case of presentation as a collodion baby, the newborn is encased in a collodion membrane. Mortality in such cases in the first months of life is high as the infant may die of complications, such as sepsis, or protein and electrolyte loss. Other patients, however, do not show collodion presentation at birth. Erythroderma is often present and large, brown, plate-like scales develop, covering the whole body [125] or only the trunk with spared extremities in others [127]. These two distinct phenotypes are most likely due to less detrimental mutations, with slightly retained transglutaminase activity in the "trunk only" phenotype. Involvement of palms and soles ranges from moderate hyperkeratosis to extensive palmar and plantar keratoderma with large fissures. The facial skin may also be affected and ectropion and eclabium are often associated features. If left untreated, severe ectropion can lead to blindness. Additionally, many patients exhibit scarring alopecia. Hypohidrosis is often present and patients should be made aware of the potential risk of hyperpyrexia, especially in hot temperatures and during physical exercise [128]. Prenatal diagnosis has been performed at 13 weeks of gestation by

TABLE 2. Autosomal Recessive Congenital Ichthyoses. Classification of Subtypes According to Distinct Clinical Features and Chromosomal Location

	LI 1	LI 2	LI 3	NCIE 1	NCIE 2	NNCI
Scales	Medium to large, brown	Very large, brown	Large, brownish, adherent	Medium to large, brown	Fine, white, semiadherent	Fine, white, mild
Erythema	Variable	None	Variable	Variable	Intense, widespread	None
Nails	Normal	Normal	Normal	Normal	Normal	?
Hair	+/− Scarring alopecia	Normal	Normal	+/− Scarring alopecia	Diffuse alopecia	?
Linkage	14q11 (*TGM1*)	2q33–35	19p12–q12	14q11	3p21	19p13

identification of TGase1 mutations by molecular analysis of chorionic villus samples in LI 1 [129] and by histological examination of fetal skin biopsies and amniotic fluid cells in the second trimester [130,131].

LI 2 is characterized by collodion presentation at birth that usually evolves to a generalized ichthyosis with involvement of the large folds. The scales are brownish in color and larger in size than those seen in LI 1 and erythroderma is absent. Ectropion may also be present and ranges from mild to moderate [132,133].

LI 3 patients usually present at birth as collodion babies. Large brownish adherent scales and palmoplantar keratoderma are typical features; erythroderma, ectropion, and eclabium may or may not be present. Hair, nails, and teeth are not affected [134].

NCIE is an inflammatory form of ichthyosis characterized by thin, white scales and prominent erythroderma (Fig. 11). In NCIE 1, presentation at birth ranges from ichthyotic skin, through erythema to collodion skin. Later, thin white scales cover the entire body. The flexures and palms and soles are involved in the majority of patients, ectropion is usually present, and alopecia may or may not be an associated feature.

NCIE 2 has collodion presentation at birth and widespread erythema with fine, superficial, semiadherent white scales that cover the entire body are characteristic features. In addition to erythema, palmoplantar hyperkeratosis, diffuse alopecia, ectropion, and eclabium are also seen in most patients [134].

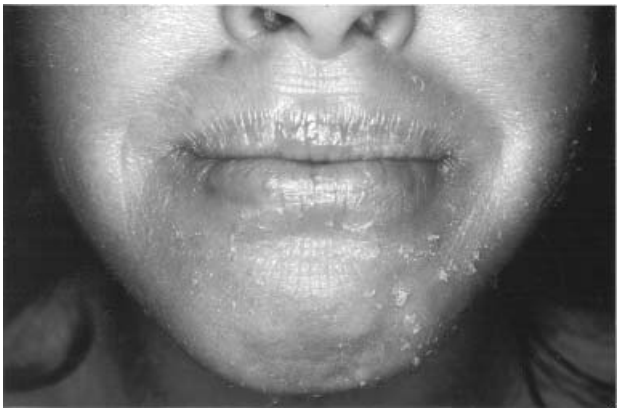

Figure 11. Nonbullous congenital ichthyosiform erythroderma with severe erythema and fine whitish nonadherent scales.

NNCI is a nonlamellar, nonerythrodermic type of ichthyosis that resembles neither lamellar ichthyosis nor erythrodermic congenital ichthyosiform erythroderma. At birth, the affected infant presents with ichthyotic skin or as a collodion baby, while later the skin develops fine, white scales, that predominate over flexural folds, ankles, and ears. The clinical picture at birth can be severe, but later develops into a remarkably mild scaling, distinct from that observed in NCIE. Palms and soles exhibit hyperlinear, hyperkeratotic skin. Erythroderma and ectropion are usually absent [135].

Histopathology in ARCI shows variable acanthosis, hypergranulosis, and hyperkeratosis. Keratinocytes of the stratum corneum have been shown to contain lipid droplets [136,137], the significance of which remains to be elucidated. Keratinocytes in culture bearing mutations in TGase 1 are unable to form cross-linked envelopes, which could serve as a tool to distinguish LI 1 and NCIE 1 from other ichthyotic diseases by a simple test [138]. Determination of TGase activity in combination with immunohistochemistry for TGase protein in frozen skin sections allows a rapid distinction between the different forms of autosomal recessive congenital ichthyosis [115].

Molecular Defect

Initially, linkage studies implicated the keratinocyte-specific transglutaminase 1 gene locus (*TGM1*) on chromosome 14q11 in LI [139] and subsequently mutations have been identified in the *TGM1* gene in patients with LI 1 and NCIE 1 [140–143]. The transglutaminase 1 (TGase1) enzyme is critical for the assembly of the cornified cell envelope in terminally differentiating keratinocytes. It is a complex enzyme, existing as both cytosolic and membrane-bound forms, and it is the membrane-bound isoform that is the major functionally active form [144]. The membrane-bound isoform catalyzes Ca^{2+}-dependent cross-linking of proteins through the transfer of the γ-carboxyl group from protein-bound glutamine to the ε-amino group of protein-bound lysyl residues. During formation of the stratum corneum, TGase1 catalyzes the cross-linking of involucrin, loricrin, SPRRs, and other minor components, to form a protein complex that builds the cornified envelope on the inner side of the plasma membrane [145].

LI patients with normal transglutaminase 1 activity have been described suggesting the involvement of other gene loci [140,146]. Indeed, a second locus for LI has

been mapped to chromosome 2q33–35 [132] and the corresponding type of lamellar ichthyosis has been designated LI 2. Three further loci have recently been identified for ARCI. A region on chromosome 19p12–q12 is linked to lamellar ichthyosis type 3 (LI 3) [134] and a region on chromosome 19p13 has been assigned to a nonlamellar, nonerythrodermic type of ichthyosis (NNCI) in a Finnish family [135]. The fifth locus that has been linked to ARCI is a region on chromosome 3p21. This locus is associated with NCIE 2, the second form of congenital nonbullous ichthyosiform erythroderma [134]. A mouse model lacking the *Tgm1* gene has been established and mutant mice have erythrodermic skin with abnormal keratinization. Skin barrier function is impaired and these mice die a few days after birth [147].

Treatment of Autosomal Recessive Congenital Ichthyoses

Systemic treatment with retinoids (acitretin and isotretinoin) has proven particularly effective in LI and NCIE. Patients should be closely monitored because continued benefit usually requires long-term treatment [94]. Regular topical treatment is very important and needs to be practiced by all means, especially in the lamellar variants. Rehydrating topical treatments should be applied at least twice daily. A recent cream formulation using Locobase^{TM} with 5% ammonium lactate and 20% propylene glycol has proven to be particularly effective [148], also, in our own experience (D. Hohl, unpublished observation). Some patients prefer cold cream with 5% ammonium lactate in a more hydrophilic cream formulation, especially for the scalp, where it is desirable that topical preparations be easily removable. Other patients, however, prefer ointments containing 12% urea, 1% dexpanthenol, and 0.03% tretinoin and many diverse commercial and magistral formulations are in use by different specialists and patient groups. In addition, scales should regularly be removed by bathing with the addition of oils (e.g., paraffinum liquidum), marine salt, and antiseptics against malodorous superinfections.

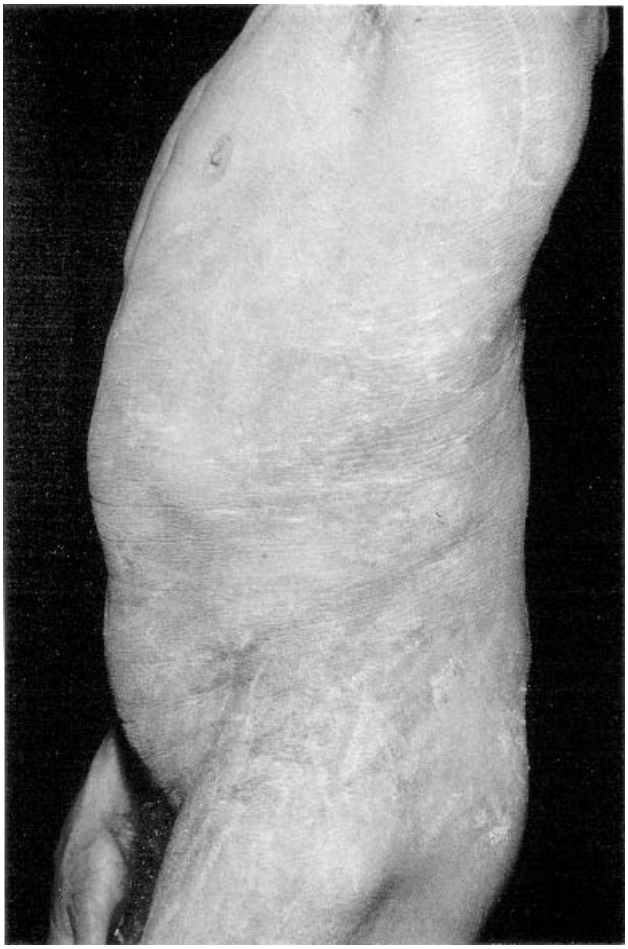

Figure 12. Sjögren-Larsson syndrome; lichenified keratotic lesions typical of adult patients.

DISORDERS OF ABNORMAL LIPID METABOLISM

Sjögren-Larsson Syndrome (SLS) (MIM 270200)

This autosomal-recessive syndrome was first described in northern Sweden where several large kindreds presented with ichthyosis that occurred in association with spastic paralysis and mental retardation [149]. SLS is distributed worldwide; however, half of all reported cases originate from northern Sweden, suggesting a founder mutation [150].

Clinical and Histological Features

The ichthyosis presents at birth with congenital erythroderma with fine to large scales and a varying degree of hyperkeratosis, predominantly over the flexural areas of the extremities, neck, and abdomen. The characteristic features develop during the first months of life and consist of yellowish to dark brown hyperkeratoses, the palms, soles, and face being spared. Hair and nails are clinically unaffected. Pruritus is a characteristic feature in SLS and continuous scratching leads to severe lichenification that is typical of the clinical picture in older patients [151] (Fig. 12). Less severe pruritus may also be observed in other cutaneous lipid disorders, but usually not in ichthyoses due to defects in keratins or transglutaminases. During the second year of life, a spastic di- or tetraplegia develops, seizures occur frequently, and mental retardation is always associated with the disease. Eye involvement is frequently observed and consists of glistening dots in the fundus due to focal microglial degeneration. Histologically, the skin in SLS cannot be distinguished from that in LI. Both diseases show orthokeratotic hyperkeratosis with parakeratosis. Ultrastructurally, lipid vacuoles in the horny layer are rarely seen and the tonofibrils have a stiffer appearance than normal [152].

Molecular Defect

The disease has been linked to chromosome 17p11.2 in Swedish pedigrees and this linkage has been confirmed in non-Swedish pedigrees [153,154]. A deficiency of fatty aldehyde dehydrogenase (FALDH) has been associated with the syndrome and mutations in the *FALDH* gene have been identified [155]. FALDH is a component of the microsomal enzyme complex, FAO (fatty alcohol: nicotinamide adenine dinucleotide (NAD) oxidoreductase), which catalyzes the oxidation of fatty alcohols to fatty acids. In SLS, there is a concomitant reduction of both FALDH and FAO activities, resulting in accumulation of fatty alcohols in blood and tissues. The incorporation of fatty alcohol into lipids alters the lipid composition and leads to the impaired cell membrane function thought to be the cause of the symptoms seen in SLS [156]. Neurological

manifestations and abnormal desquamation are the clinical correlates of abnormal lipid composition of nerve sheaths and keratinocytes. To date, more than 35 mutations have been identified in the *FALDH* gene, including deletions, insertions and point mutations [157–160]. Interestingly, a single point mutation has been identified in patients from both northern Europe and Sweden, supporting the hypothesis of a common ancestor in whom this point mutation originally occurred [158].

Gaucher Disease, Type II (Infantile Cerebral Type; Acute Neuronopathic Type) (MIM 230900)

Gaucher disease is an autosomal recessive disorder involving various organ systems (see also Chapter 22, this volume). It has been divided into three subtypes, only one of which shows skin involvement. Type II Gaucher disease, the rarest type, is progressive and fatal. Several sporadic cases have been reported in the literature in which newborns presented with ichthyotic skin at birth and later developed neurological symptoms. Death occurred within the first months of life [161,162].

Clinical and Histological Features

Newborns often show a collodion-like presentation at birth. A variation in severity of the skin phenotype may be due to differences in the levels of residual enzyme activity of β-glucocerebrosidase [163]. Upon histological and ultrastructural examination, hyperkeratosis and disruption of the lamellar structure of the stratum corneum are noted.

Molecular Defect

The genetic defect lies in the *GBA* gene encoding β-glucocerebrosidase (GCase), leading to enzyme deficiency [164,165]. GCase is located generally in lysosomes, including those in the stratum corneum of the epidermis, and catalyzes the hydrolysis of glucosylceramides to ceramides. In the skin, ceramides are a crucial component of intercellular lamellae between corneocytes and play an important role in barrier function and desquamation [166]. The persistence of glucosylceramides rather than the decreased concentration of ceramides is thought to be the cause of the structural abnormalities in the stratum corneum [163]. The lipid alteration in the intercellular lamellae may induce corneocyte adhesion through increased hydrogen bonding, consequently leading to retention of the abnormal stratum corneum [163,167,168]. An animal model for Gaucher disease has been developed that shows ichthyotic skin changes and increased transepidermal water loss corresponding to the findings in the most severely affected patients [169].

X-Linked Dominant Chondrodysplasia Punctata (CDPX2; Conradi-Hünermann Syndrome; Happle Syndrome) (MIM 302960)

The term Conradi-Hünermann syndrome has been used in the literature for the X-linked dominant form of chondrodysplasia punctata. "Happle syndrome" denotes the occurrence of X-linked dominant chondrodysplasia punctata, ichthyosis, cataract, and short stature. About 60 cases of familial X-linked dominant chondrodysplasia punctata have been described [170], most of which are sporadic, suggesting *de novo* mutations [171,172]. A mild form of chondrodysplasia punctata with autosomal dominant inheritance has been reported, in which patients had a characteristic facies and symmetric stippling of the limbs, which disappeared after childhood [173]. The existence of the autosomal dominant form has been questioned, because some cases initially described as being autosomal dominant were later shown to follow an X-linked dominant inheritance [174], while other sporadic cases could be attributed to warfarin embryopathy [175].

Clinical and Histological Features

The X-linked dominant form is characterized by asymmetric chondrodysplasia punctata, ichthyosiform erythroderma, limb skeletal defects, and segmental cataracts. The phenotype in CDPX2 females ranges from stillbirth to mildly affected individuals identified in adulthood. The X-linked dominant inheritance pattern is presumed lethal in males, although a few affected males have been reported. Genetic heterogeneity, variable gene expression, and incomplete penetrance have been proposed to explain the wide range of clinical and radiological manifestations.

The newborn presents with a generalized erythema and blaschkoid disposition of scales and follicular keratoses that are arranged in a linear pattern and diminish over the first weeks to months, leaving hypochromic areas, follicular atrophoderma, and alopecia of the scalp. At birth, segmental collodion membranes may be observed particularly over peripheral extremities. The linear cutaneous findings, which follow the lines of Blaschko, are interpreted as an example of random X chromosome inactivation in each cell (Lyonization). The skeletal abnormalities are characterized by marked asymmetry. At birth, endochondral punctate calcifications can be observed in areas of spine, ribs, pelvis, long bones and scapula, that may disappear spontaneously later in life (Fig. 13). Impaired endochondral bone formation can result in short stature, asymmetric leg length, kyphoscoliosis, and an asymmetric facial appearance due to hypoplasia on one side. Cataracts occur in one third of cases, are often unilateral, and may be confined to one quadrant of the eye as a manifestation of functional X-chromosomal mosaicism [176]. Histopathology shows prominent follicular and interfollicular compact hyperkeratosis containing specific and unique calcium deposits revealed by histochemistry [177] (Fig. 14). Electron microscopy demonstrates cytoplasmic vacuoles in keratinocytes of the granular layer, which have been shown to contain calcium crystals [178].

Molecular Defect

Inborn errors of the cholesterol biosynthetic pathway have been identified in several dysmorphogenetic syndromes, such as CDPX2, CHILD syndrome (see below), and Smith-Lemli-Opitz syndrome [179]. The X-linked semidominant mouse mutation "tattered" (*Td*) [180] has been shown to cause a phenotype in mice similar to that seen in heterozygous human females with CDPX2. The defect in *Td* mice has been identified as a single amino acid substitution in the Δ8Δ7-sterol-isomerase, also known as emopamil-binding protein (Ebp) [181]. In humans, a candidate gene (*EBP*), encoding a sterol-Δ8-isomerase has been identified and mapped to Xp11.22–p11.23. Several different mutations in the human *EBP* gene have been identified in unrelated CDPX2 patients [181,182]. The mechanism whereby cholesterol deficiency and/or accumulation of intermediates of the synthetic pathway lead to different phenotypes remains to be elucidated. It has been shown that members of the hedgehog (Hh) family of secreted signaling proteins play a role in animal development, and alteration of one of the members, "patched" (Ptc), has been associated with nevoid basal cell carcinoma syndrome (NBCCS or Gorlin syndrome) [183,184]. The discovery that "dispatched" (Disp), a novel member of this family, is required to

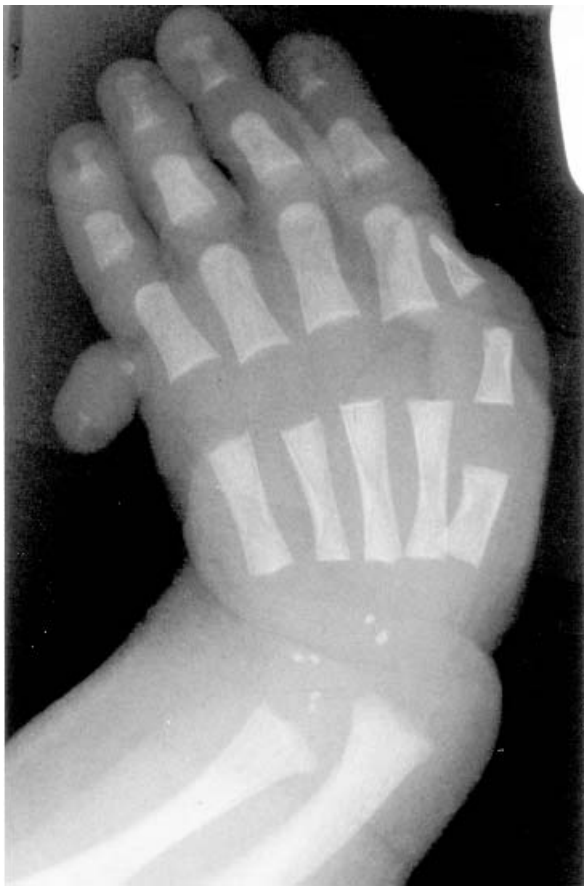

Figure 13. X-linked dominant chondrodysplasia punctata: stippled epiphyseal calcifications.

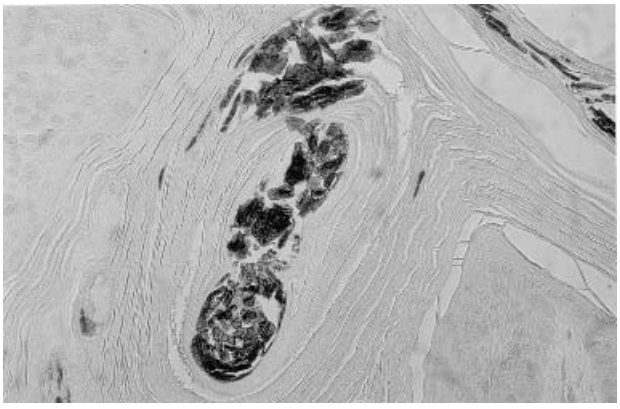

Figure 14. X-linked dominant chondrodysplasia punctata: von Kossa stain revealing calcification within the stratum corneum.

release cholesterol-anchored hedgehog to ensure effective Hh signaling [185], suggests that the cholesterol biosynthetic pathway may be closely linked to the hedgehog-patched signaling cascade. Thus, the Hh-Ptc signaling cascade might be a candidate for cellular processes that require an intact cholesterol pathway, and defects in either pathway could be associated with impaired terminal differentiation.

CHILD Syndrome (Congenital Hemidysplasia with Ichthyosiform Erythroderma and Limb Defects) (MIM 308050)

The term CHILD syndrome was introduced in 1980 by Happle to describe congenital hemidysplasia with unilateral inflammatory nevus and limb defects [186]. X-linked dominant inheritance with lethality in hemizygous male embryos has been proposed following the observation that far more females than males are affected [187]. The unilateral distribution of the defects can be explained by Lyonization.

Clinical and Histological Features

The clinical features are similar to those of rhizomelic chondrodysplasia punctata and X-linked dominant chondrodysplasia punctata. The CHILD nevus is usually present at birth, but may also develop during the first months of life. The erythematous and ichthyosiform lesion has a sharp demarcation, is usually covered with waxy scales, and is located predominantly over flexural areas and body folds. The distribution is either diffuse over one side of the body or arranged along the lines of Blaschko [188]. The skeletal anomalies are unilateral and involve mainly long bones. Epiphyseal chondrodysplasia punctata can be seen shortly after birth and can lead to skeletal defects that may vary from minimal asymmetry of limbs to the complete lack of one extremity [189]. Associated defects in other organ systems include ipsilateral hypoplasia of brain or spinal cord and ipsilateral visceral anomalies. Early death has been associated with congenital heart disease due to cardiovascular malformations [188]. Histology reveals acanthosis, areas of parakeratosis, and areas of orthohyperkeratosis. The dermis shows lymphohistiocytic infiltrates, which makes a distinction from psoriasis difficult. Ultrastructurally, the corneocytes contain lipid vacuoles.

Molecular Defect

Mutations in the gene encoding NAD(P)H steroid dehydrogenase-like protein (*NSDHL*), leading to impaired protein function, have been identified in CHILD patients [190]. NSDHL is a 3β-hydroxysteroid dehydrogenase that functions in one of the later steps of cholesterol biosynthesis upstream of EBP, the defective protein in CDPX2. It is thought that the defective enzyme is responsible for decreased synthesis of cholesterol, accounting for the skin and bone phenotype. Two X-linked semidominant mouse mutations, "bare patches" (Bpa) and "striated" (Str) have been associated with mutations in the *Nsdhl* gene [191] and can be used as animal models for the study of CHILD syndrome.

Treatment of Disorders of Abnormal Lipid Metabolism

Treatment of Sjögren-Larsson syndrome by reducing fatty acid intake is controversial [192,193]. Skin changes, including the severe pruritus of SLS, are markedly improved by moderate doses of systemic retinoids such as acitretin and etretinate [193,194]. Nevertheless, such treatment does not change the neurological symptoms. Topical treatment with moisturizing and lubricating preparations, e.g., a lipophilic lotion or cream containing 5% ammonium lactate and 20% propylene glycol, is well tolerated in our own experience in SLS (D. Hohl, unpublished observation). While type 1 Gaucher disease responds clinically to enzyme replacement, such response has not been seen in patients with type

2 disease [195]. Controversial results have been achieved with systemic retinoid treatment in CHILD patients. With isotretinoin, corneal deposits have been reported; low dosage, however, was effective in reducing the extent of erythroderma [196].

PEROXISOMAL DISORDERS

Rhizomelic Chondrodysplasia Punctata Type 1 (RCDP1) (MIM 215100)

Peroxisomes function in a number of essential metabolic pathways, especially those related to lipid metabolism. Peroxisomal proteins are synthesized in the cytosol and targeted to the peroxisome via specific targeting signals. Defects in those import routes may result in single or multiple enzyme deficiencies. In rhizomelic chondrodysplasia punctata type 1 (RCDP1), one import pathway is blocked, resulting in a deficiency of multiple peroxisomal proteins.

Clinical Features

Clinically, RCDP patients present with symmetrical proximal shortening of the limbs (rhizomelia), epiphyseal calcifications, cataract, severe mental retardation, and, less frequently, ichthyosis. The generalized discrete ichthyosis differs from the blaschkoid pattern of the follicular hyperkeratosis observed at birth in CDPX2. The ocular phenotype is also helpful in its distinction from the X-linked dominant form: in RCDP the opacities are bilateral and symmetric, whereas in the X-linked form they are usually asymmetric and unilateral [176].

Molecular Defect

Deficiency of plasmalogens from red cells and decreased dihydroxyacetonephosphate acyltransferase (DHAPAT) in platelets and fibroblasts, with concomitant elevation of phytanic acid but normal oxidation of very long chain fatty acids, is a characteristic finding in RCDP patients [197]. RCDP is classified as a protein-targeting disease characterized by the unique feature that the biogenesis of most peroxisomal proteins is normal, but a specific subset of peroxisomal matrix proteins fails to be imported from the cytosol [198–200]. Two of the targeting sequences that are required for import into peroxisomes have been identified and are referred to as peroxisome targeting sequences (PTS). The PTS2 receptor is defective in RCDP, and several mutations have been identified in the *PEX7* gene [198–200]. Several peroxisomal matrix proteins are mistargeted in this disease; consequently, RCDP is characterized by multiple enzyme deficiencies [201], accounting for the severe symptoms.

Refsum Disease (MIM 266500)

Refsum disease is prevalent among populations in or from northern Europe and Scandinavia and follows a recessive mode of inheritance. It was first described in 1946 by the Norwegian neurologist, Sijvald Refsum, who reported the symptoms in two inbred families in Norway [202].

Clinical and Histological Features

A variable ichthyosis develops during the second or third decade of life with small white scales over the trunk and extremities (Fig. 15), resembling ichthyosis vulgaris. The flexural areas and palms and soles are usually spared. Ophthalmologic and neurological manifestations include retinitis pigmentosa, peripheral neuropathy and cerebellar ataxia. Other abnormalities include cardiomyopathy, impaired renal tubular function and epiphyseal dysplasia. Several cases of fatal outcome have been reported, mostly due to cardiopulmonary complications, such as arrythmia or respiratory paralysis [203]. Histologically, vacuoles in epidermal and dermal cells are characteristic and correspond to lipid accumulation. Electron microscopy shows lipid droplets in the cytoplasm of keratinocytes and melanocytes, which are located close to degenerated mitochondria or the endoplasmic reticulum. The degenerated mitochondria, which are characteristic of Refsum disease, have a poorly developed internal membrane system [204].

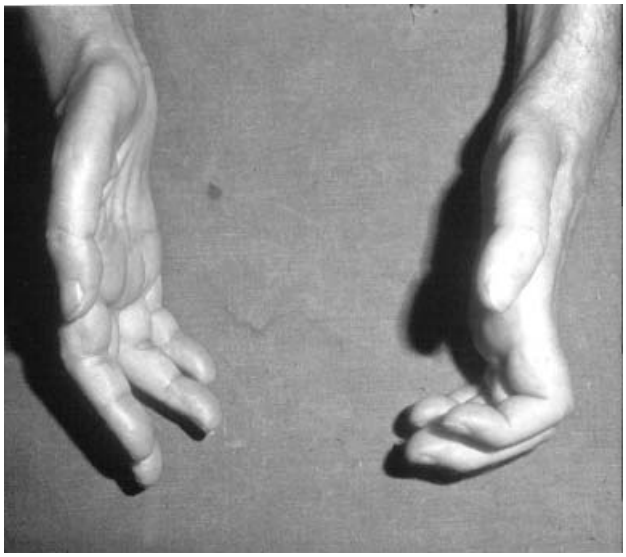

Figure 15. Refsum syndrome with mild palmar keratosis and spastic polyneuritis.

Molecular Defect

Refsum patients exhibit an accumulation of phytanic acid, an exclusively exogenous branched-chain fatty acid, that originates from the degradation of plant chlorophyll in cattle and other vegetarian animals. Phytanoyl-CoA-hydroxylase (PhyH), which normally catalyzes the conversion of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA in peroxisomes, is deficient [205]. The *PHYH* (*PAHX*) gene has been mapped to chromosome 10 [206] and inactivating mutations have been identified, including deletions and point mutations [207,208]. Thus, dietary phytanic acid accumulates in serum and tissues, where it substitutes for essential fatty acids in all lipid subfractions, especially phospholipids. In the epidermis, decreased degradation of altered epidermal lipids may account for the impaired desquamation [209]. Phytanic acid is a natural ligand for RXR (retinoid X receptor) α and promotes the formation of RXRα/RXR response element complexes similar to 9-*cis*-retinoic acid, the natural ligand for RXRα. It has been proposed that phytanic acid may play a role in the regulation of transcription of specific genes and that high levels of phytanic acid may lead to a disturbed regulation of metabolism through stimulation of RXR receptors [210,211]. However, the role of the RXR pathway in Refsum disease remains unclear.

Treatment

As phytanic acid is derived exclusively from exogenous sources, a diet lacking phytanic acid and phytol, or their precursors, thus excluding dairy products, is recommended [212]. Plasmapheresis performed regularly has been proven effective to prevent progression of the symptoms.

SULFATASE DISORDERS AND CONTIGUOUS GENE DELETION DISORDERS

Multiple Sulfatase Deficiency (MSD) (MIM 272200)

To date, 11 human sulfatases have been characterized and their corresponding genes identified [213]. Sulfatase enzymes catalyze the hydrolysis of sulfate ester bonds. Arylsulfatases represent a subset of sulfatases that additionally catalyze the hydrolysis of aromatic compounds. Most sulfatases are located in lysosomes; however, steroid sulfatase (arylsulfatase C) is microsomal in location, the recently identified arylsulfatases D and F are found in the endoplasmic reticulum, and arylsulfatase E is present in the Golgi apparatus. The sulfatase genes are clustered on the short arm of chromosome X (Xp22.3) and share a high sequence homology that suggests a gene duplication event from a common ancestral gene [214]. The sulfatase deficiencies lead to an accumulation of substrates in various tissues and this determines the clinical phenotype. Multiple sulfatase deficiency (MSD) is a fatal disorder caused by a defect of all known sulfatases, resulting in clinical features also seen in single enzyme deficiencies [215].

Clinical Features

The diagnosis of MSD is confirmed by the absence of several singular sulfatases. Consequently, glycosaminoglycans, gangliosides, and sulfatides accumulate in brain and various tissues of affected individuals. Ichthyosis, which is usually mild, is the clinical correlate of steroid sulfatase deficiency and resembles that seen in X-linked ichthyosis (see below). The scales have been shown to contain increased amounts of cholesterol sulfate. The neurological symptoms are severe and are the result of arylsulfatase A deficiency, this enzyme also being defective in metachromatic leukodystrophy (MLD). In the second year of life, psychomotor retardation develops, and shortly thereafter children exhibit motor deficits and mental regression. The neurological symptoms progress and death occurs in the first decade of life. Other clinical features like growth retardation, hepatosplenomegaly, and lumbar kyphosis can be attributed to arylsulfatase B deficiency.

Molecular Defect

A major contribution to the understanding of the pathogenesis of MSD was the identification of the lack of co- or post-translational modification of the sulfatases, a step that is required for their catalytic activity [216]. Normal sulfatases undergo the conversion of a highly conserved cysteine residue to 2-amino-3-oxopropionic acid or serine semialdehyde during post-translational modification, yielding a catalytically active sulfatase. In MSD, lack of this modification leads to the enzyme deficiency. However, an underlying mutation has not yet been identified.

X-Linked Ichthyosis (XRI) (MIM 308100)

The linkage of X-linked recessive ichthyosis to other X-linked markers was demonstrated in the early 1960s [217]. Soon thereafter, two groups discovered that patients with X-linked sulfatase deficiency developed ichthyosis and it was established that these two conditions are the same disease entity [6,7]. X-linked ichthyosis is inherited in an X-linked recessive manner, with an incidence of 1 in 2,000–6,000 males [218].

Clinical and Histological Features

At 1–3 weeks of age, peeling of the skin is observed with light and loosely attached scales covering the entire skin. These light gray scales may persist into adulthood or be replaced by large, dark scales during the following weeks, and are found typically over flexural and extensor areas of the extremities (Fig. 16). The scalp may be mildly involved and scaling is observed preauricularly, but the face, palms, and soles are usually spared. Corneal opacities that do not affect vision are present in half of all adult patients, often facilitating its distinction from other forms of ichthyosis. The deposits in the cornea have been shown to be composed of cholesterol sulfate [219]. The human placenta normally shows high concentrations of sulfatases, a deficiency of which is associated with insufficient cervical dilatation, weakness of labor, and a prolonged delivery with increased stillbirth frequency [7]. Histologically, an extended granular layer, an increased Malpighian layer, and orthohyperkeratosis with slight parakeratosis are characteristic features that enable a distinction from autosomal dominant ichthyosis vulgaris in which the granular layer is diminished or even absent [4]. However, depending on the severity of the phenotype and the site of biopsy, a clear distinction between these two ichthyoses may not always be possible.

Molecular Defect

Biochemical analysis in X-linked recessive ichthyosis demonstrates a deficiency of steroid sulfatase on chromosome Xp22. The enzyme deficiency is caused by deletions and point mutations at this locus, and 80–90% of patients have been shown to have a complete deletion. Approximately 10% of patients have point mutations that affect residues that are conserved within the sulfatase family [220–222]. Rare patients with steroid sulfatase deficiency have a complex phenotype due to deletions of additional disease genes on the short arm of the X chromosome, resulting in a contiguous gene deletion syndrome [10].

Deficiency of steroid sulfatase in the epidermis leads to impaired hydrolysis of cholesterol sulfate. The retention hyperkeratosis may be due to increased adhesiveness of the cornified cells due to the negative charge of the lipid [223], abnormal hydrogen bonding of cholesterol sulfate with plasma membrane fatty acids [224], alteration of lipid metabolism, or inhibition of proteases that would normally act upon desmosomes by high levels of cholesterol sulfate [225]. Recently, the activation of the protein kinase family by cholesterol sulfate has been appreciated [226] and should also be considered in the pathogenesis of XRI. Topical application of cholesterol to XRI skin reduces the amount of scaling [227], whereas topical application of cholesterol sulfate in hairless mice induces the XRI phenotype [228]. Recent data indicate that cholesterol sulfate accumulation rather than cholesterol deficiency is responsible for the perturbed stratum corneum [229].

Treatment

XRI can usually be controlled by topical rehydration with preparations containing either 5% ammonium lactate, 5% urea or up to 20% propylene glycol. Higher concentrations are often irritating. Nevertheless, good cosmetic results have been achieved with 12% ammonium lactate, a keratolytic agent that is effective and well-tolerated when applied twice daily [230]. Systemic retinoids (acitretine/etretinate) are well tolerated and very efficient in XRI [231]. They are an excellent treatment option in severe cases with dark and dirty appearing scales of the "ichthyose noire" phenotype.

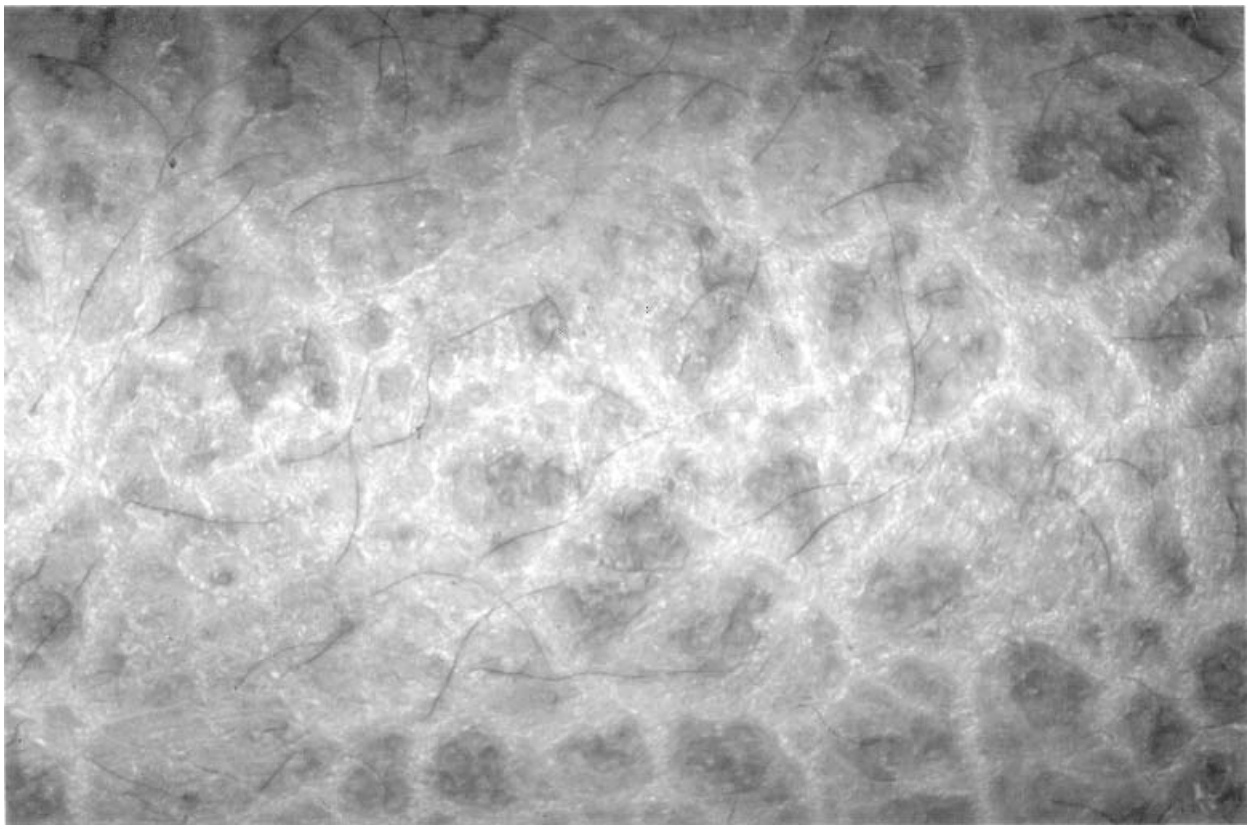

Figure 16. X-linked recessive ichthyosis with large brown-gray scales with ''dirty'' appearance.

Nevertheless, retinoids are usually not indicated in this relatively mild ichthyosis [95]. Gene therapy approaches have been tested by introducing the steroid sulfatase gene into cultured basal keratinocytes from XRI patients, thereby inducing enzyme activity in these cells [232,233].

X-Linked Recessive Chondrodysplasia Punctata (CDPX1) (MIM 302950)

Clinical Features

The X-linked recessive form of chondrodysplasia punctata (CDPX1) is a defect of bone and cartilage development and presents with symmetric ichthyosiform hyperkeratosis, alopecia, short stature, cataract and deafness. Hypoplasia of the distal phalanges as a distinctive feature in CDPX has been described in a family with atypical ichthyosis, and cholesterol levels are elevated in affected males [234]. The clinical features seen in CDPX1 resemble those observed in warfarin embryopathy [235] and vitamin K epoxide reductase deficiency [236].

Molecular Defect

The disease gene is located on the most distal part of Xp, which is pseudoautosomal [237]. In two families, a deletion at Xp22.32 was identified in affected males, their carrier mothers and several potential carrier females, and a reciprocal X-Y translocation involving the region distal to Xp22.3 was observed, thus confirming the assignment of the *CDPX1* gene to this locus [234,238]. The Xp22.3 locus contains genes of the sulfatase family that share high homology: arylsulfatases C, D, E, and F. Ancient duplications are thought to be responsible for the contiguous location of these genes of closely similar sequence and structure. This region is particularly susceptible to deletions that can extend to neighboring genes, causing a contiguous gene deletion defect. X-linked recessive chondrodysplasia punctata has been described as part of a contiguous gene deletion syndrome, and may therefore be associated with X-linked recessive ichthyosis, Kallmann syndrome, mental retardation, and short stature [237]. The gene encoding arylsulfatase E (*ARSE*) has been identified as the disease gene in CDPX1 and mutations within it have been identified, including missense and premature stop mutations that lead to accumulation of cholesterol sulfate in various tissues [239,240]. It has been shown recently that cholesterol metabolism is directly linked to the activation of the protein kinase C family of serine/threonine kinases that play critical roles in the regulation of cellular differentiation and proliferation of diverse cell types [226]. One of the isoforms of protein kinase C is activated by cholesterol sulfate, which leads to stimulation of the differentiation pathway that may account for the defective skin, bone and cartilage development in CDPX1.

Kallmann Syndrome (MIM 308700)

Kallmann syndrome describes the association of hypogonadotropic hypogonadism and anosmia. Autosomal dominant, autosomal recessive, and X-linked recessive inheritance have been reported, suggesting genetic heterogeneity. Occasionally, patients with Kallmann syndrome also manifest features of other X-linked disorders, such as ichthyosis,

chondrodysplasia punctata, short stature, mental retardation, and ocular albinism. The combination of features of several X-linked disorders is due to deletions of the distal short arm of the X chromosome, leading to a contiguous gene syndrome [237].

Clinical Features

X-linked Kallmann syndrome is characterized by a reduced secretion of gonadotropin-releasing hormone by the hypothalamus leading to hypogonadism and a eunuchoid habitus. The ability to smell can be either reduced (hyposmia) or completely absent (anosmia). Depending on the extent of the deletion on the short arm of the X chromosome, Kallmann syndrome patients may also exhibit clinical signs found in other disorders associated with Xp deletions. Thus, deletion of the Kallmann syndrome gene (*KAL1*) and the steroid sulfatase gene would lead to the association of hypogonadism, anosmia, and generalized ichthyosis. In such a case, the cutaneous phenotype would correspond to that observed in XRI. The combination of ichthyosis, Kallmann syndrome, and chondrodysplasia punctata has been described as a contiguous gene syndrome due to deletion of the terminal part of Xp, with the breakpoint at Xp22.31 [241].

Molecular Defect

The *KAL1* gene has been localized to a region on the short arm of the X chromosome (Xp22.3) in Kallmann patients and mutations identified that prove causality [242,243]. The *KAL1* gene product, anosmin, exhibits neural cell adhesion properties and appears to provide a scaffold for gonadotropin-releasing hormone (GnRH) neurons and olfactory nerves to migrate and synapse during embryogenesis. When anosmin is defective or absent, these neurons fail to synapse normally, and Kallman syndrome results [242,243].

Rud Syndrome (Ichthyosis and Male Hypogonadism) (MIM 308200)

Rud syndrome describes the association of ichthyosis with hypogonadism, retinitis pigmentosa, mental retardation and epilepsy. The Danish physician Rud described two patients in 1927 and 1929, who presented with similar symptoms: a 22-year-old male with ichthyosis, hypogonadism, epilepsy, polyneuritis, and anemia, and a 29-year-old female with ichthyosis, hypogenitalism, diabetes mellitus, and partial gigantism, respectively [244,245]. The inheritance pattern is controversial: in a family in which anosmia was an additional clinical finding, linkage with Xg was excluded [246]. However, the finding that the ratio of affected males to affected females is 2:1 suggests an X-linked pattern of inheritance [247]. Most likely, Rud syndrome also represents a contiguous gene deletion syndrome.

Clinical Features

The major findings include mental retardation, epilepsy, ichthyosis, and, in most cases, hypogonadism [246,248–251]. However, the criteria for the diagnosis of this syndrome vary greatly and different combinations of symptoms leading to a diagnosis of Rud syndrome have been reported. Two different types of ichthyosis have been reported on the basis of clinical and histological findings: one similar to that seen in X-linked recessive ichthyosis or atypical ichthyosis vulgaris [252] and another similar to lamellar ichthyosis [253], but an exact classification is sometimes not possible. Likewise, the hypogonadism has been found to be both hypo- and hypergonadotropic. Münke et al. proposed that the main symptoms of the Rud syndrome should be ichthyosis, hypogonadism, mental retardation, and epilepsy [254]. Traupe et al. reviewed the initial reports by Rud showing that the 22-year-old male was the only one affected of 16 brothers and sisters and was mentally alert. The second, female, patient had three family members who suffered from ichthyosis but had no other abnormality. He suggested that the term Rud syndrome be abandoned because it refers to a heterogeneous condition, not even described by Rud [10].

Molecular Defect

Ichthyosis with male hypogonadism was previously thought to be a distinct X-linked recessive, single gene disorder. However, it has been shown that Kallmann syndrome and X-linked ichthyosis are closely linked by demonstrating that deletions on the short arm of the X chromosome lead to the coexistence of these disorders [237]. The concurrence of hypogonadism and ichthyosis in Rud syndrome suggests in this condition also the possibility of a contiguous gene syndrome as the underlying defect. Indeed, steroid sulfatase levels are deficient in some Rud syndrome patients, but cases with normal levels do exist, suggesting genetic heterogeneity [254].

PROTEINASE DISORDERS

Netherton Disease (Comel-Netherton Syndrome) (MIM 256500)

Ichthyosis linearis circumflexa (ILC) was first characterized by Comel in 1949 [255], and Netherton described the hair abnormalities in 1958 in a patient with congenital ichthyosiform erythroderma [256].

Clinical and Histological Features

Netherton disease is an autosomal recessive syndrome that usually presents at birth or soon thereafter. It consists of hair shaft abnormalities (trichorrhexis invaginata) (Fig. 17), ichthyosis linearis circumflexa, and atopic diathesis. Abnormal cornification of the internal root sheath leads to trichorrhexis invaginata and trichorrhexis nodosa (''bamboo hair''). It is thought to be caused by an impairment of disulfide bonding in the hair shaft [257], and is often confined to the eyebrows after childhood. Ichthyosis linearis circumflexa (Fig. 18) occurs later in the disease course and is characterized by red, annular, and serpiginous patches on the trunk and extremities, which have a distinctive double-edged border. These patches are predominant in flexural areas, together with lichenification and scaling. Palms and soles are not usually affected. Ichthyosis linearis circumflexa is not a consistent feature in patients with Netherton syndrome and can be an isolated finding unrelated to Netherton syndrome. High IgE levels are commonly seen in most patients and have been considered a sign of atopy or atopic diathesis [258]. Histologically, parakeratotic cells can be seen in large areas of the stratum corneum and premature secretion of lamellar body contents with impaired transformation into mature lamellar membrane structures may account for the impaired barrier function [259].

Molecular Defect

Netherton syndrome has been mapped to chromosome 5q32 by linkage analysis [260]. The *SPINK5* gene (serine proteinase inhibitor, Kazal type 5), which encodes the serine proteinase inhibitor LEKTI (lymphoepithelial Kazal-type-related inhibitor) [261], also maps to this region and

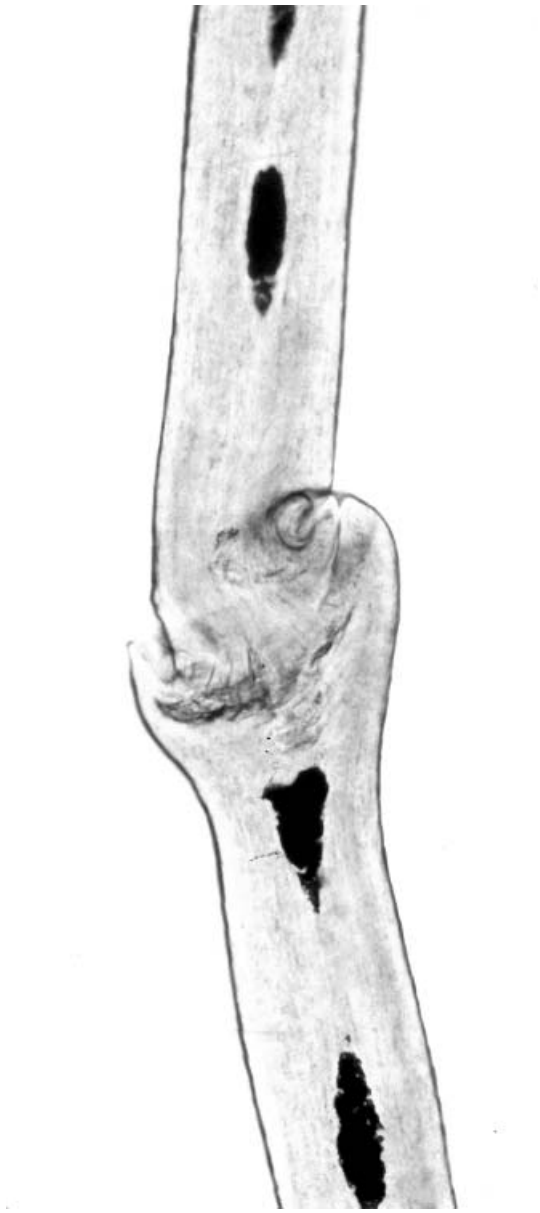

Figure 17. Trichorrhexis invaginata in Netherton syndrome.

mutations have been identified in the *SPINK5* gene in unrelated families [262]. The majority of mutations generate premature termination codons. Consistent with the recessive mode of inheritance, null expression of the mutated *SPINK5* alleles is predicted through accelerated mRNA decay. LEKTI is the first serine-proteinase inhibitor shown to be primarily involved in a skin disorder or hair morphogenesis, highlighting the significant role of the regulation of proteolysis in epithelial formation and keratinocyte terminal differentiation. Impaired LEKTI expression in the thymus of Netherton patients may account for the abnormal maturation of T lymphocytes and the severe atopic manifestations.

Treatment

Erythrodermic children are at risk of hypernatremic dehydration, especially in the neonatal period. Associated atopy may lead to nutritional intolerance. Topical treatments should be inert, because corticoid treatment is often not effective. Systemic etretinate, psoralen ultraviolet A-range therapy, and cyclophosphamide result in improvement, but the side effects of long-term treatment with these substances may outweigh their benefits [95,263].

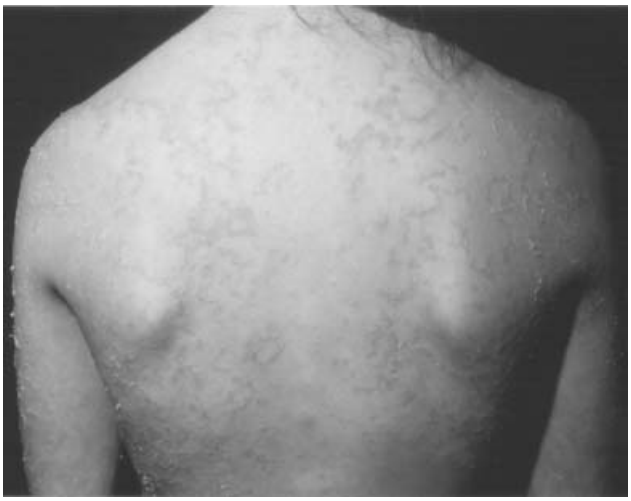

Figure 18. Comel-Netherton syndrome with erythematous, circinate, and polycyclic patches.

Papillon-Lefèvre Syndrome (PLS; Keratosis Palmoplantaris with Periodontopathia (MIM 245000)

Papillon-Lefèvre syndrome is a rare autosomal recessive disease with an incidence of 1:1,000,000, generally observed in children of consanguineous parents.

Clinical Features

Clinically, affected individuals present with diffuse palmoplantar keratoderma, often combined with hyperhidrosis and severe periodontitis leading to premature loss of both deciduous and permanent teeth [264]. The skin phenotype, varying from mild psoriasiform scaly skin to hyperkeratosis, typically develops within the first 3 years of life. Apart from the early appearance of palmoplantar lesions, Papillon-Lefèvre syndrome often involves the exposed skin over joints (knees, elbows, knuckles) and thus may be confused with psoriasis. Most patients display both periodontitis and hyperkeratosis; however, in some patients, only one or the other is encountered.

Molecular Defect

Mapping of the disease to a chromosomal locus has been undertaken in several consanguineous families and linkage to the region at 11q14–q21 has been demonstrated [265–267]. The gene for cathepsin C (*CTSC*), a lysosomal proteinase, has been identified as the disease gene and mutations in affected individuals have been identified, including nonsense, missense, and splice site mutations [268]. Cathepsins are cysteine proteinases that constitute a major component of the lysosomal proteolytic system. Loss of cathepsin C activity has been demonstrated in PLS patients, with reduced activity in obligate carriers. The skin phenotype implicates a role for cathepsin C in epithelial differentiation, which, in addition

to resulting in a reduced host response against bacteria in dental plaque, may contribute to periodontitis.

Treatment

Patients benefit from treatment with acitretin and it has been suggested that if treatment is started at an early age, the development of normal adult dentition is possible [269]. In addition, professional dental hygiene is recommended to minimize permanent dental loss. Topical treatment of the skin can be performed with diverse keratolytic agents, but should include antiseptics and treatment of the hyperhidrosis to prevent recurrent malodorous infections.

AMINO ACID METABOLISM DISORDER
Richner-Hanhart Syndrome (Tyrosine Transaminase Deficiency) (MIM 276600)

Richner-Hanhart syndrome is a rare congenital disorder of amino acid metabolism that follows an autosomal recessive inheritance. It was first described by Richner in 1938 [270] and was further characterized by Hanhart in 1947 [271].

Clinical and Histological Features

Clinical features include palmoplantar hyperkeratosis, eye involvement with bilateral dendritic keratitis, and mental retardation [272]. The skin manifestations usually develop within the first year of life; however, reports exist of the phenotype developing in early adulthood. Bullae and erosions are the first signs of skin involvement, which gradually diminish while hyperkeratoses develop. Painful palmoplantar keratoderma — probably induced by tyrosine crystals — is typical, and often accompanied by hyperhidrosis. The eye manifestations are usually present shortly after birth, comprising photophobia, redness and tearing, and develop into corneal ulcerations, neovascularizations, and corneal opacities. Mental retardation has not been described in all reported cases, suggesting that it is a variable feature. High levels of tyrosine in serum and urine are characteristic. Histological findings include a thickened granular layer with hyperkeratosis, acanthosis, and parakeratosis. On ultrastructural examination, multinucleated keratinocytes, and clumping of intermediate filaments are the striking feature, as well as intracytoplasmic tyrosine crystals [273]. Tyrosine crystals are also present in the corneal epithelium [274] and are thought to induce cross-linking of keratin filaments, leading to intermediate filament aggregation.

Molecular Defect

This rare disorder is caused by a deficiency of hepatic tyrosine aminotransferase (TAT), which is involved in amino acid metabolism and gluconeogenesis, leading to hypertyrosinemia. The gene for tyrosine aminotransferase has been mapped to chromosome 16 [275] and deletions and point mutations within it have been identified in affected individuals [276,277].

Treatment

Richner-Hanhart syndrome is the only hereditary palmoplantar keratoderma that can be treated efficiently by a dietary regimen. Restriction of dietary intake of tyrosine and phenylalanine has a positive effect and improves early skin and eye symptoms. An early diagnosis and immediate dietary intervention are crucial as regards the prevention of symptoms, especially mental retardation and reversal of the disease process [272,278]. Systemic retinoids have proven effective in the treatment of skin and eye symptoms [272,279].

DISORDER OF NUCLEOTIDE EXCISION REPAIR
Tay Syndrome (Trichothiodystrophy; TTD; IBIDS) (MIM 601675)

Tay syndrome was first described by Tay in Singapore in 1971 in three children of consanguineous parents [280]. It is also found in the literature as IBIDS, which summarizes multiple symptoms as ichthyosis, brittle hair, intellectual impairment, decreased fertility, and short stature.

Clinical and Histological Features

Patients commonly have a collodion presentation at birth. During the first months of life, the erythema subsides and a generalized ichthyosis develops that may present as fine scaling or prominent hyperkeratoses that may cover the trunk, extremities, and palms and soles, but usually spare the flexural areas. Polarized microscopy of cut hair shows transverse fracture points (trichoschisis), trichorrhexis nodosa (transverse, frayed fractures), and alternating dark and bright bands that are reminiscent of a tiger tail. It was found that the abnormal hair of these patients (Fig. 19) has a low sulfur content due to a lack of sulfur-containing amino acids, a finding termed "trichothiodystrophy" [281]. Low birth weight and short stature are striking, while psychomotor development is generally delayed and mental retardation has been present in all reported cases. In males, cryptorchidism is often found, which manifests as hypogonadism. Similarly to IBIDS, the BIDS syndrome features similar abnormalities without ichthyosis, while in the PIBI(D)S syndrome, patients present with additional photosensitivity, the ichthyosis is usually mild, and hypogonadism is absent [282]. Skin histology of TTD patients shows moderate orthokeratotic hyperkeratosis, acanthosis, and papillomatosis.

Molecular Defect

TTD has been linked to disorders of nucleotide excision repair. UV-induced DNA damage is corrected by nucleotide excision repair systems [283]. The transcription/repair factor, TFIIH, consists of several subunits, such as ERCC2 (XPD), ERCC3 (XPB) and p44. *ERCC* genes, or excision-repair cross-complementing genes, are named after the rodent repair defects that they correct, and the number designates the rodent complementary group that is corrected by the human gene. To date, the human genes for ERCC1-3 and ERCC5-6 have been isolated. The human *ERCC3* gene on chromosome 2q21 corrects nucleotide defects at an early stage in DNA excision repair in the xeroderma pigmentosum cells of complementation group B (XPB). In cells of complementation group D, the human *ERCC2* gene on chromosome 19q13.2–q13.3 has been shown to be involved in later steps of the repair pathway. Mutations in the *ERCC2* (*XPD*) gene have been associated with xeroderma pigmentosum, Cockayne syndrome, and TTD [283]. In TTD patients, mutations at different positions throughout the *ERCC2* gene have been identified [284]. A point mutation in the *ERCC3* gene has been described in siblings of a consanguineous marriage who both presented as collodion babies at birth and were later diagnosed with TTD [285]. A second excision repair complementation group has been identified in another TTD patient [286] and it has been suggested that this complementation group should be referred to as TTD-A [287]. The *TTD-A* gene product is thought to be another component of the transcription factor TFIIH. Interestingly, in a large group of TTD and XPD patients, the site of the mutation determined the nature of the phenotype [288]. A mouse model

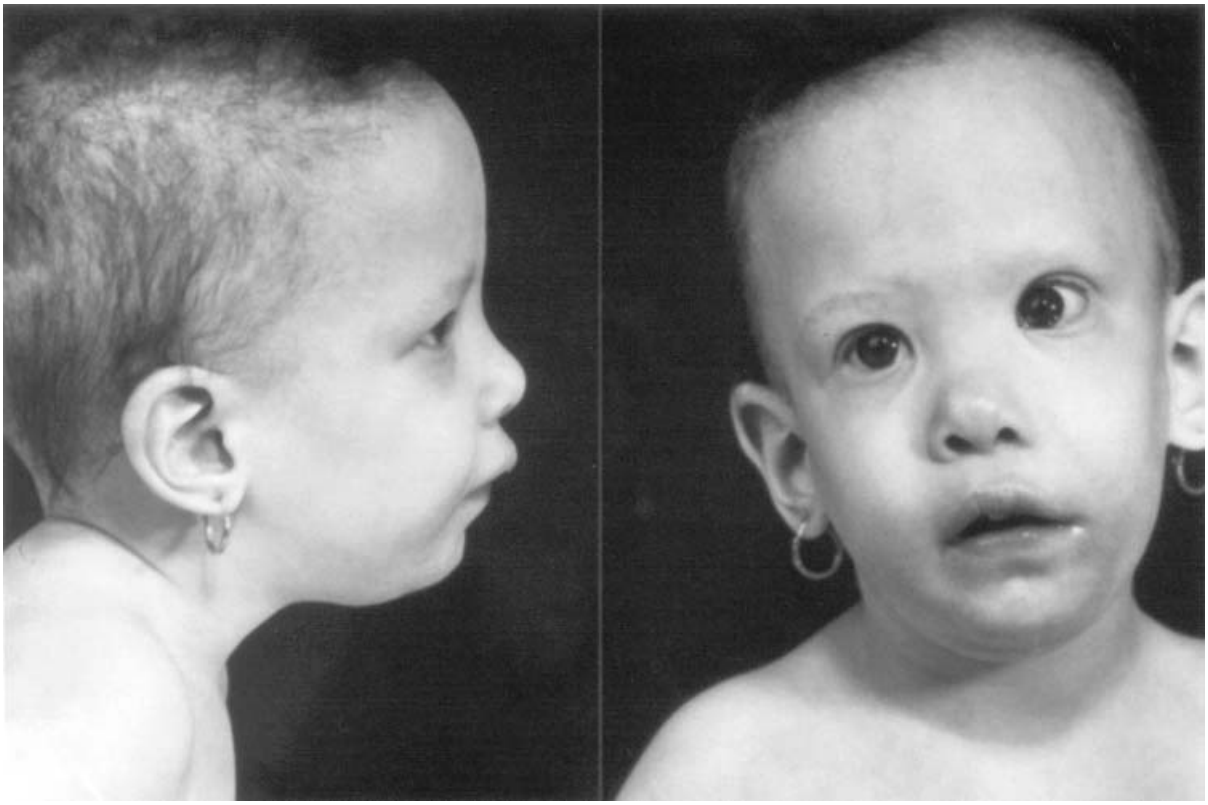

Figure 19. Tay syndrome in a 2-year-old child with sparse, brittle, and broken scalp hair.

for TTD has been generated by the introduction of an XPD point mutation previously identified in a TTD patient [289].

DEFECTS IN INTERCELLULAR COMMUNICATION

Vohwinkel Syndrome (Congenital Deafness with Keratopachydermia and Constrictions of Fingers and Toes) (MIM 124500)

This disorder was first reported by Vohwinkel in 1929 in a 24-year-old man and his 14-month-old daughter [290]. The syndrome, consisting of pseudoainhum of fingers and toes, palmar and plantar hyperkeratosis, and bilateral symmetric hearing loss of cochlear origin is referred to as Vohwinkel syndrome.

Clinical Features

Patients present with congenital deafness, honeycomb type palmoplantar keratoderma, and fibrous constricting bands at the interphalangeal joints of one or several fingers or toes. The hyperkeratoses on palms and soles develop during the first years of life and may progress during adolescence. The ring-shaped constrictions around the digits usually develop later, and can lead to autoamputation. In addition, verrucous papules and starfish-like hyperkeratoses are observed over articulations and tendons on the dorsal side of hands and feet as well as often on knees and elbows (Fig. 20). The nails, hair, and mucous membranes are usually normal [291].

Molecular Defect

Recently, the multigenic connexin family has been linked to Vohwinkel syndrome [292]. Connexins (Cx) play an important role in gap-junction mediated intercellular metabolic and electrical communication. Gap junction channels are involved in communication between cells through exchange of ions and second messengers and consist of connexin protein subunits that are encoded by a multigene family. Cx26 is located in the cochlea and has been found in the spinous and granular layers of rodent epidermis, but only in small amounts in human epidermis [293]. Mutations in *GJB2*, the gene encoding Cx26, have been identified in autosomal recessive sensorineural nonsyndromic hearing loss and in deafness associated with palmoplantar keratoderma. Mutations identified to date include nonsense mutations, missense mutations, and deletions/insertions [294–297]. The most common genetic defect is a single base pair deletion (35delG) in the *GJB2* gene, leading to a premature stop codon, and thus RNA degradation. This may suggest that the phenotype is due rather to haploinsufficiency than to a dominant negative effect. In three unrelated families affected by mutilating keratoderma and deafness, all affected individuals were shown to harbor a heterozygous non-conservative missense mutation at a highly conserved residue at the beginning of the connexin 26 molecule [298].

Erythrokeratodermia Variabilis (EKV) (MIM 133200)

Erythrokeratodermia variabilis is a very rare disorder of cornification transmitted in an autosomal dominant manner. Mendes da Costa first described this disorder in 1925, in a mother and her daughter who both showed fixed patches of hyperkeratosis and migrating erythematous areas [299]. Since then only about 30 patients have been

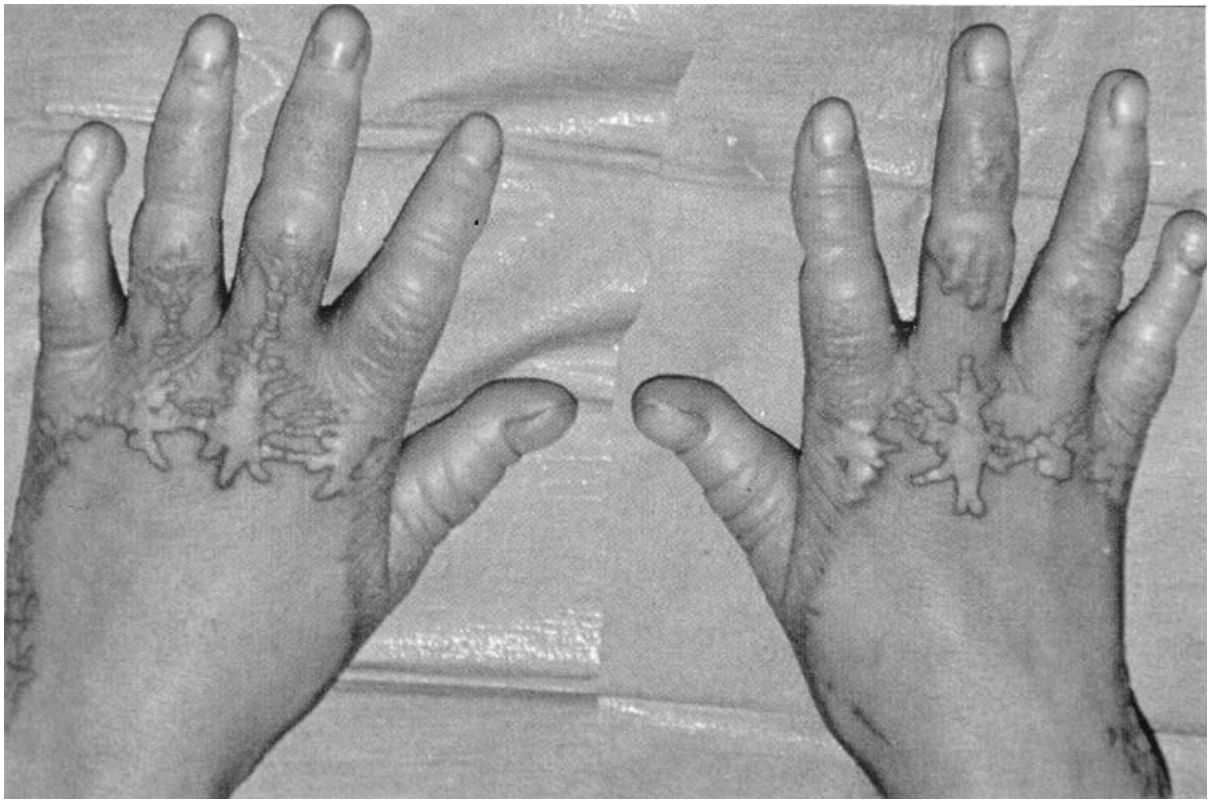

Figure 20. Vohwinkel syndrome with starfish hyperkeratoses on the knuckles.

described in the literature. Greither's disease, which primarily affects palmoplantar and neighboring surfaces, is sometimes confused with EKV, even to the extent that it is included under the same MIM number. Nevertheless, molecular data provide clear evidence that these two diseases do not cosegregate and are not allelic [300].

Clinical Features

The phenotype is present at birth or develops during the first year of life, but a delayed onset of the condition as late as early adulthood has been described. Affected individuals present either with generalized brown hyperkeratoses or with sharply demarcated hyperkeratotic plaques with fixed localization. The second distinct morphological feature includes migratory red patches (Fig. 21) that may appear or regress within minutes to hours. The development of the patches may be triggered by trauma or temperature changes. The predilection sites are the neck, the extensor surfaces of arms and legs, and the buttocks. Palmoplantar hyperkeratoses are present in about half of all cases.

Molecular Defect

After initial linkage to the Rh locus [301], EKV has been mapped to a region on chromosome 1 (1p34–p35), and the *GJB3* gene has been identified as the disease gene [302,303]. Point mutations in *GJB3*, which encodes connexin Cx31, have been identified in four families with EKV. The amino acid substitutions that were identified affected conserved residues and a dominant negative effect of the mutant connexin on the structure and function of the gap junction channel has been predicted. These findings highlight the role of connexins in epidermal differentiation. Examination of eight other EKV families did not reveal any *GJB3* mutations, indicating heterogeneity of the disorder [304]. Indeed, we have recently identified mutations in a novel human gene in EKV patients, proving molecular heterogeneity [305].

Treatment

Topical treatment using keratolytic agents such as urea, ammonium lactate, or propylene glycol, are often used. Alternatively, systemic retinoid treatment has proven effective and may be used in more severe cases [306].

Erythrokeratodermia with Ataxia (Giroux-Barbeau Type) (MIM 133190)

This syndrome was described by Giroux and Barbeau in 1972, when they discovered neurocutaneous findings in 25 members of a family in Montreal over five generations [307].

Clinical Features

Like erythrokeratodermia variabilis, erythrokeratodermia with ataxia is an autosomal dominant disease and symmetric hyperkeratotic plaques develop over extensor surfaces of the extremities. During adulthood, the skin involvement diminishes and spino-cerebellar symptoms develop. The neurological symptoms consist of severe ataxia, nystagmus, dysarthria, and decreased tendon reflexes.

Molecular Defect

The Giroux-Barbeau type of erythrokeratodermia has been mapped to the same locus on chromosome 1 as EKV [300], where a cluster of several connexin genes is located that are also expressed in the epidermis. Most likely, the Giroux-Barbeau type of EKV is not caused by *GJB3* mutations.

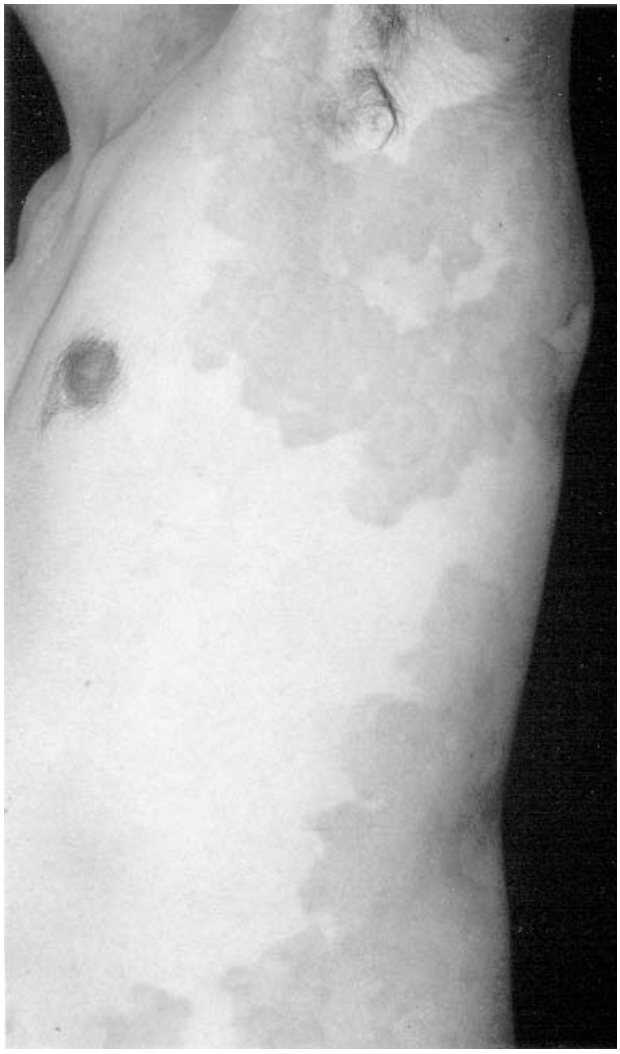

Figure 21. Erythrokeratodermia variabilis with variable, polycyclic annular, and gyrate erythema.

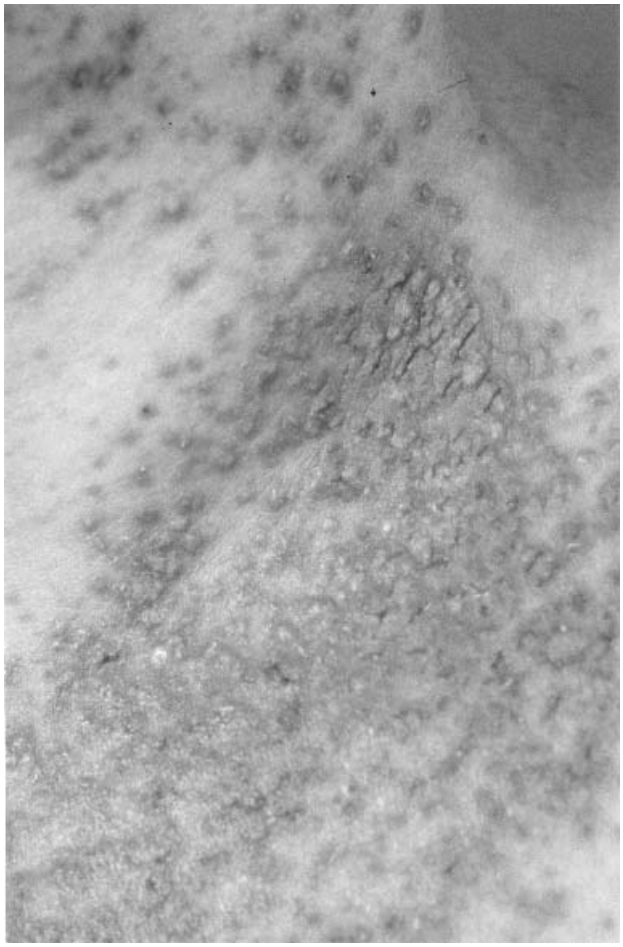

Figure 22. Darier-White disease with classical keratotic papules in the presternal area.

Darier-White Disease (DD; Keratosis Follicularis) (MIM 124200)

Darier-White disease is an autosomal dominant disorder with complete penetrance in adults, but variable expressivity. The incidence ranges from 1 in 100,000 in Denmark to 1 in 36,000 in north-east England [308,309]. It was first described by Darier and White in separate reports in 1889 [310,311].

Clinical and Histological Features

Darier-White disease typically presents between the ages of 10 and 15 years and is characterized by multiple, pruritic keratotic papules located predominantly in seborrheic areas of the skin such as flexures, scalp, face, forehead, chest, and back (Fig. 22). Lesions may, however, also occur in areas that lack sebaceous glands, such as palms and soles, and in epithelia such as mucous membranes and cornea. Mucous membranes may exhibit white, centrally depressed papules in the form of a "cobblestone" appearance. Initially, the lesions are skin colored, but are soon covered with a yellowish crust and may coalesce into large plaques and hypertrophic warty masses. The hair is usually normal; however, as a result of extensive scalp involvement with thick scales, alopecia has been observed. The nails are thin, exhibit longitudinal streaks and notches, and show a characteristic subungual thickening. Multiple plane keratoses, similar to those observed in acrokeratosis verruciformis (see below), are seen on the dorsum of the hands and feet. Disease severity ranges from mild affection of one or two areas to severe involvement of the entire skin, and several variants have been described. Unilateral, or segmental, Darier-White disease is characterized by one affected area, often with a zosteriform distribution of lesions, resembling epidermal nevus [312]. It is considered to be caused by postzygotic mutations, resulting in mosaicism. In the case of germline involvement, offspring with generalized DD can result, as described for the mosaic form of BCIE [37]. The most severe expression includes bullous, erosive and vegetating variants with involvement of the flexures or hypertrophic disease of the lower legs [313]. A hemorrhagic form with acral distribution of hemorrhagic vesicles and macules has been described [314]. Factors that precipitate exacerbations are heat and humidity, UV light, and mechanical friction. Bacterial and herpes simplex virus infections may cause exacerbations and complications [315]. Several neurological and psychiatric symptoms have been

associated with DD, including mood disorders, epilepsy, mental retardation, and progressive encephalopathy [316]. Mental retardation is usually of mild to moderate severity and may appear in up to 10–20% of patients. A few cases of slowly progressive encephalopathy have also been associated with Darier-White disease [317,318].

Histologically, suprabasal cleft formation is seen due to acantholysis within the spinous layer of the epidermis. Hyperkeratosis and dermal villi protruding into the epidermis are common. Eosinophilic, dyskeratotic round keratinocytes (*corps ronds*) in the spinous layer and the stratum corneum (grains) are characteristic of Darier-White disease. Ultrastructural examination reveals cytoplasmic vacuolization, perinuclear keratin filament clumps, and loss of desmosomal attachments. Grains occur in groups embedded in a hyperkeratotic horny layer within dyskeratotic lesions and are probably due to advanced acantholysis, whereas *corps ronds* form individually in regions lateral to grains, where hypergranulosis is prominent [319].

Molecular Defect

Extensive linkage studies have been performed on various families suggesting a region on 12q23–q24.1, close to the type II keratin gene cluster, which is located at 12q11–q13 [320–324]. Mutation analysis of several candidate genes has revealed mutations in the *ATP2A2* gene, which encodes the sarcoplasmic/endoplasmic reticulum calcium-ATPase type 2 isoform (ATP2A2 or SERCA2). SERCA pumps play a critical role in calcium signal transduction. In epithelial cells, IP_3 (inositol 1,4,5-triphosphate) acts a second messenger and triggers release of Ca^{2+} from the endoplasmic reticulum. Calcium pumps actively transport calcium back from the cytosol into the lumen of the endoplasmic reticulum by coupling ATP hydrolysis to the transfer of calcium ions [325]. Repetitive cycles of release and uptake of Ca^{2+} generate fluctuations in intracellular calcium concentration, thereby influencing gene expression and terminal differentiation [326]. The SERCA2b isoform is expressed predominantly in the epidermis and mutations are thought to result in loss of cellular adhesion and impaired differentiation [326]. The majority of mutations cause a frameshift and predict a premature termination of translation with nonsense-mediated RNA decay. The identified missense mutations cause nonconservative amino acid substitutions that result in a structurally abnormal polypeptide. This may impair specific interactions between functional domains of the ATP2A2 molecule [327]. The observation that missense mutations lead to severe or atypical clinical manifestations suggests that the corresponding domains are important for appropriate ATP2A2 function [326]. The clearest association between a missense mutation and an atypical phenotype is found with the familial hemorrhagic variant [327]. In neuropsychiatric cases, nonrandom clustering of mutations at the 3′ end of the gene, with a predominance of missense mutations, has been found [318].

Treatment

Mild variants can be treated with inert emollients and by sun avoidance. Moderate disease needs topical treatment with keratolytics, antiseptics, and antibiotics. More severe disease can be improved by topical 5-fluorouracil or tretinoin. Systemic retinoids such as acitretin at 0.25 mg/kg are initially efficient but require long-term contraception in fertile females [328,329]. Systemic antibiotic or antiviral treatment is advised in case of exacerbation by bacterial or herpes infections. All these treatments result in only transitory improvements which are followed by relapses. Surgical intervention in hypertrophic disease [330], CO_2 or YAG laser evaporization and earlier dermabrasion are also possible and may induce long-term remission, but can also be followed by relapses or even cause exacerbations.

Acrokeratosis Verruciformis (Hopf Disease) (MIM 101900)

Acrokeratosis verruciformis follows an autosomal dominant inheritance pattern and usually presents at birth or in early childhood. It was first described by Hopf in 1931, and studied in an affected pedigree by Niedelman and McKusick in 1962 [331].

Clinical Features

Small, warty hyperkeratotic lesions present predominantly on the dorsal aspect of the hands and feet and on the knees and elbows. They have a reddish-brown appearance and resemble flat warts. The palms may show punctate keratoses and the nails appear white from subungual hyperkeratosis [315]. Clinically similar or identical lesions are sometimes observed in Darier-White disease to the extent that it has been proposed that Darier-White disease and acrokeratosis verruciformis are manifestations of the same disease entity [332]. Nevertheless, the two diseases have a different histological picture and thus should not be confused. Indeed, we have observed a mother and two of her children in one of our own pedigrees who presented with isolated acrokeratosis verruciformis without associated signs of Darier-White disease and without its histological characteristics (D. Hohl, unpublished observation). Molecular analysis in such families should clarify this issue.

Hailey-Hailey Disease (HHD; Benign Chronic Pemphigus) (MIM 169600)

Hailey and Hailey first described this blistering dermatosis in 1939 [333]. It follows an autosomal dominant inheritance pattern and usually presents in the third or fourth decades of life.

Clinical and Histological Features

Hailey-Hailey disease is characterized by persistent blistering and erosions of the skin. The involvement of the skin can be limited to folds and intertriginous areas (Fig. 23), although widespread involvement is also possible [334]. Painful vesicles and blisters that develop into erosions and erythematous plaques due to friction and secondary infection present on an erythematous skin. The lesions tend to extend peripherally, thus producing a characteristic annular shape. Vesicles, blisters, and crusty and scaly areas are found over flexural areas of the body, predominantly in the neck, axilla, and groin. Lesions may also occur on the trunk, and in the antecubital and popliteal fossae, and a seborrheic dermatitis-type involvement of the scalp has been reported [335]. Painful rhagades appear in flexural areas, and these regions may become hypertrophic; bacterial overgrowth can lead to infections. Mucosal involvement is rare; however, oral, esophageal, vulvovaginal, perianal, and conjunctival involvement has been described [336]. Nail changes taking the form of asymptomatic longitudinal white bands in the fingernails may be present in up to 50% of patients and are a helpful physical sign [337]. The

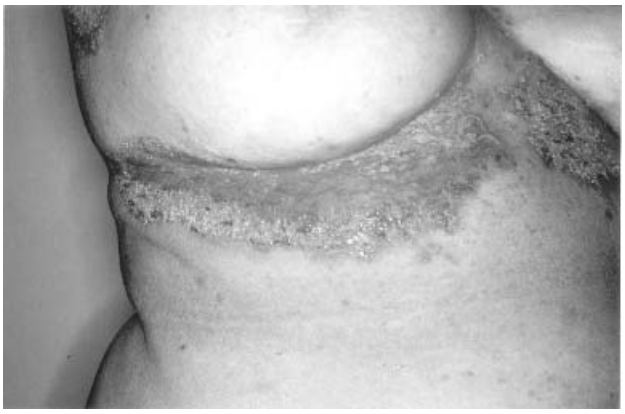

Figure 23. Hailey-Hailey disease with erythematous and exudative eroded plaques in the submammary intertriginous fold.

onset of clinical signs is usually in the second to fourth decades, but can be as soon as the first decade or delayed until after the age of 40 [337]. The course of the disease is characterized by recurrent exacerbations that can be triggered by UV light, mechanical friction, and infections, and there may be intermittent remissions that can last up to several years [337]. Histology shows the separation of suprabasal cells that leads to a loss of cohesion (acantholysis). The characteristic histological picture is reminiscent of a "dilapidated brick wall," and is due to extensive loss of intercellular bridges between keratinocytes. Ultrastructurally, aggregates of keratin filaments that are dissociated from desmosomes and are distributed perinuclearly can be seen in acantholytic cells.

Molecular Defect

Linkage analysis mapped the gene for Hailey-Hailey disease to a region on chromosome 3q [338] and mutations in a gene encoding a calcium ATPase, ATP2C1, have been identified in Hailey-Hailey patients [339]. Mutations identified include missense, nonsense, splice site, and frameshift mutations that are predicted to produce a truncated or absent protein. ATP2C1 is the human homolog of an ATP-powered pump that sequesters calcium in the Golgi apparatus in yeast. It is highly expressed in epidermal keratinocytes and may play an important role in maintaining calcium concentrations in the cytosol and within compartments of the Golgi apparatus. The regulation of cytoplasmic calcium is important in maintaining the adhesion and differentiation of stratified epithelia. In the epidermis, a calcium gradient exists with increasing concentration from the basal compartment to the granular layer, thus implying an important role for calcium in terminal differentiation. Dysfunction of the ATP2C1 pump may decrease intraluminal calcium stores in the endoplasmic reticulum and the Golgi apparatus, thus affecting post-translational modifications of key molecules of cell-to-cell adhesion that are carried out in the lumen of the Golgi apparatus. A defective calcium pump could also affect cytosolic calcium concentrations, thus impairing intracellular signalling pathways that regulate cell adhesion through the phosphorylation of target proteins or the regulation of gene transcription [340]. Dysfunction of a second ion-transport ATPase, ATP2A2, which is defective in Darier-White disease, seems to lead to similar alterations in calcium metabolism. However, dysfunction of the different calcium pumps seems to have distinct consequences in the differentiation pathway and it remains to be elucidated how *ATP2A2* mutations lead to dyskeratosis as a prominent feature in Darier-White disease and *ATP2C1* mutations cause erosions and blisters in Hailey-Hailey disease.

Treatment

Topical or systemic administration of anti-infective agents is the mainstay of therapy. Combination of antibiotics with topical corticosteroids has proven an effective treatment, especially when started early [337]. Short courses of systemic glucocorticoids seem to be useful in controlling major exacerbations, with the risk, however, of rebounds after withdrawal of the drugs [334]. Surgical intervention may be required to achieve prolonged remission or cure. Dermabrasion has been used and reepithelialization could be demonstrated to be complete after a week, followed by excellent functional and cosmetic long-term results [341]. Recently, patients have been treated with continuous CO_2 or erbium:YAG laser ablation that effectively removed lesions of both Hailey-Hailey and Darier disease and yielded excellent long-term results in chronic cases [342,343].

DISORDERS OF KERATINIZATION OF UNKNOWN MOLECULAR DEFECT

Autosomal Dominant Ichthyosis (ADI; Ichthyosis Vulgaris) (MIM 146700)

Autosomal dominant ichthyosis (ADI) is the most common of the ichthyoses and follows an autosomal dominant mode of inheritance. It has an incidence of 1 in 250 to 2000 individuals, and the onset of the disease phenotype is within the first year of life.

Clinical and Histological Features

The affected individual presents with mild xerotic and translucent scaling (Fig. 24), mostly prominent on the trunk and extensor surfaces, the flexural areas being spared. In comparison to X-linked recessive ichthyosis (XRI), the scales in ichthyosis vulgaris are smaller. Hyperlinear palms and soles with accentuated palmoplantar creases and keratosis pilaris are often associated with the disease. As with most ichthyoses, the condition improves in warm, humid weather. In 50% of cases, a family history of atopy is common [344]. This fact hides the key to why ADI has so far resisted elucidation by molecular genetic approaches. Indeed, xerotic dry atopic skin is often difficult to separate clinically from moderate "vulgar" ichthyosis. Additionally, the very frequent isolated follicular keratosis that occurs in up to 80% of the population further obscures the phenotypic classification. Past genetic linkage analysis may have failed for these reasons, suggesting that ADI might be a genetically heterogeneous group of diseases. Histological examination shows mild retention hyperkeratosis, that is, without acanthosis, and a diminished granular layer. Electron microscopy usually reveals small, abnormally shaped keratohyalin granules that may be reduced or totally absent.

Molecular Defect

Keratohyalin granules, an ultrastructural landmark in the granular layer of the epidermis, are decreased in number and structurally abnormal in ichthyosis vulgaris patients [345]. Filaggrin and its precursor, profilaggrin, are major components of keratohyalin granules. They play a role in the aggregation of keratin filaments in the stratum corneum of the epidermis and are found to be decreased

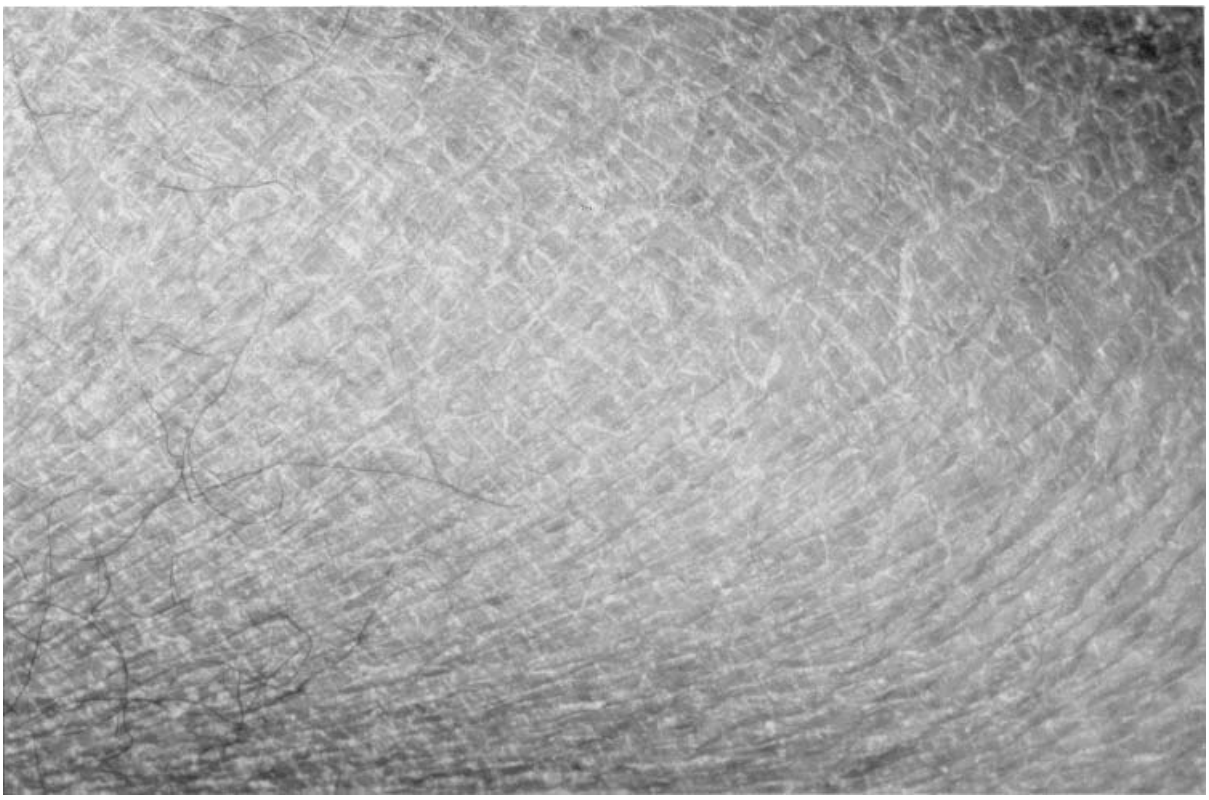

Figure 24. Autosomal dominant ichthyosis with fine adherent and translucent scaling.

in ichthyosis vulgaris [346–348]. A correlation has been established between the severity of the disease phenotype and the extent of filaggrin and profilaggrin reduction [349]. It has been proposed that the defective profilaggrin expression in ichthyosis vulgaris is the result of selectively impaired post-transcriptional control [350].

It has been demonstrated that desmosomes, which usually degrade in the granular layer, persist up to the stratum corneum in patients with ichthyosis vulgaris, although the number of desmosomes in the spinous layer of these patients is not significantly different from that in normal subjects [351]. The decreased enzymatic activities of trypsin-like and chymotrypsin-like serine proteases that are normally involved in desmosome breakdown may be responsible for the defective degradation of desmosomes that leads to the accumulation of excessive corneocytes in the stratum corneum, with the clinical formation of scales [352].

Treatment

Mild forms of ichthyosis vulgaris can be treated with creams and lotions that contain water and lipid. Urea is effective because of its water-binding capacity and keratolytics can be applied when hyperkeratoses predominate. Retinoids are not indicated in this relatively mild ichthyosis due to their potential adverse effects, especially in atopic patients.

Ichthyosis Hystrix of Curth and Macklin (IHCM) (MIM 146590)

Ichthyosis hystrix of Curth and Macklin is an extremely rare autosomal dominant disorder. Besides the family originally described by Curth and Macklin in 1954 [353], only one more family [354] and solitary cases have been reported to date [355,356]. Interestingly, the members of the originally described family exhibited both severe generalized and mild localized forms often restricted to physically exposed skin. The second family, however, presented with a mild localized form characterized by scaly plaques and mild hyperkeratoses on the knees, elbows, and ankles [354,357]. Solitary cases have all shown a generalized form of the disease.

Clinical and Ultrastructural Features

The clinical features are reminiscent of bullous congenital ichthyosiform erythroderma (BCIE), but blistering does not usually occur. The onset and clinical presentation of the disease are variable. In the generalized form, hyperkeratosis presents over extensor areas and is often widespread with a verrucous (histrix) appearance. In the mild localized form, rough skin areas or scaly plaques over extensor surfaces, such as the knees, elbows, and ankles may be the only clinical signs. Interestingly, one male patient in the family with localized IHCM [354] and one solitary case [356] exhibited undescended testes and possibly short stature in addition to the skin phenotype. Sulfatase deficiency was excluded as a potential underlying defect. It remains to be seen, however, if other genes on the short arm of the X chromosome might be defective in IHCM. Histological examination reveals severe orthokeratotic hyperkeratosis, acanthosis, and papillomatosis, similar to the findings in BCIE. Unbroken shells of tonofilament clumps surrounding the nucleus in keratinocytes of the upper spinous and granular

layers are characteristic features seen by electron microscopy. In contrast to BCIE, these concentric shells are unbroken, which allows a distinction from BCIE at the ultrastructural level.

Molecular Defect

Altered keratin expression has been suggested in the pathogenesis of IHCM [354]. However, linkage analysis has failed to identify keratin mutations as the underlying genetic defect [357]. It has been proposed that proteins involved in interactions with keratins, e.g., constituents of desmosomes, might be defective in IHCM [358].

Chanarin-Dorfman Syndrome (Neutral Lipid Storage Disease) (MIM 275630)

The Chanarin-Dorfman syndrome is a rare autosomal recessive disorder. To date, less than 30 cases have been reported in the literature. The accumulation of triglycerides in various tissues leads to multisystemic triglyceride storage. In granulocytes, fat-containing cytoplasmic vacuoles are seen upon ultrastructural examination. This phenomenon was described in two brothers with muscular dystrophy by Jordans and has been termed "Jordans' anomaly" [359]. A year after Dorfman first described "ichthyosiform dermatosis with systemic lipidosis" [360], Chanarin named the disease "neutral lipid storage disease" [361]. Today, the Chanarin-Dorfman syndrome is distinguished from a second type of neutral lipid storage disease in which ichthyosis is not present [362]. The incidence is higher among Arabic populations, which might be the result of a founder mutation many centuries ago. Heterozygotes can be identified by demonstrating vacuoles in circulating eosinophils [363].

Clinical and Histological Features

Generalized ichthyosis usually presents at birth, with fine, white scales and hyperkeratosis. Erythroderma, ectropion, and eclabium may be present. Nail and hair abnormalities in the form of nail ridging and scarring alopecia are complications in older patients. Cataracts, myopathy, fatty degeneration of the liver, and neurological symptoms, including horizontal nystagmus, ataxia and bilateral neurosensory hearing loss, are further abnormalities observed in association with the skin phenotype. These noncutaneous findings can vary in severity and may be progressive during adulthood [364]. Identification of lipid vacuoles in neutrophil granulocytes in the peripheral blood in association with ichthyosis usually leads to the diagnosis (Fig. 25). Histology reveals lipid accumulation in dermal and basal epidermal cells. Upon electron microscopic examination, lipid deposition is seen within the cytoplasm of basal and granular cells and within lamellar bodies. The stratum corneum is thickened and the broadened epidermis shows hyperkeratosis, acanthosis and papillomatosis.

Molecular Defect

The pathogenesis of Chanarin-Dorfman syndrome is poorly understood, but appears to be related to a perturbed intracellular triglyceride catabolism. The accumulation of triacylglycerol may be due to defects in synthesis and degradation of phospholipids [365].

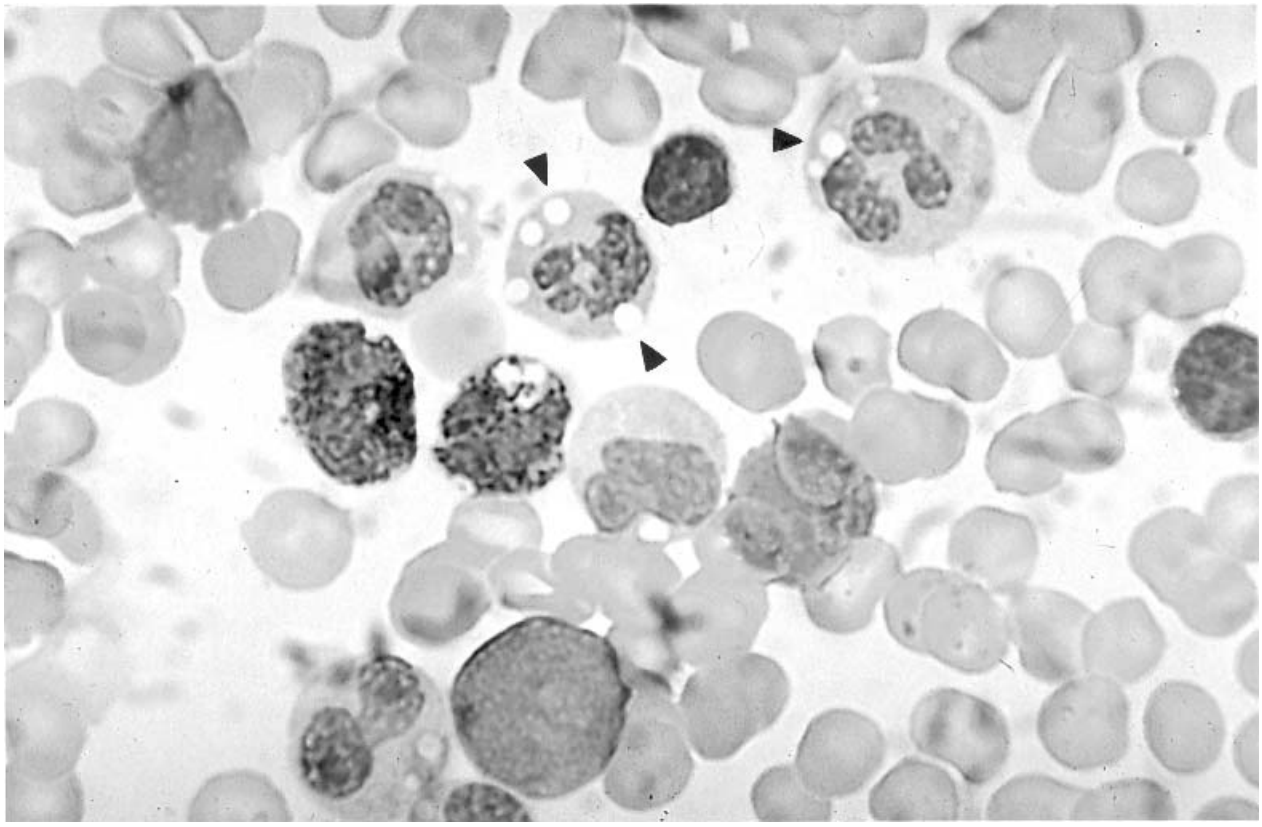

Figure 25. Chanarin-Dorfman syndrome: multiple lipid vacuoles in leucocytes (arrowheads). (Courtesy M. Landau, Tel Aviv.)

KID Syndrome (Keratitis-Ichthyosis-Deafness Syndrome) (MIM 148210)

The keratitis, ichthyosis, and deafness (KID) syndrome is a rare congenital disorder first described by Burns in 1915 [366] and called the KID syndrome by Skinner more than half a century later [367]. It affects ectodermal tissues such as epidermis, corneal epithelium, and the inner ear, which has prompted Caceres-Rios to propose renaming the syndrome as keratodermatous ectodermal dysplasia (KED) [368]. Its mode of inheritance is still unclear, and both autosomal dominant and autosomal recessive forms may exist. Three reports have suggested a dominant mode of inheritance [369–371]. Furthermore, the KID syndrome has been diagnosed in two siblings with unaffected parents, which does not exclude the possibility of gonadal mosaicism with dominant transmission [372]. In contrast, a report of two sisters born to consanguineous parents and a description of two half siblings born to the same unaffected mother favor an autosomal recessive mode of inheritance [373,374]. Spontaneous new mutations as the cause of the disease have also been proposed [375].

Clinical and Ultrastructural Features

At birth, erythroderma is usually present, but it diminishes during the first days of life. Ichthyosis usually develops during the first months and presents as a hyperkeratotic state with erythematous plaques that have a verrucous appearance. The plaques are well demarcated and predominate on the face and physically exposed areas of the limbs. Palmoplantar hyperkeratosis is almost always present. Children with KID syndrome look prematurely aged [375]. Congenital alopecia and scarring alopecia due to follicular hyperkeratosis have been described as a major feature in KID patients [368]. Eyelashes and eyebrows are often absent and nail dystrophy is frequent. Furthermore, patients suffer from neurosensory deafness and impaired vision caused by a keratitis with neovascularization of the cornea that can eventually lead to blindness [376]. Additional findings include increased susceptibility to skin infections, defective dentition, and hypohidrosis [368]. The risk of developing squamous cell carcinoma is increased [377] and oral leukoplakia, a premalignant lesion, was originally described by Burns [366]. Histologically, the stratum corneum is thickened, with acanthosis and papillomatosis, and the granular layer shows vacuolization [378]. Keratin filament clumping and accentuated insertion of intermediate filaments into desmosomes have been noted. Moreover, keratohyalin granules are distributed in a shell-like manner around the nucleus [375].

Treatment

Therapy with systemic retinoids has shown contradictory results in severely affected patients, the aromatic retinoid etretinate having brought about improvement of hyperkeratotic plaques [379] or having had only little effect [375]. Treatment with isotretinoin may result in an exacerbation of corneal neovascularization that makes close monitoring necessary [380].

HID Syndrome (Hystrix-Like Ichthyosis with Deafness) (MIM 602540)

The concurrence of ichthyosis hystrix and bilateral hearing loss was first described in a 17-year-old male [381,382]. Traupe named this syndrome hystrix-like ichthyosis with deafness (HID) [10].

Clinical and Ultrastructural Features

Clinical features include scarring alopecia and red patches that develop shortly after birth and evolve into verrucous, hyperkeratotic plaques, affecting the entire skin. The son of the individual reported in 1977 [381,382] has been diagnosed with HID syndrome: he showed the typical skin involvement in association with neurosensory hearing loss [383]. The differential diagnosis is the KID syndrome. The involvement of palms and soles is a feature characteristic of KID syndrome, and has not been reported in HID syndrome. Furthermore, the hyperkeratotic plaques seen in KID syndrome never involve the trunk, in contrast to their appearance anywhere on the skin in HID. Differentiation at the ultrastructural level is also possible. Characteristic features seen in HID include excess formation of mucous-containing granules and reduction of tonofibrils [10].

Peeling Skin Syndrome (PSS; Keratolysis Exfoliativa Congenita) (MIM 270300)

Peeling skin syndrome is a rare, autosomal recessive disorder that is characterized by lifelong peeling of the epidermis, and the easy ability to separate the stratum corneum mechanically from the rest of the epidermis [384]. PSS is, in fact, a heterogeneous group of disorders and designates several different clinical entities with large overlaps that were classified by Traupe into two distinct types, A and B [10]. This classification has been expanded to incorporate a third type, PSS type C [385].

Clinical and Histological Features

The clinical presentation in PSS type A starts at birth or in early childhood with generalized peeling of the stratum corneum. Thin, superficial flakes cover the entire skin, including the face [386]. The peeling is usually noninflammatory and asymptomatic [387]. Ultrastructural analysis shows orthohyperkeratosis and separation of corneocytes above the granular cell layer. The cleavage occurs within the cytoplasm of corneocytes, leaving the plasma membrane firmly adherent to the underlying cell, while the upper part of the cell is exfoliated [388].

In PSS type B, congenital ichthyosiform erythroderma is present at birth and develops into erythematous migratory patches with a peeling border over the entire skin, usually sparing palms and soles [384]. The erythematous lesions have an exfoliative character, and can be accompanied by pruritus [389]. Associated noncutaneous anomalies and abnormal laboratory findings include short stature, aminoaciduria and elevated IgE levels. Seasonal variations have been reported, and patients are usually more susceptible to skin infections [390]. Histologically, parakeratosis and acanthosis are seen and the granular layer is absent [384]. The entire stratum corneum separates from the underlying acanthotic epidermis and, in contrast to the A type, the split occurs intercellularly [390].

A further variant of PSS has been delineated on the basis of distinct clinical findings and designated PSS type C [385]. The onset of the skin phenotype in this case was at birth, with generalized scaling and peeling. Pruritus and an elevated IgE level were associated features and the patient was more susceptible to skin infections. In contrast to the two other types, superficial blisters formed on palms and soles early in childhood and developed into hyperkeratoses. Moreover, a severe fissured cheilitis developed and crusted keratoses were seen on the eyelids, nostrils, and lips. Ultrastructural examination showed a

cleavage at the interface between the stratum corneum and stratum granulosum, on the corneocyte side of the desmosomal plaque [385].

Another variant form of PSS was reported in a patient with features similar to the previously reported case of PSS type C [385], but with associated hair defects. The authors classified this case as a typical PSS type B [391]. In addition to generalized ichthyosiform erythroderma with peeling on hands and feet, the patient exhibited cheilitis and fissuring of the nostrils, and conjunctivitis and blepharitis. The cleavage occurred at the corneal-granular cell desmosomal connection. Additionally, this patient exhibited trichorrhexis invaginata-like changes, pili torti-like changes, and hair shaft torsions, features that have not been described in PSS before, suggesting that these dysmorphic hair changes might be an integral component of this form of PSS. Given the marked overlaps between the two cases, the question arises as to whether they are indeed different subtypes or rather different manifestations of the same PSS subtype. Analysis at the molecular level and identification of the disease gene(s) might eventually allow a clearer classification.

A localized form of PSS, with strict localization of the lesions on the palms and dorsal aspects of the fingers has also been reported [392]. Another case with involvement of arms and legs was classified as "acral PSS" [393]. The splitting was found to be in the stratum corneum without inflammatory changes, similar to the findings in PSS type A, whereas the underlying erythema is a feature of PSS type B. Due to the strict palmar localization of the disease, it was proposed that this form should be referred to as "localized PSS."

Treatment

Oral acitretin has been shown to improve the skin condition. However, a persistent erythema has been observed.

Ichthyosis Follicularis, Atrichia, and Photophobia Syndrome (IFAP Syndrome) (MIM 308205)

The IFAP syndrome is a rare neuroichthyosis that has been described to date in only 10 males [394]. It is considered to be an X-linked recessive trait.

Clinical Features

Patients with IFAP syndrome present with congenital absence of hair (atrichia), generalized ichthyotic skin changes with extensive noninflammatory follicular hyperkeratoses, and photophobia [395]. A mild collodion presentation at birth has been reported [10]; later, follicular keratosis is seen on the scalp, abdomen, and extensor surfaces of the limbs, especially the knees. Associated features are severe photophobia, retardation of growth and psychomotor development, seizures, bronchial asthma, urticaria, skin infections, and nail dystrophy [396].

Another presumably X-linked recessive disorder is the dermotrichic syndrome, which presents with similar features to IFAP syndrome, such as generalized ichthyosiform lesions and baldness from birth. Whereas photophobia and recurrent respiratory infections are characteristic of the IFAP syndrome, nail dystrophy, hypohidrosis, megacolon, and vertebral defects are characteristic of dermotrichic syndrome [397]. Histological examination in IFAP syndrome shows acanthokeratosis and a well preserved granular layer. The hair follicles are poorly developed and shortened and sebaceous glands are completely absent. The IFAP syndrome is characterized by a wide clinical variability and some features, such as growth and motor development retardation, torticollis, and proneness to infections, have only been observed in a few cases. Therefore, it has been speculated that these cases might be the expression of a contiguous gene defect [10].

RECENT DEVELOPMENTS

Mal de Meleda (MDM) (MIM 248300)

Mal de Meleda belongs to the family of hereditary palmoplantar keratodermas and is characterized by hyperkeratosis of palms and soles, keratotic skin lesions, perioral erythema, brachydactyly and nail abnormalities. The progressive lesions often lead to severe impairment of hands and feet including spontaneous auto-amputation. The disorder is transmitted in an autosomal recessive mode, and the MDM gene has recently been identified as *ARS* (component B), encoding a protein named SLURP-1 (for secreted Ly6/uPAR-related protein 1 [399]. Three different homozygous mutations were detected in 19 families of Algerian and Croatian origin, suggesting founder effects.

Members of the leukocyte antigen-6 (Ly6)/urokinase-type plasminogen activator (uPAR) superfamily are either secreted or receptor proteins and have been implicated in transmembrane signal transduction, cell activation and cell adhesion. SLURP-1 is preferentially expressed in palmoplantar skin and thought to function as a ligand for an as yet unknown receptor.

Clouston Syndrome (Hydrotic Ectodermal Dysplasia, HED) (MIM 129500)

Patients with Clouston syndrome present with diffuse palmoplantar hyperkeratosis, hair defects ranging from partial to total alopecia, and severe dystrophy of the nails. Hyperpigmentation of the skin, especially over the joints, strabismus, hearing impairment and mental deficiency occur in some.

A recent study has confirmed linkage of Clouston syndrome to chromosome 13q11-q12.1 in a large French family and demonstrated two dominant missense mutations (resulting in Gly11Arg and Ala88Val) in the gene encoding connexin 30 (*GJB6*) [400]. The *GJB6* mutations might cause Clouston syndrome by a dominant-negative effect on normal connexin 30 activity, or through haploinsufficiency of gap-junction channels. Another missense mutation in the *GJB6* gene (resulting in Thr5Met) was associated with autosomal dominant nonsyndromic sensorineural deafness [401]. To understand how different mutations of the same gene cause non-syndromic deafness and HED, respectively, it will be important to study the mutant connexins in the context of other connexins expressed in the affected tissues. It is intriguing to speculate that other genetic and environmental factors may modify the penetrance of the mutations.

Erythrokeratodermia Variabilis (EKV) (MIM 133200)

In addition to the previously reported mutations in the gene encoding connexin 31 (*GJB3*), mutations in the novel human connexin 30.3 have been identified in a form of EKV with erythema gyratum repens, highlighting the genetic heterogeneity of this disorder [402]. The mutation in the *GJB4* gene affects a highly conserved phenylalanine residue in the third transmembrane region of the connexin 30.3 molecule, known to be implicated in wall formation of the gap-junction pore. Replacement of phenylalanine by leucine may lead to a faster closure of the gap-junction channel,

thereby inhibiting the transfer of signal molecules that are important for normal epidermal growth and differentiation. In classical EKV, migratory erythematous lesions tend to turn gradually into fixed keratotic plaques. In annular EKV caused by connexin 30.3 mutations, rapidly migrating erythematous lesions often appear as figurate erythema with an annular or spiral arrangement, sometimes presenting even as erythema gyratum repens [402]. In fact, this distinct phenotype has been described previously [403,404], but has passed largely unnoted.

Ichthyosis Hystrix of Curth and Macklin (IHCM) (MIM 146590)

Even though earlier studies have provided evidence against the linkage of IHCM to the keratin genes [357], a mutation in the variable tail domain V2 of the keratin 1 molecule has been identified in a patient with IHCM [405]. The heterozygous frameshift mutation creates a premature termination codon and is predicted to result in a truncated protein. Loss of a region that is almost exclusively composed of glycine and hydrophilic residues results in different chemical characteristics of the mutant protein. Structural analysis has revealed a failure of keratin filament bundling, retraction of the cytoskeleton from the nucleus, and failed translocation of loricrin to the desmosomal plaque. It is proposed that the V2 domain of keratin 1 mediates the supramolecular organization of keratin intermediate filaments and facilitates the translocation of loricrin to the cell periphery during cornified cell envelope formation.

Autosomal Recessive Congenital Ichthyoses (ARCI)

Linkage to a sixth locus has been described for the group of autosomal recessive congenital ichthyoses [406]. In pedigrees from Germany and Turkey a new ARCI locus has been identified on chromosome 17p. It could be shown, however, that the disease in two Arab families could neither be linked to 17p, nor to one of the five loci described previously, suggesting further heterogeneity.

ELECTRONIC DATABASES

- OMIM™, Online Mendelian Inheritance in Man, available at *http://www.ncbi.nlm.nih.gov/omim/*
- McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University, Baltimore, MD, available at *http://hopkins.med.jhu.edu/HealthcarePros/referral.new/dept/med/genetic/*
- National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD, available at *http//www.ncbi.nlm.nih.gov/*

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Chapter 25

Alport Syndrome

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SUMMARY

- **Alport syndrome is a progressive hereditary nephritis characterized by hematuria. Extrarenal complications, such as sensorineural hearing loss and ocular abnormalities, are often associated with the disease. Typical ultrastructural changes, such as thinning, thickening, and splitting of the glomerular basement membrane (GBM) can be observed by electron microscopy.**
- **Alport syndrome is mainly inherited as an X-linked dominant trait that affects males more severely than females. The disease is caused by mutations in the *COL4A5* gene that encodes the basement membrane-specific type IV collagen α5 chain.**
- **In the rarer autosomal recessive and dominant forms of Alport syndrome, males and females are equally severely affected. However, the symptoms are milder in the autosomal dominant form. Mutations have been found in the *COL4A3* and *COL4A4* genes encoding the type IV collagen α3 and α4 chains, respectively.**
- **Numerous deletions involving the 5′ ends of both the *COL4A6* gene and the adjacent *COL4A5* gene have been reported to cause a rare disorder, diffuse leiomyomatosis, associated with Alport syndrome.**
- **For unknown reasons, a minority of Alport syndrome patients develop a severe anti-GBM nephritis after transplantation, leading to eventual rejection of the allograft.**
- **About 300 different mutations have been reported in type IV collagen genes in Alport syndrome patients. These include large and small deletions and insertions, as well as single base changes. Different mutations cause different phenotypes, but it is difficult to predict the consequences of a particular mutation. However, in most cases, mutations can be considered somehow to disturb the normal formation of basement membrane.**
- **Studies with both produced and naturally occurring animal models of Alport syndrome are of help for better understanding the molecular pathology of the disease.**
- **Promising results have been achieved in animals concerning development of gene therapy for Alport syndrome.**

INTRODUCTION

Alport syndrome, also termed hereditary nephritis, was the first basement membrane disease the pathogenesis of which was elucidated at the gene level [1,2]. The disease was initially described in 1927 by A.C. Alport as an inherited kidney disease characterized by hematuria and sensorineural deafness [3]. Later, ocular lesions were also noted to be associated with the syndrome and, with the introduction of the electron microscope, irregularities and disruptions in the glomerular basement membrane (GBM) were shown to be typical of this disorder as well [4]. The disease is progressive, usually leading to renal failure during adolescence or before middle age. Based on the age of onset of end-stage renal disease (ESRD), Alport syndrome is divided into two clinical subgroups. In the juvenile type the mean age of ESRD in men is about 18 years, while in the adult type it is about 37. A good dividing line between the two types is 31 years of age [5].

Alport syndrome is mainly inherited as an X-linked dominant trait (MIM 301050) with an estimated gene frequency of 1:5,000, but autosomal forms also exist [4]. The defective gene in X-linked Alport syndrome was localized in 1988 and 1989 to the long arm of the X chromosome [6–8]. In 1990, the *COL4A5* gene encoding the type IV collagen α5 chain was discovered and localized to the Alport gene region on chromosome X, and this was soon followed by the identification of mutations in this gene in Alport patients [1,2,9]. More recently, mutations have also been reported in the genes for the α3 and α4 type IV collagen chains in the rarer autosomal forms of Alport syndrome [10–13]. Presently, about 300 different mutations have been identified in type IV collagen genes in Alport patients, almost all of them in the *COL4A5* gene [14–19,22]. These mutations can be considered responsible for abnormalities in the structural framework of the GBM, with resultant kidney manifestations.

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CLINICAL FEATURES, DIAGNOSTIC CRITERIA, AND FORMS OF ALPORT SYNDROME

Clinical Findings and Natural History

The clinical picture of Alport syndrome is somewhat heterogeneous [4,5]. Characteristically, Alport patients have recurrent microscopic or gross hematuria in childhood, earlier in males than in females. It usually leads to end-stage renal disease in affected males and, in rare cases, also in females. The hearing loss is sensorineural and primarily affects high tones. Electron microscopy usually reveals thinning and thickening of the GBM with longitudinal splits into thin layers with a basket-weave pattern (Fig. 1). These changes are most evident in male patients, except in boys of a very young age. Lenticonus, a conical protrusion of the substance of the crystalline lens, is also frequently observed in Alport patients. Thrombocytopenia is sometimes associated with the syndrome. The disease is inherited and there is a family history in 85% of cases, the remaining 15% probably representing new mutations [4].

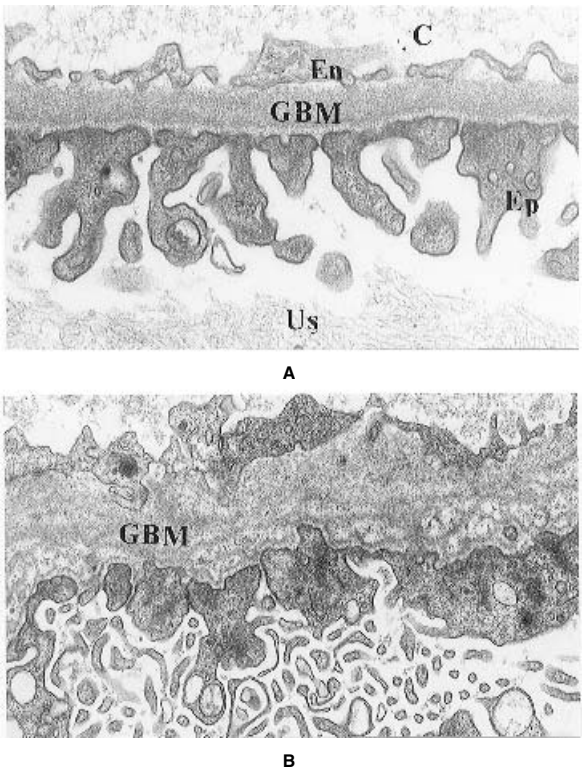

Figure 1. Electron microsopic picture of the glomerular basement membrane (GBM) of normal and Alport syndrome kidneys. **A**, The normal filtration barrier consists of the fenestrated endothelial layer (En) of the glomerular capillary (C), the ~300–350-nm-thick GBM, and the epithelial podocytes (Ep) partially covering the GBM on the urinary space side (Us). The podocytes are separated by ~25-nm-wide filtration slits. Note the uniform thickness and even amorphous pattern of the GBM. **B**, Electron micrograph of a glomerular capillary loop showing characteristic glomerular basement membrane (GBM) lesions in an Alport syndrome patient. The GBM has irregular thinning and thickening, as well as lamination of the structure. C, capillary lumen; En, endothelial cell; Ep, epithelial podocyte; Us, urinary space. Magnification ×22,000. (Courtesy, Dr. Finn P. Reinholt, Nephropathology Laboratory, Huddinge Hospital and Karolinska Institutet, Sweden.)

Diagnostic Criteria

For decades, physicians have expected hearing loss to be an essential part of hereditary nephritis. Flinter et al. [20] proposed that a diagnosis of Alport syndrome should be made in hematuric patients who fulfilled three of the following criteria; (a) familial history, (b) hearing loss, (c) ocular lesions, and (d) GBM abnormalities. However, recent data have shown that there are numerous examples of patients with progressive renal disease and mutations in type IV collagen genes who do not fulfil these criteria. Thus, inheritance cannot always be demonstrated, as *de novo* mutations may account for as many as 15% of cases. Also, it has been demonstrated that quite a large proportion of patients with renal failure and mutations in type IV collagen genes do not have hearing loss or eye manifestations [14,21,22]. So, how should Alport syndrome be defined? We have recently proposed that the disease should be defined as progressive hematuric hereditary nephritis caused by mutations in type IV collagen genes [22]. As discussed above, present data indicate that these genes are those encoding the α3(IV), α4(IV), and α5(IV) chains of type IV collagen, i.e., *COL4A3*, *COL4A4*, and *COL4A5*, respectively. However, as pointed out by Gregory et al. [5], physicians do not always have enough information to make a genetic diagnosis, and need an operational definition as a basis for action. Based on the new information accumulated on the disease, they have, therefore, proposed a new clinical definition requiring 4 out of 10 criteria (Table 1). This definition probably provides the diagnosis needed for clinical action. The ultimate diagnosis will, however, always require DNA analysis.

TABLE 1. Criteria for the Clinical Diagnosis of Alport Syndrome According to Gregory et al. [5]: 4 of the 10 Criteria Should be Fulfilled to Provide a Positive Diagnosis

1. Family history of nephritis or unexpected hematuria in a first degree relative of the index case or in a male relative linked through any number of females.
2. Persistent hematuria without evidence of another possibly inherited nephropathy such as thin GBM disease, polycystic kidney disease, or IgA nephropathy.
3. Bilateral sensorineural hearing loss in the 2000–8000 Hz range. The hearing loss develops gradually, is not present in early infancy, and commonly presents before the age of 30 years.
4. A mutation in *COL4A3*, *COL4A4*, or *COL4A5*.
5. Immunohistochemical evidence of complete or partial lack of the Alport epitope in glomerular, or epidermal basement membranes, or both.
6. Widespread GBM ultrastructural abnormalities, in particular thickening, thinning, and splitting.
7. Ocular lesions including anterior lenticonus, posterior subcapsular cataract, posterior polymorphous dystrophy, and retinal flecks.
8. Gradual progression to ESRD in the index case and in at least two other members of the same family.
9. Macrothrombocytopenia or granulocytic inclusions.
10. Diffuse leiomyomatosis of the esophaghus or female genitalia, or both.

Autosomal Recessive Alport Syndrome (MIM 203780)

As discussed above, Alport syndrome is mainly transmitted as a dominant X-linked trait. However, in about 15% of kindreds, autosomal transmission is obvious, including both autosomal recessive and, in very rare cases, autosomal dominant modes. Autosomal recessive disease should be suspected in cases of symptomatic individuals lacking a positive family history, especially when females are as severely affected as males [23]. In the much less common autosomal dominant form of the disease, males and females are equally severely affected. Still, individuals show a milder phenotype than is usually found in the X-linked form, i.e., an older age of onset of ESRD, minimal deafness, and an absence of eye signs [24].

Alport Syndrome–Associated Diffuse Esophageal Leiomyomatosis (MIM 308940,303631)

There is presently no direct evidence showing that mutations in the α6(IV) collagen chain *COL4A6* gene alone can cause Alport syndrome. Numerous deletions in the 5′ end of the *COL4A6* gene extending into the adjacent *COL4A5* gene have been reported to cause an Alport syndrome and diffuse leiomyomatosis (DL-AS) association first described by Garcia-Torres and Guarner in 1983 [25]. DL is a rare disorder characterized by benign proliferation of smooth muscle cells in the esophageal wall [26,27]. Both sporadic and hereditary cases have been described [27–30]. In addition to the esophagus, other organs such as the female genital tract and tracheobronchial tree may be affected. The clinical features and molecular genetics of DL have been reviewed by Antignac and Heidet [27]. The first report on mutations in DL-AS involved three patients with deletions at the 5′ end of the *COL4A5* gene and extending beyond it [31]. Later, it was shown that the upstream breakpoints were located within intron 2 of the *COL4A6* gene, which lies in a head-to-head fashion with *COL4A5* only 450 bp upstream [32]. Because *COL4A6* has been shown to be expressed in smooth muscle, it has been hypothesized that inactivation of this gene is the cause of DL-AS [32]. However, larger deletions of *COL4A6* associated with deletions initiating in *COL4A5* have been reported in a few patients with Alport syndrome without DL [33]. These results indicate other potential explanations for the development of DL than simply inactivation of *COL4A6*. For example, it is possible that there are other as yet unidentified genes located within the 5′ ends of the *COL4A5* or *COL4A6* genes. Intron 2 of *COL4A5* and intron 3 of *COL4A6* are possible locations as these introns are huge in size, the latter being estimated to be 340 kb [34–36] (see also Chapter 2, Part II, this volume).

Alport Syndrome with Antiglomerular Basement Membrane Disease (MIM 301050,203780)

Electron microscopic studies carried out in the late 1960s already indicated molecular abnormalities in a structural component(s) of the GBM. In 1976, McCoy et al. [37] showed by immunohistochemical staining with autoantibodies from Goodpasture syndrome patients that some Alport patients lacked the Goodpasture antigen, a finding that was confirmed later in several studies. The Goodpasture antigen has been shown to be located in the NC1 domain of the α3(IV) chain [38]. This NC1 domain, which can be purified from the GBM after digestion with bacterial collagenase, has a molecular weight of 28 kDa. This led researchers to believe that the α3(IV) chain gene was defective in Alport syndrome. However, as shown later and described above, this could not be the case in the X-linked form of the disease, since this gene resides on chromosome 2. Indications for the existence of another as then unidentified component of type IV collagen that might be involved in Alport syndrome came from studies by McCoy et al. [39], and later by several other investigators, showing that some Alport patients developed antibodies against renal allografts. Some of these antibodies have been shown actually to react with the 28 kDa NC1 domain of the α3(IV) chain, but some identify an NC1 domain-like protein of 26 kDa [40]. This 26 kDa component is now known to represent the NC1 domain of the α5(IV) chain, which is known to be mutated in a number of patients with X-linked Alport syndrome. Recently, Brainwood et al. [41] studied the targets of the alloantibodies of 12 Alport patients who had developed post-transplant anti-GBM disease. Western blot analysis of recombinant NC1 domains produced in insect cells showed selective binding to α5(IV)NC1 in nine cases. In one case, antibody showed binding to both α5(IV)NC1 and α3(IV)NC1, while two cases showed predominant binding to α3(IV)NC1. Seven of the patients who had X-linked inheritance all showed anti-α5 reactivity, and one with α3 reactivity had a *COL4A3* mutation. In spontaneous anti-GBM disease (anti-GBM or Goodpasture syndrome, MIM 233450), the major target of autoantibodies is the α3(IV)NC1 domain [42–44], but the primary target in most Alport anti-GBM patients is the NC1 domain of the α5 chain of type IV collagen [41].

STRUCTURE AND MOLECULAR BIOLOGY OF THE GLOMERULAR BASEMENT MEMBRANE

General

The basement membrane is a thin, ubiquitous, sheet-like type of extracellular structure that is located at the interphase of cells of organized tissues and the connective tissue. Basement membranes are found beneath all epithelia and endothelia, as well as surrounding muscle fibers, fat cells and peripheral nerves (Fig. 2). They perform important biological roles by providing structural support for cells, and mediating differentiation, adhesion, and migration, as well as proliferation of cells. They also function as filters for macromolecules, as exemplified by the renal glomerular basement membrane (GBM), and they are important in tissue regeneration, such as reepithelialization and renervation [45].

The thickness of a basement membrane can vary between 50 and 350 nm. By electron microscopy, it is often observed to contain two morphologically distinct layers, the lamina lucida (rara) and lamina densa. Some basement membranes, such as the GBM, have three layers, the lamina rara interna, lamina densa, and lamina rara externa, as this particular type of basement membrane is formed by two oppositely located cell layers, the capillary endothelium and the epithelial podocytes of the urinary space (Fig. 1). These layers may be artefacts of the routine fixing techniques, as studies of tissue samples made by the quick-freeze method have shown the basement membranes not to contain any distinct layers [46].

The Glomerular Filtration Barrier

The major complications of Alport syndrome, hematuria and proteinuria, are caused by structural alterations and consequent malfunction of the glomerular filtration barrier. The renal glomerulus functions as the ultra-filtration unit for plasma (Fig. 3). There are about one million glomeruli

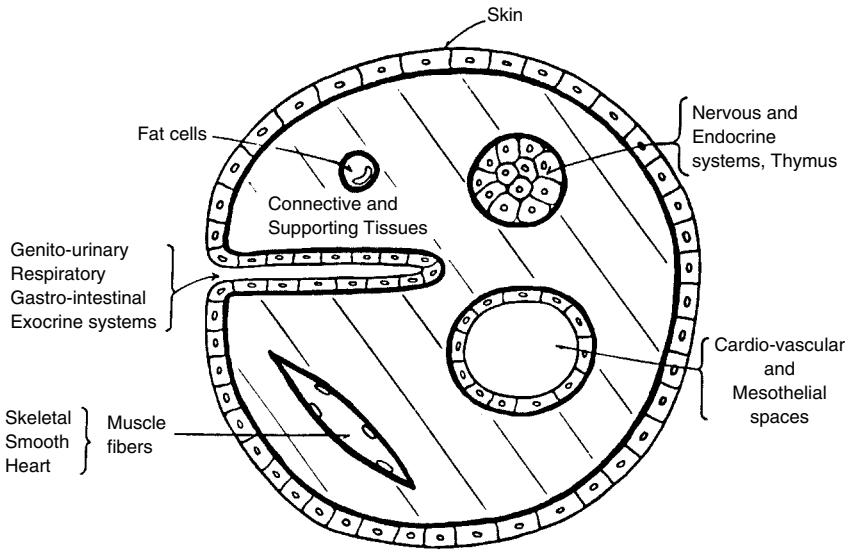

Figure 2. Schematic illustration of the distribution of basement membranes in the human body. The basal laminae are shown as heavy lines located in a characteristic manner between organized tissue cells and the interstitial connective tissue. The organ cells include all epithelial cells of epidermis and epidermal appendages of the respiratory, gastrointestinal, and genitourinary tracts, all exocrine glands, endothelial cells of the cardiovascular system, mesothelial cells of body cavities, cells of the central and peripheral nervous systems, endocrine glands, muscle fibers, and fat cells. The connective tissue (dashed area) includes interstitial stroma, bone, and cartilage and their associated cells. (Reprinted, with permission from [45].)

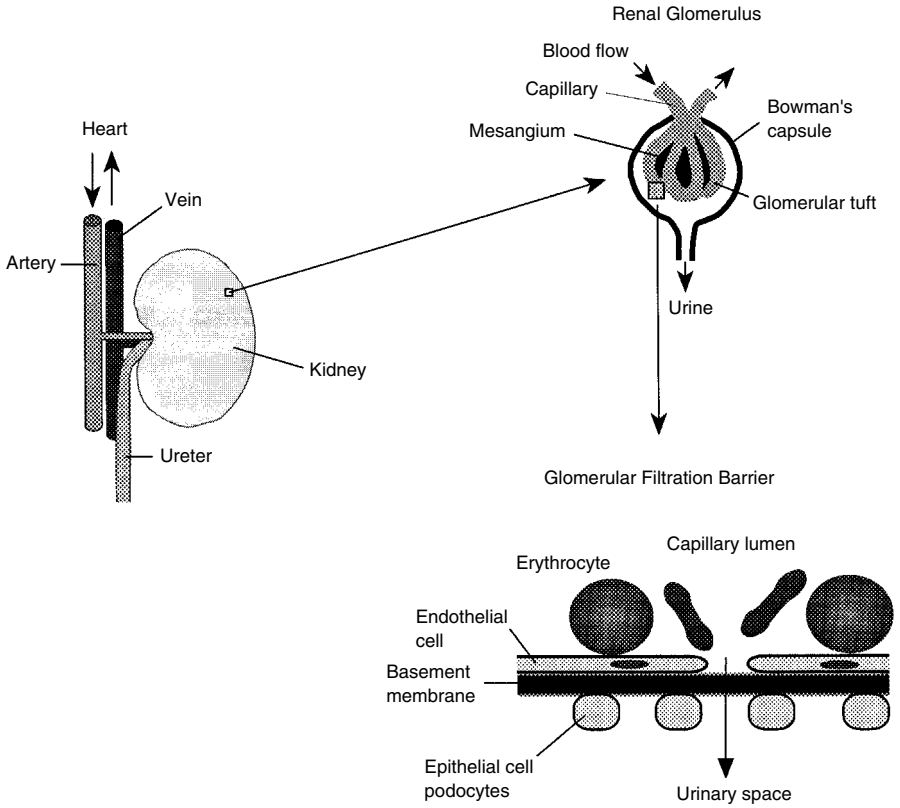

Figure 3. Schematic illustration of the kidney, a filtration unit (glomerulus), and the glomerular filtration barrier. Each kidney contains about one million ball-shaped glomeruli in which the filtration of blood occurs. The glomerular capillaries divide and form a set of loops (glomerular tuft) which are located inside Bowman's capsule. The filtration of blood occurs in the capillaries so that only small proteins and waste products together with water can pass into the urinary space. The actual filtration barrier consists of the fenestrated capillary endothelium, the glomerular basement membrane, and the filtration slits located between the foot processes (pedicels) of the glomerular epithelial cells (podocytes). The illustrations are not drawn to scale.

per human kidney. During morphogenesis, a glomerulus is formed by the invagination of a capillary with mesenchymal mass into the epithelial lining of a Bowman's capsule. The capillary divides into several loops (glomerular tuft). The inner part of the capillary faces the mesangium, while the outer part forms the filter. The filtration barrier consists of three layers: (a) a fenestrated endothelial layer, (b) a basal lamina (glomerular basement membrane; GBM) of about 300–350 nm in thickness, and (c) podocytes, interdigitating epithelial cells, that almost completely cover the external surface of the GBM. The adjacent processes are separated by 20–30 nm wide slit pores, or filtration slits, which are covered by thin diaphragms [47]. Under normal conditions, the filtration barrier practically completely excludes from the filtrate plasma proteins of the size of albumin (67 kDa) or larger, while exhibiting an extremely high permeability to water and small solutes. Filtration across the barrier depends on the size of the molecules, i.e., it is a size-dependent permeability barrier [48]. Filtration decreases with increasing molecular radius. Additionally, the filtration barrier discriminates between molecules according to their charge, cationic and neutral molecules traversing the membrane more readily than anionic molecules of the same size [49].

Molecular Composition of the Glomerular Basement Membrane

In general, the molecular structure of the GBM does not differ from that of basement membranes in other tissues. Type IV collagen is the major structural component that forms a tightly cross-linked meshwork connected through entactin (nidogen) to a less dense laminin network. In addition to contributing to the three-dimensional structure, laminin is a major adhesion molecule which also plays a role in cell differentiation and migration (see also Chapter 5, this volume). Type IV collagen and laminin are both complex heterotrimeric proteins that exist in numerous more or less tissue-specific isoforms. Other minor components include BM40 (osteonectin, SPARC), fibulins, and agrin, that are present in some specialized types of basement membranes [50,51].

Type IV Collagen

The scaffold of the basement membrane is a dense, tightly cross-linked protein meshwork made up of type IV collagen (see also Chapter 2, Part I, this volume), which is specific to the basal lamina [52]. The type IV collagen molecules that form the building blocks of the basement membrane are typical elongated triple-helical collagen molecules composed of three α chains. Each α chain is composed of an approximately 1,400-residue collagenous domain with Gly–X–Y repeats interrupted at several sites by short noncollagenous sequences, an aminoterminal ~15-residue noncollagenous segment and a carboxyl-terminal ~230-residue noncollagenous (NC1) domain (see Fig. 4A). The noncollagenous amino terminus and its short neighboring cysteine-rich collagenous sequence is referred to as the 7S domain [53]. The presence of a glycine residue as every third amino acid in the collagenous domain is essential, as it is the only amino acid small enough to fit into the center of the collagen triple helix. The interruptions in the Gly–X–Y repeat sequences are thought to provide the triple helical molecules with flexibility. The type IV collagen α chains are highly glycosylated, with about 50 hydroxylysine-linked disaccharide units and an asparagine-linked oligosaccharide unit in the 7S domain [54,55].

Six genetically distinct type IV collagen α chains, α1(IV)–α6(IV), have been characterized. The most abun-

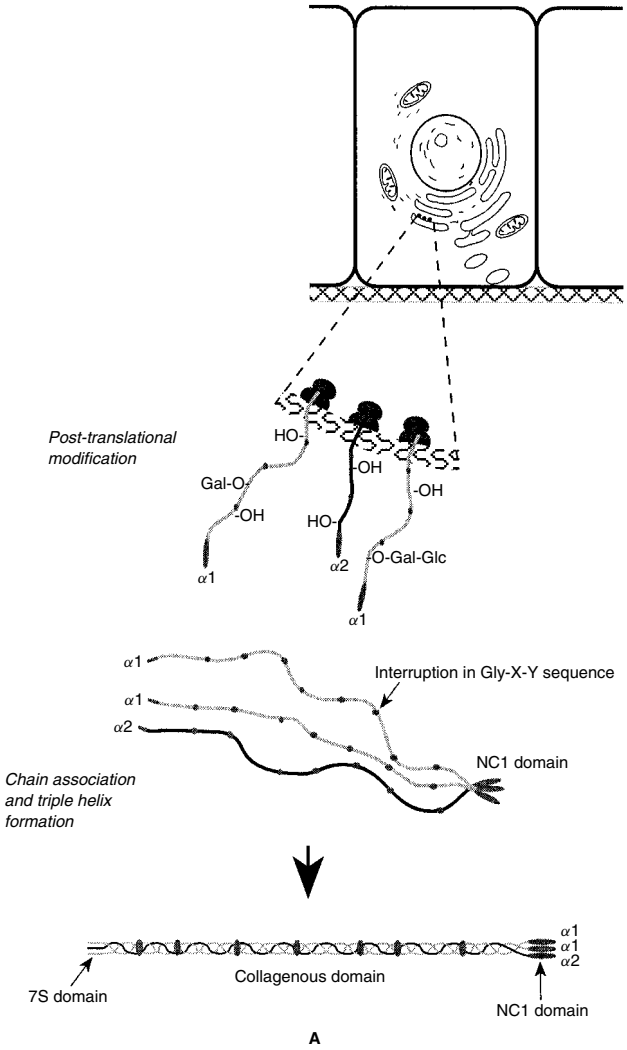

Figure 4. A. Biosynthesis of type IV collagen in a cell resting on a basement membrane follows the general pattern of synthesis of collagenous proteins [70] (see also Chapter 2, Part I, this volume). After transcription and processing of the primary transcript, the mRNA is transported to the cytosol to ribosomes upon which translation occurs. Post-translational prolyl and lysyl hydroxylation and hydroxylysyl glycosylation reactions take place on the nascent α chains in the cisternae of the rough endoplasmic reticulum. This is followed by chain association, disulfide bonding and formation of the triple helix of three appropriate α chains. Single chains not incorporated into triple helices are degraded, while the triple-helical molecules are transported through the Golgi apparatus to the extracellular space where supramolecular assembly occurs. **B.** Schematic illustration of the structure and supramolecular assembly of type IV collagen. Individual monomers associate into dimers via disulfide bonds between NC1 domains or into tetramers through disulfide bonds between the amino termini. A supramolecular network is formed by the assembly of dimers and tetramers and strengthened by the lateral association and partial winding of some molecules around each other. Other basement membrane components — such as laminin, proteoglycans, and nidogen — are bound into the type IV collagen meshwork. (Modified from [205].)

(*continued overleaf*)

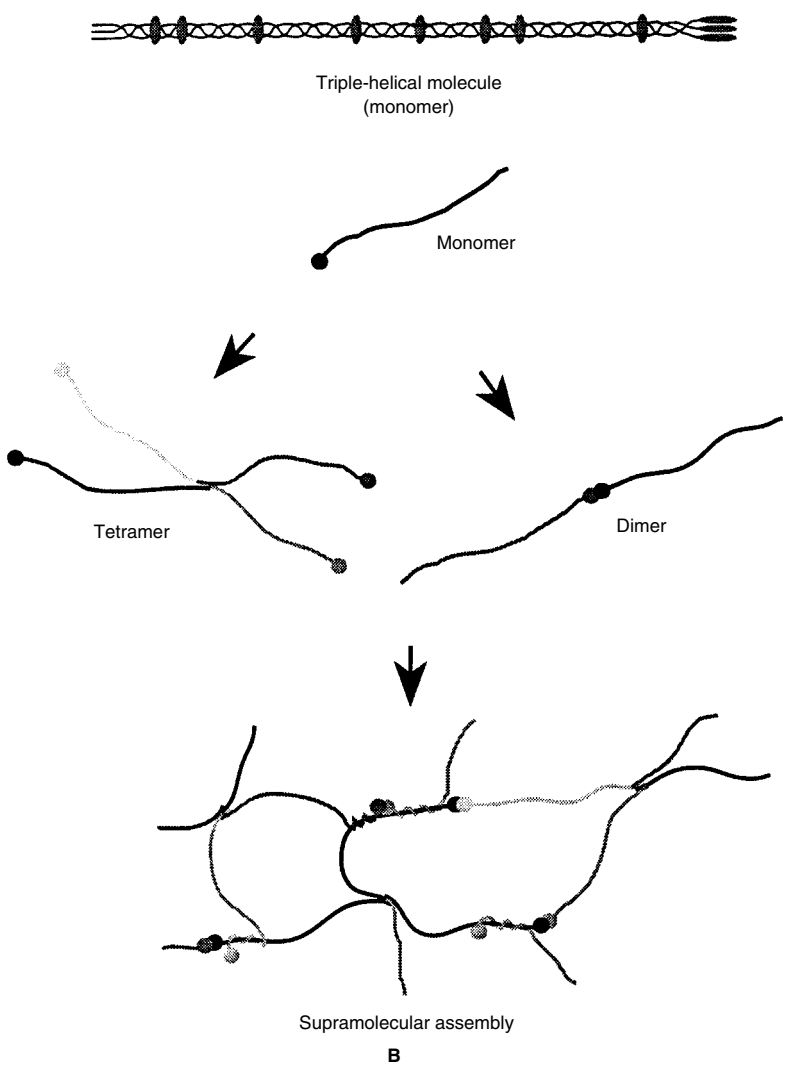

Figure 4. (*Continued*)

dant α1(IV) and α2(IV) chains have been sequenced in man [56,57], mouse [58,59] and *Caenorhabditis elegans* [60,61]. Additionally, the α1(IV) sequence is known for *Drosophila* [62] and sea urchin [63], and the α2(IV) sequence in *Ascaris suum* [64]. The primary sequences of the tissue-restricted α3(IV) [65], α4(IV) [66], α5(IV) [34,67], and α6(IV) [68,69] chains have been determined in man. The collagen IV chains are very homologous to each other and can be divided into two classes. The α1(IV), α3(IV), and α5(IV) chains belong to an α1-like class, and the α2(IV), α4(IV), and α6(IV) to an α2-like class.

The biosynthesis of type IV collagen involves similar post-translational modifications to those of collagenous proteins in general (Fig. 4A) [70] (see also Chapter 2, Part I, this volume). The secreted triple-helical type IV collagen monomers self-assemble into a complex network structure (Fig 4B). Several modes of interaction have been demonstrated in *in vivo* studies. Linear dimers are formed through association of the NC1 domains of two molecules. The resulting hexameric NC1 complex is mostly stabilized by interchain disulfide bond formation [71]. Tetrameric structures are formed through noncovalent interactions, namely parallel and antiparallel associations of the aminoterminal ends of four monomers. The dimers and tetramers further assemble, forming a network of highly branched filaments that include laterally aligned molecules and molecules twisting around each other [50,72]. The result is a three-dimensional, tightly meshed structure to which other basement membrane components bind. The short, noncollagenous interruptions in the collagenous domain of the type IV collagen molecules presumably provide the network structure with flexibility.

Several different molecules with variable compositions are possible, because of the existence of six different α chains. The most common form of type IV collagen, present in most basement membranes, is a heterotrimer containing two α1(IV) chains and one α2(IV) chain [53]. Homotrimers of α1(IV) chains have also been found [42,73]. The existence of combinations of the other four α chains (α3–α6) has been intensively investigated and evidence for different networks has been presented. Heterotrimers of two α3(IV) chains and one α4(IV) chain, and homotrimers of α3(IV) chains appear to exist in the glomerular basement membrane [42,74].

Recently, Gunwar et al. [75] identified a novel disulfide-cross-linked network of the α3(IV), α4(IV) and α5(IV) chains of type IV collagen in the kidney. This finding explains several features of the GBM abnormalities in Alport syndrome.

Other Glomerular Basement Membrane Components

Laminin. The laminins are a group of complex extracellular glycoproteins that are ubiquitous components of the basement membrane [76–79] (see also Chapter 5, this volume). Laminin was initially isolated and characterized from a murine basal lamina matrix-forming Engelbreth-Holm-Swarm (EHS) tumor as a trimeric protein containing 400 kDa, 220 kDa, and 200 kDa subunit chains, referred to as A, B1, and B2 chains, respectively [80]. Later, laminin was shown actually to represent a large protein family existing in numerous isoforms [76–78]. With the identification of multiple new laminin subunit chains and trimeric isoforms, a new, more systematic nomenclature has been adopted [81]. According to this new nomenclature, the A, B1, and B2 chains are now designated α, β, and γ, respectively. Similarly, the genes for these chains are termed A, B, and C, respectively. The laminins have been shown to perform numerous biological functions, which are general but also specific for certain cell-lineages and tissues. These functions include cell adhesion, migration, differentiation, and proliferation [76–78] and, furthermore, laminin is part of the structural framework [82]. In addition to its physiological roles, it has been implicated in tissue repair and in cell migration events such as occur during tumor invasion [76,83,84].

Laminin molecules are heterotrimers composed of α, β, and γ chains that vary in size, being 165–400 kDa, 140–220 kDa, 130–220 kDa, respectively. These chains are multidomain polypeptides that all share certain structural features, although they clearly form three distinct classes. To date, five α, three β, and two γ chains have been identified, these chains forming ten, or even more, different types of heterotrimers (isoforms) [76]. The sequences of all the human chains, except for the laminin α5 chain, have now been determined [85–93]. All the chains have a region at the carboxyl terminal end (domains I and II) that assembles with the other chains to form a coiled coil structure, the long arm. Additionally, the α chains have a large carboxyl terminal globular domain (G-domain) not present in the β and γ chains. The amino termini of the chains have a varying number of cysteine-rich laminin repeats and globular domains.

Knowledge of the tissue-specificity of laminin variants implies unique cell and tissue specific functions for different laminin isoforms. The laminin α chains are particularly interesting, because they have been shown to have a remarkably limited tissue distribution. For example, as determined by Northern analysis and *in situ* hybridization, the α1 chain appears to be important in the early embryo, as it is expressed in the embryo primarily in the brain and to some extent also in epithelial cells of the forming proximal nephron [86], while little expression is observed postnatally. The laminin β1 and β2 chains seem to be mutually exclusive [94], the β1 chain, however, having almost ubiquitous expression. It is of interest that the β2 chain is expressed in a restricted manner during late development of the kidney, being particularly prominent in the GBM, as well as in the tubular basement membrane [95]. It is also expressed in endoneural and perineural basement membrane, as well as in the synaptic basement membrane, blood vessels and trophoblasts. Inactivation of the gene in transgenic mice resulted in a phenotype showing severe proteinuria and renal failure, as well as ultrastructural abnormalities in the synaptic basement membrane [96].

Proteoglycan. Proteoglycans are extracellular proteins containing large carbohydrate (glycosaminoglycan) side chains that increase the volume of tissues by binding water through their hygroscopic properties (see also Chapter 4, this volume). The basement membrane has been shown to contain proteoglycans which differ from those of other connective tissues such as stroma and cartilage. The best studied and most abundant basement membrane proteoglycan is a large heparan sulfate proteoglycan termed perlecan [97,98]. The human perlecan core protein belongs amongst the largest polypeptides in the human body with a size of 467 kDa [99]. The human core protein contains five distinct domains; an amino-terminal domain I containing three attachment sites for heparan sulfate side chains; domain II containing two regions of homology to LDL receptor-like repeats and one IgG-like domain; domain III with cysteine-rich repeats similar to those in domains III and V of laminin as well as regions similar to the globular domains IV and VI of laminin; domain IV containing 21 copies of IgG-like repeats; and domain V containing three subdomains similar to the five subdomains present in the G-domain of laminin α chains.

Perlecan is likely to serve several functions in basement membrane, but present knowledge as to what they might be, is still very limited. However, Farquhar and co-workers have shown strong evidence for heparan sulfate side chains being important for the filtration function of the glomerular basement membrane due to their high anionic charge [100–102]. Loss of heparan sulfate has also been reported in the GBM in many diseases characterized by proteinuria. Heparan sulfate chains on other proteoglycans have also been proposed to provide a kind of extracellular reservoir of growth factors, such as fibroblast growth factors, which bind heparan sulfate. Due to its complex multidomain structure, it seems likely that perlecan has multiple functions within the basement membrane.

Nidogen. Nidogen [103], also described as entactin [104], is abundant in all basement membranes (see also Chapter 5, this volume). It has a molecular weight of 148 kDa including 5% of carbohydrates [105,106]. The human cDNA-derived protein sequence [107], together with electron microscope analysis [108], shows the secreted protein to contain three globular domains, G1, G2, and G3, separated by two rod-like domains, which consist of cysteine-rich EGF-like repeats.

Nidogen is probably essential for a functional basement membrane, as it appears to maintain the three-dimensional structure by linking the independent type IV collagen and laminin meshworks. The carboxyl-terminal end of the nidogen molecule has been shown to bind to a single, internal cysteine-rich repeat in domain III of the laminin γ1 chain [109–111], which is present in the basement membrane of most, if not all tissues in the body. The G2 domain of nidogen, in turn, binds to type IV collagen, but in a more complex manner, and possibly to several sites of that molecule [112,113]. Nidogen has been shown to interact, also, with the core protein of perlecan [114], so nidogen may truly function as a major link between the various structural proteins of the basement membrane. Nidogen has also been shown to bind *in vitro* to fibronectin and fibrinogen, which are not authentic basement membrane proteins [108].

Other Components. Other proteoglycans have been identified in basement membrane, but knowledge of their structure and other properties is still very limited. Several other proteins such as fibronectin, BM-40 [115] (also termed

osteonectin [116] or SPARC [117]) and fibulin have been localized to the basement membrane, but they are also, or mainly, present in other extracellular components and their actual role in basement membrane function is not known.

Type IV Collagen Genes

The six mammalian type IV collagen genes have an exceptional arrangement in that they are located pairwise in a head-to-head fashion on three different chromosomes (Fig. 5); (for the gene structure of collagens, see also Chapter 2, Part II, this volume). This arrangement implies that they have evolved from a common ancestor gene, and careful analysis of the sequences of the six α-chains, together with the available data on the structure of the genes, has allowed us to envisage the probable evolutionary pattern of the genes. Present data predict that the first event involved a duplication and inversion of the ancestor gene at the initial locus, so that the genes came to lie head-to-head. This was followed by extensive divergence of the two genes into α1-like and α2-like genes. Later, the gene pair was duplicated in its entirety to another chromosome, giving rise to the *COL4A3*/*COL4A4* gene pair (on human chromosome 2) coding for the α3(IV) and α4(IV) chains, respectively [118]. Still later, the original gene pair was duplicated to chromosome X to form the *COL4A5*/*COL4A6* gene pair encoding the α5(IV) and α6(IV) chains, respectively [32]. The original gene pair became the *COL4A1*/*COL4A2* genes on chromosome 13 that code for the α1(IV) and α2(IV) chains, respectively [119]. It is to be noted that a similar evolution has knowingly not taken place in lower organisms, as the two type IV collagen *COL4A1* and *COL4A2* genes in *C. elegans* are located on two different chromosomes [120], for example.

The 5′ ends of each gene pair are adjacent to each other, separated by sequences which contain motifs involved in the regulation of transcriptional activity [121]. The 5′ ends of the *COL4A1* and *COL4A2* genes overlap each other, and the region between transcription start sites is only about 130 bp. The genes are transcribed from opposite DNA strands and share a common bidirectional promoter [122]. The arrangement of the *COL4A5* and *COL4A6* genes is similar, but the distance between transcription start sites is longer, 442 base pairs. In addition, the *COL4A6* gene has two alternative promoters, indicating usage of different regulatory elements for the expression of the gene, resulting in two distinct transcripts in a tissue-specific manner [123]. Studies of the murine genes have shown the bidirectional operation model for the promoter elements of the α3(IV) and α4(IV) genes [124]. Recently, Momota and co-workers [125] showed that the human *COL4A3* and *COL4A4* genes are also transcribed in opposite directions and share the same promoter region. Additionally, the *COL4A4* gene has two alternative transcripts, the expression of which appears to be tissue-specific. One transcription start site of *COL4A4* is only 5 bp away from the transcription start site of *COL4A3* and the other starts 373 nucleotides downstream from the first. Two kinds of transcript are generated that differ in the 5′ untranslated region, thus resulting in the same deduced amino acid sequences.

All the genes encoding the human type IV collagen α1 to α6 chains are very large in size, because of the large sizes of the introns. It has been estimated by macrorestriction mapping that the size of the α1(IV) gene is 100–160 kb, as is that of the α2(IV) gene [126]; the α5(IV) gene is 230–250 kb, and the α6(IV) gene is at least 425 kb [127]. The combined size of the *COL4A3*/*COL4A4* gene pair has been estimated to be about 500 kb [118], the *COL4A4* gene being at least 113 kb [13]. The α1(IV) gene contains 52 exons, the sizes of which vary from 27 to 213 base pairs [128]. The number of exons in the *COL4A2* gene is 47, the smallest being 36 bp and the largest 287 bp [129]. Previously the exons encoding the most 3′ end of the *COL4A3* and *COL4A4* genes had been characterized [130,131], but recently Boye et al. [13] showed the *COL4A4* gene to have 48 exons, of which the smallest is 9 bp and the largest 287 bp. The type IV collagen α5 chain gene contains 51 exons and they are almost the same size as those of the α1(IV) gene, the smallest being 27 bp and the largest 213 bp [34]. The *COL4A6* gene contains 46

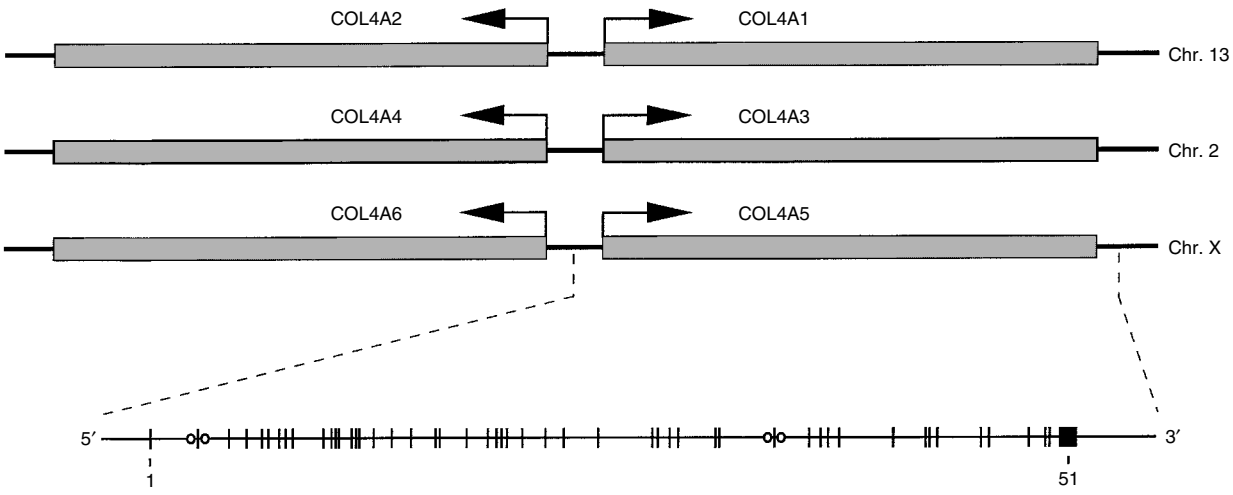

Figure 5. Illustration of the organization and chromosomal location of the human type IV collagen genes. The genes encoding the six type IV collagen chains are located pairwise in a head-to-head fashion on three different chromosomes. The genes are designated by boxes and flanking sequences by solid lines. The chromosome number is shown on the right. For the *COL4A5* gene, the arrangement of exons is shown beneath by vertical bars, and that of introns by the horizontal line. The exons are numbered from the 5′ end of the gene. Intron sequences of unknown size are indicated by circles. (Modified from [52].)

exons including exon 1′ which is alternatively transcribed with exon 1 from alternative promoters. The sizes of exons vary from 36 to 222 bp [35].

TISSUE DISTRIBUTION OF THE TYPE IV COLLAGEN α CHAINS

The cloning of the six type IV collagen α chains and the production of recombinant proteins as antigens, has enabled the generation of chain-specific nucleotide probes and antibodies which have revealed cell-specific expression and tissue location of the different α chains. These studies have demonstrated that the six type IV collagen α chains differ quite remarkably in their tissue distribution (Table 2). This, in turn, suggests that the different isoforms serve different functions *in vivo*. The α1(IV) and α2(IV) chains that form the most common $[\alpha1(IV)]_2\alpha2(IV)$ isoform are present in practically all basement membranes where they are frequently the only chains present. This emphasizes a major general role of this isoform in basement membranes. The other, minor chains have a highly restricted distribution [52].

Normal Tissue Distribution

The α3, α4, and α5 chains of type IV collagen are expressed in the kidney, and their expression is highly enriched in the glomerular basement membrane, but they are also found in a subset of tubular basement membranes, i.e., Bowman's capsule and the distal tubules [132,133]. The α5(IV) chain is present, furthermore, in the collecting ducts. α6(IV) chain expression is almost identical to that of α5(IV) throughout the kidney, but it is notably absent from the GBM [132]. In the glomerulus, the α1(IV) and α2(IV) chains are present in the mesangium, where expression of the α3(IV)–α6(IV) chains cannot be seen [133,134] (Table 2). Those basement membranes that are known to be affected in Alport syndrome express the α3(IV), α4(IV), and α5(IV) chains. In addition to the GBM, these include the anterior lens capsule, the internal limiting membrane of the retina, Bruch's membrane, and Descemet's membrane in the eye, as well as several basement membranes of the cochlea [23]. Type IV collagen chains are also known to be present in extrarenal non-Alport target tissues; the α3(IV) and α4(IV) chains are expressed in lung, brain [135], and synaptic fibers [94] and the α5(IV) and α6(IV) chains in lung, esophagus, and skin [135].

Immunohistology of Basement Membranes in Alport Syndrome

Immunohistochemical studies have shown that the type IV collagen α3, α4, and α5 chains are absent from the GBM of many male patients with X-linked Alport syndrome, whereas the expression of the α1(IV) and α2(IV) chains

TABLE 2. Distribution of the Six Type IV Collagen α Chains in Normal tissues and in Tissues of Alport Patients

Tissue	α1(IV)	α2(IV)	α3(IV)	α4(IV)	α5(IV)	α6(IV)	References
Normal distribution							
Kidney							23,132–137
GBM embryonic	+	+	−	−	−	−	
GBM postnatal	(+)	(+)	+	+	+	−	
Bowman's capsule	+	+	+	+	+	+	
Distal tubules	+	+	+	+	+	+	
Collecting ducts	+	+	−	−	+	+	
Mesangium	+	+	−	−	−	−	
Eye							139
Anterior lens capsule	+	+	+	+	+	?	
Internal limiting membrane	+	+	+	+	+	?	
Bruch's membrane	+	+	+	+	+	?	
Descemet's membrane	+	+	+	+	+	?	
Cochlea	+	+	+	+	+	?	23,135
Skin	+	+	−	−	+	+	135
Esophagus	+	+	−	−	+	+	135
Brain	+	+	+	+	−	−	135
Lung	+	+	+	+	+	+	135
Muscle synaptic fibers	+	+	+	+	?	?	94
Distribution in X-linked AS (mutations in the α5 chain gene)							
Kidney							23,132–137
GBM	+	+	−	−	−	−	
Bowman's capsule	+	+	−	−	−	−	
Distal tubules	+	+	−	−	−	−	
Eye							139
Anterior lens capsule	+	+	−	−	−	?	
Skin	+	+	−	−	−	−	133,138
Distribution in autosomal recessive AS (mutations in the α3 and α4 chain genes)							
Kidney							140
GBM	+	+	−	−	−	−	
Bowman's capsule	+	+	−	−	+	?	
Distal tubules	+	+	−	−	+	?	
Skin	+	+	−	−	+	?	140

is even increased [132,136]. Expression of the α6(IV) chain is normally seen in Bowman's capsules and tubules but not in such patients; this is also the case with the α3(IV), α4(IV), and α5(IV) chains [133]. Women who are heterozygous for the X-linked disease exhibit segmental expression of the α3(IV), α4(IV), and α5(IV) chains in the GBM, while expression of the α1(IV) and α2(IV) chains is preserved [137].

Immunohistochemical analyses of skin tissue from males with X-linked Alport syndrome have shown a complete absence of α5(IV) and α6(IV) chain expression. A discontinuous or mosaic staining for these two chains is seen in the epidermal basement membrane (EBM) of female heterozygotes [133,138]. Cheong et al. [139] have studied the expression of type IV collagen α chains in another Alport target tissue, the anterior lens capsule, in X-linked cases, and demonstrated the same features as in the GBM, namely, a lack of the α3(IV) to α5(IV) chains (Table 2).

The distribution of α(IV) chains in autosomal recessive cases of Alport syndrome has been studied by Gubler et al. [140]. Usually, no expression of the α3(IV), α4(IV), and α5(IV) chains can be seen in the GBM, but deviating from the X-linked expression pattern, the α5(IV) chain is normal in Bowman's capsule and distal tubular basement membrane. A similar expression pattern can be seen in both sexes. In addition, the α5(IV) chain shows normal staining in the EBM, while the α3(IV), α4(IV), and α5(IV) chains are absent from the GBM (Table 2). This combination cannot be seen in X-linked Alport syndrome, as it is well known that only the α5(IV) chain of these three is present in the EBM [140]. This differential pattern of staining may be a useful method for distinguishing between X-linked and autosomal recessive forms of Alport syndrome [23].

MOLECULAR PATHOLOGY OF THE ALPORT SYNDROME

General

As discussed above, Alport syndrome is caused by abnormalities in the genes encoding the basement membrane-specific type IV collagen α3, α4, and α5 chains—i.e., *COL4A3*, *COL4A4*, and *COL4A5*, respectively. In the most common, X-linked dominant form of Alport syndrome, close to 300 different mutations in the *COL4A5* gene have thus far been identified (Tables 3 and 4). Large deletions spanning the 5′ ends of the *COL4A5* and *COL4A6* genes have been shown to be associated with a rare combination of Alport syndrome and diffuse esophageal and/or genital leiomyomatosis, a condition characterized by benign smooth muscle cell proliferation [27]. The rarer autosomal inheritance, including both autosomal recessive and autosomal dominant cases, has been estimated to account for up to 15% of affected families. Autosomal disease is caused by abnormalities in the *COL4A3* and *COL4A4* genes, and to date 17 different mutations have been characterized in these genes [13,18]. Recently, Lemmink et al. [141] detected a glycine substitution in the collagenous domain of the *COL4A4* gene in a case of autosomal dominant benign hematuria.

Mutations in the *COL4A5* Gene in X-linked Alport Syndrome

Since the first mutations described in the type IV collagen α5 chain gene in X-linked Alport syndrome by Barker et al. [2], nearly 300 mutations have been elucidated by different groups [18,19]. Southern blot analysis using cDNA probes has been utilized to detect large rearrangements of the *COL4A5* gene. The main method of screening of small mutations has been SSCP analysis of PCR amplified products. However, direct sequencing of PCR amplified exon regions has revealed the highest mutation detection frequency [19].

The *COL4A5* mutations identified in the disease to date are listed in Tables 3 and 4. They include large and small gene rearrangements such as deletions, insertions, inversions, and duplications, as well as single base changes. Furthermore, complete loss of the gene has been reported in two cases [33,142,143]. A striking finding is that the mutations identified in *COL4A5* so far are highly dispersed in this huge gene, which contains 51 exons and exceeds 250 kb in size [34,144]. The same mutation has been found in only a few cases in two or more unrelated kindreds. This contrasts sharply with findings in other diseases such as cystic fibrosis, in which approximately 70% of patients have the same mutation [145]. The dispersion of mutations in *COL4A5* resembles the situation reported for osteogenesis imperfecta, a brittle bone disease that involves a variety of mutations in type I collagen genes [146] (see also Chapter 8, this volume).

The mutations in the *COL4A5* gene in Alport syndrome can, in most cases, well explain the structural and pathophysiological changes in the GBM. They can result in changes that affect many of the steps in type IV collagen biosynthesis and assembly, such as: (a) synthesis of the primary transcript, (b) post-transcriptional modification of the transcript, such as splicing, (c) translation of the mRNA, (d) post-translational modifications of nascent α chains, (e) assembly and folding of three α chains into a triple-helical molecule, (f) the stability of the helical structure, and (g) the formation of cross-links between individual triple-helical molecules. Large gene rearrangements can result in a total loss of expression of the gene, and small mutations in regulatory elements can have the same effects. Large and small mutations can also affect splicing of the primary transcript. Abnormal splicing and skipping of exons can cause two types of change. First, if the exon skipping is in frame, the result is a shortened polypeptide lacking a segment while, second, if the exon skipping occurs out of frame, the result is a protein containing a nonsense amino acid sequence. Duplications of the gene segments can lead to abnormally long α chains.

All mutations leading to absence of the α5(IV) chain or extensive alterations in protein size would certainly lead to the abnormal structure of any type IV collagen network in which the α5(IV) chain is required as a normal component. Complete loss, or the presence of an abnormal α5(IV) chain, may explain the clinical manifestations observed in Alport syndrome. In the kidney, the α5(IV) chain is practically present only in the GBM as shown by immunostaining techniques, with a similar GBM staining pattern observed for the α3(IV) and α4(IV) chains. Although it is not known in what molecular chain combinations the α5(IV) chain is present in the GBM, it was recently shown by Gunwar et al. [75], that an α3:α4:α5 network is formed in the GBM. Thus, the absence of a normal α5(IV) chain would most probably weaken the structural network of the GBM, leading to leakage of large proteins or even large blood cells into the urinary space. It is important to note, however, that heterozygous females usually exhibit only mild phenotypes and seldom develop end stage renal disease. This suggests that the synthesis of low amounts of a normal α5(IV) chain is sufficient to maintain normal GBM function, even though an abnormal chain may be present. This fact is particularly

TABLE 3. Small Mutations of the *COL4A5* Gene in Alport Syndrome Patients with X-Linked Disease

Mutation[a]	Effect on Coding Sequence[b]	Location of Mutation	Predicted Effect on *COL4A5* Protein	Juvenile/ Adult	Hearing Loss	Eye Lesions	References
203A→G	Met1Val	Exon 1	Other start codon, shorter protein	J	+	Maculopathy	15,18
344-1G→A	3′ splice signal	Intron 2	Incorrect splicing	J	+	Cataracts	206
353insA/358insT	Frameshift Gly51	Exon 3	Premature stop, shorter protein	J	+	—	18,22
363G→A	Gly54Asp	Exon 3	Gly–X–Y interruption	A	+	Lens opacity	15,18,171
452/454delG	Frameshift Pro85	Exon 4	Premature stop, shorter protein	J			206
479-1G→T	3′ splice signal	Intron 4	Incorrect splicing	J	−		206
524-1G→A	3′ splice signal	Intron 5	Incorrect splicing	J	+		206
588G→A	Gly129Glu	Exon 7	Gly–X–Y interruption, possibly incorrect splicing	J	+		18
588G→T	Gly129Val	Exon 7	Gly–X–Y interruption, possibly incorrect splicing	J	+	—	18,22
590G→T	Glu130Stop	Exon 7	Premature stop, shorter protein	J	+	Posterior lenticonus, Macular flecks	206
632G→A[c]	Gly114Ser	Exon 7	Gly–X–Y interruption				206
644/648delC	Frameshift Pro148/149	Exon 8	Premature stop, shorter protein	J	+	Cataracts	19
722G→C	Gly174Arg	Exon 9	Gly–X–Y interruption				15,18
731G→C	Gly177Arg	Exon 9	Gly–X–Y interruption	A	+	Myopia	15,18
734/735delC	Frameshift Pro178	Exon 9	Premature stop, shorter protein	J	+	—	17
750+1G→A	5′ splice signal	Intron 9	Incorrect splicing	A	+	Maculopathy	17
750+3insT	5′ splice signal	Intron 9	Incorrect splicing	A	+	—	17
751/752insC	Frameshift Gly183/Leu184	Exon 10	Premature stop, shorter protein	J	+	—	19
812-2T→A	3′ splice signal	Intron 10	Incorrect splicing	J	+	Maculopathy, lenticonus	17
813G→T	Gly204Val	Exon 11	Gly–X–Y interruption	J	+		17
836/837delC	Frameshift Pro212	Exon 11	Premature stop, shorter protein	J	+	—	15,18
848-3C→A	3′ splice signal	Intron 11	Incorrect splicing, Exon 12 spliced out	J	−	—	18,172,206
848G→A	Gly216Arg	Exon 12	Gly–X–Y interruption, possibly incorrect splicing	J	+	—	18,22
857G→A	Gly219Ser	Exon 12	Gly–X–Y interruption	J	−	—	15,18
889+1G→A	5′ splice signal	Intron 12	Incorrect splicing	J	+	Unspecified	18,22
890-3C→G	3′ splice signal	Intron 12	Incorrect splicing	J	+	Maculopathy	17
890G→C	Gly230Arg	Exon 13	Gly–X–Y interruption	J	+		17
891delG	Frameshift Gly230	Exon 13	Premature stop, shorter protein	J	+	Lenticonus	17
918G→A	Gly239Glu	Exon 13	Gly–X–Y interruption			Maculopathy	17

(*Continued overleaf*)

TABLE 3. (*Continued*)

Mutation[a]	Effect on Coding Sequence[b]	Location of Mutation	Predicted Effect on *COL4A5* Protein	Juvenile/ Adult	Hearing Loss	Eye Lesions	References
962del2	Frameshift Glu254	Exon 13	Premature stop, shorter protein	J	+	Unspecified	18,22
962del2	Frameshift Glu254	Exon 13	Premature stop, shorter protein	J	−	—	16,18
983-1del7	3′ splice signal	Intron 13	Incorrect splicing	A	−	—	16,18
983-1del7	3′ splice signal	Intron 13	Incorrect splicing	J			18,136
992G→C	Gly264Arg	Exon 14	Gly–X–Y interruption	A	+	—	17
1036+1G→A	5′ splice signal	Intron 14	Incorrect splicing	A	+	—	17
1061G→T	Glu287Stop	Exon 15	Premature stop, shorter protein	J	+		18
1061G→T	Glu287Stop	Exon 15	Premature stop, shorter protein	J	+	Unspecified	18,22
1068G→T[c,d]	Gly289Val	Exon 15	Gly–X–Y interruption	J	−		18,173
1077G→T	Gly292Val	Exon 15	Gly–X–Y interruption	J	+	—	18,22
1087delC	Frameshift Gly296	Exon 15	Premature stop, shorter protein	J	+	Unspecified	18,22
1093+1G→A	5′ splice signal	Intron 15	Incorrect splicing	J	+	—	17
1094-2A→G	3′ splice signal	Intron 15	Incorrect splicing, shorter protein	A	+	—	18,22
1094-1G→C	3′ splice signal	Intron 15	Incorrect splicing, shorter protein	J	+	Unspecified	18,22
1094G→A	Gly298Ser	Exon 16	Gly–X–Y interruption				17
1115G→T	Glu305Stop	Exon 16	Premature stop, shorter protein	J	+		206
1147insT	Frameshift Gly316	Exon 17	Premature stop, shorter protein	J	+	—	18,19,22
1175G→A	Gly325Arg	Exon 17	Gly–X–Y interruption		+		18,174
1175G→T	Gly325Stop	Exon 17	Premature stop, shorter protein		−	—	16,18
1175G→A	Gly325Arg	Exon 17	Gly–X–Y interruption	A	+		15,18
1175G→A	Gly325Arg	Exon 17	Gly–X–Y interruption		−	—	18,22
1176G→A	Gly325Glu	Exon 17	Gly–X–Y interruption		−	—	18,175,176
1194G→T	Gly331Val	Exon 18	Gly–X–Y interruption	J	+	—	206
1234del4	5′ splice signal	Intron 18	Incorrect splicing	J	+	—	18,22
1234del4	5′ splice signal	Intron 18	Incorrect splicing	J	+	Unspecified	18,22
1234+3del4	5′ splice signal	Intron 18	Incorrect splicing	J	+	—	17
1272/1276delA	Frameshift Lys358/Glu359	Exon 19	Premature stop, shorter protein	J	+	Maculopathy, lenticonus	17
1272/1276delA	Frameshift Lys358/Glu359	Exon 19	Premature stop, shorter protein				16,18
1294del9	Gly365-Pro367 deleted	Exon 19	Shorter protein	J	−	—	18,22
1296G→A	Gly365Glu	Exon 19	Gly–X–Y interruption	J	+	—	18,22
1314G→A	Gly371Glu	Exon 19	Gly–X–Y interruption	J	+	Maculopathy	15,18
1319C→T	Arg373Stop	Exon 19	Premature stop, shorter protein	J	+	Unspecified	18,22
1319C→T	Arg373Stop	Exon 19	Premature stop, shorter protein	J	+	—	15,18
1319C→T	Arg373Stop	Exon 19	Premature stop, shorter protein	J			17
1323G→C	Gly374Ala	Exon 19	Gly–X–Y interruption		−		15,18
1350G→A	Gly383Asp	Exon 19	Gly–X–Y interruption	J	+	—	16,18
1367+1G→A	5′ splice signal	Intron 19	Incorrect splicing		+	Corneal dystrophy	15,18
1367+2T→G	5′ splice signal	Intron 19	Incorrect splicing	J	+	Myopia	15,18
1401G→A	Gly400Glu	Exon 20	Gly–X–Y interruption	A	+	—	18,172,206
1416insA	Frameshift Arg405	Exon 20	Premature stop, shorter protein	J	+	Maculopathy	17
1419G→T	Gly406Val	Exon 20	Gly–X–Y interruption		+	—	18,172,206
1419G→T	Gly406Val	Exon 20	Gly–X–Y interruption	A	+	Cataracts	15,18

1419ins4	Frameshift Gly406	Exon 20	Premature stop, shorter protein	J	+	Anterior lenticonus	15,18
1421C→T	Gln407Stop	Exon 20	Premature stop, shorter protein	J	+		19
1428G→A	Gly409Asp	Exon 20	Gly–X–Y interruption	J	+		15,18
1437G→T	Gly412Val	Exon 20	Gly–X–Y interruption	A	+		17
1461G→T	Gly420Val	Exon 20	Gly–X–Y interruption		–	—	19
1461G→A	Gly420Glu	Exon 20	Gly–X–Y interruption	J	+	—	17
1466/1467delC	Frameshift Pro422	Exon 20	Premature stop, shorter protein				17
1470G→A	Gly423Glu	Exon 20	Gly–X–Y interruption		+		17
1482insA	Frameshift Pro428	Exon 20	Premature stop, shorter protein	J	+	Maculopathy	15,18
1533T→G	Ile444Ser	Exon 20	Unknown	J	+	Retinal abnormalities	16,18
1533T→G	Ile444Ser	Exon 20	Unknown	J	–	—	16,18
1533T→G	Ile444Ser	Exon 20	Unknown		–	—	16,18
1542-2A→G	3′ splice signal	Intron 20	Incorrect splicing		+		18
1550delAT	Frameshift Ile450	Exon 21	Premature stop, shorter protein	J	+	Anterior lenticonus	206
1551del2	Frameshift Ile450	Exon 21	Premature stop, shorter protein	J	+	Unspecified	18,172
1567del9	Pro456-Pro458 deleted	Exon 21	Shorter protein				15,18
1567del9	Pro456-Pro458 deleted	Exon 21	Shorter protein	A	+	Unspecified	19
1573/1578delC	Frameshift Pro458/459	Exon 21	Premature stop, shorter protein	J	+	—	17
1599G→A	Gly466Glu	Exon 21	Gly–X–Y interruption		+	—	16,18
1616G→A	Gly472Arg	Exon 21	Gly–X–Y interruption	J	+	Anterior lenticonus	206
1625+1G→A	5′ splice signal	Intron 21	Incorrect splicing	A	+	Anterior lenticonus, retinal flecks, myopia	19
1674G→A	Gly491Glu	Exon 22	Gly–X–Y interruption	J	+	Maculopathy, lenticonus	17
1683G→A	Gly494Asp	Exon 22	Gly–X–Y interruption		+	Maculopathy	15,18
1685del36	Gln495-Pro505 deleted	Exon 22	Shorter protein	J	+	—	15,18
1685del36	Gln495-Pro505 deleted	Exon 22	Shorter protein	J	+	—	15,18
1691G→T	Gly497Cys	Exon 22	Gly–X–Y interruption	A	+		17
1744delAG	Frameshift Gly515	Exon 23	Premature stop, shorter protein		+	Anterior lenticonus	206
1763G→T	Gly521Cys	Exon 23	Gly–X–Y interruption	J	+	—	18,67
1768/1769delA	Frameshift Ala522/Thr523	Exon 23	Premature stop, shorter protein				15,18
1773G→A	Gly524Asp	Exon 23	Gly–X–Y interruption	A	+	—	17
1783del16	Exon/Intron boundary deleted	Exon 23/Intron 23	Incorrect splicing	J	+		206
1783del16	Exon/Intron boundary deleted	Exon 23/Intron 23	Incorrect splicing	J	+		206
1789+1G→A	5′ splice signal	Intron 23	Incorrect splicing	J	+	Anterior lenticonus	19
1789+1G→A	5′ splice signal	Intron 23	Incorrect splicing	J	+	Unspecified	206
1789+1delG	5′ splice signal	Intron 23	Incorrect splicing	J	+	Maculopathy	17
1835G→C	Gly545Arg	Exon 24	Gly–X–Y interruption		+	Unspecified	206
1836G→T	Gly545Val	Exon 24	Gly–X–Y interruption	J	–	—	206
1883G→A	Gly561Arg	Exon 24	Gly–X–Y interruption	J	+		206
1902G→C	Gly567Ala	Exon 24	Gly–X–Y interruption	J	+	—	15,18
1920G→A	Gly573Asp	Exon 24	Gly–X–Y interruption	J	+		19
1937G→A	Gly579Arg	Exon 24	Gly–X–Y interruption	J	+		206
1981+1G→T	5′ splice signal	Intron 24	Incorrect splicing				15,18
2027G→C	Gly609Arg	Exon 25	Gly–X–Y interruption	J	+		17
2028G→T	Gly609Val	Exon 25	Gly–X–Y interruption	J	+		18

(*Continued overleaf*)

TABLE 3. (*Continued*)

Mutation[a]	Effect on Coding Sequence[b]	Location of Mutation	Predicted Effect on *COL4A5* Protein	Juvenile/ Adult	Hearing Loss	Eye Lesions	References
2063G→T	Gly621Cys	Exon 25	Gly–X–Y interruption	J	+	Maculopathy	17
2073G→A	Gly624Asp	Exon 25	Gly–X–Y interruption		–	—	19
2097G→A	Gly632Asp	Exon 25	Gly–X–Y interruption		+		17
2106G→A	Gly635Asp	Exon 25	Gly–X–Y interruption	J	+	Cataracts, astigmatism ''warped lenses''	19
2106G→A	Gly635Asp	Exon 25	Gly–X–Y interruption		+		206
2114G→A[c]	Gly638Ser	Exon 25	Gly–X–Y interruption				206
2115G→T	Gly638Val	Exon 25	Gly–X–Y interruption		+	Anterior lenticonus	18,172,206
2115G→C	Gly638Ala	Exon 25	Gly–X–Y interruption	J	–	—	18,172,206
2159G→A	Gly653Arg	Exon 26	Gly–X–Y interruption	J	+	Macular flecks	18,172,206
2159/2162delG	Frameshift Gly653/Asp654	Exon 26	Premature stop, shorter protein	J	+		17
2208G→C	Gly669Ala	Exon 26	Gly–X–Y interruption				206
2220delG	Frameshift Arg673	Exon 26	Premature stop, shorter protein				15,18
2243+1G→T	5′ splice signal	Intron 26	Incorrect splicing		+	—	19
2244G→A	Gly681Asp	Exon 27	Gly–X–Y interruption	J	+		17
2252G→T	Gly684Stop	Exon 27	Premature stop, shorter protein	J	+	Unspecified	206
2253G→T	Gly684Val	Exon 27	Gly–X–Y interruption	A	+	Retinal abnormalities	16,18
2262G→A	Gly687Glu	Exon 27	Gly–X–Y interruption	J	+		206
2348G→C	Gly716Arg	Exon 27	Gly–X–Y interruption, possibly incorrect splicing	J	+	Retinal abnormalities	16,18
2349-3C→G[c]	3′ splice signal	Intron 27	Incorrect splicing		–	—	19
2349/2349-1delG	Frameshift Gly716	Exon 28	Premature stop, shorter protein, possibly incorrect splicing	J	+	Unspecified	19
2421G→A	Gly740Glu	Exon 28	Gly–X–Y interruption	J			16,18
2430G→A	Gly743Asp	Exon 28	Gly–X–Y interruption		–		206
2447-1G→A	3′ splice signal	Intron 28	Incorrect splicing	J	+	Unspecified	19
2517G→A	Gly772Asp	Exon 29	Gly–X–Y interruption	J	–	—	16,18
2534G→A	Gly776Ser	Exon 29	Gly–X–Y interruption	A	+		206
2549/2550delC	Frameshift Pro783	Exon 29	Premature stop				19
2588G→A	Gly796Arg	Exon 29	Gly–X–Y interruption		+		18,172,206
2597+2delT	5′ splice signal	Intron 29	Premature stop	J	+	Maculopathy	15,18
2605del18	Gly802-Pro807 deleted	Exon 30	Shorter protein	J	+	—	19
2606G→A	Gly802Arg	Exon 30	Gly–X–Y interruption	J	+	Maculopathy	17
2625G→A	Gly808Glu	Exon 30	Gly–X–Y interruption	A	+		206
2634G→T	Gly811Val	Exon 30	Gly–X–Y interruption	J	+		17
2636del8/2636ins30	Frameshift Pro812	Exon 30	Premature stop, shorter protein	J	+	Anterior lenticonus	19
2665del9	Gly822-Pro824 deleted	Exon 30	Shorter protein	J	–		206
2756G→A	Gly852Arg	Exon 31	Gly–X–Y interruption		–	—	16,18
2757G→A	Gly852Glu	Exon 31	Gly–X–Y interruption				206
2799G→A	Gly866Glu	Exon 31	Gly–X–Y interruption	A	+		15,18
2799G→A	Gly866Glu	Exon 31	Gly–X–Y interruption		–	—	15,18
2807G→A	Gly869Arg	Exon 31	Gly–X–Y interruption		+		18,172,206
2807G→A	Gly869Arg	Exon 31	Gly–X–Y interruption	J	+		206

2807G→A	Gly869Arg	Exon 31	Gly–X–Y interruption	J	+		206
2807G→A	Gly869Arg	Exon 31	Gly–X–Y interruption	A	+	—	206
2807G→A	Gly869Arg	Exon 31	Gly–X–Y interruption	A	+		206
2807G→A	Gly869Arg	Exon 31	Gly–X–Y interruption	J	−	—	15,18
2807G→A	Gly869Arg	Exon 31	Gly–X–Y interruption		+	Lens opacity	15,18
2807G→A[c]	Gly869Arg	Exon 31	Gly–X–Y interruption			Maculopathy	17
2807G→A	Gly869Arg	Exon 31	Gly–X–Y interruption		−	—	19
2807G→A	Gly869Arg	Exon 31	Gly–X–Y interruption	J	+	—	19
2816G→C	Gly872Arg	Exon 31	Gly–X–Y interruption		+		18,172,206
2827delA	Frameshift Gly875	Exon 31	Premature stop, shorter protein	J	+	Anterior lenticonus	18,172,206
2835G→T	Gly878Arg	Exon 31	Gly–X–Y interruption	J	+		206
2948insAT/del13/2961dup27[c]	Frameshift Ser916	Exon 32	Premature stop, shorter protein				206
3004insT	Frameshift Gly935	Exon 33	Premature stop, shorter protein	J	+		19
3023G→T	Gly941Cys	Exon 33	Gly–X–Y interruption	A	+		19
3025del3	Ser942 deleted	Exon 33	Gly–X–Y interruption, shorter protein				15,18
3060G→T[e]	Gly953Val	Exon 33	Gly–X–Y interruption	J	+		17
3119+1G→C	5′ splice signal	Intron 33	Incorrect splicing	J	+		17
3120-1G→T	3′ splice signal	Intron 33	Incorrect splicing	J	+	—	17
3129delAG[c]	Frameshift Val977	Exon 34	Premature stop, shorter protein				206
3142/3145delA	Frameshift Pro980/Leu981	Exon 34	Premature stop, shorter protein	J	+	Lenticonus	15,18,177
3161del27	Pro987-Gln992 deleted	Exon 34	Shorter protein	A			206
3164/3165delG	Frameshift Gly988	Exon 34	Premature stop, shorter protein		+		18
3246G→T	Gly1015Val	Exon 35	Gly–X–Y interruption			—	17
3246G→A[c]	Gly1015Glu	Exon 35	Gly–X–Y interruption				17
3248C→T	Gln1016Stop	Exon 35	Premature stop, shorter protein		+	Lenticonus	17
3258/3259delT	Frameshift Leu1019	Exon 35	Premature stop, shorter protein	J	+	Maculopathy, lenticonus	17
3290G→A	Gly1030Ser	Exon 35	Gly–X–Y interruption	A	+		19
3309-2A→G	3′ splice signal	Intron 35	Incorrect splicing	J	+		19
3309G→T	Gly1036Val	Exon 36	Gly–X–Y interruption		+	Maculopathy, lenticonus	17
3317G→A	Gly1039Ser	Exon 36	Gly–X–Y interruption	J	+	Maculopathy	17
3371G→T	Gly1057Stop	Exon 36	Premature stop, shorter protein	J	+		17
3380G→T	Gly1060Stop	Exon 36	Premature stop, shorter protein	J	+	Anterior lenticonus, cataracts	19
3383C→T	Gln1061Stop	Exon 36	Premature stop, shorter protein		+	—	206
3398G→A	Gly1066Ser	Exon 36	Gly–X–Y interruption	J	+	—	19
3398G→C	Gly1066Arg	Exon 36	Gly–X–Y interruption		+	Maculopathy	17
3414C→G	Ser1071Stop	Exon 36	Premature stop, shorter protein	J	+	—	19
3513G→T	Gly1104Val	Exon 37	Gly–X–Y interruption		−	—	16,18
3491A→T	Lys1097Stop	Exon 37	Premature stop, shorter protein	J	+	Myopia	19
3521G→A	Gly1107Arg	Exon 37	Gly–X–Y interruption				206
3532/3533delA	Frameshift Thr111	Exon 37	Premature stop, shorter protein				15,18
3539insCCTG	Frameshift Gly1113	Exon 37	Premature stop, shorter protein		−	—	15,18
3629G→A	Gly1143Ser	Exon 38	Gly–X–Y interruption	A	+	Lens opacity	15,18,178
3630G→A	Gly1143Asp	Exon 38	Gly–X–Y interruption	J	−	—	18,179

(*Continued overleaf*)

TABLE 3. (*Continued*)

Mutation[a]	Effect on Coding Sequence[b]	Location of Mutation	Predicted Effect on *COL4A5* Protein	Juvenile/ Adult	Hearing Loss	Eye Lesions	References
3630G→A	Gly1143Asp	Exon 38	Gly–X–Y interruption	J	–	—	19
3656+1G→C	5′ splice signal	Intron 38	Incorrect splicing, Exon 38 spliced out	J	+	—	18,180
3656+1G→C[c]	5′ splice signal	Intron 38	Incorrect splicing	J	+	—	19
3683G→A	Gly1161Arg	Exon 39	Gly–X–Y interruption	J			206
3710/3711delG	Frameshift Gly1170	Exon 39	Premature stop, shorter protein		+	—	15,18,181
3728/3729delG	Frameshift Gly1176	Exon 39	Premature stop, shorter protein	J	+	Unspecified	19
3740C→T	Gln1180Stop	Exon 39	Premature stop, shorter protein		+	—	17
3745del7	Frameshift Lys1181	Exon 39	Premature stop, shorter protein	J	+	—	15,18,182
3746G→C	Gly1182Arg	Exon 39	Gly–X–Y interruption	J	+	Retinal abnormalities	16,18
3756-9C→G	3′ splice signal	Intron 39	Incorrect splicing	J	+	Anterior lenticonus	19
3756-3C→G	3′ splice signal	Intron 39	Incorrect splicing	A	+	Anterior lenticonus, cataracts	19
3756-1G→A	3′ splice signal	Intron 39	Incorrect splicing				17
3788G→A	Gly1196Arg	Exon 40	Gly–X–Y interruption	J	+	—	19
3807-2A→T	3′ splice signal	Intron 40	Incorrect splicing	J	+	—	17
3807-1G→A	3′ splice signal	Intron 40	Incorrect splicing	J	+		17
3815G→T	Gly1205Cys	Exon 41	Gly–X–Y interruption	J	+		17
3814/3816delG	Frameshift Gly1205	Exon 41	Premature stop, shorter protein	J	+		18,183
3819del4	Frameshift Asp1206	Exon 41	Premature stop, shorter protein	J	+	Anterior lenticonus	15,18,182
3833G→C	Gly1211Arg	Exon 41	Gly–X–Y interruption		+		206
3834G→A[e]	Gly1211Glu	Exon 41	Gly–X–Y interruption				17
3848/3849delC	Frameshift Pro1216	Exon 41	Premature stop, shorter protein		+		17
3861G→A	Gly1220Asp	Exon 41	Gly–X–Y interruption				206
3892/3894delC	Frameshift Pro1231	Exon 41	Premature stop, shorter protein	J	+	—	17
3912del52	Frameshift Pro1237	Exon 41	Premature stop, shorter protein		–	—	16,18
3912insCC	Frameshift Gly1238	Exon 41	Premature stop, shorter protein				206
3923G→T[c]	Gly1241Cys	Exon 41	Gly–X–Y interruption		–	—	18,172,206
3956G→A	Gly1252Ser	Exon 41	Gly–X–Y interruption	A	–	—	17
3959ins4	Frameshift Asn1253	Exon 41	Premature stop, shorter protein		+	—	16,18
3984G→A	Gly1261Glu	Exon 41	Gly–X–Y interruption	J	+	—	19
3992/3992+1delG	5′ splice signal	Intron 41	Incorrect splicing		–		17
3993-1G→A	3′ splice signal	Intron 41	Incorrect splicing, Exon 42 spliced out, premature stop, shorter protein	J	+		18,152,183
4010G→A	Gly1270Ser	Exon 42	Gly–X–Y interruption				18
4016/4017delC	Frameshift Pro1272	Exon 42	Premature stop, shorter protein	J	+		15,18,181

4058del7	Frameshift Asn1286	Exon 42	Premature stop, shorter protein	J	+		18,151
4122delT	Frameshift Leu1307	Exon 42	Premature stop, shorter protein	J	+		17
4127-1G→A	3′ splice signal	Intron 42	Incorrect splicing	J	+		206
4193/4194delT	Frameshift Phe1331	Exon 43	Premature stop, shorter protein			—	15,18
4199G→A	Gly1333Ser	Exon 43	Gly–X–Y interruption				206
4200-2A→G	3′ splice signal	Intron 43	Incorrect splicing, Exon 44 spliced out	J			15,18
4245G→A/4251del6	Gly1348Glu/Pro1350-Gly1351 deleted	Exon 44	Gly–X–Y interruption		+	—	15,18
4271G→A	Gly1357Ser	Exon 44	Gly–X–Y interruption	J			206
4271/4271+1G→A	Gly1357Ser	Exon 44	Gly–X–Y interruption, possibly incorrect splicing	J	+	—	19
4271+1G→A	5′ splice signal	Intron 44	Incorrect splicing	J	+	Cataracts	206
4273delT	Frameshift Gly1357	Exon 45	Premature stop, shorter protein	J	+		17
4304delAG	Frameshift Ser1368	Exon 45	Premature stop, shorter protein	J	+	—	206
4338G→T	Gly1379Val	Exon 45	Gly–X–Y interruption				15,18
4363ins4[c]	Frameshift Gly1388	Exon 45	Premature stop, shorter protein				18,176
4430C→T	Arg1410Cys	Exon 46	Unknown	J	+	—	15,18
4463G→T	Gly1421Trp	Exon 46	Gly–X–Y interruption	A	–	—	15,18
4467C→T[c,d]	Arg1422Cys	Exon 46	Unknown				18,173
4482G→T	Gly1427Val	Exon 46	Gly–X–Y interruption		–	—	206
4517dup36	Dup 12 codons from Gly1439	Exon 47	Longer protein				15,18,184
4527G→A	Gly1442Asp	Exon 47	Gly–X–Y interruption	J	–	—	206
4546insT	Frameshift Pro1449	Exon 47	Premature stop, shorter protein	J	+	—	15,18
4553G→A	Gly1451Ser	Exon 47	Gly–X–Y interruption		–		18
4637del2	Frameshift Gly1479	Exon 47	Premature stop, shorter protein	J	+		18,185
4639/4640delA	Frameshift Thr1480	Exon 47	Premature stop, shorter protein	J	+	Unspecified	206
4659G→C	Gly1486Ala	Exon 47	Gly–X–Y interruption	A	+		206
4665C→T	Ser1488Phe	Exon 47	Unknown				17
4686C→A	Ala1498Asp	Exon 47	Unknown	A	–		18,186
4712+1G→C	5′ splice signal	Intron 47	Incorrect splicing, Exon 47 spliced out	J	+	—	18,187
4712+1G→C	5′ splice signal	Intron 47	Incorrect splicing	J	–	—	16,18
4749insT	Frameshift Met1516	Exon 48	Premature stop, shorter protein	J	+		18,188
4751C→A	Pro1517Thr	Exon 48	Unknown	J	+		18,152
4751C→A	Pro1517Thr	Exon 48	Unknown		–	—	15,18
4815G→C	Trp1538Ser	Exon 48	Unknown	A	–		18,153
4816G→A	Trp1538Stop	Exon 48	Premature stop, shorter protein		+		17
4889C→T	Arg1563Stop	Exon 48	Premature stop, shorter protein	J	+		18,154
4889C→T	Arg1563Stop	Exon 48	Premature stop, shorter protein		+	—	17
4889C→T	Arg1563Stop	Exon 48	Premature stop, shorter protein	J	+		18

(*Continued overleaf*)

TABLE 3. (*Continued*)

Mutation[a]	Effect on Coding Sequence[b]	Location of Mutation	Predicted Effect on *COL4A5* Protein	Juvenile/ Adult	Hearing Loss	Eye Lesions	References
4890G→C	Arg1563Gln	Exon 49	Unknown	A	+		18,154
4890G→A	5′ splice signal	Exon 48	Incorrect splicing	J	+		18,154
4890G→A	5′ splice signal	Exon 48	Incorrect splicing		+		18,154
4890G→A	5′ splice signal	Exon 48	Incorrect splicing, Exon 48 spliced out	J	+		18,152,183
4890G→A	5′ splice signal	Exon 48	Incorrect splicing	J	+	Maculopathy, lenticonus	17
4891-3del32	3′ splice signal	Exon 49	Incorrect splicing	A	−	—	16,18
4893G→T	Cys1564Ser	Exon 49	Unknown	A	+		9
4901T→C	Cys1567Arg	Exon 49	Unknown	J	+	Maculopathy	17
4951del7	Frameshift Pro1584	Exon 49	Premature stop, shorter protein	A	+		206
4989G→A	Gly1596Asp	Exon 49	Unknown		+	—	15,18
4991insA	Frameshift Tyr1597	Exon 49	Premature stop, shorter protein	J	+	—	18,189
4992A→G	Tyr1597Cys	Exon 49	Unknown		+	—	190
4993T→A	Tyr1597Stop	Exon 49	Premature stop, shorter protein		+	—	19
5005G→A	Met1601Ile	Exon 49	Incorrect splicing, Exon 49 spliced out	J	+	—	206
5005G→C	5′ splice signal, Met1601Ile	Exon 49	Incorrect splicing, multiple transcripts with premature stops	J	−	—	16,18,191
5005+1Gins10	5′ splice signal, frameshift Met1601	Exon 49	Incorrect splicing	A	+		18,152,183
5005+1G→A	5′ splice signal	Intron 49	Incorrect splicing	A	+	—	17
5033/5034delG	Frameshift Gly1611	Exon 50	Premature stop, shorter protein	J	+	—	15,18,182
5148T→G	Leu1649Arg	Exon 50	Unknown		−	—	18,19,192
5170delC[c]	Frameshift Asp1656	Exon 50	Premature stop, shorter protein		−		18,193
5178G→A	5′ splice signal	Exon 50	Incorrect splicing	J	+	Maculopathy, lenticonus	17
5179-2A→G	3′ splice signal	Intron 50	Incorrect splicing	J	+	—	18,187
5191del8/5195del8	Frameshift Thr1665	Exon 51	Premature stop, shorter protein		+		18,194,206
5222C→T	Arg1674Stop	Exon 51	Premature stop, shorter protein		+	—	190
5231C→T	Arg1677Stop	Exon 51	Premature stop, shorter protein	J	+	Unspecified	19
5231C→T	Arg1677Stop	Exon 51	Premature stop, shorter protein		+		19
5231C→T	Arg1677Stop	Exon 51	Premature stop, shorter protein	J	+		206
5232G→A	Arg1677Gln	Exon51	Unknown	A	+	—	195
5237C→T	Glu1679Stop	Exon 51	Premature stop, shorter protein		−	—	18,189
5244G→A	Cys1681Tyr	Exon 51	Unknown	J	+	Anterior lenticonus	190

[a]Mutations are designated according to Beaudet and Tsui [196].

[b]Nucleotide and amino acid residue numbering is based on Zhou et al. [34].

[c]Female patient.

[d,e]Another mutation found in same patient.

TABLE 4. Major Rearrangements of the *COL4A5* Gene in Alport Syndrome Patients with X-Linked Disease

Location of Mutation	Mutation[a]	Predicted Effect on *COL4A5* Protein	Juvenile/ Adult	Hearing Loss	Eye Lesions	Remarks	References
Exons 1–51	del 470 kb	No protein	J	+		del exons 1–3 *COL4A6*	18,33,143
Exons 1–51	del exons 1–51	No protein	J	+			18,142
Exons 1–32	del 210 kb[b]	No protein			Congenital cataract	del exons 1–2 *COL4A6*, DL[c]	18,33
Exons 1–32[d]	del 290 kb[b]	No protein			Congenital cataract	del exons 1–2 *COL4A6*, DL	18,33
Exons 1–30	del 330 kb[b]	No protein	J	+		del exons 1–3 *COL4A6*	18,33,143
Exons 1–30	ND[e]	No protein	J	+	Anterior lenticonus, macular flecks		206
Exons 1–18	del 210 kb[b]	No protein		–	Bilateral cataract	del exons 1–2 *COL4A6*, DL esophageal	18,31–33,143
Exons 1–4	del 75 kb[b]	No protein			Congenital cataract	del exons 1–2 *COL4A6*, DL	18,33,197
Exon 1	del 420 kb	No protein		+		del exons 1–4 *COL4A6*	18,33,143
Exon 1	ND	No protein	J	+	Anterior lenticonus, macular flecks, cataract	del exons 1–3 *COL4A6*	206
Exon 1	del 120 kb[b]	No protein		+	Cataract	del exons 1–2 *COL4A6*, DL esophageal	18,31–33,143
Exon 1[d]	del 90 kb[b]	No protein				del exons 1–2 *COL4A6*, DL	18,33,197
Exon 1	del 15–19 kb	No protein	J	+	Cataract	del exons 1–2 *COL4A6*, DL esophageal	18,198,199
Exon 1	del <10 kb[b]	No protein	J	–	Bilateral cataract	del exons 1–2 *COL4A6*, DL esophageal	18,31–33,143
Exon 1	del Exon 1	No protein	J	+	Cataract	del exons 1–2 *COL4A6*	18,32
Exon 1	ND	No protein	J	+	Cataract	del exons 1–2 *COL4A6*, DL	18,197
Exon 1	ND	No protein		+		del exons 1–2 *COL4A6*, DL	18,197
Exon 1[d]	ND	No protein		–		DL	18,197
Exon 1[d]	ND	No protein		+		DL	18,197
Exons 2–51	del 300 kb	Truncated protein	J	+	—		206
Exons 2–51	del >500 kb	Truncated protein	J	+	Anterior lenticonus, macular flecks		206

(*Continued overleaf*)

TABLE 4. (*Continued*)

Location of Mutation	Mutation[a]	Predicted Effect on *COL4A5* Protein	Juvenile/ Adult	Hearing Loss	Eye Lesions	Remarks	References
Exons 2–36	del exons 2–36	Truncated protein	J	+			18,199
Exons 2–19	del exons 2–19	Truncated protein		+			18,199
Exons 2–8	del exons 2–8[b]	Truncated protein	J	–	—		19
Exons 4–47	del exons 4–47	No protein	J	+			18,200
Exons 4–13	del exons 4–13	Truncated protein	J				18,199
Exons4/5–26	del exons 4/5–26[b]	Truncated protein	J	+			18,143
Exons 14–51	del exons 14–51	Truncated protein	J	+			18,200
Exon 17	del Exon 17	Truncated protein	J	+			18,199
Exons 19–22	del exons 19–22	Truncated protein	J	+			18,199
Exons 20–26	del exons 20–26[b]	Truncated protein		+			18,143
Exons 20–21	del exons 20–21	Truncated protein	J	+			18,199
Exon24/Intron25	del 1.4 kb	Truncated protein	J	+	Lenticonus, macular flecks		206
Exon 22/Exon 38	del exons 22–28, del exons 38–51	Truncated protein	J	+			18,153
Exons 26–29	ND	Aberrant protein					206
Exons 28–32	dup exons 28–32	Aberrant protein	J	+			18,199
Exons 34–51	del 300 kb	Truncated protein					18,201
Exons 37–45	ND	Aberrant protein		+	Posterior lenticonus		206
Exons 38–41	ND	Aberrant protein	J	+			206
Exons 38–51	del 34 kb	Truncated protein	J	+			18,142
Exons 38–51	dup 35 kb	Aberrant protein	A	+	Macular flecks		18,144,202,206
Exons 38–46	ins exons 38–46	Truncated protein					18,201
Exon 40	del Exon 40	Truncated protein		+			18,199
Exons 42–47	del 15 kb	Truncated protein	J	+			2,18,179,203
Exons 45–51	del 450 kb	Truncated protein	J	+			18,144,202,206
Exons 46/47	ins 10–15 kb, del 25 kb	Aberrant protein		+			18,144,202, 206
Exons 49–51	del 10 kb	Truncated protein	J	+			18,204

[a]Mutations are designated according to Beaudet and Tsui [196].

[b]Precise location of 5′ and 3′ breakpoints has not been determined.

[c]DL, diffuse leiomyomatosis.

[d]Female patient.

[e]ND means no data available for the deleted area.

important when considering future possibilities for gene therapy.

Single base mutations in exons leading to amino acid changes in the α5(IV) chain can be expected to render the protein malfunctional in most, but not necessarily all cases, because such changes can affect proteins in a number of ways. Therefore, the question is how can we know whether an amino acid substitution actually causes disease instead of being silent? This is an important question, especially from a clinical point of view when making a decision about the termination of pregnancy after a mutation has been identified in a fetus. This is also a very difficult question to answer and, in fact, the only definite way of proving that a mutation is causative for the disease is to generate transgenic animals with the same mutation and observe if it results in an Alport syndrome-like phenotype. However, it is possible that a mutation causing disease in man does not cause the same disease in mice. Therefore, one has to rely on strong "circumstantial" evidence when predicting the potential pathogenic effect of mutations. Such evidence can be linkage or the segregation of a mutation with the phenotype in an Alport kindred, knowledge of the role of a certain amino acid in the function of the protein, or the conservation of an amino acid during evolution. It is assumed that amino acid residues conserved in a protein between widely distant species during evolution have such functional importance that their substitution cannot be tolerated. This can be illustrated by several examples.

The approximately 230 residue amino acid sequence of the C-terminal NC1 domain of type IV collagen α chains consists of two homologous halves, with about 50% of amino acid residues being conserved in all six mammalian chains and also in type IV collagen α chains of *Drosophila*, *C. elegans*, and sea urchin [1,57–60,62,63,147–151]. The conserved amino acid residues include 12 cysteines, 6 in each homologous half of the NC1 domain. It is apparent that the NC1 domain of all type IV collagen chains has the same biological function and that the highly conserved amino acids and three-dimensional structure of the NC1 domain are essential for the correct alignment of three α chains prior to their folding and twisting into a triple helix, and subsequently for the formation of intermolecular cross-links in the extracellular space. Therefore, substitution of conserved amino acids in this domain may affect these functions. Thus far, several single base mutations leading to amino acid substitutions in the NC1 domain of the α5(IV) chain have been published in relation to Alport syndrome. For example, one mutation resulted in the change of cysteine1564 to serine [9]. The mutation would abolish one of the intra-chain disulfide bonds, destroying either that between cysteine residues 1476 and 1564, or that between cysteines 1509 and 1564 (Fig. 6). The mutation, which would alter the conformation of the NC1 domain, could have several effects. One possibility is that the mutation would interfere with the alignment of chains and formation of the triple helix so that helix formation would be slowed down, abolished completely, or the alignment of Gly–X–Y repeats would be out of register. Alternatively, the triple-helical molecule may be formed and the protein secreted from the cell, but the loss of the cysteine residue might cause weak intermolecular cross-links in the type IV collagen in the basement membrane meshwork.

The effects of amino acid changes in the NC1 domain other than of cysteine are more difficult to predict as the function of those amino acids is not known. Examples of such mutations are Pro1517Thr, Trp1538Ser, and Arg1563Gln (Table 3) [152–154]. In such cases, one could, if possible, (a) study whether the mutation co-segregated in the families with the Alport phenotype, (b) determine whether the mutation was the result of a normal polymorphism and existed in normal individuals or, (c) examine whether the amino acid had been conserved during evolution. As can be seen in Figure 7, each of the amino acids listed above is conserved in all known type IV collagen α chains, even between distant species. Consequently, it may be assumed that these amino acids are essential for the normal function of the NC1 domain and that their change cannot be tolerated.

A large number of mutations converting a glycine residue in the Gly–X–Y repeat-containing collagenous domain to another amino acid have been described (Table 3). These mutations undoubtedly affect the stability of the triple helix of the molecule because, as mentioned above, glycine is the only amino acid small enough to fit in the center of the triple helix, and an uninterrupted Gly–X–Y repeat sequence is essential for maintenance of the triple-helical conformation. The result of glycine substitution would, therefore, be that the helix was destabilized, creating kinks in the molecule which would not be tolerated. Although this has not been demonstrated at the protein level for type IV collagen, a number of similar glycine mutations, either inhibiting helix formation or causing kinks in the molecule, have been described in type I collagen in osteogenesis imperfecta [146] (see also Chapter 8, this volume).

The dispersion of mutations throughout the *COL4A5* gene is a characteristic of Alport syndrome. Thus far, no "hot spots" particularly prone to mutagenic changes have been found, and are not likely to be. Regions highly susceptible to mutation would be of significant clinical value because they could greatly facilitate the search for mutations in Alport patients.

Different *COL4A5* Mutations Cause Different Phenotypes

Alport syndrome is a heterogeneous disease, with onset of end-stage renal disease at varying ages, and with or without manifestations such as hearing loss or deafness, ocular lesions, and thrombocytopathy. Because so many mutations have been identified in the *COL4A5* gene, it is of interest from a clinical point of view to try to correlate them with the phenotypes they produce. As can be seen from Table 3 and references therein, such an analysis is disappointing in the sense that it does not seem to be possible to predict the phenotype on the basis of the nature of a mutation. For example, the point mutations that result in a substitution of glycine residues in the collagenous domain or of conserved amino acids in the NC1 domain generate quite a wide spectrum of phenotypes. Most, but not all patients have a juvenile onset of end-stage renal disease, and the presence of hearing loss and ocular lesions varies between individuals.

A similarly heterogeneous picture is seen for large changes in the gene (Table 4). Even the entire loss of the gene does not necessarily produce more severe disease than a single glycine substitution in the collagenous domain of the α5(IV) chain. Although disappointing, this does not come as a surprise as the same phenomenon has been observed with defects in the genes for fibrillar collagens resulting in osteogenesis imperfecta, chondrodysplasia, Stickler syndrome, and Ehlers-Danlos syndrome [146] (see also Chapters 8,9,23, this volume). One particularly interesting Alport phenotype displays diffuse leiomyomatosis in addition to the classical

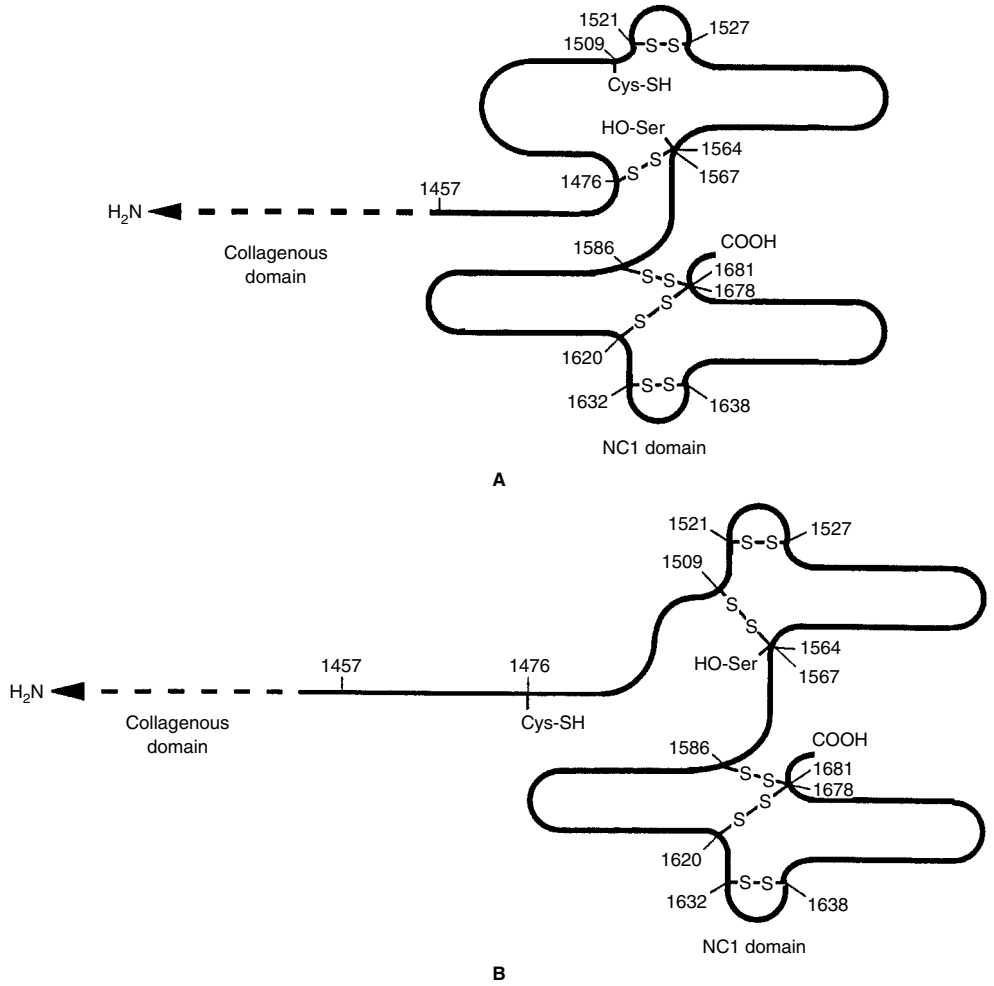

Figure 6. Hypothetical consequences of a Cys1564Ser mutation in the *COL4A5* gene. The mutation abolishes a disulfide bond connecting cysteine1564 to either cysteine1509 (**A**) or cysteine 1476 (**B**). This would, in turn, cause a change in the conformation of the NC1 domain, with a possible rearrangement of intrachain bonds and subsequent interference in helix formation. The mutation may also seriously influence the rearrangement of the disulfide bonds and formation of interchain bonds, with the simplest change reducing the number of cross-links from eight to six. However, the mutation might interfere more seriously with dimerization by even completely inhibiting the formation of interchain cross-links between NC1 domains. (Modified from [9].)

	First half			Second half		
	1517	1538	1563	1628	1648	1677
patient 1, W1538S	..STMPFMF..YSYSLSTP..FISRCAVCE..			..SAPFIECH..SYSFWLA..RISRCQVCM..		
patient 2, P1517T	..STMTFMF..YSYWLSTP..FISRCAVCE..			..SAPFIECH..SYSFWLA..RISRCQVCM..		
patient 3, R1563Q	..STMPFMF..YSYWLSTP..FISQCAVCE..			..SAPFIECH..SYSFWLA..RISRCQVCM..		
Human α5(IV)	..STMPFMF..YSYWLSTP..FISRCAVCE..			..SAPFIECH..SYSFWLA..RISRCQVCM..		
Human α1(IV)	..STMPFLF..YSYWLSTP..FISRCAVCE..			..SAPFIECH..AYSFWLA..HVSRCQVCM..		
Mouse α1(IV)	..STMPFLF..YSYWLSTP..FISRCAVCE..			..SAPFIECH..AYSFWLA..HVSRCQVCM..		
Human α2(IV)	..STMPFLY..KSYWLSTT..YISRCSVCE..			..ATPFIECN..KYSFWLT..HISRCQVCM.		
Mouse α2(IV)	..STMPFLY..KSYWLSTT..YISRCSVCE..			..ATPFIECN..KYSFWLT..HISRCQVCM..		
Human α3(IV)	..TTMPFLF..YSYWLSTP..YISRCTVCE..			..ASPFIECH..SYSFWLA..IISRCQVCM..		
Bovine α3(IV)	..TTMPFLF..YSYWLSTP..YISRCTVCE..			..ASPFIECH..SYSFWLA..IISRCQVCM..		
Human α4(IV)	..STLPFAY..RSYWLASA..YVSRCAVCE..			..SSPFIECQ..KYSFWLT..KISRCQVCV..		
c elegans α1(IV)	..STMPFLF..KSYWLSTS..YISRCAVCE..			..ATPFIECN..KFSFWLT..RVSRCQVCC..		
c elegans α2(IV)	..NTMPFMF..YSFWLSTD..YISRCAVCE..			..AVPFIECH..NHGFWPS..RVSRCQVCL..		
Sea urchin α1(IV)	..STMPFLF..YSYWLSTT..FISRCVVCE..			..SSPFIECH..TYSFWLS..RVSRCAVCL..		
Drosophila α(IV)	..STLPVLS..KTFWLTTN..YISRCVVCE..			..ATPFIECN..MTSFWMY..HVSRCQVCM..		

Figure 7. Comparison of substituted amino acids in three Alport patients with corresponding residues in the homologous halves of the NC1 domain in all known type IV collagen α chains [14]. Gaps (..) have been introduced to maintain alignment. Capital letters indicate the amino acid residues. Amino acids that are conserved through evolution are shown in boxes. The proline, tryptophan, and arginine residues (boldface) that are mutated in the three patients [152–154] are conserved in both symmetrical halves of the NC1 domain in all known type IV α chains.

symptoms [31]. A clinically important group is the ~15% of post-transplantation Alport patients who develop anti-GBM antibodies. About one-third of those patients subsequently reject the transplant. Initial studies on Alport mutations and phenotypes gave hope that, depending on the nature of the mutation, it might be possible to predict which patients would develop anti-GBM nephritis and, thus, who should receive a transplant and who not. It seemed reasonable to presume that a mutation deleting the highly antigenic NC1 domain of the α5(IV) chain would cause a patient to recognize the corresponding domain in an allograft as a foreign protein and should, therefore, not receive an allograft as he would develop anti-GBM nephritis. However, more recent data on transplanted patients with similar kinds of gene defects have shown that this expectation is not entirely valid. In the light of current, still limited knowledge, it is not yet possible to predict which patients are likely to develop anti-GBM nephritis following kidney transplantation.

Mutations in the *COL4A3* and *COL4A4* Genes in Autosomal Recessive Alport Syndrome

A distinct form of autosomal recessive Alport syndrome was suspected for several years by French investigators, because involvement of the *COL4A5* gene was excluded by polymorphism studies of certain pedigrees [155]. It was logical to search for mutations in the *COL4A3* and *COL4A4* genes, encoding the α3(IV) and α4(IV) collagen chains, respectively, in the autosomal form of the disease, as these chains are also prominent in the GBM. Such mutations have now been found in both the *COL4A3* and *COL4A4* genes in different Alport kindreds (Table 5).

The first mutation in the *COL4A3* gene was found in a girl with juvenile Alport syndrome accompanied by sensorineural deafness [10]. To date, five different mutations have been found in the *COL4A3* gene [10–12,156], two of them in two unrelated patients. These have been found as either homo- or heterozygous mutations in different patients. The parents have been shown to be unaffected carriers, indicating an autosomal recessive pattern of inheritance. Two of the *COL4A3* mutations are nonsense, two are small deletions in exons and one is a splice site mutation. All five mutations create premature translation stop codons, which would lead to a truncated malfunctional polypeptide.

The first mutations in the *COL4A4* gene were reported by Mochizuki and co-workers in two families with Alport syndrome (Table 5) [10]. One mutation is a glycine substitution in the collagenous domain with the probable impairment of triple helix formation. The other causes a premature stop codon in the collagenous domain and a truncated protein as a result. Recently, Boye et al. [13] analyzed the *COL4A4* gene in 31 unrelated autosomal recessive Alport syndrome patients and found ten novel mutations among eight of them (Table 5). Five of the mutations result in premature stop codons or a shift in the

TABLE 5. Mutations in the *COL4A3* and *COL4A4* Genes in Recessive Alport Syndrome

Mutation[a]	Effect on Coding Sequence[b]	Location of Mutation[c]	Mutation Status: Homozygous Change/ Heterozygous Change	Consanguinity in the Family	References
COL4A3					
G → T[d]	Frameshift Arg1643	Intron 1	Homozygous	(+)	156
4559C → G	Ser1524Stop	Exons 4,5	Heterozygous	−	11
4441C → T	Arg1481Stop				
4441C→T	Arg1481Stop	Exon 5	Homozygous	+	10
4414del5/ 4419del5	Frameshift Leu1474	Exon 5	Homozygous	−	10
4414del5/ 4419del5	Frameshift Leu1474	Exon 5	Heterozygous	−	11
4346del7	Frameshift 1449Thr	Exon 5	Homozygous	−	12
COL4A4					
5131C → A	Cys1641Stop	Exons 48,44	Heterozygous	+	13
4337C → T	Arg1377Stop				
4337C → T	Arg1377Stop	Exons 44,13	Heterozygous	+	13
1013/6insA	Frameshift Gly270				
4923C → T	Pro1572Leu	Exon 47	Heterozygous	−	13
4542−23A → G	3′ splice signal	Intron 45	Homozygous	+	13
3921C → A	Ser1238Stop	Exon 40	Homozygous	−	10
3854/6delG	Frameshift Ser1217	Exons 39,20	Heterozygous	+	13
1527del18	Pro441-Ala446 deleted				
3809G→A	Gly1201Ser	Exon 39	Homozygous	+	10
3297G→T	Gly1030Val	Exon 33	Heterozygous	−	13
2009delC	Frameshift Pro601	Exon 24	Homozygous	+	13
1122del46	5′ splice signal	Exon 14/Intron 15	Homozygous	+	13

[a]Mutations are designated according to Beaudet and Tsui [196].

[b]Nucleotide and amino acid residue numbering is based on Mariyama et al. [65] for *COL4A3* and on Boye et al. [13] for *COL4A4*.

[c]Exon numbering of *COL4A3* starts at the 3′ end.

[d]Exact location not known; splice-mediated insertion of an *Alu* fragment leading to two different mRNA transcripts.

reading frame, and are therefore potential null mutations. One mutation probably causes a deletion of two Gly–X–Y repeats in the collagenous region, thereby shortening the protein. Two mutations affect splicing and two are missense mutations, one a glycine substitution in the collagenous region and the other a highly conserved proline to leucine substitution in the carboxyterminal NC1 domain.

Defects in the *COL4A3* and *COL4A4* Genes in Autosomal Dominant Alport Syndrome

A much less common autosomal dominant form of the disease had already been described in 1985 [157], but recently Jefferson et al. [24] performed genetic linkage analysis of a large family with autosomal dominant Alport syndrome. X-linkage was excluded by negative results with *COL4A5* markers. Polymorphic markers across the *COL4A3/COL4A4* region showed strong linkage. Furthermore, SSCP analysis of five exons from the 3′ end of the *COL4A3* gene resulted in a band shift in exon 5, which segregated with one of the maternal haplotypes and not with the disease. Sequencing revealed a missense mutation in exon 5, changing the evolutionarily conserved leucine to proline. Lemmink et al. [11] have previously reported this mutation as nonpathogenic in two families; in this case the mutation is not pathogenic in itself, but it may lead to a more severe phenotype in affected patients who carry this abnormality. In the autosomal dominant form, males and females within a family are equally severely affected, but the phenotype is usually milder than that found in the X-linked dominant form.

ANIMAL MODELS OF ALPORT SYNDROME

To understand better the molecular pathology of Alport syndrome, several animal models have been studied. *Col4a3* knockout mice have also been produced as a model for the autosomal recessive form of the disease. In addition, there is a naturally occurring model of this same type in the English cocker spaniel. X-linked hereditary nephritis in Samoyed dogs and bull terrier hereditary nephritis are also naturally occurring models of Alport syndrome.

X-Linked Dominant Inheritance

Canine X-linked hereditary nephritis is an animal model of human X-linked Alport syndrome. Clinical manifestations are similar to those in the human disease. Studies of affected male dogs [158] showed persistent hematuria at 3–4 months of age and the occurrence of end-stage renal failure at the age of 8–15 months. By electron microscopy, bilamellar splitting of the GBM could already be seen at one month of age, progressing to GBM multilamellation and confluency by 3–4 months of age.

Genetic study of this model revealed a single base substitution, changing a conserved glycine codon (GGA) to a stop codon (TGA) in exon 35 of the α5(IV) gene [159]. Canine α5(IV) cDNA is greater than 90% identical to human α5(IV) cDNA at both the nucleotide and amino acid levels. The highly conserved noncollagenous domain (NC1) shows 93% identity at the DNA level and 97% identity at the protein level between the species. Furthermore, the sequences of the other α(IV) NC1 domains are ≥88% identical at the DNA level and ≥92% identical at the protein level to the respective human α(IV) NC1 domains [160].

The glycine substitution creating a premature stop codon is associated with an approximately 90% reduction in the amount of α5(IV) mRNA in the kidneys of affected male dogs. The single family of Samoyed dogs has provided an opportunity to study the effects of a selective absence of the α5(IV) chain on other basement membrane components. It was shown that the levels of mRNA for the α3(IV), α4(IV), and α5(IV) chains are all significantly reduced in affected dog kidney. Supporting evidence in human disease is not available, most probably as a consequence of the limited amount of material available from patient biopsies. The mechanisms for this kind of coordination are currently not understood, and as speculated by Thorner et al. [160], could include factors acting at either the transcriptional or the translational level. Western blot analysis with chain-specific antibodies revealed that α3(IV), α4(IV), and α5(IV) chains are present in the GBM of normal dogs, but undetectable in affected male dogs. Thus, a comparable situation exists between canine and human X-linked disease [160].

Autosomal Recessive Inheritance

Col4a3 Knockout Mouse

The collagen *Col4a3* knockout mouse is a model of the autosomal form of Alport syndrome [161]. These mice are homozygous for a mutation leading to the development of a progressive glomerulonephritis with microhematuria and proteinuria. By clinical examination, microhematuria was already observed at the age of two weeks, and proteinuria at about 5 weeks of age. The disease progressed to end-stage at the age of 14 weeks. By transmission electron microscopy of the GBM, it was seen that focal multilaminated thickening and thinning begins in the external capillary loops at 4 weeks and spreads throughout the GBM by 8 weeks. Half of the glomeruli were fibrotic with collapsed capillaries by end-stage.

Immunofluorescence analysis of the GBM showed the absence of the α3, α4, and α5 chains of type IV collagen. According to Northern blot data, the α3(IV) mRNA was absent from the kidneys of *Col4a3* knockout mice, whereas the α4(IV), and α5(IV) mRNAs were intact and present at levels observed in normal littermates. This is consistent with a mechanism that involves processes downstream of the mRNA and does not support the argued suprastructure destabilization theory [140], which suggests that a mutated *COL4A* gene in Alport patients might be translated into an abnormal protein that is incorporated into the suprastructure in a destabilizing manner, leading to a loss of all three components.

An absence of the α3(IV) and α4(IV) chains throughout the membranous labyrinth was revealed by immunohistochemical analysis of the *Col4a3* knockout mouse cochlea [161]. The α5 chain was absent from all cochlear basement membranes except those in the vessels of the stria vascularis. Electron microscopic examination of the cochlear membranes showed significant thinning of the basement membranes from the spiral limbus up to the spiral prominence [161], and thickening of the basement membranes surrounding the vessels of the stria vascularis. All in all, these results illustrate changes in the basement membranes of the strial vessels that bear a resemblance to Alport GBM pathology.

English Cocker Spaniel

Hereditary nephritis in English cocker spaniel dogs is a naturally occurring animal model of the autosomal recessive form of Alport syndrome. Affected dogs have proteinuria and juvenile-onset chronic renal failure. Lees et al. [162] studied 10 affected dogs, 3 obligate carriers, and 4 unaffected dogs. The dogs appeared healthy at 3–4 months of age, and proteinuria was the first abnormality detected at 5–8

months of age. Hematuria was associated with proteinuria. No signs of impaired hearing or ocular abnormalities were observed in the dogs despite a thorough examination. The disease progressed to terminal renal failure at 8–27 months of age. Ultrastructural examination of the kidneys showed thinning and thickening of the GBM in 6-month-old dogs. In dogs nearing the end-stage, marked GBM thickening and multilamellation was seen.

Immunohistochemical study showed no expression of the α3(IV) or α4(IV) chains in the kidneys of affected dogs. Interestingly, labeling for the α5(IV) chain was observed in the GBM; however, its intensity was reduced compared with normal staining. Additionally, GBM staining was positive for the α6(IV) chain in all affected dogs. Furthermore, unaffected dogs showed variable α6(IV) labeling. Adolescent dogs had no GBM labeling for the α6(IV) chain, but in older dogs labeling was segmental or diffuse. In humans, the α6(IV) chain is absent from the GBM [132], but in these cocker spaniels the α5(IV) and α6(IV) chains seemed to be co-expressed.

Autosomal Dominant Inheritance

Hereditary nephritis in bull terriers is a model of autosomal dominant Alport syndrome [163]. Common clinical features resemble those in X-linked Alport syndrome, except the lack of hearing impairment. Hematuria is much more likely to occur in animals with proteinuria than in those without, but still less than half of animals with proteinuria have hematuria. In contrast, in humans, hematuria is an early and preceding indication of the subsequent development of significant proteinuria. The ultrastructural findings of the kidney sections were identical to those seen in X-linked Alport syndrome. Immunohistologic study of kidney sections of affected bull terriers with monoclonal antibodies against Goodpasture and Alport antigens showed positive staining for both the α3(IV) and the α5(IV) chains [163].

THERAPY AND GENE THERAPY

To date, there is no satisfactory and curative conservative treatment available for Alport syndrome [164,165]. Patients developing end-stage renal disease are treated by hemodialysis, and also by kidney transplantation whenever possible. However, about 5% of transplanted patients develop anti-GBM nephritis and reject the allografted kidneys.

As a result of recent advances in molecular genetics, gene therapy may be developing into a real possibility for the treatment of a variety of hereditary diseases in the future. Although gene therapy has not yet come of age as a real therapeutic alternative for human diseases, extensive research efforts are being made in that direction. Alport syndrome, which primarily affects the renal glomeruli, is an attractive disease target for gene therapy for two reasons [165]. First of all, as it almost solely affects the kidney glomeruli, extrarenal complications not being life-threatening or occurring in all patients, the therapy can, at least initially, be targeted to the kidney alone. Secondly, the "isolated" kidneys with their well separated circulatory system lend themselves extremely well to organ-targeted gene transfer. Extensive research still needs to be carried out before we can expect to be able to do successful gene therapy of Alport syndrome in man, and there are numerous questions that need to be answered before we know if gene therapy is even theoretically possible for this disease. In the following, some of the key questions [165] concerning the possibility of gene therapy of Alport syndrome are discussed.

Can Genes be Efficiently Transferred to Renal Glomerular Cells?

The GBM is located between layers of endothelial and epithelial cells, which are both believed to contribute to the biosynthesis of the GBM type IV collagen. Due to a lack of sensitivity, methods such as *in situ* hybridization have not confirmed if one or both cell types produces the type IV collagen isoform, α3:α4:α5, characteristic of the GBM. However, from the gene therapy point of view, these cells are considered the prime targets.

In our recent work, we have shown that it may not be impossible to target gene transfer into these cells, as we have been able to achieve transfer of the β-galactosidase reporter gene into an estimated 85% of the glomeruli in pigs using an adenovirus vector (Fig. 8) [165]. The key to this high transfer efficacy was prolonged perfusion of the kidney, which allowed a long contact time between the cells and the viruses. In contrast, intra-arterial injections of the virus have not yielded efficient gene delivery to the glomeruli, as has been noted by others [166–168]. The perfusion method is relatively easy to apply to the kidney; it can be applied *in*

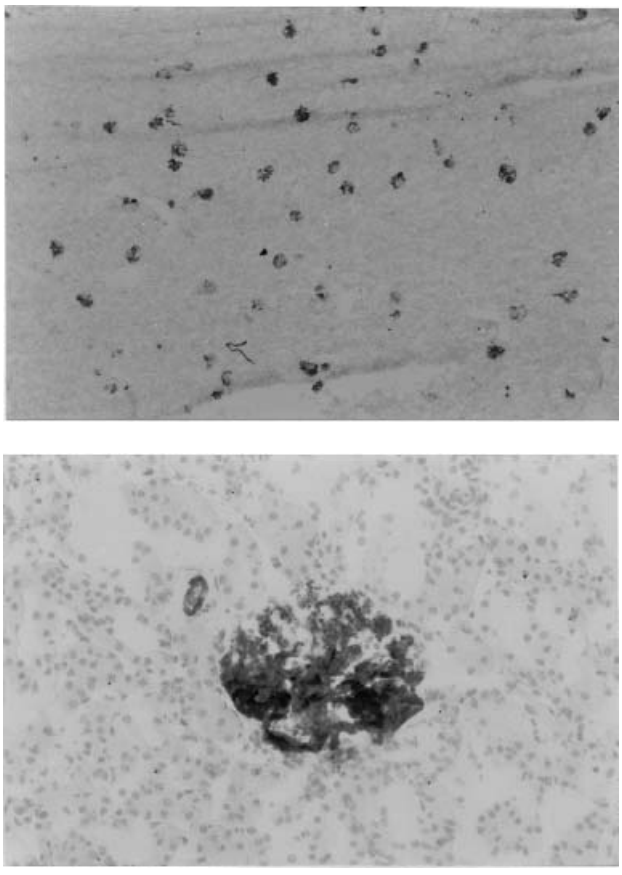

Figure 8. Expression of the β-galactosidase gene in pig kidneys following organ perfusion with a solution containing adenovirus carrying the β-galactosidase reporter gene. **A**, Intense expression can be seen in a large proportion of the glomeruli of the kidney cortex, while little if any expression is observed in other regions of the kidney. Magnification ×21. **B**, Expression of the reporter gene is generally achieved in all regions of the glomerulus, including the epithelial podocytes [165]. Magnification ×214.

situ, and may also prove useful for the transfer of genes to other organs with a well-separated blood supply.

Is It Possible to Obtain Life-Lasting and Controlled Expression of the Transferred Gene?

This is a serious problem that is the main hurdle of gene therapy in general, and it poses one of the main challenges that research in this field faces today. The adenovirus used in our experiments carried out in pigs, in its current form, provides expression for only 6–8 weeks, as it remains extrachromosomal and is not integrated into the genome [165]. Even though the half-life of type IV collagen is estimated to be between 1 and 2 years, this means that the treatment would need to be frequently repeated during life. This is not easy as, at least presently, it requires an operation. Retroviruses would theoretically be better, but they cannot integrate large DNA inserts and, also, they are only integrated into replicating DNA which is not appropriate for the stationary glomerular cells. Therefore, successful gene therapy of Alport syndrome in man will have to depend on future developments in research on gene transfer vectors.

Will a Transferred Type IV Collagen cDNA or Gene Coupled to an Appropriate Promoter be Expressed in a Controlled Manner, and Will the Corrected Chain be Incorporated into Normal α3:α4:α5 Heterotrimers?

We have already been able to express the full-length α5 chain transferred by adenoviruses in cultured cells, as well as in pig glomeruli *in vivo* (unpublished data). The fact that GBM type IV collagen is a heterotrimer is probably favorable, because it means that the limiting factor for intracellular synthesis of the trimer is likely to be the availability of the two other partner chains. Thus, if one chain is produced in excess, it will be degraded intracellularly. It has been shown that α1(IV) mRNA can exist in 3- to 15-fold excess over α2(IV) mRNA, even though the ratio of the respective chains in the protein is 2:1 [169]. If production of the defective chain can be corrected to some extent, the secretion of normal molecules is likely to occur, even in the presence of some abnormal malfunctional molecules. However, only experimental work will prove or disprove such a speculation.

Can Corrected Type IV Collagen Trimers be Incorporated into the GBM and Restore Its Structure and Function?

This is still an open question that will have to be addressed experimentally in animal models. However, observations from heterozygous female dogs have shown that although the glomeruli are chimeric for the gene defect, it appears that normal molecules can spread within the GBM to restore its structure [170]. Thus, it can be anticipated that this may not turn out to be a major obstacle to gene therapy of Alport syndrome.

RECENT DEVELOPMENTS

The fairly late onset of end-stage renal disease (ESRD) in Alport syndrome can be explained by a failure of the developmental switch between the fetal α1:α2 type IV collagen network and the adult α3:α4:α5 network in the GBM, as demonstrated by Harvey et al. [207]. Their studies of glomerular development in the X-linked canine model indicated that the α1:α2 network is essential for normal development, whereas the α3:α4:α5 network is essential for the long-term stability of the GBM and the maintenance of glomerular function. Thus, progression to ESRD, at least in X-linked Alport syndrome, results from a congenital malformation of the GBM which, alone, is not sufficient to cause disease. Rather, the GBM composed of the "fetal" collagen network is more prone to postnatal deterioration that, in the long term, leads to kidney dysfunction.

How a mutation in one of the genes coding for one of three α(IV) chains in the GBM leads to malfunction of the whole α3:α4:α5 network remains an open question. To clarify this issue, Boutaud et al. [208] studied the NC1 domain of type IV collagen and noted that there are recognition sequences within the NC1 monomers that specify the selection of chains and monomers, and thereby regulate the assembly of two different networks in the GBM. Mutations in the NC1 domain may, then, interfere with the assembly of the α3:α4:α5 network through a failure in the assembly of the NC1 hexamer. Further studies with the NC1 domains of smooth muscle basement membrane [209] has revealed an identical mechanism for specifying the chain-specific assembly of the α1:α2 and α1:α2:α5:α6 networks. The failure of such a mechanism when applied to a network containing both the α5 and the α6 chain, may explain the loss of both of these chains from basement membranes in X-linked Alport syndrome patients in whom the α5 chain is mutated.

Thus far, prediction of the phenotype produced by a particular mutation in the *COL4A5* gene has been difficult. Jais et al. [210] have now reported a multicenter study of 195 European Alport syndrome families demonstrating genotype-phenotype correlations in males. All patients had hematuria and the rate of progression to ESRD and deafness correlated with the type of mutation. Those affected male patients with large deletions, nonsense mutations, or small mutations changing the reading frame, had a 90% probability of developing ESRD before 30 years of age. The comparable risk was 70% in patients with splice-site mutations and 50% in those with missense mutations. The risk of developing hearing loss before the age of 30 years was 60% in male patients with missense mutations, and 90% in those with the other types of mutations. Three of the 118 male patients who had received kidney transplants, each of whom had a large *COL4A5* deletion, developed anti-GBM nephritis. Nevertheless, even if it is the case that some types of mutations may constitute a higher risk, it is still unclear which factors would influence the initiation and elaboration of the immune response to an allograft.

Even with the most powerful methods currently available for mutation analysis of the *COL4A5* gene, about 10% of mutations have remained unidentified in documented cases of X-linked Alport syndrome. We determined to see if an alternatively used 18 bp sequence, described in the type IV collagen α5 chain mRNA from human kidney [211], might provide any explanation for the situation in such cases [212], and have shown this 18 bp sequence to be composed of two separate, novel 9 bp exons. The predicted amino acid sequence encoded by each of these exons contains a Gly–X–Y repeat sequence, either one or both of which is used depending on the tissue [212]. Mutation analysis has not revealed any sequence variations in the two exons to date.

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Chapter 26, Part I

Corneal Dystrophies Due to Mutations in the Kerato-Epithelin Gene (β*ig-h3*)

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CLINICAL ASPECTS

Groenouw type I (MIM 121900) and lattice type I (MIM 122200) corneal dystrophies are the most frequent heritable blinding diseases of the cornea. The phenotypes are characterized by autosomal dominant transmission with complete penetrance but variable morbidity. Painful corneal erosions usually develop in the second decade, resulting in progressive visual impairment often necessitating corneal transplantation. White granular opacities form in granular corneal dystrophy Groenouw type I (GCD), often accumulating subepithelially and between stromal lamellae [1]. In lattice type 1 corneal dystrophy (CDL1), linear, branching, fibrillar amyloid deposits opacify the cornea [2]. In the combined Avellino (ACD) granular-lattice phenotype, both granular and lattice alterations occur [3,4]. The Reis-Bucklers (CDRB) type (MIM 121900) appears mainly to affect Bowman's membrane and the superficial stroma [5]. Compelling genetic and DNA sequence evidence prove that all of these dystrophies are caused by mutations in βig-h3 (called kerato-epithelin in the ophthalmologic literature) as follows: Arg555Trp in GCD, Arg555Gln in CDRB, Arg124Cys in CDL1, and Arg124His in ACD [6]. Recently, the late-developing lattice IIIA type characterized by thick, ropy branching lattice lines has been shown to be caused by a Pro501Thr missense mutation (see Fig. 1) [7].

There have been relatively few studies directly linking βig-h3 expression to abnormal deposits in the dystrophic corneal stroma. By *in situ* hybridization of human cornea with Fuchs' dystrophy (MIM 136800), Hirano localized βig-h3 mRNA to both the corneal epithelium and endothelium. Immunohistochemical studies demonstrated positive antibody staining of the subepithelial matrix, an abnormal Descemet's membrane, and a posterior collagenous layer [8]. In a study of CDL1, Takacs showed positive staining of subepithelial matrix deposits, and, in addition to a 68 kDa protein band, a 42 kDa band was detected by Western blot analysis using a βig-h3 polyclonal antibody [9]. Corneas from patients with GCD showed βig-h3 reactive subepithelial deposits as well [10]. In the most comprehensive localization study to date, Streeten et al. [11] demonstrated that in normal corneas, immunoreactivity for βig-h3 was strongest in Bowman's membrane, and next strongest along stromal interlamellar junctions and attachment sites of collagen to Descemet's membrane. Antibody binding was intense on dystrophic aggregates found in GCD, CDL1, ACD, and CDRB. Collectively, these data support the hypothesis that βig-h3 is a major component of the deposits, if not the main one. However, there is clearly a need for more definitive information concerning the expression of βig-h3 during normal corneal development and progression of the dystrophic conditions.

MOLECULAR ASPECTS

Identification and Cloning of β*ig-h3*

cDNA encoding the protein, βig-h3, was originally isolated by differential screening of a cDNA library prepared from the human adenocarcinoma cell line, A549, which had been treated with TGF-β1 [12]. The encoded 70 kDa protein of 683 amino acids contains a secretory signal at the amino terminus and is composed largely of four repeats of approximately 135 residues. Subsequently, a highly homologous murine protein was cloned [13]. The human gene was localized to chromosome 5q31 and the mouse gene to the syntenic chromosome 13 region B to C1 [13]. A number of cultured cell lines were shown to express the protein, usually at low levels, and TGF-β1 markedly up-regulated expression in many, but not all, cells tested. Northern analysis demonstrated that βig-h3 was expressed in a variety of human and mouse tissues with the highest levels found in uterus, but easily detectable levels of mRNA were found in heart, mammary gland, prostate, skeletal muscle, testis, thyroid gland, kidney, liver, and stomach. Expression was absent in brain, spleen, and parathyroid gland. At the time of its original identification, the only protein exhibiting limited homology to βig-h3 was fasciclin-1, a glycoprotein expressed in the grasshopper and *Drosophila* central nervous systems which may be involved in growth cone guidance [14].

In 1996, Gibson et al. [15] demonstrated that the sequences of five peptides isolated from a protein found in bovine ligamentum nuchae, a tissue particularly rich in elastic fibers, were very similar to sequences in βig-h3. This suggested that this protein, designated MP70, was a bovine

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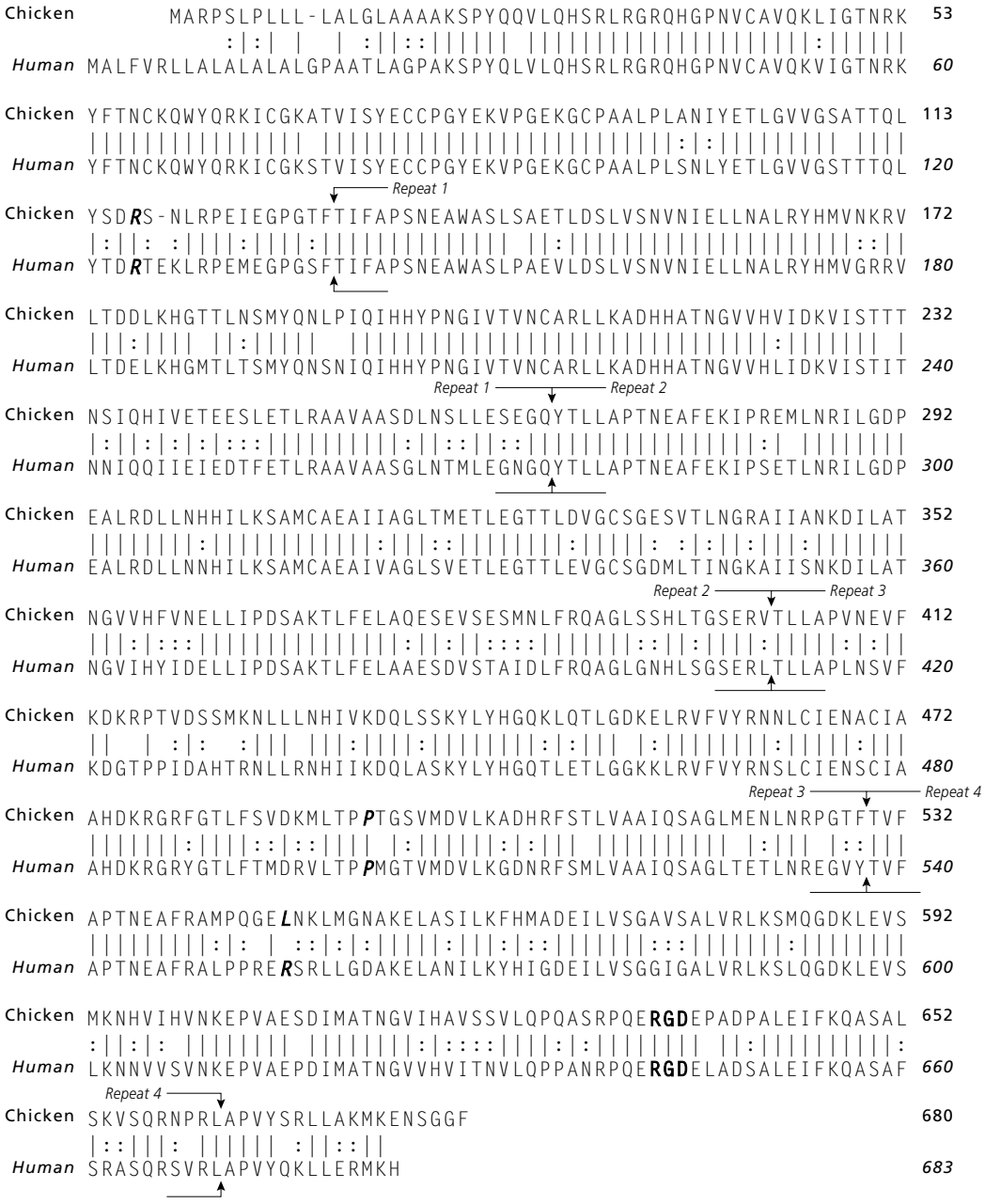

Figure 1. Comparison of the amino acid sequences of human and chick βig-h3 with the four repeat domains indicated. Within each repeat, there are two highly conserved sequences of 10 (H1) and 11 (H2) residues that are not shown in the figure. A vertical line (|) indicates complete identity, a colon (:) indicates amino acid residues with similar characteristics, and a space indicates no homology. The bolded RGD (Arg–Gly–Asp) is the consensus sequence for integrin binding. Italicized and bolded are residues 124, 501, and 555 in the human sequence which are mutated in the corneal dystrophies.

form of βig-h3. The isolation procedure used by Gibson et al. [15] was designed specifically to solubilize the microfibrillar component of elastic fibers and MP70 has tentatively been identified as a microfibrillar protein.

cDNAs encoding chick, pig, and rabbit βig-h3 have also been cloned [16–18]. Sequence analysis has demonstrated that the mammalian protein is highly conserved (~90–92% identity among species), and that there is strong homology between mammalian and chick βig-h3 (~77% identity). Such strong structural conservation argues for an important, although presently unknown, function(s). Other proteins having more limited homology to βig-h3 include osteoblast-specific factor 2 (OSF2) and several bacterial proteins [19,20].

Analysis of βig/h3 Primary Structure

Figure 1 contains a comparison of the amino acid sequence of human and chick βig-h3 with the four repeat domains indicated. Within each repeat, there are two highly conserved sequences of 10 (H1) and 11 (H2) residues (not identified in

the figure). The three residues of the human sequence (124, 501, and 555) shown to be mutated in corneal dystrophies are also identified in bold. Note that while residues 124 and 501 are conserved between species, residue 555 is not. A Leu residue is found in this position in the chick, while Arg is found in all other species analyzed. Interestingly, as discussed above, mutation of this residue to Trp or Gln appears causal for corneal granular dystrophies Groenouw type 1 and Reis-Bucklers, respectively. Note that the presence of a Leu in chick indicates that at least one type of hydrophobic residue in this position is compatible with normal function and it remains a challenge to determine why others are not.

Potential Functions of βig-h3

βig-h3 has been shown to enhance the attachment and spreading of dermal fibroblasts, suggesting that it functions as an extracellular attachment protein in skin [21]. Indeed, the protein contains an RGD cell attachment/integrin recognition site in its C-terminal region. The distribution of βig-h3 has been examined in fetal bovine tissues by immunohistochemical analysis [22]. This analysis showed that βig-h3 was associated with collagen fibers in developing nuchal ligament, aorta, lung, and mature cornea. Immunoreactive material was also present in reticular fibers in fetal spleen, as well as in capsular and tubular basement membranes in developing kidney. From this study, it was concluded that the staining pattern closely resembled that of Type VI collagen microfibrils. Type VI collagen copurified with βig-h3 upon extraction from rabbit corneas [18] and was colocalized in double immunolabeling experiments [8]. In addition, βig-h3 has been reported to bind in a non-ionic fashion to types I, II and IV collagens [16], and to the globular domain of type VI collagen [23]. These data suggest that βig-h3 may act as a link protein interconnecting specific matrix components, such as collagens, with each other and resident cells.

Expression of βig-h3 in the Cornea

The most detailed studies of the expression of βig/h3 expression have been carried out in the cornea. The major function of the corneal stroma is to act as a strong transparent structure that is responsible for greater than 50% of the refraction required to focus light on the retina. Thus, the biochemical composition and molecular interactions of its extracellular components, including βig-h3, are crucial to its function. Similar levels of βig-h3 mRNA were detected by reverse transcriptase/polymerase chain reaction in 23-day fetal and neonatal rabbit corneas, while it was not detectable in adult corneas [24]. In normal human corneas, Hirano et al. [8] showed that βig-h3 was present in Bowman's membrane and that it appeared to be associated with collagen in the stroma, while Escribano et al. [25] and Takacs et al. [9] showed that βig-h3 was preferentially localized to the corneal epithelium. It is likely that βig-h3 is expressed by corneal epithelium and endothelium as well as by stromal fibroblasts. Further studies are needed to define more precisely the expression of βig-h3 during normal development and to delineate its function.

RECENT DEVELOPMENTS

βig-h3

The murine gene has been cloned and characterized [26]. The gene spans 30 kb on mouse chromosome 13 and is very similar in structure to the human gene, with conservation of exon/intron organization. In the developing mouse, expression is observed as early as day 11.5 in the first and second branchial arches, and the gene is strongly expressed in many mesenchymal tissues throughout development. Expression is seen at day 11.5 in the mesenchyme surrounding the optic stalk, by day 14.3 in the sclera and choroid, and by day 17.5 in the cornea.

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Chapter 26, Part II

Progressive Pseudorheumatoid Dysplasia

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INTRODUCTION

Progressive pseudorheumatoid dysplasia (PPD) (MIM 208230) was initially reported by Wynne-Davies et al. as spondyloepiphyseal dysplasia tarda with progressive arthropathy [1], and by Spranger et al. [2] as progressive pseudorheumatoid arthropathy of childhood. Several additional patients and families have since been described [3–15]. The hallmark features of this syndrome are childhood-onset joint swelling, stiffness, contractures, and pain. Joint involvement is noninflammatory. Radiographic examination indicates a spondyloepiphyseal dysplasia; however, radiographic changes may not be present in very young children. There are no extraskeletal manifestations in PPD. In contrast to other forms of spondyloepiphyseal dysplasia tarda, the joint involvement in PPD is unremitting and often debilitating. The disorder has been mapped to human chromosome 6q22 [16]. Locus homogeneity appears likely [17,18] and a responsible gene, *WISP3*, has been identified [19]. This gene encodes a member of the CCN (connective tissue growth factor, cysteine-rich protein 61, nephroblastoma overexpressed) family of secreted growth regulators [20–23]. The precise function of WISP3 during skeletal growth and homeostasis is not known.

CLINICAL FEATURES

Nearly all affected patients appear normal at birth and during infancy. Signs and symptoms of disease usually have their onset between three and 11 years of age. Signs in young children may include muscle weakness, easy fatiguability, and difficulty walking. Several affected children have come to medical attention because of a waddling or awkward gait, or bowed legs. Fusiform swelling or an enlargement of finger joints, particularly the proximal interphalangeal joints, often precedes other joint complaints (Fig. 1). This feature suggests a diagnosis of juvenile rheumatoid arthritis; however, patients have neither local nor systemic inflammatory disease. Patients have normal intelligence, a normal facial appearance, and no extraskeletal findings. With advancing age, multiple joints become affected. Stiffness and contractures occur first, followed by unremitting pain, particularly in the hips and knees. By young adulthood, nearly all joints are affected (Fig. 1). Clinical spinal involvement may manifest as scoliosis, lordosis, or kyphosis. Joint pain is often debilitating and poorly controlled by steroidal or nonsteroidal anti-inflammatory agents. Affected patients often become wheelchair dependent by their second and third decades of life. Surgical release of joint contractures does not appear to provide long-term relief. In contrast, joint replacement surgery has been performed in several patients and has been noted to improve mobility and to alleviate pain.

HISTOPATHOLOGY

Results of an iliac crest biopsy have been reported in a single patient [2]. This biopsy revealed abnormal clustering of chondrocytes in the resting and proliferating cartilage. Growth zone cell columnization was defective and many cells had pycnotic nuclei. Skin biopsies and synovial biopsies have been normal in several patients.

RADIOGRAPHIC FEATURES

Because signs and symptoms have not been apparent in most affected patients until mid-childhood, few published reports describe pre-symptomatic radiographic findings. At least three children with PPD have had limited radiographic studies in infancy for unrelated respiratory disease. None had a recognized skeletal dysplasia.

Evidence of skeletal dysplasia becomes apparent with advancing age. By late childhood, most children will have clear radiographic signs of PPD. These include large-appearing epiphyses and metaphyses (particularly in the hips, hands, and knees), joint space narrowing, and peri-articular osteopenia. Platyspondyly develops in all patients, but may not be apparent in younger children [12]; for example, a child presumed to be affected with PPD had platyspondyly at 9 years of age, but a normal-appearing spine at 7 years of age [24].

DIFFERENTIAL DIAGNOSIS

The common joint disorder juvenile rheumatoid arthritis (JRA) must be considered in all young children. In contrast to children with JRA, children with PPD have no evidence of inflammatory joint disease. With advancing age, radiographic findings clearly differentiate PPD from JRA and other inflammatory arthropathies [7,11].

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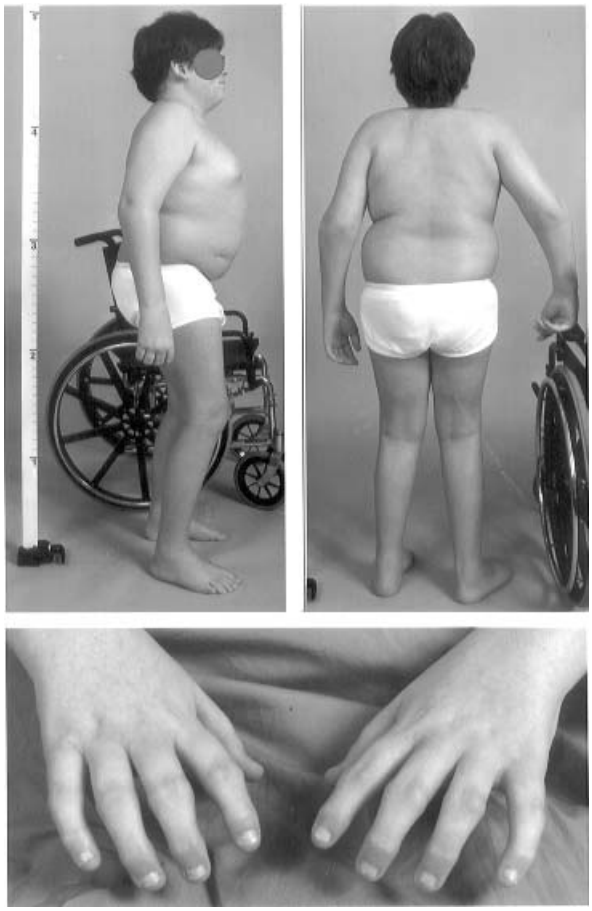

Figure 1. Progressive pseudorheumatoid dysplasia in a 12-year-old boy. **Top left**: Flexion contractures affecting hips and knees. Also note wheelchair reliance due to severe large joint pain. **Top right**: Bony enlargement and flexion contractures at the elbows. **Bottom**: Bony enlargement affecting all proximal and distal interphalangeal joints. (Photographs courtesy of Dr. J. Kenneth Herd.)

Patients with type II and type XI collagenopathies can also have enlarged epiphyses and signs of a spondyloepiphyseal dysplasia (see also Chapter 23, Part II, this volume). Such patients often have extraskeletal features such as mild facial dysmorphism, myopia, retinal detachment, a bifid uvula, cleft palate, or sensorineural hearing loss. However, in contrast to collagenopathies that lead to precocious osteoarthritis necessitating joint replacement surgery, the joint pain in PPD appears more severe and is more rapidly progressive.

X-linked spondyloepiphyseal dysplasia also can present in mid-childhood to late childhood. Boys are principally affected and the clinical course is generally much milder than that of PPD [25,26] (see also Chapter 23, Part II, this volume).

GENETICS

PPD is an autosomal recessive disorder. Its prevalence has been estimated at one per million in the United Kingdom [1]. It is likely to be greater in countries with higher rates of consanguinity [27]. The majority of published patients with PPD have been offspring of consanguineous unions. Penetrance appears complete. The locus for PPD has been mapped to human chromosome 6q22 using DNA from a single large consanguineous kindred [16]. Linkage studies in eight other families are consistent with this map location and suggest that PPD is locus homogeneous [17–19].

Linkage analysis suggested the PPD candidate interval would be 2 million megabases in size. A sequenced bacterial artificial chromosome could be placed within this interval because it contained a genetic marker that was fully linked to the PPD phenotype. This bacterial artificial chromosome also contained a putative gene that was similar to, but clearly distinct from, the gene encoding connective tissue growth factor. This new gene, *WISP3*, was tested as a candidate for PPD, with the result that nine published and several unpublished disease-causing mutations were identified in affected patients [19].

Mutations include both large and small deletions, reading frame-shifts, and nonsense and missense mutations. The latter typically alter one of the protein product's highly conserved cysteine residues. It is hypothesized, but not yet proven, that the disease-causing mutations result in a loss of WISP3 function. Whether dominant skeletal phenotypes may result from dominant-negative mutations in this protein is not known. While the PPD work was in progress, the gene was independently identified and named *WISP3* (Wnt-induced secreted protein-3) by investigators interested in downstream genes in the Wnt signaling pathway that may be involved in malignant transformation [20]. WISP3 coding sequence is contained within five exons. Coding sequence mutations have been found in most, but not all, patients that are clinically diagnosed with PPD [19]. At this time, it is not known whether the inability to find mutations in all patients reflects locus heterogeneity, phenocopy, or mutations in as yet uncharacterized *WISP3* regulatory elements.

WISP3 PROTEIN

WISP3 is a member of the CCN (connective tissue growth factor, cysteine-rich protein 61, nephroblastoma overexpressed) protein family [21]. At present, this family has six different members (CTGF, Cyr-61, Nov, WISP1, WISP2, WISP3), all of which appear to be cysteine-rich, secreted proteins likely to be involved in regulating cell growth and differentiation and morphogenesis [22,23]. The different patterns of expression and the effects upon cells in culture of CCN family members indicate that they have nonredundant roles. WISP3 mRNA is not detected by standard Northern blot analysis; however, it can be amplified by reverse-transcription PCR from several tissues including cartilage, synovium, kidney, prostate, and testis [19,20]. The precise function of WISP3 during skeletal growth and homeostasis is not presently known.

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Chapter 26, Part III

The Camptodactyly-Arthropathy-Coxa Vara-Pericarditis Syndrome

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INTRODUCTION

Familial arthropathy associated with congenital flexion deformity of the fingers was described by Jacobs in 1965 [1]. Additional families have since been reported and the phenotypic spectrum of this autosomal recessive disease (MIM 208250) has broadened [2–11]. The hallmark features of the syndrome remain congenital or early-onset camptodactyly and noninflammatory, childhood-onset, large-joint arthropathy. Other features can include progressive coxa vara deformity, noninflammatory pericarditis, and occasionally pleuritis and peritonitis. The disorder has been mapped to chromosome 1q25 and it appears to be locus homogeneous [10]. The responsible gene, *CACP*, has been identified [12], and encodes a large, highly glycosylated protein that is synthesized and secreted by synoviocytes and superficial zone chondrocytes [12–14]. The precise function of this protein is unknown; however, it may be involved in the lubrication of joints and tendons and in the regulated growth of intimal cells.

CLINICAL FEATURES

Camptodactyly principally affects the first metacarpophalangeal joint and the proximal interphalangeal joints (Fig. 1). Camptodactyly, which has occurred in all published cases, has either been present at birth or has been recognized during the first few months of life. Involvement is bilateral, but need not be symmetrical. Finger contractures are generally painless, but may be progressive. Tendon releases have improved function. Toes can also be affected.

Arthropathy begins during early childhood and is most apparent in large joints, such as the wrists, hips, knees, elbows, and ankles (Fig. 1). It has been present in all published cases. Affected joints appear swollen and feel "boggy" due to thickening of the synovium, but do not appear inflamed and are neither red nor warm to the touch. Joint swelling and a reduced range of motion are early signs of the arthropathy. Symptoms of pain and stiffness are reported by some patients. Little information is available regarding the long-term prognosis of the arthropathy. Joint pain is temporarily relieved by nonsteroidal anti-inflammatory drugs and by physiotherapy. Synovectomy has been performed in some patients; however, the long-term effects of this procedure have not been reported. Joint involvement is not debilitating in many patients; however, some patients do require hip and/or knee joint replacement surgery for progressive joint disease.

Coxa vara deformity has been observed in 60% of published cases and is progressive. It can be detected by radiographic examination in younger patients and is often suspected on the basis of clinical examination in older patients. The precise cause of the deformity is unknown. It may represent a primary dysplasia or a secondary abnormality of femoral neck growth associated with the arthropathy.

Clinical pericarditis has been observed in 30% of published cases. Siblings have been discordant for this feature, yet concordant for other component features. Pericarditis does not respond to anti-inflammatory agents. It may clinically disappear or it may progress to life-threatening constrictive heart failure. The latter has been successfully treated by pericardectomy.

HISTOPATHOLOGY

The absence of inflammation in synovial aspirates and synovial biopsies distinguishes the camptodactyly-arthropathy-coxa vara-pericarditis syndrome (CACP) from the more common childhood disorder, juvenile rheumatoid arthritis. Patients with CACP can have synoviocyte hyperplasia, diffuse villous hypertrophy with necrosis, and islands of free floating villi in synovial fluid. Multinucleate giant cells are occasionally seen; however, there is no other evidence of inflammation, such as infiltrating lymphocytes, plasma cells, neutrophils, or perivascular cuffing. Scarring between tendon and tendon sheath has been described in pathological material removed during tenosynovectomy for camptodactyly. Affected fibrous pericardium has shown both intimal cell hyperplasia and subintimal thickening, again without evidence of inflammation. Fibrin deposition has been observed on the intimal surfaces of synovium, tenosynovium, and pericardium.

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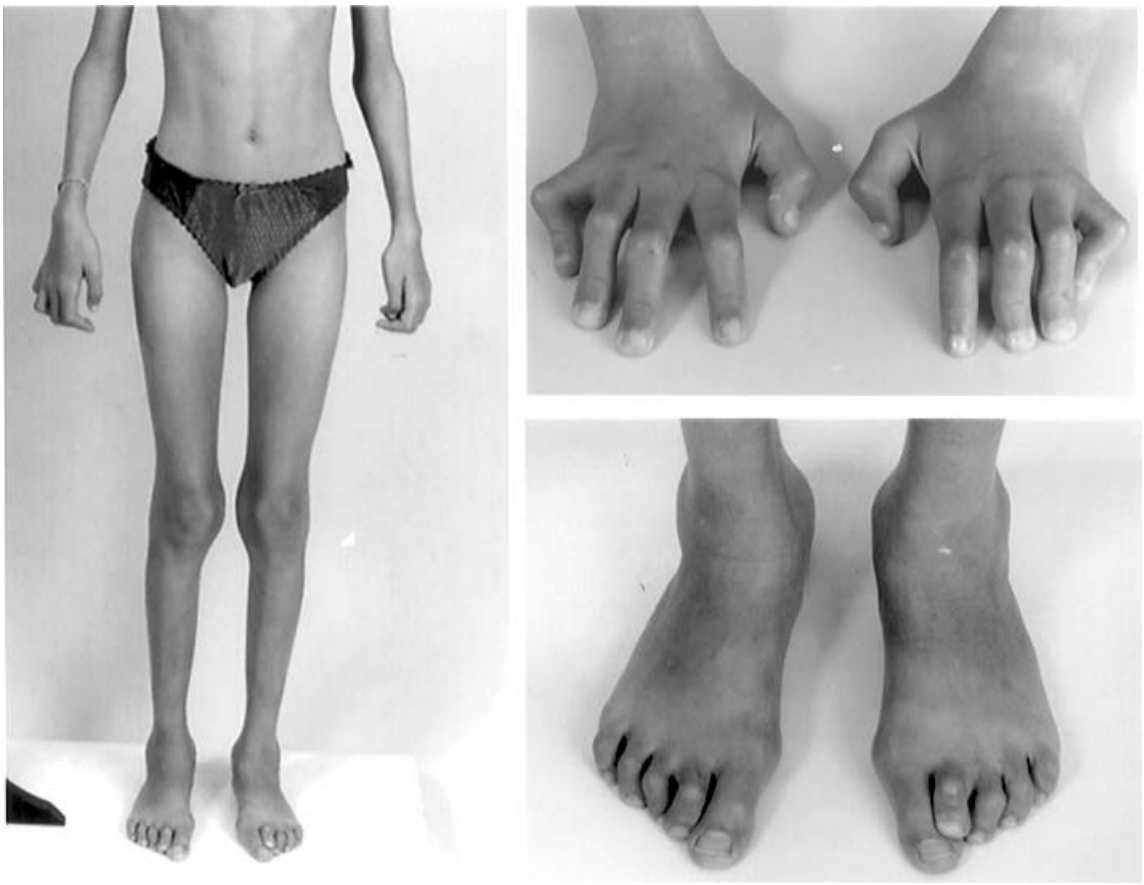

Figure 1. Camptodactyly-arthropathy-coxa vara-pericarditis syndrome in a 9-year-old girl. **Left**: Noninflammatory swelling at knees and ankles. **Top right**: Flexion contractures affecting multiple fingers. **Bottom right**: Flexion contractures affecting multiple toes and prominent ankle swelling. (Photographs courtesy of Dr. Chong Ae Kim.)

RADIOGRAPHIC FEATURES

Coxa vara deformity has been observed in the majority of patients and is progressive. Subchondral bone cysts may occur in the femoral head and acetabulum. Periarticular osteoporosis may occur at affected joints. Significant skull, spine, and apical extremity involvement have not been described.

DIFFERENTIAL DIAGNOSIS

CACP is a noninflammatory, nongranulomatous disease. It is thus distinguished from the more common disorder, juvenile rheumatoid arthritis, and rare autosomal dominant disorders such as Blau syndrome [15] (MIM 186580), familial arthritis and camptodactyly [16] (MIM 108050), and familial erosive arthritis with camptodactyly [17]. Patients with CACP have a normal facial appearance, and do not show signs of respiratory distress, which distinguishes CACP from the syndrome of progressive laryngotracheal stenosis with short stature and arthropathy [18] (MIM 603391).

GENETICS

CACP is an autosomal recessive disorder, the precise incidence of which is not known. Many patients affected with CACP have been the offspring of consanguineous unions. The number of published cases remains small (less than 50 patients); penetrance appears complete, whereas clinical expression is variable. The locus for CACP was mapped to human chromosome 1q25 using DNA from four consanguineous kindreds. Unpublished linkage analyses and mutation detection studies in other affected families suggest that the disorder is locus homogeneous.

The identification of the responsible gene, *CACP*, was facilitated by random sequencing of a bacterial artificial chromosome contig across the 2 mb CACP candidate interval on chromosome 1. Sequence encoding the megakaryocyte stimulating factor precursor protein was found. Because this sequence was also represented as an expressed sequence tag (that is, a cDNA fragment) in a synoviocyte cDNA library, it was tested positively as a candidate for causing CACP [12]. To date, seven published, and several unpublished, disease-causing mutations have been identified within it, all of which have been reading frame-shifts or nonsense mutations causing truncation of the protein. Coding sequence mutations have not been identified in all patients with clinical CACP, for which there are two likely explanations. First, one of the 12 coding exons within the gene is quite large and difficult to sequence rigorously. Second, noncoding regulatory regions of the gene are not known and therefore have not been systematically screened for mutations. A

third possible explanation for the failure to find all disease-causing mutations is locus heterogeneity; however, this is least likely, since several affected patients have had one of their two mutant *CACP* alleles detected and other patients with undetected mutations still demonstrate genetic linkage to human chromosome 1q25.

CACP PROTEIN

The precise biologic function of the *CACP* gene is not known. Its mRNA is highly expressed in synoviocytes and pericardiocytes, but it is also expressed in several other tissues, including liver. Because the protein product was initially identified as a megakaryocyte stimulating factor [19], one of its roles may be to regulate cell growth. The *CACP* gene encodes a 1404 amino acid residue polypeptide with a predicted molecular weight of 151 kDa; however, its megakaryocyte stimulating factor activity is contained within a 25-kDa fragment. The *CACP* gene product has also been identified in bovine and human joints as a proteoglycan expressed by superficial zone chondrocytes [20,21] and as a major boundary lubricant [14]. The *CACP* gene product has a large mucin-like domain that is rich in serine and threonine residues and highly glycosylated with O-linked oligosaccharides. Other protein domains may be involved in cell surface binding or matrix binding. Taken together, these biochemical and biophysical properties of the *CACP* gene product suggest it will be involved in lubricating and protecting cells on the surfaces of tendons and joints. The protein may also have this role at other intimal cell surfaces such as those of pericardium, pleura, and peritoneum.

RECENT DEVELOPMENTS

Faivre et al. [22] have reported clinical and linkage data for 12 individuals affected with CACP from eight unrelated families. The authors note that the earliest description of the condition may be that of Didier, in 1953 [23], and that spine and hip involvement may be more common than previously thought. In providing a follow-up of Didier's patient after more than 40 years, they observed "joint involvement gradually increased and tended to become incapacitating with age" in this individual. In addition to adding to the clinical delineation of CACP, the authors also support locus homogeneity for CACP based upon their genetic linkage analyses.

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Chapter 26, Part IV

Bruck Syndrome

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CLINICAL FEATURES

Bruck syndrome (BRKS; MIM 259450) presents with symmetrical joint contractures and pterygia of elbows and knees at birth. Recurrent fractures of ribs and long bones reminiscent of osteogenesis imperfecta occur in infancy and early childhood, as well as bowing of long bones, dimpling of the skin, osteopenia, Wormian bones and basilar impression. Normal anthropometric measurements are seen at birth and in infancy. Growth deficiency from early childhood results in short stature. Kyphoscoliosis is seen in all patients. In contrast to osteogenesis imperfecta (OI), most patients do not have blue sclerae, hearing loss or dentinogenesis imperfecta; also atypical of OI is the presence of clubfoot which occurs in Bruck syndrome. None of the reported patients is able to walk without aids. Some older patients have died from restrictive lung disease. The presence of pterygia suggests that lack of movement of the affected joints commences early during embryological development.

Bruck syndrome is exceptionally rare. Mackenzie [1], in a series of 37 cases of arthrogryposis multiplex congenita, found only one infant with associated osteogenesis imperfecta, who seems to have been the first reported case of Bruck syndrome, although no clinical description was given. The first clinical description of a patient with Bruck syndrome among the total of 21 patients known today was reported from India [2]; among the other 20 are one from India [3], four from South Africa and one from Zimbabwe [4], two from Pakistan [5], one from Germany [6], one Caucasian from North America [7], five from Kurdistan [8], one from Egypt [9], three from Belgium [10], and one from Australia [11].

Viljoen et al. [4] believed that Bruck [12] was the first to describe the particular combination of clinical manifestations of arthrogryposis multiplex congenita and osteogenesis imperfecta, and eponymously attached Bruck's name to the disorder. Steinmann [13], however, pointed out that Bruck's patient had a normal postnatal stature and quite different progressive bone changes (fractures, deformations), and joint contractures starting only at the ages of 14 and 18 years, respectively, and thus did not have what we now call Bruck syndrome.

INHERITANCE

Bruck syndrome is most likely an autosomal recessively inherited disorder because of its multiple occurrence in sibs of both sexes and the existence of several patients born to consanguineous parents. It has a wide geographic distribution (see above). Obligate heterozygotes seem to be asymptomatic although no densitometric studies of bone have been performed.

The disease locus has been localized by homozygous mapping in the family reported by Breslau-Siderius et al. [8] to chromosome 17p12, within a region of maximally 18 cM between markers D17S969 and D17S2196 [14], but the gene itself has not yet been characterized (R.A. Bank, personal communication 2001). In two families, including that of the patient of Sillence et al. [11], linkage analysis suggests a second location other than 17p12 (D. Sillence and R. Bank, personal communications 2001); the patients in these two families, therefore, justify a splitting of Bruck syndrome to include a type II form contrasting with the type I form in the family reported by Bank et al. [14]. The urinary pyridinoline profile is the same in patients with either type (see below). In the two Bruck syndrome type II patients, two different disease-causing mutations in a conserved region of a lysyl hydroxylase gene have been identified (R. Bank, personal communication, 2001).

PATHOGENESIS

Bank et al. [14] provided convincing, though indirect, evidence that a bone-specific collagen telopeptidyl lysyl hydroxylase is deficient in Bruck syndrome. In bone collagen I from patients with the type I form of the disorder, substantial amounts of bifunctional cross-links were found, indicating that collagen in the extracellular matrix met the spatial requirements for cross-linking. However, the relative abundances of different bifunctional (lysinonorleucine [LNL] and hydroxylysinonorleucine [HLNL]) and trifunctional cross-links (lysyl pyridinoline [LP] and hydroxylysyl pyridinoline [HP]) were completely different from what is observed in normal bone, in that there was a preponderance of cross-links derived from lysyl residues in the telopeptides compensating for the very low amounts of

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telopeptidyl hydroxylysine-derived cross-links. In contrast, the normal levels of unmodified and glycosylated hydroxylysine in the triple helix indicated that the hydroxylation defect was limited to the telopeptides and thus different to that in the kyphoscoliotic type of the Ehlers-Danlos syndrome (EDS VI; see Chapter 9, this volume). The most likely explanation for these results is that Bruck syndrome is caused by the lack of an enzyme capable of hydroxylating telopeptidyl lysyl residues. The normal cross-linking in ligament (collagen I) and cartilage (collagen II) indicate a normal telopeptidyl lysyl hydroxylation in these tissues. Thus, Bruck syndrome not only provides circumstantial evidence for the existence of a telopeptidyl-specific lysyl hydroxylase; it also suggests the existence of a tissue-specific form, in that only bone collagen I is affected. Identification of the specific gene, the possibility of its transcriptional/translational control by a gene on chromosome 17p12, and mutations in each of these in Bruck syndrome type II and type I, respectively, provide further very strong support for the existence of the enzyme postulated by Royce and Barnes [15] and Steinmann et al. [16].

In the turkey leg tendon mineralization model, major changes occur in the cross-link profile of collagen I during transition of the tendon from unmineralized to mineralized tissue [17], this being characterized in the latter by a high proportion of bifunctional cross-links, a relatively low HP content, and the presence of significant amounts of LP. These findings suggest that correct cross-linking is critical for proper mineralization, a suggestion supported by the observation that, in the almost complete absence of HP and LP, a deficiency of mineral in relation to the organic matrix is seen, as well as an increase in size of the hydroxyapatite crystals [6]. The bone brittleness observed in Bruck syndrome is most likely caused by a combination of diminished cross-linking via the telopeptidyl hydroxylysine route and subsequent defective mineralization.

Earlier reported findings are compatible with those of Bank et al. [14]. Collagen extracted from bone with pepsin is normal with regard to hydroxylysine content and the relative proportions and electrophoretic mobilities of the α1(I), α2(I), β, and γ chains of collagen I [6,14], and collagens produced by fibroblasts are also normal in these respects [7,8,10].

DIAGNOSIS, DIFFERENTIAL DIAGNOSIS, AND MANAGEMENT

The diagnosis of Bruck syndrome is suspected clinically, and confirmed by a low urinary excretion of total pyridinolines (LP + HP) expressed in relation to creatinine, a lower than normal ratio of LP/HP (R.A. Bank, personal communication 1999; normal ratio 0.20 ± 0.10; see Chapter 9 and Appendix II, this volume), and the finding of collagens produced by fibroblasts being normal with regard to relative proportions and electrophoretic mobilities upon SDS-PAGE.

A differential diagnosis comprises osteogenesis imperfecta, mainly types III and IV, and, among these, especially the rare autosomal recessively inherited forms (see Chapter 8, this volume), the various forms of arthrogryposis multiplex, and congenital contractural arachnodactyly (CCA; MIM 121050).

The management of Bruck syndrome is the same as that of osteogenesis imperfecta (see Chapter 8, this volume). Bisphosphonate has been given to one patient with apparent success [11]; the patient died, however, from complications resulting from progressive cervical kyphosis (D. Sillence, personal communication 2001).

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Chapter 26, Part V

Osteoporosis-Pseudoglioma Syndrome

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CLINICAL FEATURES

The osteoporosis-pseudoglioma syndrome (OPPG, OPS; MIM 259770) is characterized by visual loss at birth or in infancy and by a fracturing bone disease that presents in childhood. The term "pseudoglioma" alludes to the histological appearance of the eyes, which were sometimes mistakenly removed because of presumed retinoblastoma (for review, see [1]), and is a nonspecific term for any condition more or less mimicking retinoblastoma.

Many OPPG patients are born with severe eye involvement such as phthisis bulbi (shrinkage and wasting of the eyeball), microphthalmia, corneal opacity, absence of the anterior eye chamber, iris atrophy, secondary glaucoma, lens opacity, posterior synechiae, and leukokoria (white retrolental masses behind the pupil resembling retinoblastoma); in such patients the eyelids are constantly closed. In patients with milder eye involvement there is persistent hyperplasia of the primary vitreous, which has led to speculation that the ocular pathology results from a defect in the normal involution of the primary vitreal vasculature that begins during the 13th gestational week [2]. In patients with delayed blindness, an acute episode (such as "eye infection" or "glaucoma") is often reported as immediately preceding final loss of vision [1]. The sclerae are white.

Patients have fractures in the long bones and axial skeleton with subsequent deformations, multiple crush fractures, bone pain, kyphosis, and short stature, and become progressively immobile and wheelchair-bound. Radiological findings are generalized osteopenia affecting the axial as well as the appendicular skeleton, decreased bone density, thin cortical and coarse trabecular structures, a thin cortex, and often Wormian bones. The teeth are normal.

Intelligence and hearing are normal in most cases; sometimes microcephaly, muscular hypotonia and ligamentous laxity are noted. Interfamilial and even intrafamilial variability may be considerable. The natural history of the disease has not yet been well described.

CHEMICAL AND HISTOLOGICAL FINDINGS

OPPG patients have normal anabolic and catabolic hormone levels, calcium homeostasis, endochondral growth, and bone turnover, as indicated by normal urinary excretion of pyridinolines [3] (and B. Steinmann, unpublished results). Collagen synthesis by fibroblasts is also normal [4,5] (and B. Steinmann, unpublished results). Histology of bone biopsies reveals a deficient trabecular bone volume, but a normal surface density and appearance of osteoblasts and osteoclasts on bone surfaces [6]. In the eyes, pseudogliomatous tissue is observed (for review, see [1]). Collagen fibrils in the dermis are ultrastructurally normal [5].

INHERITANCE AND MOLECULAR DEFECT

OPPG is a rare disease which is autosomal recessively inherited as evidenced by the occurrence of affected sibs of both sexes, and of several patients born to consanguineous parents. Although Frontali et al. [1] mentioned that the disease may be more prevalent in Mediterranean countries, actual review of the literature indicates that it occurs world-wide [1,5–7]. Some, but not all, obligate carriers of OPPG display an increased incidence of osteoporosis-related fractures and a reduced bone mass (dominant effect of the mutation) [6,7].

The OPPG locus was earlier assigned to chromosome 11q13 [7]. Using the positional candidate approach, the gene responsible for OPPG has now been identified as *LRP5*, a member of the low density lipoprotein (LDL) receptor-related family [6].

LRP5 comprises 23 exons that span >100 kb and has an open reading frame of 4.9 kb [8,9]. The gene encodes a 29 amino acid residue signal peptide, 20 Tyr–Thr–Trp–Asp spacer repeat domains interspersed with four epidermal growth factor (EGF)-like domains, a transmembrane domain, and a 200 amino acid residue intracytoplasmic domain. Gong et al. [6] have described six different homozygous frameshift or nonsense mutations and two different homozygous missense mutations in unrelated patients born to consanguineous parents, and four heterozygous nonsense, frameshift, or missense mutations in patients from non-consanguineous families; (in these latter four cases the other mutant allele has not yet been determined). The different mutations indicate that there is no ancestral mutation or founder effect.

PATHOGENESIS

LRP5 is expressed by osteoblasts *in situ* and its expression pattern changes over time when mesenchymal cells differentiate to osteoblasts *in vitro* [6]. LRP5 localizes

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to cell membranes when expressed in transfected human fibroblasts and is capable of binding Wnt-4. These observations, in combination with similar phenotypic findings in an *Lrp5* knockout mouse model (see below), suggest that accrual of bone mass occurs during Wnt-mediated osteoblastic proliferation and differentiation [6].

Loss of function seems to be the general mechanism for the mutational effect in OPPG. This is especially convincing in respect of the homozygous nonsense codon at the 10th amino acid (Trp10Stop) within the signal peptide [6] in two affected siblings described by Oexle et al. [10]. Furthermore, transiently transfected COS-7 cells containing either wild-type *LRP5* or two disease-causing alleles (Gln853Stop and 1270fs1438stop) synthesize normal and mutant proteins, respectively [6]. In contrast to this, COS-7 cells containing an artificial LRP5 construct lacking the transmembrane domain do secrete mutant LRP5; conditioned medium containing this truncated protein reduced bone formation in calvarial explant cultures. Thus, this artificial LRP5 is able to inhibit bone growth, presumably by binding to, and interfering with, secreted growth factors [6].

The precise cause of low bone mass in OPPG patients is not known. However, several independent observations suggest that LRP5 regulates osteoblasts at the level of their proliferation and differentiation. Since reduced LRP5 expression appears to regulate bone mass negatively, it may be speculated that other *LRP5* mutations, or common polymorphisms, which enhance the expression of LRP5, will positively regulate bone mass. In this context, it is of interest that a genetic high bone mass phenotype (bone mineral density variation 1; MIM 601884) [11] and a bone mass quantitative trait locus (bone mineral density variation 2; MIM 605833) [12] have also been mapped to the genetic interval that contains *LRP5*; in the former condition, a mutation in this gene has been described [12a].

Gong et al. [6] suspect that transient expression of LRP5 by cells within the vitreal vasculature is responsible for the involution process and that in OPPG failure of this process leads to fibrosis and contracture. Traction on other ocular structures by this fibrotic process affects vision by creating retinal folds, retinal detachment, and, in extreme cases, phthisis bulbi.

Two other autosomal dominantly inherited ocular disorders leading to blindness, familial exudative vitreoretinopathy (EVR1; MIM 133780) and neovascular inflammatory retinopathy (VRNI; MIM 193235) also map to an interval containing *LRP5* ([13] and [14], respectively). EVR1 appears to result from impaired growth of the peripheral retinal vasculature, with the resultant formation of fibrovascular vitreal membranes that leads to retinal traction and detachment, while VRNI appears to result from an inflammatory process leading to occlusion of the peripheral retinal vasculature and subsequent new vessel growth. It is reasonable to speculate that different *LRP5* mutations, in addition to causing OPPG, also cause EVR1 and VRNI via different mechanisms.

LRP5 is expressed in several tissues [8,15]. It is, therefore, surprising that the phenotypic consequences of mutations are restricted to the skeleton and the eye; it is possible that LRP6, which is also broadly expressed, shares a functional redundancy with LRP5 in many tissues [16].

DIAGNOSIS, DIFFERENTIAL DIAGNOSIS, AND MANAGEMENT

The importance of establishing the correct diagnosis and distinguishing OPPG from osteogenesis imperfecta is critical in the context of genetic counseling of the healthy parents, the risk of recurrence in a subsequent child being 25%. The demonstration of ocular calcification by computed tomography or a persistent hyperplastic primary vitreous by color Doppler imaging [17] in a blind patient with generalized osteopenia supports the diagnosis of OPPG, which can now be proven at the molecular level.

OPPG is not always easily distinguished from osteogenesis imperfecta and there is phenotypic confusion between the two which is reflected by historical reports (for review see [1]); older synonyms, such as the "ocular form of osteogenesis imperfecta" [18] and "pseudoglioma with bone fragility", should no longer be used [19]. The visual problems typically begin early in life, before osteopenia has become pronounced. This makes the early diagnosis of OPPG difficult because the differential diagnosis of leukokoria is quite elaborate. In the absence of microphthalmia it includes retinoblastoma (MIM 180200), retrolental fibroplasia of prematurity, posterior cataract, congenital retinal detachment, and coloboma of iris, choroid and retina (MIM 120200); while in the presence of microphthalmia, it includes Norrie disease (congenital progressive oculo-acousticocerebral degeneration; MIM 310600), Coat disease, Reese retinal dysplasia (MIM 266400), toxoplasmosis, and congenital cataract. Calcifications of the eye are seen in retinoblastoma, toxoplasmosis, and OPPG.

The management of brittle bones in OPPG is similar to that in osteogenesis imperfecta (see Chapter 8); bisphosphonates have also been tried with apparent success [3] and are under current trial. In OPPG patients with mild eye involvement, treatment is symptomatic.

ANIMAL MODEL

Analysis of the *Lrp5* knockout mouse supports a role for Lrp5 in osteoblast proliferation and differentiation, and confirms the observation that *LRP5* mutations may have recessive as well as dominant effects, such as reduced bone mass in the heterozygous state. Homozygous *Lrp5* knockout mice have ocular abnormalities (G. Karsenty, personal communication 2001).

Interestingly, the same investigator also finds that statins (HMG Co-A reductase inhibitors) can abrogate the skeletal phenotype of *Lrp5* deficiency. (Statins have also been reported to increase bone mass in mice [20] and postmenopausal women [21]). Although the mechanism by which they do so remains unclear, it is tempting to speculate that reducing cellular cholesterol leads to an increased expression of LRP5 or other superfamily members, and that this, in turn, directs uncommitted cells into an osteoblastic differentiation pathway.

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Chapter 26, Part VI

Myopathies Due to Defects in Collagen VI

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BETHLEM MYOPATHY (BENIGN CONGENITAL MYOPATHY WITH CONTRACTURES; MIM 158810)

Clinical Features

The eponym "Bethlem myopathy" was coined by Mohire [1] to describe a mild and slowly progressive congenital myopathy affecting mainly the proximal muscles, with contractures predominantly of the distal joints (elbows, wrists, fingers, and ankles), but often without significant disability. It is inherited as an autosomal dominant trait. The features of Bethlem myopathy can be present from birth onwards, manifesting in some patients as torticollis and contractures or neonatal floppiness, suggesting a congenital nature. Many patients, however, do not develop significant weakness before the first years of life and gross motor development may be normal. Patients have moderate weakness and atrophy of trunk and limb muscles, the proximal muscles being more involved than the distal ones, and the extensors more so than the flexors [1–4].

In addition to a limb-girdle type of muscular weakness, the extensor digitorum communis muscle is invariably involved. Flexion contractures of the elbows, the interphalangeal joints of the last four fingers — a hallmark of this myopathy [5] — and the ankle joints are found frequently. There is no relationship between contractures and the severity or duration of the muscular weakness [1,3]. Some patients experience muscle cramps following exercise [6].

Cardiac and facial muscles are not involved [1,2,7]; at autopsy, the hearts of two patients were normal in all respects [1], and moderate facial involvement has been reported only once [8]. Despite earlier reports of an absence of respiratory muscle involvement, progressive respiratory compromise has been reported [9] in a patient from the family described by Pan et al. [10], which expands the clinical spectrum of the disorder. Life expectancy is normal [1,2,5].

Bethlem myopathy appears to be a rare condition; however, careful examination may reveal that it is more common than assumed. In one study [5], nearly half of the individuals labeled by the authors as affected were unaware of their disease. Sporadic or familial oligosymptomatic individuals with mere clumsiness may escape medical attention.

Despite a relatively uniform clinical picture, variations do exist between patients from different families and also between patients within single families [11]. The onset of weakness may occur before 5 years of age, with mildly delayed motor milestones [1,3,11–13], or it may occur at a later stage, in the fourth [14] or sixth decades [11]. As some adults remain unaware of their weakness, an age of onset cannot always be reliably established [5]. The investigation of two novel kindreds and a reevaluation of previously reported families [2,3] as part of a genetic linkage study [15], disclosed aspects that differed from previous observations, shedding new light on the onset and natural course of Bethlem myopathy [16]. The analysis showed that nearly all affected children exhibit weakness or contractures and torticollis during the first 2 years of life. Early features include diminished fetal movements, neonatal hypotonia, slightly delayed motor milestones, and congenital torticollis and contractures which are of a dynamic nature during childhood. A slight worsening of the muscular weakness often ensues, to be followed by relative recovery during puberty. Apart from contractures, many patients are almost asymptomatic during early adult life. From middle age onwards, muscular weakness becomes obvious and sufficiently severe to warrant a wheelchair in more than two-thirds of patients over 50 years of age [16]. Thus, the course of Bethlem myopathy in adults is less benign than previously thought.

Biochemical, Histologic, Electromyographic, and Computed Tomographic Findings

Serum creatine phosphokinase is usually normal or moderately elevated, especially in younger patients [1,2,5,6,14,16]. Histopathological findings are nonspecific, showing a marked variation in muscle fiber diameter, a moderate increase in the number of fibers with internal nuclei, lobulated type I fibers, and a marked increase in the endomysial fatty tissue, but no significant fibrosis. There is a normal distribution of type I and type II fibers [1,2,4,5,11]. In one large family [5], Merlini et al. [17] found an age-related progressive deficiency of laminin β1 in the muscle fiber basal lamina. The expression of laminin β1, however, was well preserved in the blood vessels, demonstrating that the reduction of this protein in muscle fiber basal lamina represents a secondary and selective defect (see below).

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Electromyography has shown myopathic abnormalities in several families [1,4,5,11,18], whilst computed tomography has shown that muscle impairment is mild but more diffuse than clinically apparent and demonstrates an unexpected progressive fatty replacement of paravertebral muscles (the gluteal muscles are relatively preserved) [4,5,11], a finding which clinical examination has failed to show [5].

Genetics

Bethlem myopathy is inherited in an autosomal dominant fashion and is genetically heterogeneous. Linkage studies by Jöbsis et al. [19] and Speer et al. [20] first suggested that collagen VI was involved, and the disorder is now known to be due to mutations in the three genes coding for the α(VI) chains of collagen VI, i.e., *COL1A6* and *COL2A6*, located on chromosome 21q22.3, and *COL3A6*, located on 2q37.

Eight mutations have been characterized to date, five of which are substitutions for glycine. Except for one Gly substitution found in the NH_2-terminal globular domain of the α3(VI) chain, the other missense mutations are all localized in the triple helical regions of the three chains, and thus, most likely, exert a dominant negative effect. The remaining three mutations are splice-site mutations in the *COL6A1* gene, one of which leads to a premature termination codon (PTC), the other two of which lead to exon skipping; all three splice-site mutations cause haploinsufficiency (see below).

The eight described mutations and their consequences (in brackets) are as follows:

- α1(VI) Gly286Val [15] (N.B., the numbering is based on +1 being the first amino acid of the secreted chain without the signal peptide; if the ATG codon were considered as +1, the mutation would be Gly305Val); α1(VI) IVS14 + 1G > A (skipping of exon 14) [21]; α1(VI) IVS14 + 2T > C (skipping of exon 14) [18]; α1(VI) IVS11-1delG (PTC) [22];
- α2(VI) Gly250Ser [15] in two kindreds (N.B., the numbering in these cases also is based on +1 being the first amino acid of the secreted chain without the signal peptide);
- α3(VI) Gly1679Glu [10]; α3(VI) Gly2056Arg [23] (in these cases the numbering is based on ATG being +1).

Pathogenesis

Collagen VI, a major protein of the interstitial matrix, is composed of three genetically distinct α(VI) chains, [α1(VI)α2(VI)α3(VI)]. Tetrameric aggregates of collagen VI trimers are secreted and form the basic unit of collagen VI microfibrils deposited in the extracellular matrix. They aggregate end-to-end through interaction of their "outer-terminal" globular domains, giving rise to characteristic long, thin, beaded filamentous structures which have a widespread tissue distribution and localize close to cells, nerves, blood vessels, and large collagen fibrils, and are considered to have an anchoring function, linking cells and the extracellular matrix (see Chapter 2, Parts I and II, this volume).

Collagen VI consists of a central, short collagenous region flanked by several von Willebrand factor type A modules at the N- and C-terminal ends. Both types of domain participate in specific molecular interactions. The collagenous domain binds cell membranes through integrins α1β1 and α2β1, as well as such matrix constituents as proteoglycans such as perlecan, decorin, biglycan and fibromodulin, along with collagen II, whereas the type A modules bind collagens I, IV and VI, as well as fibronectin, heparin, and hyaluronic acid.

In general, muscular dystrophies are caused by a disturbance of the attachment of muscle cells to their basement membranes [24,25]. The normal attachment structure in muscle consists of a submembranous dystrophin network, a dystrophin-associated glycoprotein complex (composed of α-, β-, γ-, and δ-sarcoglycans and α- and β-dystroglycans), and laminin (merosin) and collagen IV, which are both basement membrane components. A second attachment structure has been described, the α7β1 integrin receptor that also binds to (extracellular) laminin [26]. Kuo et al. [27] have shown strong binding of collagen VI to collagen IV *in vitro*, a general co-distribution of collagens IV and VI in human muscle by double-label immunofluorescence microscopy, and an intimate association of collagen VI with basement membranes surrounding muscle fibers by immunoelectron microscopy. Collagen VI is, therefore, part of the muscle cell attachment structure that provides an important physical connection between muscle cells and their extracellular matrix and may also play a role in myofiber regeneration.

Haploinsufficiency due to mutant *COL6A1* genes is a well documented pathogenic mechanism in Bethlem myopathy, as shown in three separate families [18,21,22]. In one large family, Lamandé et al. [22] characterized a G deletion at the conserved splice acceptor site in intron 11 of *COL6A1* (IVS11-1delG), and as a consequence thereof, a PTC due to a splicing defect in the mRNA leading to nonsense-mediated mRNA decay. (Residual protein expression was predicted to result in a 37 kDa truncated product). While the α(VI) chains produced by fibroblasts from affected individuals migrated normally under reducing conditions in SDS-polyacrylamide gels, analysis under non-reducing conditions revealed the accumulation of a disulfide-bonded intermediate which was more abundant and migrated faster than that observed in normal cells; this observation is consistent with the association of α2(VI) and α3(VI) chains proceeding normally, but further assembly being limited by a reduced availability of α1(VI) chains. Such an α2(VI)-α3(VI) assembly intermediate containing only two chains could not be triple-helical and would not be secreted, and thus the net effect of α1(VI) haploinsufficiency is the assembly and secretion of reduced amounts of collagen VI. Reduced amounts of deposited collagen VI have been documented by immunofluorescent staining of the matrix laid down by Bethlem fibroblasts in long-term culture [22].

In two further families, Lamandé et al. [21] and Pepe et al. [18], respectively, showed that skipping of exon 14 led to truncated α1(VI) chains (lacking 18 amino acids together with a unique cysteine residue thought to be involved in dimer formation) which were clearly detectable within Bethlem fibroblasts, but not in their medium, that only half amounts of dimeric and tetrameric aggregates were formed, and that trimers containing a mutant α1(VI) chain accumulated intracellularly. Hence, the shortened mutant α1(VI) chains were able to assemble with normal chains to form monomeric trimers, but the mutant chain-containing trimers were unable to form disulfide-bonded dimeric and tetrameric aggregates, probably for sterical reasons or the absence of a necessary cysteine residue, and were not secreted. Because stable monomeric trimers of helical collagen VI only form with an equimolar stoichiometry of α1(VI), α2(VI), and α3(VI) chains, half of the monomeric trimers contained the mutant α1(VI) chain and were not secreted, resulting

in a functional haploinsufficiency of collagen VI in the extracellular matrix. It is worthy of note that members of the two different families displayed markedly different severe phenotypes despite the fact that all have the same shortened α1(VI) chain; the affected family members reported by Pepe et al. [18] have the most severe phenotype reported so far. Haploinsufficiency of mutant *COL6A2* genes has not yet been observed (see below and Ullrich Disease, this chapter).

Single glycine substitutions in one of the three α(VI) chains may also cause Bethlem myopathy [10,15,23]. However, biosynthetic studies have not been performed in such cases and it remains to be determined whether the mutations interfere with intracellular assembly and secretion, thereby resulting in collagen VI haploinsufficiency, or whether the disease may result from the presence of structurally abnormal collagen VI in the extracellular matrix exerting a dominant negative effect, as may be inferred from the effects of Gly substitutions in the triple-helical domains of several other collagens (see Chapters 8, 9, 15, 23 Part II, 25, and 26 Part VII, this volume). A Gly1679Glu mutation in the N2 motif of the N-terminal globular domain of *COL6A3* is at a conserved residue in the center β sheet of the von Willebrand A motif [10], and recombinant protein and molecular modeling studies suggest that it is likely to impair protein folding, consistent with radioimmunoassay data showing reduced levels of N2-epitopes in cultured fibroblasts from two patients possessing the mutation [28].

All these findings identify collagen VI as a contributor to normal skeletal muscle integrity and suggest that a reduced collagen VI microfibrillar network may no longer adequately anchor muscle cells to the surrounding connective tissue, and that collagen VI may also play a role in myofiber regeneration. The age-related secondary decrease in laminin β1 expression that occurs in individuals with Bethlem myopathy is restricted to skeletal muscles [17] (see Animal Model and Cell Expression Studies, below).

It appears that the interaction of collagen VI with basement membranes involves multiple interaction sites since the basement membrane proteoglycan perlecan also interacts with α2(VI) and there are other binding partners. It is, therefore, not possible to say exactly which interactions will be disturbed in Bethlem myopathy. The fact that there are multiple binding sites on basement membranes for collagen VI could also explain the mild phenotype, since the connection would not be completely broken, but only weakened, depending on the type of mutant collagen VI. This, together with the existence of various differently spliced isoforms (with distinct tissue-specific distribution and probably subtly different functional properties) may explain the distinct pattern of muscular involvement as well as the sparing of involvement of other tissues.

Considering the wide distribution of collagen VI, it is surprising that mutations within it produce a muscle-specific disease rather than a more general one. Collagen VI is also abundant in skin and cornea and is found in cartilage and bone; however, these tissues are apparently not affected in patients with Bethlem myopathy. It may be worthwhile examining affected individuals in greater detail with reference to, for example, the thickness, stiffness, deformability, and turbidity of the cornea, which normally contains a considerable amount of collagen VI (~25% dry weight [29]) (see Ullrich Disease and Animal Model and Cell Expression Studies, below).

Diagnosis, Differential Diagnosis, and Management

The detection of Bethlem myopathy depends on recognition of the clinical pattern and, given its autosomal dominant inheritance, the careful examination of family members, with a systematic search for contractures, the use of muscle computed tomography, and measurements of muscle strength.

In contrast to Bethlem myopathy, the dominant limb-girdle muscular dystrophies (LGMD1A-LGMD1D) show no early contractures and onset is in adulthood. In Emery-Dreifuss muscular dystrophy (MIM 310300), contractures of the cervical spine and the elbows are combined with cardiac conduction defects. The rigid spine syndrome (MIM 602771) is mostly sporadic, preferentially affects males, and presents with scapulohumero-pelvic weakness, progressive scoliosis, and reduced life span due to respiratory failure. Congenital muscular dystrophies are characterized by autosomal recessive inheritance, proximal muscular weakness, generalized muscular hypertrophy, usually grossly elevated creatine phosphokinase, and early respiratory failure.

The management of Bethlem myopathy is symptomatic. Tenotomy for lengthening of the Achilles tendon is necessary in many patients. From middle age onwards, slow deterioration becomes apparent in sufficient degree to warrant aids such as a walking stick, orthopedic shoes, adjustments to the home such as a raised toilet seat or a staircase with lowered steps, and a wheelchair.

ULLRICH DISEASE (SCLEROATONIC MUSCULAR DYSTROPHY; MIM 254090)

Clinical Features and Pathogenesis

The autosomal recessive disorder, Ullrich disease, a severe congenital muscular dystrophy, is allelic to Bethlem myopathy [30]. In 1930, Ullrich [31] described two unrelated patients with congenital muscular dystrophy (MCD). The disorder was defined as "scleroatonic muscular dystrophy". Peculiar clinical manifestations were proximal joint contractures and striking distal hyperextensibility, especially of wrists and ankles, leading to severe disability, while intelligence was normal. Since then, more than 20 patients with similar clinical manifestations have been reported worldwide [25,32], and thus recognized as having a distinct form of MCD. Some familial cases showed more than one sibling affected and a high incidence of consanguinity among unaffected parents, compatible with autosomal recessive inheritance [32]. Creatine phosphokinase is only moderately elevated [30,32]. Most patients die of respiratory failure with diaphragmatic paralysis in the first decade of life, and it is noteworthy that the diaphragm is particularly involved in *Col6a1* knockout animals (see below). De Paillette et al. [32] stated that diagnosis is easy and that the condition may not be as rare as suggested by the paucity of reports, as undiagnosed cases may be included in reported series of other congenital muscular dystrophies.

Vanegas et al. [30] described 4 patients from three families who all had homozygous or compound heterozygous mutations in the *COL6A2* gene. Patient A had a homozygous insertion of a C nucleotide leading to a premature termination codon (PTC) in exon 16 of the triple-helical domain; the healthy, consanguineous parents in their fifties were heterozygous carriers of the mutation. In patient B, there was an α2(VI) IVS17-2A>G substitution and, as a consequence,

activation of a cryptic acceptor site in exon 18, deletion of 28 nucleotides, and the introduction of a PTC due to a frame shift; the second mutation consisted of an α2(VI) IVS23-1G>A substitution with skipping of exon 24. Both mutations were present in a similarly affected brother. Again, the parents were healthy carriers. In patient C, only a mutation in one allele, the same α2(VI) IVS17-2A>G substitution as in patient B, could be detected and appeared to be a *de novo* mutation, as it was not found in her parents. A near total absence of collagen VI in the matrix of fibroblast cultures (patients A and B) and muscle sections (patients B and C) was demonstrated by immunofluorescence. A similar absence of collagen VI in muscle and skin was demonstrated in two further patients [33]. These results clearly demonstrate that Ullrich disease is caused by recessive mutations leading to a severe reduction of collagen VI. It is even more intriguing that, in Ullrich disease, a total lack of collagen VI should produce a muscle-specific disease rather than a more general phenotype consistent with its wide distribution, than it is that a haploinsufficiency should have the same effect in Bethlem myopathy.

It is somewhat surprising that both parents in family A and the mother in family B, all of whom carry heterozygous *COL6A2* mutations leading to premature termination codons, are clinically healthy and do not have Bethlem myopathy. It is noteworthy that no patients with Bethlem myopathy due to *COL6A2* haploinsufficiency have been reported to date, and that the *COL6A1* and *COL6A2* genes are regulated differently. Up-regulation of a normal *COL6A2* allele could compensate for the lack of expression of a mutant *COL6A2* allele and rescue the carriers from a Bethlem myopathy phenotype, whereas such a compensatory mechanism may not be operative for the *COL6A1* gene [30].

For differential diagnostic considerations, see Voit [25] and the kyphoscoliotic type of Ehlers-Danlos syndrome (EDS VI) (Chapter 9, this volume).

ANIMAL MODEL AND CELL EXPRESSION STUDIES

Bonaldo et al. [34] inactivated the mouse *Col6a1* gene by targeted gene disruption. As a consequence of the subsequent α1(VI) chain deficiency, homozygous mutants lacked collagen VI in all tissues, and neither could triple-helical molecules consisting of α2(VI) and α3(VI) chains be detected. Histological findings included fiber necrosis and phagocytosis and a pronounced variation in muscle fiber diameter. Muscles also showed signs of enhanced fiber regeneration. At all stages examined, necrotic fibers were particularly common in the diaphragm and auxiliary respiratory muscles. Similar, although milder, alterations were detected in heterozygous mutant mice, indicating haploinsufficiency of the *Col6a1* gene function. It seems that this is an animal model of both Bethlem myopathy and Ullrich disease, although the distribution of affected muscles is different. Amazingly, given the fact that collagen VI is widely distributed in the body, no other clinical signs were apparent, even in −/− mice. However, the corneal stroma especially of embryonic −/− mice does show disorganization of lamellae, fibril packing, and cells (D. Birk, personal communication 2001).

To explore further the consequences of collagen VI triple helical deletions on collagen VI biosynthesis, and to determine whether mutations in subunits other than the α1(VI) chain also affect intracellular multimer assembly, Lamandé et al. [21] transfected SaOS-2 human bone cells with an α3(VI) cDNA expression construct containing cDNA for a protein product containing a 202 amino acid deletion within the triple helical domain. Untransfected SaOS-2 cells produced α1(VI) and α2(VI) mRNAs at levels comparable to those of skin fibroblasts but were deficient in α3(VI) transcription and produced no stable collagen VI [35]. Normal collagen VI biosynthesis, however, could be restored in SaOS-2 cells by stable transfection with an α3(VI) cDNA expression construct, under which circumstances the α3(VI) chains produced rescued the α1(VI) and α2(VI) chains from intracellular degradation, and were incorporated into collagen VI trimers that were efficiently secreted. However, after transfection with mutant α3(VI) cDNA, collagen VI trimers containing truncated α3(VI) chains were almost entirely retained within the cells. The collagen VI patterns produced by fibroblasts expressing a naturally occurring truncated α1(VI) chain in Bethlem fibroblasts and SaOS-2 cells expressing a truncated α3(VI) chain were identical [21].

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Chapter 26, Part VII

Knobloch Syndrome

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CLINICAL FEATURES, DIAGNOSIS, AND DIFFERENTIAL DIAGNOSIS

Knobloch syndrome (KNO; Retinal Detachment and Occipital Encephalocele; MIM 267750) is characterized by high myopia, vitreoretinal degeneration with retinal detachment, cataracts, atypical occipital encephalocele (or congenital occipital scalp defects rather than true encephalocele), and normal intelligence. The condition seems to be rare as only 24 families have been reviewed [1] since its first description in 1971 [2]. The clinical manifestations may also include lens subluxation, abnormal macular pigmentation, neovascular glaucoma, phthisis bulbi, a flat nasal bridge, midface hypoplasia, and mild generalized hypermobility of the joints. Sniderman et al. [1] reported a patient with a midline defect of the frontal region. It is noteworthy that one of the five affected siblings of the initial family [2] had only high myopia and vitreous syneresis but no history of occipital encephalocele, and that the size of the encephalocele may vary among affected individuals from the same family [3].

The encephalocele may be visualized by magnetic resonance imaging and computerized tomography. Histological examination of the scalp defect has demonstrated heterotopic neuronal tissue in most patients, but no true brain tissue, hence the designation "scalp defects" rather than true encephalocele [4]. A differential diagnosis for syndromes with encephaloceles is given by Cohen [5].

INHERITANCE

The disorder is inherited as an autosomal recessive trait because of the occurrence of affected sibs of both sexes born to healthy parents who have often been consanguineous. The gene for the disorder was mapped to chromosome 21q22.3 [6] on the basis of homozygosity mapping in the largest kindred described [3]. The disorder in this family is due to a homozygous mutation at the AG consensus acceptor splice site, IVS1-2A>T, of the *COL18A1* gene coding for the α1(XVIII) chain of collagen XVIII [7]. In three of five unrelated families, Sertié et al. [7] identified different frameshift changes, each predicting a premature stop codon, in exons 10, 36, and 41, respectively. Menzel et al. [8] excluded chromosome 21q22.3 as the locus for Knobloch syndrome in a small New-Zealand family [9], and proposed that the condition be subdivided into KNO1 and KNO2 for the two variants, linked and unlinked to chromosome 21q22.3, respectively.

PATHOGENESIS

Collagen XVIII is expressed in three variant forms, with large differences between them in the size and sequence of their N-terminal regions (see also Chapter 2, Part I, this volume). The short isoform of collagen XVIII is expressed in brain, retina, and vascular and epithelial basement membranes throughout the body, whereas the long isoforms are not expressed in retina or brain. Collagen XVIII exists as a heparan sulfate proteoglycan, and proteolytic cleavage within its hinge region results in release of the last 184 carboxyl terminal amino acid residues, which correspond to a 20 kDa fragment, endostatin, that can be detected in plasma and tissue extracts, and that inhibits angiogenesis and tumor growth.

It has been proposed that collagen XVIII acts as a local, basement membrane-associated regulator of angiogenesis. It is also likely that the triple-helical, heparan sulfate-containing portion plays a role in the assembly and maintenance of the structural integrity of the basement membranes in which it is found, or in their cell-binding properties. Since collagen XVIII is expressed in the developing and postnatal eye, along with collagens II and XI, it is possible that it is part of a connective tissue scaffold that is essential for maintaining a normal vitreous structure and functional retina.

The pathogenetic mechanism leading to Knobloch syndrome seems to be haploinsufficiency. The α1(XVIII) IVS1-2A > T mutation described by Sertié et al. [7] predicts the creation of a stop codon in exon 4 and, therefore, truncation of the α1(XVIII) collagen short isoform which indicates that the short isoform has a major role in determining retinal structure as well as in the closure of the neural tube, and that its absence impairs embryonic cell proliferation and/or migration as a primary or secondary effect. It is intriguing that ocular anomalies and occipital encephalocele are the only major clinical alterations observed in patients in spite of the ubiquitous presence of the collagen XVIII short isoform throughout the body.

ANIMAL MODEL

In mice with inactivated *Col18a1* alleles, the normal postnatal regression of hyaloid vessels along the inner limiting

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membrane of the retina is greatly delayed, and the outgrowth of the retinal vasculature is abnormal. The results are consistent with the hypothesis that collagen XVIII/endostatin plays a negative role in the regulation of angiogenesis in specific anatomical locations, and this is further supported by enhanced vessel outgrowth in aortic explants from homozygous mutants. The lack of collagen/endostatin also causes alterations in the matrix of the vitreous. All these alterations provide an explanation for the high myopia, vitreoretinal degeneration, and retinal detachment seen in patients with Knobloch syndrome [10].

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Appendix I

International Nomenclature of Constitutional Disorders of Bone: Osteochondrodysplasias

	*Mode of Inheritance	MIM Syndrome	Chromosomal Locus	Gene	Protein	MIM Gene/ Protein
1. Achondroplasia group						
Thanatophoric dysplasia, Type I (includes San Diego type)	AD	187600	4p16.3	*FGFR3*	FGFR3	134934
Thanatophoric dysplasia, Type II	AD	187610	4p16.3	*FGFR3*	FGFR3	134934
Achondroplasia	AD	100800	4p16.3	*FGFR3*	FGFR3	134934
Hypochondroplasia	AD	146000	4p16.3	*FGFR3*	FGFR3	134934
Hypochondroplasia	AD	146000	other			
SADDAN (severe achondroplasia, developmental delay, acanthosis nigricans)	AD		4p16.3	*FGFR3*	FGFR3	134934
2. Spondylodysplastic and other perinatally lethal groups						
Lethal platyspondylic skeletal dysplasias (Torrance type, Luton type)	SP	270230, 151210				
Achondrogenesis type 1A	AR	200600				
SMD Sedaghatian Type	AR	250220				
3. Metatropic dysplasia group						
Fibrochondrogenesis	AR	228520				
Schneckenbecken dysplasia	AR	269250				
Metatropic dysplasia (various forms)	AD	156530				
4. Short-rib dysplasia (SRP) (with or without polydactyly) group						
SRP type I/III	AR	263530/510				
SRP type II, Majewski	AR	263520				
SRP type IV, Beemer-Langer	AR	269860				
Asphyxiating thoracic dysplasia (Jeune)	AR	208500				

*AD, autosomal dominant; AR, autosomal recessive; XLD, X-linked dominant; XLR, X-linked recessive; SP, sporadic.

(*Continued Overleaf*)

	Mode of Inheritance	MIM Syndrome	Chromosomal Locus	Gene	Protein	MIM Gene/ Protein
Chondroectodermal dysplasia (Ellis-van Creveld dysplasia)	AR	225500	4p16	*EVC*		604831
Synpolydactyly	AD	186000	2q31–q32	*HOXD13*		142989
Greig cephalopolysyndactyly		175700	7p13	*GLI3*		165240
Pallister-Hall		146510	7p13	*GLI3*		165240
Postaxial polydactyly, type A1		174200	7p13	*GLI3*		165240
Thoracolaryngopelvic dysplasia (Barnes)	AD	187760				
5. Atelosteogenesis-omodysplasia group						
Atelosteogenesis type I (includes "Boomerang dysplasia")	SP	108720				
Omodysplasia I (Maroteaux)	AD	164745				
Omodysplasia II (Borochowitz)	AR	258315				
Otopalatodigital syndrome type II	XLR	304120				
Atelosteogenesis Type III	SP	108721				
de la Chapelle dysplasia	AR	256050				
6. Diastrophic dysplasia group						
Diastrophic dysplasia	AR	222600	5q32–q33	*DTDST*	Sulfate transporter	
Achondrogenesis 1B	AR	600972	5q32–q33	*DTDST*	Sulfate transporter	
Atelosteogenesis type 2	AR	256050	5q32–q33	*DTDST*	Sulfate transporter	
Autosomal recessive MED4 or EDM4	AR	226900	5q32–q33	*DTDST*	Sulfate transporter	
7. Dyssegmental dysplasia group						
Dyssegmental dysplasia, Silverman-Handmaker type	AR	224410	1p36.1	*PLC* (*HSPG2*)	Perlecan	142461
Dyssegmental dysplasia, Rolland-Desbuquois type	AR	224400				
8. Type II collagenopathies						
Achondrogenesis II (Langer-Saldino)	AD	200610	12q13.1–q13.3	*COL2A1*	Type II collagen	120140
Hypochondrogenesis	AD	200610	12q13.1–q13.3	*COL2A1*	Type II collagen	120140
Kniest dysplasia	AD	156550	12q13.1–q13.3	*COL2A1*	Type II collagen	120140
Spondyloepiphyseal dysplasia (SED) congenita	AD	183900	12q13.1–q13.3	*COL2A1*	Type II collagen	120140
Spondyloepimetaphyseal dysplasia (SEMD) Strudwick type	AD	184250	12q13.1–q13.3	*COL2A1*	Type II collagen	120140
SED with brachydactyly	AD		12q13.1–q13.3	*COL2A1*	Type II collagen	120140
Mild SED with premature onset arthrosis	AD		12q13.1–q13.3	*COL2A1*	Type II collagen	120140
Stickler dysplasia type I (arthro-ophthalmopathy)	AD	108300	12q13.1–q13.3	*COL2A1*	Type II collagen	120140
SED Namaqualand type	AD	142670	12q13.1–q13.3	*COL2A1*	Type II collagen	120140
9. Type XI collagenopathies						
Stickler dysplasia type II	AD	184840	1p21	*COL11A1*	Type XI collagen	120280
Otospondylomegaepiphyseal dysplasia (OSMED) (Stickler without ocular involvement)	AR	215150	6p21.3	*COL11A2*	Type XI collagen	120290
Marshall syndrome	AD		6p21.3	*COL11A2*	Type XI collagen	120290

10. Other spondyloepi-(meta)-physeal [SE(M)D] dysplasias						
X-linked spondyloepiphyseal dysplasia tarda	XLD	313400	Xp22.2–p22.1	*SEDL*	Sedlin	300202
Other late-onset spondyloepi(meta)physeal dysplasias (Irapa)	AR	271650				
Progressive pseudorheumatoid dysplasia	AR	208230	6q22–q23	*WISP*		603400
Dyggve-Melchior-Clausen dysplasia	AR	223800				
Wolcott-Rallison dysplasia	AR	226980	2p12	*EIF2AK3*		
Immuno-osseous dysplasia-Schimke	AR	242900				
Opsismodysplasia	AR	258480				
Schwartz Jampel Syndrome	AR	255800	1p36.1	*PLC (HSPG2)*	Perlecan	142461
Spondyloepiphyseal dysplasia with joint laxity	AR	271640				
SEMD short limb-abnormal calcification	AR	271665				
SEMD Pakistani type	AR	603005	10q23–24	*PAPSS2*	PAPSS2	603005
11. Multiple epiphyseal dysplasias & pseudoachondroplasia						
Pseudoachondroplasia	AD	177170	19p12–13.1	*COMP*	COMP	600310
MED (Fairbanks and Ribbing types) or EDM2	AD	600204	1p32.2–33	*COL9A2*	Type IX collagen	120260
MED or EDM3	AD	600969	20q13.3	*COL9A3*	Type IX collagen	120270
MED or EDM6	AD		6q13	*COL9A1*	Type IX collagen	120210
MED or EDM5	AD		2p23–24	*MATN3*	Matrilin 3	602109
Familial hip dysplasia (Beuke)	AD	142669	4q35			
12. Chondrodysplasia punctata (stippled epiphyses group)						
Rhizomelic CDP type 1	AR	215100	6q22–q24	*PEX7*	Peroxin-7	601757
Rhizomelic CDP type 2	AR	222765	1q42	*DHPAT*	DHPAT	602744
Rhizomelic CDP type 3	AR	600121	2q31	*AGPS*	ADHAPS	603051
Zellweger syndrome	AR	214100	6p21.1	*PEX6*	Peroxin-6	601498
	AR	214100	7q11.23	*PEX1*	Peroxin-1	602136
	AR	214100	12	*PEX5*	Peroxin-5	
	AR	214100	8q21.1	*PEX2*	Peroxin-2	170993
Conradi-Hünermann type	XLD	302950	Xp11.23–11.22	*EBP*	EBP	300205
X-linked recessive type	XLR	302940	Xp22.32	*ARSE*	Neutral arylsulfatase E	302950
Tibial-metacarpal type	AD	118651				
Vitamin K-dependent coagulation defect	AR	277450			Vitamin K-epoxide reductase	
Other acquired and genetic disorders including Warfarin embryopathy						
Hydrops-ectopic calcification-motheaten appearance HEM (Greenberg dysplasia)	AR	215140				
Dappled diaphyseal dysplasia (Strudwick)	AR	184250				
13. Metaphyseal dysplasias						
Jansen type	AD	156400	3p22–p21.1	*PTHR1*	Parathyroid receptor1	168468
Blomstrand type	AR	215045	3p22–p21.1	*PTHR1*		168465
Schmid type	AD	156500	6q21–q22.3	*COL10A1*	COL10 α chain	120110
Cartilage-hair hypoplasia, McKusick type	AR	250250	9p13	*RMRP*	MRP RNAse	157660
Metaphyseal anadysplasia (various types)	AD/XLR	309645				

(*Continued Overleaf*)

	Mode of Inheritance	MIM Syndrome	Chromosomal Locus	Gene	Protein	MIM Gene/ Protein
Metaphyseal dysplasia with pancreatic insufficiency and cyclic neutropenia (Shwachman Diamond)	AR	260400				
Adenosine deaminase deficiency	AD	102700	20q–13.11	*ADA*	Adenosine deaminase	102700
Metaphyseal chondrodysplasia—Spahr type	AR	250400				
Acroscyphodysplasia (various types)	AR	250215				
14. Spondylometaphyseal dysplasias (SMD)						
Spondylometaphyseal dysplasia Kozlowski type	AD	184252				
Spondylometaphyseal dysplasia (Sutcliffe type)	AD	184255				
SMD with severe genu valgum (includes Schmidt and Algerian types)	AD	184253				
SMD Sedaghatian type	AR	250220				
15. Brachyolmia spondylodysplasias						
Hobaek (includes Toledo type)	AR	271530–630				
Maroteaux type	AR					
Autosomal dominant type	AD	113500				
16. Mesomelic dysplasias						
Dyschondrosteosis (Leri-Weill)	AD	127300	Xpter–p22.32	*SHOX*		312865
Langer type (homozygous dyschondrosteosis)	AR	249700	Xpter–p22.32	*SHOX*		312865
Nievergelt type	AD	163400				
Kozlowski-Reardon type	AR					
Reinhardt-Pfeiffer type	AD	191400				
Werner type	AD					
Robinow type, dominant	AD	180700				
Robinow type, recessive	AR	268310	9q22	*ROR2*	Orphan receptor tyrosine kinase	602337
Mesomelic dysplasia, Verloes type	AD	600383				
Mesomelic dysplasia, Kantaputra type	AD	156232	2q24–q32			
Mesomelic dysplasia, Savarirayan type	SP	605274				
17. Acromelic and acromesomelic dysplasias						
Acromicric dysplasia	AD	102370				
Geleophysic dysplasia	AR	231050				
Weill-Marchesani dysplasia	AR	277600				
Cranioectodermal dysplasia	AR	218330				
Trichorhinophalangeal dysplasia, type I/III	AD	190350	8q24.12	*TRPS1*	Zinc finger protein	604386
Trichorhinophalangeal dysplasia, type II (Langer-Giedeon)	AD	150230	8q24.11–q24.13	*TRPS1* + *EXT1*	(+ exostosin 1)	133700

Grebe dysplasia	AR	200700	20q11.2	*CDMP1*	Cartilage-derived morphogenic protein 1	601146
Hunter-Thompson dysplasia	AR	201250	20q11.2	*CDMP1*	Cartilage-derived morphogenic protein 1	601146
Myhre dysplasia		139210				
Brachydactyly type A1	AD	112500	2q35	*IHH*	Indian hedgehog	600726
Brachydactyly type A2	AD	112600				
Brachydactyly type A3	AD	112700				
Brachydactyly type B	AD	113000	9q22	*ROR2*	NTRKR2	602337
Brachydactyly type C	AD	133100	21q11.2	*CDMP1*	Cartilage-derived morphogenic protein 1	601146
Brachydactyly type D	AD	113200	12q24			
Brachydactyly type E	AD	113000				
Pseudohypoparathyroidism (Albright hereditary osteodystrophy), various types			20q13	*GNAS1*	Guanine nucleotide binding protein of adenylate cyclase α-subunit	139320
Acrodysostosis	SP(AD)	101800				
Saldino-Mainzer dysplasia	AR	266920				
Brachydactyly-hypertension dysplasia (Bilginturan)	AD	112410	12p12.2–p11.2			
Craniofacial conodysplasia	AD					
Angel-shaped phalango-epiphyseal dysplasia (ASPED)	AD	105835				
Acromesomelic dysplasia	AR	201250				
Camptodactyly arthropathy coxa vara pericarditis (CACP)	AR	208250	1q24–q25	*PRG4*	Proteoglycan 4	604283
Acromesomelic dysplasia, type Campailla-Martinelli	AR	201250	20q11.2			
Acromesomelic dysplasia, type Ferraz/Ohba	AD					
Acromesomelic dysplasia, type Osebold Remondini	AD	112910				
18. Dysplasias with prominent membranous bone involvement						
Cleidocranial dysplasia	AD	119600	6p21	*CBFA1*	Core binding factor α1-subunit	600211
Craniosynostoses						
Apert	AD	101200	8p11.2–p11.1	*FGFR1*	FGFR1	136350
Crouzon	AD	123500	8p11.2–p11.1	*FGFR1*	FGFR1	136350
Pfeiffer	AD	101600	10q26	*FGFR2*	FGFR2	176943
Saethre-Chotzen Syndrome	AD	101400	7p21	*TWIST*	Transcription factor	601622
Osteodysplasty, Melnick-Needles	XLD	309350				
Precocious osteodysplasty (ter Haar dysplasia)	AR					
Yunis-Varon dysplasia	AR	216340				
Parietal foramina (isolated, cranium bifidum)	AD	168500	11p11.2	*ALX4*		123101
Parietal foramina (isolated)	AD	168500	5q34–35	*MSX2*		605420

(*Continued Overleaf*)

	Mode of Inheritance	MIM Syndrome	Chromosomal Locus	Gene	Protein	MIM Gene/ Protein
19. Bent-bone dysplasia group						
± Campomelic dysplasia (± campomelia)	AD	114290	17q24.3–q25.1	*SOX9*	SRY-box 9	211970
Kyphomelic dysplasia	?AR	211350				
Cumming syndrome	AR	211890				
Stüve-Wiedemann dysplasia (neonatal Schwartz-Jampel)	AR	601559				
20. Multiple dislocations with dysplasias						
Larsen syndrome	AD	150250	3p21.1–p14.1			
Larsen-like syndromes (including La Réunion Island)	AR	245600				
Desbuquois dysplasia	AR	251450				
Pseudodiastrophic dysplasia	AR	264180				
21. Dysostosis multiplex group						
Mucopolysaccharidosis IH	AR	252800	4p16.3	*IDA*	α1-Iduronidase	
Mucopolysaccharidosis IS	AR	252800	4p16.3	*IDA*	α1-Iduronidase	
Mucopolysaccharidosis II	XLR	309900	Xq27.3–q28	*IDS*	Iduronate-2-sulfatase	
Mucopolysaccharidosis IIIA	AR	252900	17q25.3	*HSS*	Heparan sulfate sulfatase	
Mucopolysaccharidosis IIIB	AR	252920	17q21		N-Ac-α-D-glucosaminidase	
Mucopolysaccharidosis IIIC	AR	252930			Ac-CoA: α-glucosaminidase-N-acetyltransferase	
Mucopolysaccharidosis IIID	AR	252940	12q14	*GNS*	N-Ac-glucosamine-6-sulfatase	
Mucopolysaccharidosis IVA	AR	230500	16q24.3	*GALNS*	Galactose-6-sulfatase	
Mucopolysaccharidosis IVB	AR	230500	3p21.33	*GLBI*	β-Galactosidase	
Mucopolysaccharidosis VI	AR	253200	5q13.3	*ARSB*	Arylsulfatase B	
Mucopolysaccharidosis VII	AR	253200	7q21.11	*GUSB*	β-Glucuronidase	
Fucosidosis	AR	230000	1p34	*FUCA*	α-Fucosidase	
α-Mannosidosis	AR	248500	19p13.2–q12	*MAN*	α-Mannosidase	
β-Mannosidosis	AR	248510	4q22–q25	*MANB*	β-Mannosidase	
Aspartylglucosaminuria	AR	208400	4q23–q27	*AGA*	Aspartylglucosaminidase	
GM1 Gangliosidosis, several forms	AR	230500	3p21–p14.2	*GLB1*	β-Galactosidase	
Sialidosis, several forms	AR	256550	6p21.3	*NEU*	α-Neuraminidase	
Sialic acid storage disease	AR	269920	6q14–q15	*SIASD*		
Galactosialidosis, several forms	AR	256540	20q13.1	*PPGB*	β-Galactosidase protective protein	
Multiple sulfatase deficiency	AR	272200			Multiple sulfatases	
Mucolipidosis II	AR	252500	4q21–23	*GNPTA*	N-Ac-Glucosamine-phosphotransferase	
Mucolipidosis III	AR	252600	4q21–23	*GNPTA*	N-Ac-Glucosamine-phosphotansferase	
22. Osteodysplastic slender bone group						
Type I osteodysplastic dysplasia	AR	210710				
Type II osteodysplastic dysplasia	AR	210720				
Microcephalic osteodysplastic dysplasia	AR	210730				

23. Dysplasias with decreased bone density						
Osteogenesis imperfecta I (without opalescent teeth)	AD	166200	17q21	*COL1A1*	α(1) I procollagen	120150
Osteogenesis imperfecta I (with opalescent teeth)	AD	166240	17q21	*COL1A1*	α(1)I procollagen	120150
	AD	166240	7q22.1	*COL1A2*	α(2)I procollagen	120160
Osteogenesis imperfecta II	AD	166210	17q21	*COL1A1*	α(1)I procollagen	120150
	AD	166210	7q22.1	*COL1A2*	α(2)I procollagen	120160
	AR	259400	17q21	*COL1A1*	α(1)I procollagen	120150
Osteogenesis imperfecta III	AD	259420	17q21	*COL1A1*	α(1)I procollagen	120150
	AD	259420	7q22.1	*COL1A2*	α(2)I procollagen	120160
	AR	259420	7q22.1	*COL1A2*	α(2)I procollagen	120160
	AR	259420				
Osteogenesis imperfecta IV (without opalescent teeth)	AD	166220	7q22.1	*COL1A2*	α(2)I procollagen	120160
	AD	166220	17q21	*COL1A1*	α(1)I procollagen	120150
Osteogenesis imperfecta IV (with opalescent teeth)	AD	166220	7q22.1	*COL1A2*	α(2)I procollagen	120160
	AD	166220	17q21	*COL1A1*	α(1)I procollagen	120150
Osteogenesis imperfecta V	AD					
Osteogenesis imperfecta VII	AR		3q22			
Cole-Carpenter dysplasia	SP	112240				
Bruck dysplasia, type I	AR	259450	17p12			
Bruck dysplasia, type II	AR		3q22	*PLOD2*	Lysyl hydroxylase 2	601865
Singleton-Merton dysplasia	AR					
Osteopenia with radiolucent lesions of the mandible	AD	166260				
Osteoporosis-pseudoglioma dysplasia	AR	259770	11q12–q13	*LRP5*	Low density lipoprotein receptor-related protein 5	603506
Geroderma osteodysplasticum	AR	231070				
Hyper IgE syndrome with osteopenia	AR	147060				
Idiopathic juvenile osteoporosis	SP	259750				
24. Dysplasias with defective mineralization						
Hypophosphatasia-perinatal lethal and infantile forms	AR/AD	241500	1p36.1-p34	*TNSALP*	Tissue non-specific alkaline phosphatase	171760
Hypophosphatasia adult form	AD	146300	1p36.1–p34			
Hypophosphatemic rickets	XLD	307800	Xp22.2–p22.1	*PHEX*	X-linked phosphate-regulating endopeptidase	171760
Hypophosphatemic rickets	AD	605380	12p13.3	*FGF23*	Fibroblast growth factor 23	605380
Pseudovitamin D deficiency rickets	AR	264700	12q14	*CYP27B1*	25-α-OH cholecalciferol-1-hydroxylase	
Vitamin D-resistant rickets	AR	277440	12q12–q14	*VDR*	1,25-α-$(OH)_2$ vit. D receptor	601769
Neonatal hyperparathyroidism	AR	239200	3q13.3–q21	*CASR*	Calcium sensing protein	601199
Transient neonatal hyperparathyroidism with hypocalciuric hypercalcemia	AD	145980	3q13.3–q21	*CASR*	Calcium sensing protein	601199
25. Increased bone density without modification of bone shape						
Osteopetrosis						
Precocious type	AR	259700	11q12–13	*TCIRG1*	Vacuolar pump α3 subunit	604592
Infantile form	AR	259700	16p13.3	*CLCN7*	Chloride channel pump	602727
With infantile neuroaxonal dysplasia	AR?	600329				

(*Continued Overleaf*)

	Mode of Inheritance	MIM Syndrome	Chromosomal Locus	Gene	Protein	MIM Gene/ Protein
With ectodermal dysplasia and immune defect (OLEDAID)	XL	300301	Xq28	*IKBKG* (*NEMO*)	NF-κB signalling	300248
Cranial osteosclerosis with bamboo hair (Netherton)	AR	256500				
Delayed type	AD	166600	1p21			
Intermediate type	AR	259710				
With renal tubular acidosis	AR	259730	8q22	*CA2*	Carbonic anhydrase II	
Bone mineral density variation 1	AR	601884	11q12–q13	*LRP5*		603506
Axial osteosclerosis						
Osteomesopyknosis	AD	166450				
With bamboo hair	AR	266500				
Pyknodysostosis	AR	265800	1q21	*CTSK*	Cathepsin K	601105
Osteosclerosis Stanescu type	AD	122900				
Osteopathia striata						
Isolated	SP					
With cranial sclerosis	AD	166500				
Sponastrime dysplasia	AR	271510				
Melorheostosis	SP	155950				
Osteopoikilosis	AD	166700				
Mixed sclerosing bone dysplasia	SP					
Ankylosing vertebral hyperostosis with tylosis	AD	106400				
26. Increased bone density with diaphyseal involvement						
Diaphyseal dysplasia Camurati Engelmann	AD	131300	19q13.1	*TGFB1*	Transforming growth factor β1	190180
Craniodiaphyseal dysplasia	?AR	218300, 122860				
Lenz Majewski dysplasia	SP	151050				
Endosteal hyperostosis, van Buchem type	AR	239100	17q12–21			
Worth type	AD	144750				
Sclerosteosis	AR	269500	17q12–21	*SOST*	Sclerostin	605740
With cerebellar hypoplasia	AR	213002				
Sclero-osteo-cerebellar dysplasia	AR	213002				
Kenny Caffey dysplasia, type I	AR	244460	1q41–q42			
Kenny Caffey dysplasia, type II	AD	127000				
Osteoectasia with hyperphosphatasia (Juvenile Pagets)	AR	239000				
Diaphyseal dysplasia with anemia	AR	231095				
Diaphyseal medullary stenosis with bone malignancy (Hardcastle)	AD	112250	9p21–22			
27. Increased bone density with metaphyseal involvement						
Pyle dysplasia	AR	265900				
Craniometaphyseal dysplasia						
Severe type	AR	218400				

Mild type	AD	123000	5p15.2–p14.2	*ANK*	Pyrophosphate transporter	605145
Other types						
Frontometaphyseal dysplasia	XLR	305620				
Dysosteosclerosis	AR	224300				
	XLR					
Oculodentoosseous dysplasia	AD	164200	6q22–24			
Trichodentoosseous dysplasia	AD	190320	17q21	*DLX3*	Distal-less 3 protein	600525
Osteodysplasty, Melnick-Needles	XLD	309350				
Precocious osteodysplasty (ter Haar dysplasia)	AR	249420				
Otopalatodigital syndrome, type I	XLD	311300	Xq28			
Otopalatodigital syndrome, type II	XLR	304120				
28. Neonatal severe osteosclerotic dysplasias						
Blomstrand dysplasia	AR	215045				
Raine dysplasia	AR	259775				
Prenatal onset Caffey disease	?AR	114000				
29. Lethal chondrodysplasias with fragmented bones						
Greenberg dysplasia	AR	215140				
Dappled diaphyseal dysplasia	AR	184250				
Astley-Kendall dysplasia	AR					
30. Disorganized development of cartilaginous and fibrous components of the skeleton						
Dysplasia epiphysealis hemimelica	SP	127800				
Multiple cartilaginous exostoses	AD	133700	8q23–q24.1	*EXT1*	Exostosin-1 } heparan sulfate	
	AD	133701	11p12–p11	*EXT2*	Exostosin-2 } glycosyltransferases	
	AD	600209	19p	*EXT3*	Exostosin-3 }	
Enchondromatosis, Ollier	SP	166000				
Enchondromatosis with hemangiomata (Maffucci)	SP	166000				
Spondyloenchondromatosis	AR	271550				
Spondyloenchondromatosis with basal ganglia calcification	AR					
Dysspondyloenchondromatosis						
Metachondromatosis	AD	156250				
Osteoglophonic dysplasia	AD	166250				
Genochondromatosis	AD	166000				
Carpotarsal osteochondromatosis	AD	127820				
Fibrous dysplasia (McCune-Albright and others)	SP mosaic	174800	20q13	*GNAS1*	Guanine nucleotide protein, α subunit	139320
Jaffe Campanucci	SP					
Fibrodysplasia ossificans progressiva	AD	135100	4q27–31			
Cherubism	AD	118400	4p16.3	*SH3BP2*	SH3 binding protein 2	602104
Cherubism with gingival fibromatosis	AR	135300				
31. Osteolyses						
Multicentric predominantly carpal & tarsal in the hand						
Multicentric carpal-tarsal osteolysis with and without nephropathy	AD	166300				

(*Continued Overleaf*)

	Mode of Inheritance	MIM Syndrome	Chromosomal Locus	Gene	Protein	MIM Gene/ Protein
Shinohara carpal-tarsal osteolysis						
Multicentric predominantly carpal, tarsal and interphalangeal						
Francois syndrome	AR	221800				
Winchester syndrome	AR	277950				
Torg syndrome	AR	259600	16q12–21	*MMP2*	MMP2	120360
Whyte Hemingway carpal-tarsal phalangeal osteolyses	AD					
Predominantly distal phalanges						
Hajdu-Cheney syndrome	AD	102500				
Giacci familial neurogenic acroosteolysis	AR	201300				
Mandibulo acral syndrome	AR	248370				
Predominantly involving diaphyses and metaphyses						
Familial expansile osteolysis	AD	174810	18q21.1–q22	*TNFRS11A*	RANK	603499
Juvenile hyaline fibromatosis	AR	228600				
32. Patella dysplasias						
Nail patella dysplasia	AD	161200	9q34.1	*LMX1B*	LIM homeodomain	602575
Scypho-patellar dysplasia	AD					
Ischiopubic patellar dysplasia	AD	147891				
Genitopatellar syndrome		606170				
Ear patella short stature syndrome (Meier Gorlin)	AR	224690				
33. Miscellaneous Disorder						
Opitz syndrome 1	XL	300000	Xp22.3	*MID1*		
Opitz syndrome 2	AR		22q11.2			
Spondylocostal dysostosis	AR	277300	19q13	*DLL3*	Delta-like 3	602768
Oro-facial-digital syndrome, type I	XL	311200	Xp22.3–p22.2	*OFD1*		300170
Limb mammary syndrome		603543	3q27	*p63*		603273
EEC3 (ectrodactyly, ectodermal dysplasia, cleft palate)		604292	3q27	*p63*		603273
Hay-Wells (ankyloblepharon-ectodermal defects-cleft lip/palate)		106260	3q27	*p63*		603273
Acro-dermato-ungual-lacrimal tooth (ADULT)		103285	3q27	*p63*		603273
Cleft lip/palate-ectodermal dysplasia	AR	225060	3q27	*p63*		603273
Lacrimo-auricular-dental-digital syndrome (LADD)		149730	3q27	*p63*		603273
Acheiropodia	AR	200500	7q36	*C7ORF2*		605522
Radioulnar synostosis with megakaryocytic thrombopenia	AD?	605432	7p15–p14.2	*HOXA11*		142958
Keutel syndrome	AR	245150	12p13.1–12.3	*MGP*	Matrix Gla protein	
Hand-foot-genital syndrome	AD	140000	7p15–p14.2	*HOXA13*		142259
Ulnar-mammary syndrome	AD	181450	12q21.1	*TBX3*		601621
Holt-Oram syndrome	AD	142900	12q24.1	*TBX5*		601620
Townes-Brock syndrome	AD	107480	16q12.1	*SALL1*		602218

Appendix II

Extracellular Collagen Metabolites in Body Fluids

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INTRODUCTION

In recent years, several methods have been developed and subsequently validated for use in clinical laboratories that allow the quantitative measurement of various structural domains of procollagens and collagens in body fluids, in particular in blood samples. As such assays are based on immunological detection, they offer superior sensitivity and specificity to traditional biochemical methods. Although the typical samples used in the clinic are serum or urine, measurements can also be carried out in relevant tissue fluids and thus allow the assessment of metabolite concentrations *in situ*. In fact, the development of assay technology has made it possible to attain better insight into the physiology of fibrillar collagens and their precursors, procollagens, in the human organism. This methodology has mostly been used in research into, and clinical management of, acquired diseases, whereas its application to genetic disorders of connective tissue has so far been relatively rare [1–3]. Table 1 summarizes the advantages of the immunoassay approach in the quantitative assessment of the synthesis and degradation of connective tissue proteins.

To interpret correctly the results of a quantitative immunoassay, it is necessary to understand the chemical nature of the antigenic determinant recognised by the assay. This is particularly important in the case of genetic diseases in which not only the amount but also the structure of a connective tissue protein may be altered. For fibrillar collagens, the varying degrees of the posttranslational modifications and differing extents of the cross-linking process provide additional sources of variation.

TABLE 1. Advantages of Quantitative Immunoassays for Connective Tissue Metabolites

- Noninvasiveness: sample usually serum or urine
- Quantitative results in absolute terms, transferable from one laboratory to another
- Generally available, if commercialized, and relatively inexpensive
- Can be performed in ordinary hospital laboratories: regular and professional quality control
- Applicable to cell cultures [4] and tissue studies (e.g., suction blisters of the skin [5])

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Here we first briefly describe the collagen metabolites and the assays that are generally available for them, and comment on some other assays that have also been described in the literature. This section is followed by tables listing the reference intervals for the metabolites of types I and III collagens in different kinds of biological samples. In this part, we concentrate on those metabolites for which commercial assays are generally available and for which adequate reference materials have been published. Because the immunoassays, when carried out in a hospital laboratory, are reliable, reproducible, relatively simple, and inexpensive, it seems justifiable to include some of them in the basic evaluation of patients with suspected osteogenesis imperfecta or other heritable disorders which affect type I or type III collagen.

BY-PRODUCTS OF COLLAGEN SYNTHESIS

Assay Principles

There is an elegant possibility of noninvasively assessing the ongoing synthesis of the fiber-forming interstitial collagen types I to III in humans, because these proteins are synthesized as procollagens, which lose their propeptide domains *en bloc*. The free propeptides, in principle, reflect the amount of the respective collagen that is being deposited in the tissue at any one time in a stoichiometric 1 : 1 fashion. The propeptides are measurable by immunological techniques, and a number of biological fluids can be used as samples. However, it has turned out that when applying such a method to a new type of sample it is essential to characterize the antigen measured, at least with respect to its size and the shape of the inhibition curve it produces in comparison with the standard antigen [1].

The locations of the antigenic determinants and the conformational requirements of the propeptide immunoreactions have been relatively well characterized. As procollagens contain three polypeptide chains, the intact propeptides are also trimeric structures. In this chapter, the term "propeptide" and the abbreviations for the individual propeptides denote such trimeric protein fragments. The molecular properties of the intact propeptides of human type I procollagen are given in Table 2, together with the main characteristics of the circulating antigens related to these protein fragments. The primary structures of the amino terminal and carboxyl terminal propeptide domains of other fibrillar procollagens are homologous to the respective domains of type I procollagen,

TABLE 2. Properties of the Propeptides of Type I Procollagen and Corresponding Circulating Antigens

	PICP	PINP
Chemical nature	Glycoprotein, contains oligosaccharides of the high-mannose type	Partly collagenous, phosphorylated at serine residues
Molecular mass	100,000	35,000
Molecular shape	Globular	Elongated
Related circulating antigens		
Homogeneity	Homogeneous	Heterogeneous
Size	As intact PICP	Major antigen as intact PINP, minor antigen smaller
Clearance by	Liver endothelial cells	Liver endothelial cells, smaller antigen by the kidneys
Clearance receptor	Mannose receptor	Scavenger receptor

PICP = carboxyl terminal propeptide of type I procollagen; PINP = amino terminal propeptide of type I procollagen;
The data relate to the intact, trimeric propeptides as they are released from the native procollagen molecule, not to the corresponding domains in the individual polypeptide chains. Although not directly studied, the half-lives of the propeptides in human serum can be estimated to be about one hour. This conclusion is based on their rates of response after glucocorticoid treatment in humans [6] and on comparison of PINP with *in vivo* clearance studies of the amino terminal propeptide of type III procollagen (PIIINP), which is removed from the circulation by the same receptors as PINP [7].

but at the protein level only the amino terminal propeptide of type III procollagen (PIIINP) has so far been available for protein chemical studies and commercial immunoassay. PIIINP resembles the amino terminal propeptide of type I procollagen, PINP, in its molecular shape, charge and metabolic properties. However, the metabolism of PIIINP is more complex, in particular because of its transglutaminase-mediated cross-linking [8]. On the other hand, PINP is less stable than PIIINP because of its lack of interchain disulfide bridges. An assay for the carboxyl terminal propeptide of type II procollagen, also known as chondrocalcin, is being used in studies on acquired cartilage diseases, but this assay is not commercially available (see below).

Although the two propeptides, amino terminal propeptide (PINP) and carboxyl terminal propeptide (PICP) of type I procollagen originate from the two ends of the same procollagen molecule and are thus produced and released in equimolar amounts, in reality their relative quantities as measured in serum show some variation. Initially, the cleavage of PINP may be delayed and, indeed, some amino terminal propeptide can be found on the surface of growing fibrils in, e.g., the osteoid of mineralizing bone. Also their clearance differs. In particular, in infants and children there is an excess of PINP, in comparison with PICP (Fig. 1). After puberty the equimolar ratio typical of the sera of adults is reached. A disproportionate increase in the circulating PINP concentration also takes place in active Paget's disease of bone [10] and aggressive breast carcinoma [11]. An inherited disorder, Camurati-Engelmann disease (MIM 131300) that resembles Paget's disease (MIM 167250 and 602080) in some of its pathophysiological features, may also display such a characteristic [12].

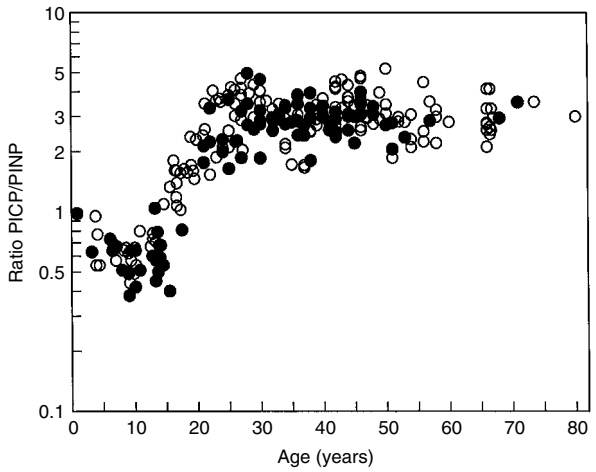

Figure 1. Ratio of the concentrations (in μg/L) of the carboxyl terminal (PICP) and amino terminal (PINP) propeptides of type I procollagen in human serum as a function of age of the individual. The open symbols denote females and the closed symbols males. (Redrawn from [9], with permission.)

Metabolic Fates of the Propeptides

Once the propeptides have been cleaved from a procollagen molecule, they do not seem to be significantly further metabolized *in situ*, but find their way into the circulation. For the propeptides set free in soft tissue, this route involves passage via the lymphatic system, whereas those originating in the skeleton obviously have direct access to the blood. Consequently, the relative concentration of the amino terminal propeptide of type III procollagen, PIIINP, is about ten times higher in lymph than in serum, whereas for the carboxyl terminal propeptide of type I procollagen, PICP, both concentrations are equal [13].

The half-lives of the circulating propeptides are of the order of minutes when studied in the rat [14,15] or up to an hour in the pig [7]. Their own metabolism takes place in the liver, where they are taken up by two different receptor-mediated systems (Table 2). These data from experimental and domestic animals and cultured liver endothelial cells have been substantiated by measurements in different vascular beds in man [16]. The scavenger function of the sinusoidal endothelial cells must be distinguished from the metabolic functions of the hepatocyte. In the case of the most common liver damage, the former is rarely affected, and thus a mild to moderate increase in the serum concentrations of transaminases is not usually accompanied by impaired

propeptide clearance. However, the deterioration of the scavenger function of the sinusoidal endothelial cells, e.g., in primary biliary cirrhosis, as indicated by an increased PIIINP concentration in the serum, predicts poor survival [17].

The propeptides can be measured in a number of tissue fluids, such as wound fluid [18] or suction blister fluid [5] of the skin, where they reflect the local collagen synthesis rate. The production of suction blisters is a relatively noninvasive method that has been used, for example, to demonstrate the effect of glucocorticoids on skin collagen synthesis [5] and in the diagnosis of sporadic cases of Ehlers-Danlos syndrome type IV [19]. The latter (see Chapter 9, this volume) is associated with a decreased production of type III procollagen as measured by PIIINP. In these fluids the propeptide antigens resemble, with respect to both homogeneity and molecular size, the authentic propeptides as they are released *en bloc* from the procollagen molecule. The amniotic fluid, at least close to the term of a pregnancy, can be regarded as an *in situ* system in this respect, as most of the intact propeptides in it must have originated from the fetal membranes (Fig. 2). However, these membranes have the same genotype as the other fetal tissues; i.e., they will produce genetically defective collagens in the case of an inherited collagen disorder.

In contrast to the propeptides at their sites of origin, in the blood the antigens related to the amino terminal propeptides of the fibrillar procollagens are heterogeneous in size and metabolic behavior (Fig. 3). The smallest antigenic forms are eliminated via the kidneys [16]. The origin of these is at least partially the degradation of the corresponding pN-collagen in tissues, in addition to the possibility that the propeptide itself is partially digested or denatured [21]. For PINP, the different assays described in the literature differ with respect to their sensitivities to such degradation products [21,22]. In the case of PIIINP the situation is even more complex, as the additional circulating antigen forms partially appear even larger than the intact, trimeric propeptide. One explanation for this could be that PIIINP contains a transglutaminase cross-linking site [8] allowing the formation of higher aggregates of PIIINP itself or with other proteins. Another reason could be the fact that a substantial proportion of PIIINP is still found as tissue type III pN-collagen, the degradation of which could also produce large antigenic fragments. In fact, the most abundant form of PIIINP in the blood of healthy adults is form B (Fig. 3). Such complexity is not a problem for PICP as it is always present as one form with properties identical to those of the intact PICP.

Tables 3 and 4 list the assays for the propeptides of type I and type III procollagens, respectively, that have been described in the literature and that have been or are available commercially.

Stability of the Propeptide Antigens

All the propeptide antigens are very stable in serum if samples are taken, handled and stored according to good clinical laboratory practice. There are a number of reports in the literature on the analysis of PINP, PICP or PIIINP in frozen samples that were collected several years previously; the antigens also resist repeated freezing and thawing cycles.

The thermal stability of PIIINP is clearly higher than that of PINP because there are interchain disulfide bridges within the collagenous domain of the former but not within that of the latter. Their *in vitro* stabilities are comparable to each other, i.e., there is no change in the serum concentration of either if samples are kept for 7 days at room temperature. However, denaturation of the PINP antigen may occur if samples are handled improperly, e.g., by keeping them for days at +37°C [21]. As an antigen, PICP is more sensitive to extreme conditions, such as acidic pH, but if samples are handled according to the normal practices of clinical laboratories, antigen stability is not a problem.

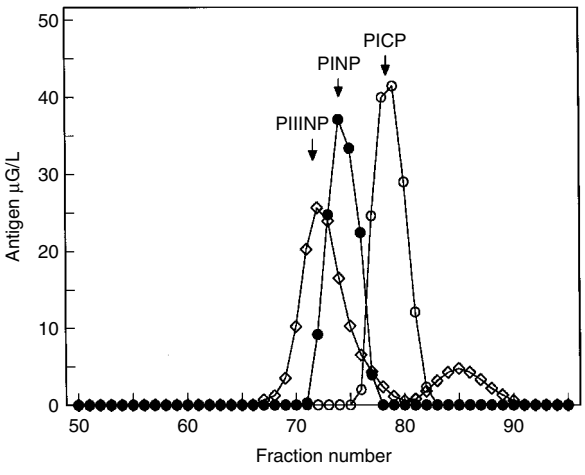

Figure 2. Sizes of the propeptide antigens of type I and III procollagens in amniotic fluid at term. Gel filtration on Sephacryl S-300 of amniotic fluid from full-term labor. The elution positions of standard PIIINP, PINP, and PICP are indicated by arrows. PIIINP and PINP elute earlier than PICP, because the former are elongated, partially collagenous structures, whereas PICP has a compact, globular conformation. Open circles = PICP; closed circles = PINP; open squares = PIIINP. (Reproduced from [20], with permission).

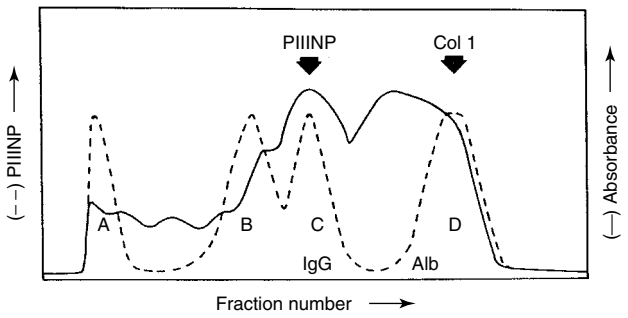

Figure 3. Schematic representation of a gel filtration analysis of different antigenic forms of the amino terminal propeptide of type III procollagen (PIIINP) in serum, in which up to four antigenic peaks (A–D) can be detected [1]. Form C has the size of the authentic PIIINP. In normal adult serum, form B, a possible dimer of C, is the most abundant. Form A is possibly a conjugate with LDL or some other large plasma protein. Depending on the assay used, a considerable amount of form D can be measured, which behaves similarly to the monomeric degradation product of the propeptide after bacterial collagenase digestion, the elution position of which is indicated by Col 1. IgG and Alb denote the elution positions of immunoglobulin G and serum albumin, and indicate that the intact PIIINP (form C) elutes in the position of a protein as large as 150,000 kDa, in spite of the fact that its molecular mass is only 42,000. This is due to its elongated, partially collagenous structure.

TABLE 3. Assays for the Propeptides of Type I Procollagen

Assays Commercially Available		
Assay/Supplier	Antigenic Determinant/Assay Principle	Reference
PICP/Orion Diagnostica (previously Farmos Diagnostica) +DiaSorin (previously Incstar) +Chugai Diagnostic Science	Conformational, requires three polypeptide chains/RIA, polyclonal antiserum	[23]
PICP/Metra Biosystems (Prolagen-C®)	Conformational, requires three polypeptide chains/IEMA, monoclonal and polyclonal antibodies	[24]
PINP/Orion Diagnostica	Antigenic determinant in the most aminoterminal part of PINP, specific, detects the major circulating antigen in serum/RIA, polyclonal antiserum	[22]
"In-house" Methods That Have Been Described in the Literature		
Reference	Comment	
[25]	PINP assay based on a synthetic peptide; antiserum most likely nonspecific and detects unrelated material in serum, since concentrations 100 times too high	
[26]	PINP assay based on a synthetic peptide; specific; probably detects both circulating forms of PINP	
[27]	PINP assay based on PINP derived from a single proα1 chain; antigen isolated from amniotic fluid; specific; detects both circulating forms of PINP	

RIA = radioimmunoassay; IEMA = immunoenzymometric assay.

TABLE 4. Assays for the Aminoterminal Propeptide of Type III Procollagen

Assay/Supplier	Antigenic Specificity/Assay Principle[a]	Reference
P-III-P		
Hoechst-Behringwerke RIA-Gnost P-III-P (no longer available)	Antigenic determinant in the most aminoterminal part of the propeptide; specificity D = B,C/RIA, polyclonal antiserum	[28]
Fab-assay (no longer available)	Specificity D ≫ B,C/RIA with Fab-fragments of a polyclonal antiserum	[29]
IRMA (now from CIS International)	Specificity D ≫ B,C/IRMA, two monoclonal antibodies	Not published
PIIINP/Orion Diagnostica	Specificity B,C ≫ D/RIA, polyclonal antiserum	[30]

RIA = radioimmunoassay; IRMA = immunoradiometric assay.

[a]See Figure 3 for the key to the letters.

Effects of Physiological Changes and Acquired Diseases

The rate of collagen metabolism changes in many physiological situations and is affected by many kinds of treatment. Normal growth is associated with rapid collagen turnover, and the catch-up growth during the recovery of a child from a debilitating disease enhances collagen synthesis and turnover even further. Circulating propeptide concentrations are at their highest in infants, decrease rapidly thereafter and then remain almost constant until puberty. Large changes can be seen during puberty, coinciding with the pubertal growth spurt, the age at which the maximal increase in propeptide markers occurs in girls preceding that in boys by 2 years. In children up to puberty there is also a still unexplained difference between the two type I

procollagen propeptides, PICP and PINP. Before puberty the ratio PICP/PINP, when expressed in μg/L, is around 1, but after puberty it increases close to the ratio of 3–4 : 1. Since the molecular mass of PICP is about three times that of PINP (Table 2), the latter ratio is close to the equimolar concentrations expected (Fig. 1). Glucocorticoid treatment decreases the rates of type I and III collagen synthesis rapidly and specifically. Other drugs that slow down bone turnover, even though their primary effect would be toward matrix degradation, such as the bisphosphonates, decrease the circulating concentrations of PICP and, in particular, of PINP, because of the coupling of bone formation with bone resorption [3].

Propeptides in Genetic Diseases

At present, several hundred different genetic alterations have been described in the *COL1A1*, *COL1A2*, and *COL3A1* genes, leading to different biochemical defects (see Chapters 8 and 9, this volume). Although there are already some reports on the use of biochemical markers, in osteogenesis imperfecta, for example, clearly more work is needed before it is possible to generally predict what changes different defects can be expected to cause in the circulating concentrations of the procollagen propeptides. However, there is already some information suggesting that these noninvasive tests can aid in the diagnosis of certain heritable disorders of type I and III collagens.

Osteogenesis Imperfecta

Several reports have shown that in osteogenesis imperfecta (see Chapter 8, this volume) the serum concentration of PICP is low or within the lower part of the reference interval, indicating that the rate of synthesis of type I procollagen is low in this disorder (Table 5). Fewer studies have included the use of PINP and published studies have, unfortunately, been carried out with two assays that differ with respect to their antigen specificity. The assay for intact PINP was more sensitive in distinguishing an abnormality than that for intact PICP in one study in which the same patient was tested *in utero* (Fig. 4) and after birth at the age of 1–2 years [20]. In another study, an assay for PINP was used which measures both the intact propeptide and its monomeric form [34]. The latter may be derived either from the denaturation of the propeptide itself [21] or from the degradation of tissue type I pN-collagen — this may explain why the total PINP assay was less sensitive than that for PICP in distinguishing osteogenesis imperfecta patients from controls. The lower than normal circulating concentration of PICP in type I osteogenesis imperfecta has been a general finding, and is excellently demonstrated by a study in a large Italian kindred [32] (Fig. 5).

In principle, the diagnosis of osteogenesis imperfecta could be made by serum propeptide analysis in those cases (mostly type I) in whom there is a quantitative decrease in the rate of synthesis of type I procollagen as a result of a regulatory defect. Similarly, a qualitative change in the structure of the procollagen that leads to a decreased rate of synthesis, but does not affect the structure of the propeptides, can be detected by either the PICP or the PINP analysis. However, if the structure of one propeptide is altered, both propeptides of type I procollagen should be measured to detect any discrepancy in the ratio of PICP/PINP. It would also be interesting to know how lack of the proα2(I) chain is reflected in the apparent concentrations of the circulating propeptides, since this occurs in certain patients with osteogenesis imperfecta. At present it is not known how well

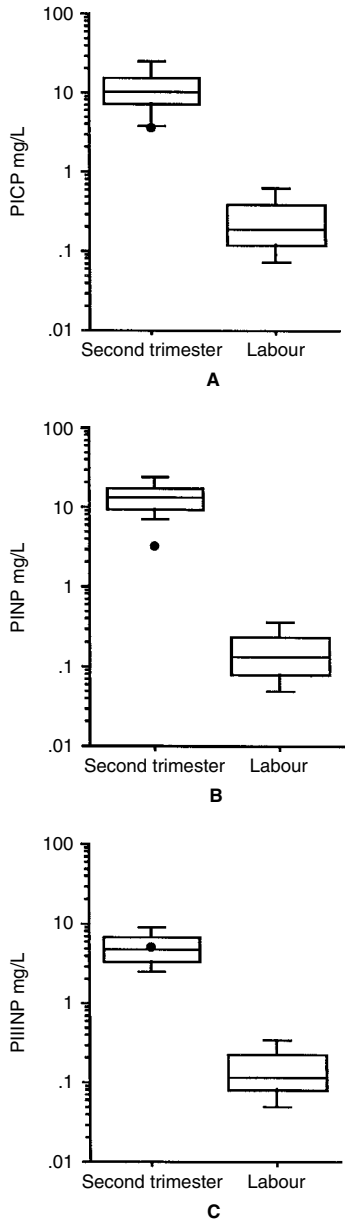

Figure 4. Concentrations of the intact trimeric propeptides of type I (PINP and PICP) and type III procollagens (PIIINP) in amniotic fluid. The reference intervals in the second trimester ($n = 105$) and at term ($n = 40$) are indicated in the form of box-and-whiskers diagrams. The box indicates the lower and upper quartiles and the central line is the median. The ends of the "whiskers" are the 2.5% and 97.5% values. The black dots indicate the normal PIIINP, decreased PICP, and particularly low PINP values in the amniotic fluid of a mother carrying a fetus with mild osteogenesis imperfecta. (Reproduced from [20], with permission.)

the immunoassay for PICP is able to detect the corresponding propeptide derived from the α1-homotrimer variant of type I collagen, although the assay for PINP equally recognizes both the normal and the homotrimer variant. In this respect, it is interesting that a polymorphism in an SP1 binding site in the *COL1A1* gene has been suggested to lead to some synthesis

TABLE 5. Propeptides of Type I Procollagen in Osteogenesis Imperfecta (OI)

Finding	Reference	Comment
Diagnostic potential		
PICP lower than −2 SD of the controls in type I OI, less low in types III and IV OI	[31]	Assay from [23], suggested for pedigree screening in type I OI
PICP decreased in type I OI	[32]	Assay from [23]
PICP decreased in types I, III and IV OI	[33]	Assay from [23]
PICP decreased in type I OI and less so in types III and IV; total PINP low in children with OI, not in adults with OI	[34]	Both PICP [23] and PINP [27] generally lower in mild OI with a quantitative defect than in severe OI with a qualitative defect
Effects of treatment		
PICP decreased in type I OI, some increase in response to growth hormone treatment	[35]	Assay from [23]
PICP probably decreases during bisphosphonate treatment of OI	[36]	Assay from [23]
Potential for prenatal diagnosis		
PICP and PINP decreased in amniotic fluid of a fetus with type I OI (mild phenotype)	[20]	PICP [23], PINP assay [22]

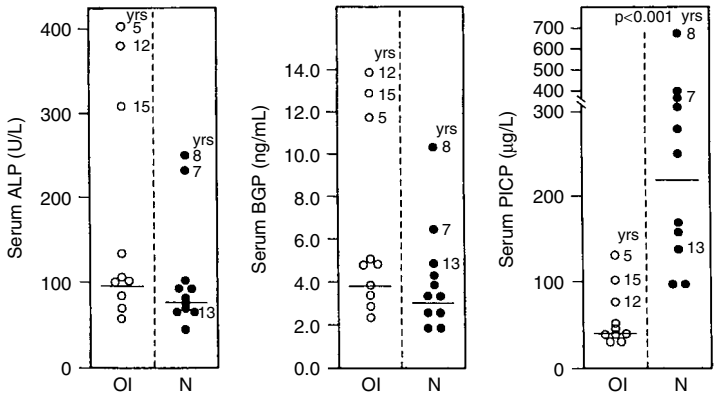

Figure 5. Concentrations of the carboxyl terminal propeptide of type I procollagen (PICP) and osteocalcin/bone Gla-protein (BGP) and the activity of alkaline phosphatase (ALP) in affected (open circles) and unaffected (closed circles) members of a family with type I osteogenesis imperfecta. The three markers measured represent different aspects of bone formation, matrix formation (PICP), matrix maturation (ALP), and matrix mineralization (BGP). Only the concentration of PICP is decreased due to a deficiency in the rate of synthesis of type I collagen, whereas the other aspects of bone formation appear normal. (Reproduced from [32], with permission.)

of the α1-homotrimer type I collagen in bone in apparently healthy people, increasing its fragility [37].

Propeptide measurements could be used as the first tests to distinguish overt osteogenesis imperfecta from suspected child abuse. An ordinary serum sample is sufficient and the results are obtained quickly and inexpensively. However, because healing of recent fractures increases the concentrations of the propeptides, the patient should be tested also later. In any case, a follow-up of suspected cases is otherwise recommendable and the simplicity of the biochemical procedure could justify it. Until recently, cell culture studies requiring a skin biopsy sample and taking a long time to perform have been the only biochemical tools to be used in this differentiation [38].

The Ehlers-Danlos Syndrome

The defective production of type III collagen in Ehlers-Danlos syndrome type IV (see Chapter 9, this volume) leads to a lower than normal circulating concentration of the amino terminal propeptide of type III procollagen, PIIINP (Table 6). The general view is complicated by the fact that several different PIIINP assays have been used in the literature that differ with respect to whether they detect exclusively the intact propeptide, as would be desirable here. There is also one example of a measurement in skin suction blister fluid verifying diagnosis [19].

In Ehlers-Danlos syndrome type VII, the various forms of which lead to impaired release of the amino terminal propeptide of type I procollagen by several mechanisms, the concentration of intact PINP in the serum should, in principle, be decreased, in the presence of a normal PICP concentration. However, in one adult patient with EDS VIIB studied in our laboratory (case 1 in [41]), the serum concentration of PINP was 32 µg/L and that of PICP 160 µg/L, with a PICP/PINP ratio of 5.0. Thus, the lack of a cleavage site in the α2 chain of type I procollagen in this condition does not seem completely

TABLE 6. Aminoterminal Propeptide of Type III Procollagen in Ehlers-Danlos Syndrome Type IV (EDS IV)

Finding	Reference	Comment
Low in serum in atypical case of EDS IV	[39]	Assay from [28], no longer available
Low in serum in some patients with EDS IV	[40]	As above
Decreased concentration in suction blister fluid in the skin, with normal PINP concentration, and low in serum (sporadic case of EDS IV)	[19]	Assay from [30]

to prevent the *in vivo* liberation of the amino terminal propeptide. However, the possibility still remains that such a defect could have a more pronounced effect on the PICP/PINP ratio in children.

A Genetic Trait Increasing the Circulating PICP Concentration

Individuals with surprisingly high circulating concentrations of PICP have sporadically been found in several populations. Values in such individuals typically exceed the upper limits of the age-adjusted reference intervals by two standard deviations or more and are seen in persons with no known pathology of bone or other specific tissues. This trait is inherited in an autosomal dominant fashion [42], and is not related to any abnormality in the structure or metabolism of type I procollagen itself. The molecular explanation of this finding is not yet known, but it is probably related to the further metabolism of the PICP fragment via the mannose receptor, as the circulating PINP is always normal (Fig. 6). It is worth noting that such grossly elevated values change in response to the known modifiers of type I collagen metabolism, e.g., growth, pregnancy [43], or medical treatment. In the rare case that a person with type I osteogenesis imperfecta had also this genetic trait, the circulating PICP concentration might be considered normal. However, defective type I collagen synthesis should then be detectable by PINP measurement.

Nonfibrillar Collagens

The possibility of specific measurement of the synthesis of any nonfibrillar collagen is very limited at present. Most such collagens are known not to be derived from procollagens that would also give rise to by-products allowing direct quantification of the synthesis rate. Specific immunoassays have been described for the 7S [44] and NC1 [45] domains of type IV collagen and for type VI collagen [46]. However, the composition of the antigens used is not completely clarified with respect to what is at present known about the chain composition of these collagens. Furthermore, changes in the antigen concentrations measured with such assays are not simple to interpret. They may reflect different metabolic phases; for example, newly synthesized molecules that have leaked into the circulation before their deposition into the tissues, antigenic fragments originating from the breakdown of tissue forms, or both.

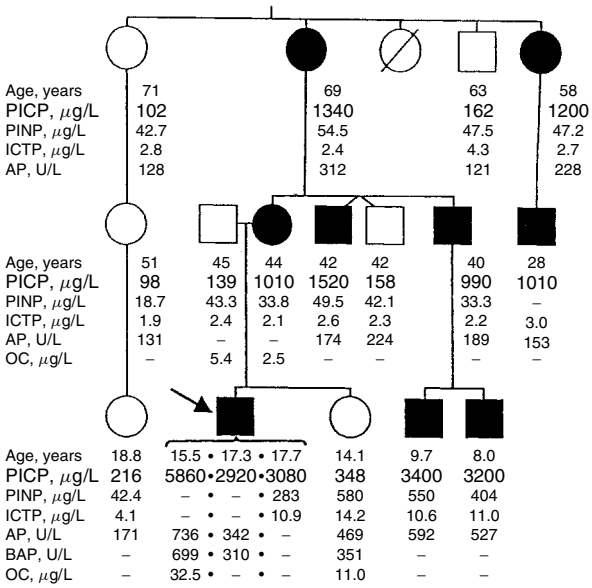

Figure 6. Elevated circulating concentration of the carboxyl terminal propeptide of type I procollagen (PICP) as a hereditary trait in a Finnish family. Members having the trait are represented by closed symbols. The arrow indicates the proband, whose high circulating PICP concentration was noticed when he participated in a study concerning the possible effects of glucocorticoid medication for asthma on the skeleton of growing individuals. PINP = amino terminal propeptide of type I procollagen, ICTP = cross-linked carboxyl terminal telopeptide of type I collagen, AP = alkaline phosphatase, BAP = bone-specific alkaline phosphatase, OC = osteocalcin. (Reproduced from [42], with permission.)

Markers of Cartilage Metabolism

Cartilage matrix contains several collagens, the most abundant of these being type II collagen which, as a fibre-forming collagen, closely resembles type I and III collagens in its structure, biosynthesis and cross-linking, together with large amounts of proteoglycans and several other proteins (see Chapters 1, Part II, and 4, this volume). Quantitative assays for a number of these components have been reported, but they are mostly still so-called "in-house" methods, i.e., they are not commercially available [47]. Their possible utility in the diagnosis and follow-up of hereditary cartilage disorders has not been systematically tested, although the information provided by them in investigating the courses of rheumatoid arthritis and osteoarthritis has been documented on several occasions [48]. The methods have mostly been applied to *in situ* measurements in synovial fluid, but often also to serum studies [48].

The carboxyl terminal propeptide of type II procollagen — PIICP according to the nomenclature used here, but originally known as chondrocalcin — can be measured in both synovial fluid and serum [49,50]. In a study of severe osteoarthritis due to a point mutation in the type II collagen gene, the concentration of the propeptide in the serum of affected family members did not differ from that in their healthy relatives [51]. Reference intervals for Canadian children and adults have been reported [50]; the serum content of the propeptide is higher in infants and children than in older age groups and also reflects the pubertal growth spurt. The mean concentration in adults, 5.7 μg/L, is about

5% of the corresponding concentration of the carboxyl terminal propeptide of type I procollagen, PICP. Several antibodies that recognize precisely defined structures in partially degraded type II collagen have also been characterized [47].

Aggrecan is the most abundant proteoglycan in cartilage, and a number of monospecific antibodies have been described that recognize well-characterized epitopes in the core protein and in the glycosaminoglycan side chains [47]. Some of these antibodies can be used for quantitative measurement of the corresponding antigens in synovial fluid and serum. There is a lot of structural and metabolic complexity as the differing extents of the various posttranslational modifications make the glycosaminoglycan side chains heterogeneous, and because several proteolytic enzymes are involved in the degradation of aggrecan. The chondroitin sulfate epitope, 846, is considered a marker of newly synthesized aggrecan, while another chondroitin sulfate epitope, 3-B-3, may indicate irreparable cartilage destruction [47]. Methods also exist for keratan sulfate which detect this glycosaminoglycan in serum.

Hyaluronan is present in cartilage, synovial tissue and other connective tissues; increased circulating concentrations have been associated with inflammatory joint disease and liver disease [52]. Hyaluronan that enters the circulation from the joints is partially taken up and degraded by lymph nodes and partially enters the liver endothelial cells by a receptor-mediated mechanism [52].

Cartilage oligomeric matrix protein, COMP, can be detected in synovial fluid and serum, where its presence in increased amounts in early rheumatoid arthritis indicates a poor prognosis [53]. This protein is also present in other connective tissues than cartilage.

The protein known previously as YKL-40 or human cartilage gp-39, for which there is a new immunoassay kit [54] available under the trademark Chondrex (Quidel), is produced by chondrocytes and synovial cells [55], and also by other tissues including the liver and malignant tumors. The protein belongs to the chitinase family, but its assumed role in the cartilage matrix has not been as thoroughly investigated as those of aggrecan, hyaluronan, and cartilage oligomeric matrix protein.

PRODUCTS OF COLLAGEN DEGRADATION

The assessment of collagen degradation has progressed from assays of hydroxyproline and other low molecular weight compounds that can be derived from any collagenous protein to the measurement of specific, often complex, telopeptide sequences derived from a known collagen type. Samples most frequently used include urine and, more recently, mainly due to unsatisfactorily large variations in urinary excretion, serum. Collagen degradation products have only rarely been measured in tissue fluids or extracts, but the peptide-related methods lend themselves well to such an approach, if it is reasonable to assume that such degradation products are set free in the tissue concerned.

In principle, mutations involved in heritable disorders of connective tissue may affect the structure of either an extracellular matrix protein, i.e., a substrate, or an enzyme involved in the degradation of various components of the matrix. An example of the former, in addition to the mutations in collagens themselves, is homocystinuria (see Chapter 13, this volume), in which the cross-linking of type I collagen seems to be impaired to the extent that the circulating concentration of one of its degradation products, the ICTP antigen (Table 7; see below) [56], is decreased to one third of that in healthy individuals [57]. An example of the latter is pycnodysostosis, a disease in which the osteoclastic enzyme cathepsin K is deficient [58] (see also Chapter 22, this volume). This disease leads to impaired release of the degradation products of bone type I collagen that normally contain epitopes produced by cathepsin K, such as those detected by the CrossLaps™ and NTx assays (see below) [59]. However, the serum ICTP concentration is paradoxically increased [59], indicating that the degradation of bone collagen is performed under such circumstances by other enzymes, most likely those belonging to the family of matrix metalloproteinases [60].

Collagen-Derived Amino Acids, Cross-Links, and Peptides

Urinary excretion of hydroxyproline (more specifically 4-hydroxyproline) has been widely used in assessing the degradation of collagen in spite of its limitations: only around 10% of hydroxyproline is excreted, a proportion is derived from the synthesis of fibrillar collagens (amino-terminal propeptides), other proteins may contain it (e.g., the C1q component of complement), and it can be absorbed from the diet.

To overcome the fact that hydroxyproline excretion can also be affected by the degradation of newly synthesized collagen before it is even deposited into fibers, the next development was to measure stable cross-links, which can only be derived from the degradation of collagen fibers. The two cross-links usually measured have a pyridinium structure and are generally known as pyridinoline (correctly, hydroxylysyl pyridinoline) and deoxypyridinoline (lysyl pyridinoline) (see Chapter 2, this volume). They differ in their extent of lysyl hydroxylation, which is one of the post-translational modifications of the collagens. Although these cross-links are found in all fibrillar collagens, there is a relative abundance of lysyl pyridinoline in bone collagen. However, there is also a lot of lysyl pyridinoline in the type I collagen of muscle and many internal organs, although not in that of skin. Since the type III collagen of soft tissues contains a significant quantity of hydroxylysyl pyridinoline at the amino terminus, the overall ratio of hydroxylysyl pyridinoline to lysyl pyridinoline is also for this reason lower in bone, which lacks type III collagen, than generally in soft connective tissues. In any case, the fully hydroxylated variant normally predominates in bone, most soft tissues and urine. Despite the fact that only pyridinolines are usually measured, other mature cross-links are also known to exist, some with pyrrolic [61] and others with still uncharacterized structures [62].

Initially, the total excretion of pyridinoline cross-links was measured by high performance liquid chromatography with fluorescence detection, but more recently it has also been possible to quantify the cross-links by specific immunoassays. In principle, either of these methods can be used to detect total (including free and peptide-bound) or free cross-links, depending, respectively, on whether the sample is first hydrolyzed or not. It should also be noted that acid hydrolysis destroys the glycosylation of the cross-links, whereas the immunoassays for the free forms may also detect cross-links that still carry carbohydrates. Some reaction also by small cross-linked peptides cannot be excluded. Specific assays have also been developed for measuring the urinary excretion of

TABLE 7. Assays for Degradation Products of Type I Collagen Fibers

Assay/Supplier/Sample	Antigenic Determinant/Assay Principle	Reference
ICTP/Orion Diagnostica +DiaSorin (previously Incstar) +Chugai Diagnostic Science/serum	Phenylalanine-rich domain in the carboxyl terminal telopeptide of type I collagen; trivalent cross-link necessary/RIA, polyclonal antiserum	[56,60]
CrossLaps™/Osteometer/serum	Two β-isomerized EKAHDGGR-peptides from the carboxyl terminal telopeptide; a trivalent cross-link must be present/ELISA, two monoclonal antibodies; automated version also available (Roche Diagnostics)	[65]
Urine (β assay)	Linear EKAHDGGR-peptide, β-isomerization of Asp residue necessary (?)/ELISA, polyclonal antiserum	[66]
Urine (α assay)	Linear EKAHDGGR-peptide, no β-isomerization of Asp residue/RIA, monoclonal antibody	[67]
NTx/Ostex International/	Trivalently cross-linked aminoterminal telopeptide of type I collagen (with a chain combination of α1 − α2 − α2)	
Urine	ELISA, monoclonal antibody	[68]
Serum	CLIA, monoclonal antibody	[69]
Serum	EIA, monoclonal antibody; automated versions also available	
Pyridinoline and Deoxypyridinoline/ in-house methods		
Urine	Reversed-phase HPLC after acid hydrolysis (total cross-links) or without hydrolysis (free fraction)	[70]
Free deoxypyridinoline/		
Quidel (previously Metra Biosystems)/urine	Direct assay of the free cross-link/ELISA, monoclonal antibody	[71]
Chiron Diagnostics/urine	Automated chemiluminescence assay, other automated versions soon available	[72]

ICTP = cross-linked carboxyl terminal telopeptide of type I collagen (see Fig. 7); NTx = trademark for assay of cross-linked aminoterminal telopeptide of type I collagen; RIA = radioimmunoassay; ELISA = enzyme-linked immunosorbent assay; CLIA = chemiluminescence immunoassay; EIA = enzyme immunoassay; HPLC = high-performance liquid chromatography

glycosylated hydroxylysines [63] that could be helpful in cases of defective lysyl hydroxylation or glycosylation.

Since a proportion of the urinary cross-links were known to be in peptides, the next phase was to develop immunoassays for the telopeptide regions of the collagen molecules where the cross-links are located. An in-house assay has also been described that measures a degradation product of type I collagen originating in its central, triple-helical domain and detects similar antigens in human serum [64].

Cross-Linked Telopeptides

At present, assays based on three kinds of antigenic determinant in the cross-linked telopeptides of type I collagen are commercially produced and generally available (Table 7), two of these being for the carboxyl terminal and one for the amino terminal telopeptide. The structures of the antigenic determinants required by the assays are depicted in Fig. 7. The size of the type I collagen fragments released depends on the nature of the degradative enzymes involved. The CrossLaps and NTx assays reflect cathepsin K-mediated bone resorption, whereas the ICTP is most likely derived via the matrix metalloproteinase route [60]. A further complication is caused by the fact that some aspartyl residues in the telopeptides are subject to L → D racemization and β isomerization of the subsequent peptide bond. Such aspartic acid residues are located within the epitope of the CrossLaps™ assay (Table 7). Recently, β-isomerization has been described also in the amino terminal telopeptide of type I collagen [73]. As the extents of these spontaneous modification reactions increase with time, the relative proportions of the different variants will vary, depending on the age of the collagen fiber being degraded. At the carboxyl terminus of type I collagen only the α1 chains take part in cross-link formation, whereas at the amino terminus the α2 chain also has an important role. The NTx assay is specific for such variants of the cross-linked N-telopeptides that involve the amino terminal telopeptide part of the α2-chain.

Collagen Degradation and Inherited Diseases

Conflicting results have been obtained with the type I collagen telopeptide assays in osteogenesis imperfecta [74]. The NTx assay has been reported to show increased type I collagen degradation in types I, III, and IV of this disease [75], whereas the ICTP assay [34] sometimes shows normal or decreased levels, as might be expected from

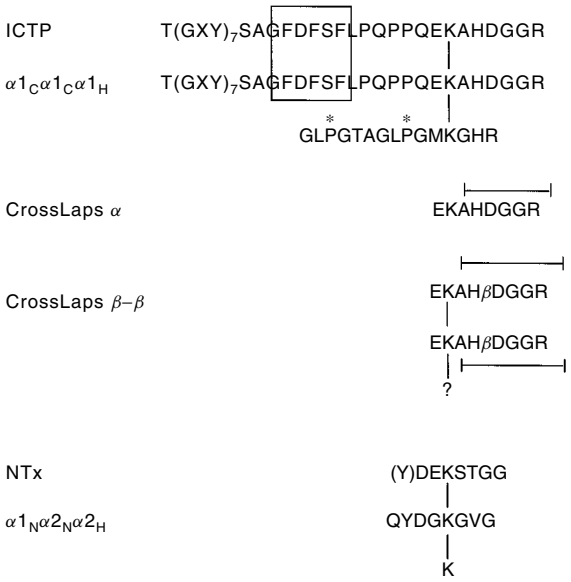

Figure 7. Antigenic determinants of different immunoassays recognizing the telopeptides of type I collagen. The CrossLaps and NTx assays reflect cathepsin K-mediated bone resorption, whereas the ICTP is most likely derived via the matrix metalloproteinase route [60]. In pycnodysostosis due to cathepsin K deficiency, the urinary excretion of the CrossLaps and NTx antigens is low, whereas the serum ICTP concentration is increased [59]. The ICTP assay detects only fragments having two phenylalanine-rich domains (box in the figure): to bring them together, a trivalent cross-link must be present. The asterisks denote proline residues (deduced from the DNA sequence) that in the protein are usually posttranslationally hydroxylated to 4-hydroxyproline. The CrossLaps assays detect linear, eight amino acid-long sequences, in which the aspartic acid residue (D) is in either α-or β-configuration, and can also racemize from an L- to a D-amino acid. The NTx assay requires the sequence QYDGKGVG, in which K is incorporated in a trivalent cross-link. The $\alpha1_C$ denotes the carboxyl terminal telopeptide, the $\alpha1_N$ and $\alpha2_N$ the aminoterminal telopeptide, and $\alpha1_H$ the peptide derived from the helical region of another collagen molecule. (Reproduced from [3], with permission.)

the decreased rate of synthesis of type I procollagen and, presumably, the smaller total amount of type I collagen in the body. There is some indication that qualitative defects in type I collagen are associated with more active collagen degradation [34,75] — in addition to NTx and ICTP, such a trend can also be seen in the excretion of the pyridinoline cross-links [34]. On the other hand, if the rate of type I collagen turnover is increased, the degradation of immaturely cross-linked collagen is not detected by the ICTP assay [60]. In addition, structures that are recognized by these assays can be derived from poorly mineralized bone matrix, which may be turned over at a high rate, as well as from the soft tissues.

Ehlers-Danlos Syndrome

An interesting, simple way to diagnose Ehlers-Danlos syndrome type VI has been suggested that can be carried out by determining the urinary excretion of hydroxylysyl pyridinoline and lysyl pyridinoline. Because the enzyme lysyl hydroxylase is defective, the amount of hydroxylysine is lower than normal. Three hydroxylysine residues are needed for the adequate formation of the hydroxylysyl pyridinoline. Consequently, the ratio of the less hydroxylated (lysyl pyridinoline or deoxypyridinoline) to the fully hydroxylated pyridinoline (hydroxylysyl pyridinoline or pyridinoline) cross-link has been found to differ dramatically between healthy individuals and patients with Ehlers-Danlos syndrome type VI [76,77] (see also Chapter 9, this volume).

Bruck Syndrome

Bruck syndrome (MIM 259450) (see also Chapter 26, Part IV, this volume) is characterized by the underhydroxylation of the telopeptides of type I collagen in bone, but not in that of ligaments, and not in those of type II collagen in cartilage [78]. Because telopeptidyl hydroxylysine is necessary for the formation of mature pyridinoline cross-links, the contents of hydroxylysyl and lysyl pyridinolines are low in bone collagen. Accordingly, the urinary excretion of lysyl pyridinoline is decreased and the ratio hydroxylysyl pyridinoline/lysyl pyridinoline is increased. However, hydroxyproline excretion is also increased, indicating an increased turnover of bone collagen (Ruud A. Bank, personal communication).

REMARKS ON THE USE OF THE REFERENCE INTERVAL TABLES (8–12)

The reference interval (previously known as normal range) of an analyte is generally defined as the range of concentrations that includes the central 95% of the values obtained in a given healthy population. For variables that show a Gaussian (normal) distribution, this is the same as the range defined as two (exactly 1.96) standard deviations to each side of the mean value. If the distribution is skewed, it can often be normalized by substituting the primary values by their logarithms, a procedure known as logarithmic transformation. If this is carried out, the limits of the reference interval are calculated using the logarithms and these are then changed back to the primary values.

Although a number of age groups from different countries have been studied with the collagen/procollagen assays, the quality and thus the usefulness of the reference values published is variable. Unfortunately, authors often do not disclose whether the distributions of the variables were found to be normal or not. Without this information it is impossible to know if it is legitimate to use, for example, the mean and standard deviation given to calculate the reference interval. Because of this diversity, we always indicate what parameter has been reported. Despite the lack of uniform reference intervals we felt justified in giving the information in the Tables as it was reported by the respective authors.

For the analytes measured in urine, there is an additional problem due to the great variation in urinary volume [79]. It is common practice to express the excretion of urinary collagen metabolites in relation to the amount of creatinine excreted in the sample. This is based on the assumption that creatinine excretion is relatively stable both within an individual and between individuals. However, when dealing with handicapped patients, often with decreased mobility, it is worth noting that creatinine excretion is related to muscle mass. A 24-hour collection of urine is generally considered ideal, but in practice a spot urine is mostly used, in particular for children. For such reasons, serum assays are generally preferred in modern clinical chemistry and these have also recently been developed for the collagen telopeptide variants. However, the kidneys concentrate the small excreted molecules and most likely also further catabolize the cross-linked peptides. Thus, free lysyl pyridinoline is practically unmeasurable in the blood, and urine should be used.

TABLE 8. Reference Intervals for Concentration of the Carboxyl Terminal Propeptide of Type I Procollagen (PICP)

Sample, Sex, Age	Concentration (μg/L)	Comment
Systemic Monitoring of Connective Tissue Metabolism		
Amniotic fluid		
Second trimester	3790–25,440 (2.5th and 97.5th centiles)	Finnish [20]
Labor at full term	70–630	
14 to 40 weeks of gestation	556–706[1] (95% confidence interval of adjusted mean in the appropriate-for- gestational-age group)	Caucasian and African American [80]
Cord blood serum (full-term)	1408 ± 419	American [81]
Serum		
Infants, 1 month	1408–5201 (95% confidence interval)	American [82]
3 months	998–3999	
6 months	961–2462	
9 months	763–1721	
12 months	829–1649	
18 months	710–1648	
Children, 1–5 years	294–402 (95% confidence interval)	Nigerian[83]
Boys, 0–2 years	784 ± 329	Chinese [84]
3–9 years	223 ± 118	
10–14 years	268 ± 113	
15–18 years	155 ± 20	
Girls, 0–2 years	557 ± 260	
3–8 years	216 ± 93	
9–12 years	243 ± 65	
13–17 years	129 ± 29	
Boys, 0.1–1.0 years	950 ± 86	Italian [85]
1.1–2.0 years	680 ± 87	
2.1–4.0 years	433 ± 58	
4.1–6.0 years	350 ± 57	
6.1–8.0 years	219 ± 40	
8.1–10.0 years	278 ± 35	
10.1–12.0 years	355 ± 25	
12.1–14.0 years	446 ± 37	
14.1–16.0 years	525 ± 46	
16.1–18.0 years	282 ± 29	
Girls 0.1–1.0 years	905 ± 82	
1.0–2.0 years	674 ± 90	
2.1–4.0 years	419 ± 50	
4.1–6.0 years	340 ± 42	
6.1–8.0 years	229 ± 21	
8.1–10.0 years	359 ± 24	
10.1–12.0 years	447 ± 36	
12.1–14.0 years	397 ± 32	
14.1–16.0 years	272 ± 24	
16.1–18.0 years	218 ± 27	
Boys, 2–14 years	52–544 (mean ± 2SD, normalized to body surface)	Italian [86]
Girls, 2–12 years	18–546	
Boys, 4–16 years	193–716 (95% reference interval)	Irish [87]
17–18 years	105–452	
Girls, 4–12 years	225–676	
13–14 years	108–567	
15–18 years	82–285	

(*Continued Overleaf*)

TABLE 8. (*Continued*)

Sample, Sex, Age	Concentration (μg/L)	Comment
Young adults, 20–50 years		Chinese [84]
men	116 ± 11	
women	82 ± 22	
Adults		
men	38–202 (95% reference interval)	Finnish [23]
women	50–170	
Adults		
men	109 ± 32	Italian [85]
women	110 ± 31	
***In situ* Monitoring of Collagen Metabolism in**		
Skin		
Suction blister fluid		
Men, 19–21 years	703 ± 141	[93]
Wound fluid		
Day 1 postsurgery	207 ± 92	[18]
Day 2	908 ± 469	
Day 3	3246 ± 2858	
Day 7	79330 ± 54151	
Meninges		
Cerebrospinal fluid		
1.5–14.8 years	20.0–92.4 (95% reference interval)	[94] Age-dependence curve similar to that for circulating concentration
Mouth		
Crevicular fluid	4200 (Mean before treatment of periodontal disease; not detectable in most healthy adults)	[95]
Pathological Fluids		
Ascitic fluid		
Malignant disease	420–7220 (range)	[96] Ovarian carcinoma
Cyst fluid		
Malignant disease	3520 (mean)	[96] ovarian cysts (malignant/benign)
Benign disease	735 (mean)	

[1] Expressed as mg/L in the publication, but obviously should be μg/L

Unless otherwise indicated, values are means ± SD (standard deviation).

In addition to the articles mentioned above, reference values have also been published for Japanese children and adolescents (serum [88], EDTA plasma [89]), and for Danish girls and boys [90], British girls [91] and Dutch girls and boys [92] according to the Tanner stages of puberty (serum).

TABLE 9. Reference Intervals for Concentration of the Amino Terminal Propeptide of Type I Procollagen (PINP)

Sample, Sex, Age	Concentration (μg/L)	Comment
Systemic Monitoring of Connective Tissue Metabolism		
Amniotic fluid		
Second trimester	7,050–23,640 (2.5th and 97.5th centiles)	Finnish [20]
Labor at full term	50–360	
Serum		
Children, 1–5 years	355–449 (95% confidence interval)	Nigerian [83]
Adults,		
Men >25 years	49.9 ± 15.8	Finnish [9]
Women >20 years	39.8 ± 14.7	

TABLE 9. (*Continued*)

Sample, Sex, Age	Concentration (μg/L)	Comment
Men	20–76 (95% reference interval)	Finnish [22]
Women	19–84	
Women, 60–90 years	43 ± 22	American [97]
***In situ* Monitoring of Collagen Metabolism in**		
Skin		
Suction blister fluid		
Men, 19–21 years	898 ± 384	[93]
Joints		
Synovial fluid	118–5,017 (range in patients with rheumatoid arthritis)	[98]
Ratio PICP/PINP		
Amniotic fluid		
Second trimester	0.82 ± 0.31	Finnish [20]
Labor at full term	1.64 ± 0.40	
Serum		
Children, 1–5 years	0.72–0.9 (95% confidence interval)	Nigerian [83]
Adults		
Men > 25 years	2.94 ± 0.58	Finnish [9]
Women > 20 years	3.19 ± 0.80	

Unless otherwise indicated, values are means ± SD (standard deviation).

TABLE 10. Reference Intervals for Concentration of the Amino Terminal Propeptide of Type III Procollagen (PIIINP)

Sample, Sex, Age	Concentration (μg/L)	Comment
Systemic Monitoring of Connective Tissue Metabolism		
Amniotic fluid		
Second trimester	2,490–9,150 (2.5th and 97.5th centiles)	Finnish [20]
Labor at full term	50 –350	
Serum[a]		
Children, 1–5 years	9.8–11.6 (95% confidence interval)	Nigerian [83]
Boys, 4–11 years	5.1–12.0 (95% reference interval)	Irish [87]
12–16 years	5.1–20.8	
17–18 years	3.0–17.0	
Girls, 4–10 years	5.4–13.6	
11–12 years	5.8–21.1	
13–14 years	4.4–15.1	
15–18 years	3.0–7.8	
Adults	1.7–4.2 (95% reference interval)	Finnish [30]
***In situ* Monitoring of Collagen Metabolism in**		
Skin		
Suction blister fluid		
Men, 19–21 years	215 ± 117	[93]
Wound fluid		
Day 1 postsurgery	70 ± 61	[18]
Day 2	86 ± 88	
Day 3	180 ± 129	
Day 7	17, 812 ± 9, 839	

(*Continued Overleaf*)

TABLE 10. (*Continued*)

Sample, Sex, Age	Concentration (μg/L)	Comment
Meninges		
Cerebrospinal fluid		
1.5–16 years	2.0–14.2 (range)	[99] age-dependence curve similar to that for circulating concentration
Adults	3.6–5.7 (range)	
Mouth		
Crevicular fluid	162 (mean in untreated periodontal disease; not detectable in most healthy adults)	[100]
Joints		
Synovial fluid	278–4,781 (range in patients with rheumatoid arthritis)	[98]
Pathological Fluids		
Ascitic fluid		
Malignant disease	25–3,880 (range)	[96] ovarian carcinoma
Cyst fluid		
Malignant disease	260 (mean)	[96] ovarian cysts
Benign disease	160–230 (mean)	

[a]In addition to the articles mentioned, reference values have also been published according to the Tanner stages of puberty for Danish children [90].

Unless otherwise indicated, values are means ± SD (standard deviation).

TABLE 11. Reference Intervals for Concentration/Excretion of Degradation Products of Type I Collagen Fibers

Analyte, Sample, Sex, Age	Concentration	Comment
ICTP (Concentration in μg/L)[a]		
Amniotic fluid	3.49–4.17[b] (95% confidence interval of adjusted mean in the appropriate-for-gestational-age group)	Caucasian and African American [80]
Cord blood serum (full-term)	63.7 ± 13.5	American [81]
Serum		
Children, 1–5 years	10.6–13.2 (95% confidence interval)	Nigerian [83]
Boys, 2–14 years	6.1–24.5 (95% reference interval, normalized by body surface)	Italian [86]
Girls, 2–12 years	6.8–22.9	
Boys, 4–11 years	5.1–17.0 (95% reference interval)	Irish [87]
12–16 years	7.5–22.8	
17–18 years	2.7–15.3	
Girls, 4–8 years	5.7–14.9	
9–13 years	7.2–20.0	
14–15 years	2.9–8.5	
Adults	1.6–4.6 (95% reference interval)	Finnish [56]
Women, 60–90 years	3.0 ± 1.6	American [97]
Crevicular fluid	425 (mean in untreated periodontal disease) 148 (mean in periodontitis-free adults)	[101]

TABLE 11. (*Continued*)

Analyte, Sample, Sex, Age	Concentration	Comment
Synovial fluid	2.8–96.9	[98]
	(range in patients with rheumatoid arthritis)	
NTx[c,d]		
Serum (concentration nmol BCE/L)		
Women, 60–90 years	14.1 ± 5.5	American [97]
Urine (spot) (concentration nmol BCE/mmol creatinine)		
Newborn, 1st void (full term)	846 ± 639	Italian [102]
24 hours	1171 ± 841	
48 hours	1492 ± 990	
Babies, ≤2 months	1895 ± 665	Austrian [103]
Boys, 10 months	1495 ± 772	French [104]
24 months	934 ± 488	
Girls, 10 months	1249 ± 616	
24 months	1377 ± 504	
Men, 21–30 years	48 ± 22	American [105]
31–86 years	33 ± 15	
25–79 years	13.5–72.2 (from the 2.5th to the 97.5th percentile)	Japanese [106]
Women >25 years,		
Premenopausal	11.1–69.1 (from the 2.5th to the 97.5th percentile)	Japanese [106]
Postmenopausal	27.4–116.9	
Women	4.1–61.0	Japanese [107]
Premenopausal	(95% confidence interval)	
Postmenopausal	14.0–89.0	
CrossLaps™[e]		
Serum (concentration pmol/L)		
Women		Danish [65]
Premenopausal	1748 ± 740	
Postmenopausal	2952 ± 1325	
Urine (spot) (concentration μg/mmol creatinine)		
Boys, 10 months	1492 ± 685	French [104]
24 months	1510 ± 446	
Girls, 10 months	1705 ± 612	
24 months	1849 ± 611	

[a]In addition to the articles mentioned below, reference values have also been published for EDTA-plasma for 10–15 years old Japanese children [89], and for serum according to the Tanner stages of puberty for British girls [91].

[b]Expressed as mg/L in the publication, but obviously should be μg/L.

[c]NTx = trademark for the assay of the cross-linked amino terminal telopeptide of type I collagen (see Figure 7)

[d]In addition to the articles mentioned below, reference values for NTx (spot urine samples) have been given for Italian children and adolescents between 4 and 20 years of age [108].

[e]CrossLaps = trademark for the assay of the cross-linked carboxyl terminal telopeptide of type I collagen (see Figure 7)

Unless otherwise indicated, values are means ± SD. ICTP = cross-linked carboxyl terminal telopeptide of type I collagen (see Fig. 7); BCE = bone collagen equivalent.

TABLE 12. Reference Intervals for Concentration/Excretion of Pyridinium Cross-Links

Analyte, Sample, Sex, Age	Concentration nmol/mmol Creatinine	Comment
Pyridinoline (hydroxylysyl pyridinoline)		
Urine (spot)		
Total pyridinoline (HPLC)		
Newborn	1262 ± 434	Austrian [103]
Infants, 1 week	411 ± 81	Japanese [109]
1 month	730 ± 157	
4 months	462 ± 80	
7 months	368 ± 98	
Boys, 10 months	366 ± 120	French [104]
Girls, 10 months	407 ± 135	
Children, 2–4.9 years	112.7 (median)	British [110]
5–7.9 years	116.7	
8–10.9 years	129.5	
11–13.9 years	97.6	
≥14 years	89.3	
Children, 4.0–10.0 years	79–213 (from the 5th to the 95th percentiles)	German [111]
10.1–12.0 years	79–387	
12.1–14.0 years	111–326	
14.1–16.0 years	39–330	
16.1–18.0 years	34–183	
Young adults, 20–25 years	12–84	
Deoxypyridinoline (lysyl pyridinoline)[a]		
Urine (spot)		
Total deoxypyridinoline (HPLC method)		
Newborn	165 ± 67	Austrian [103]
Infants, 1 week	63 ± 11	Japanese [109]
1 month	108 ± 27	
4 months	73 ± 15	
7 months	57 ± 20	
Boys, 10 months	63 ± 22	French [104]
Girls, 10 months	71 ± 27	
Children, 2–4.9 years	29.6 (median)	British [110]
5–7.9 years	35.5	
8–10.9 years	38.2	
11–13.9 years	35.2	
≥14 years	22.1	
Children, 4.0–10.0 years	20.5–65.9 (from the 5th to the 95th percentiles)	German [111]
10.1–12.0 years	24.8–104	
12.1–14.0 years	26.3–74.3	
14.1–16.0 years	6.4–105	
16.1–18.0 years	8.8–51.6	
Young adults, 20–25 years	2.6–25.9	
Total deoxypyridinoline (RIA method)[b]		
Boys, 10 months	82 ± 33	French [104]
24 months	61 ± 22	
Girls, 10 months	77 ± 25	
24 months	75 ± 18	
Free deoxypyridinoline (RIA method)[b]		
Boys, 10 months	31 ± 12	French [104]
24 months	24 ± 7	
Girls, 10 months	26 ± 8	
24 months	29 ± 9	

Unless otherwise indicated, values are means ± SD.

[a]In addition to the articles mentioned below, reference values have also been published for British girls according to the Tanner stages of puberty [91].

[b]In addition to the articles mentioned below, reference values for total pyridinoline and deoxypyridinoline (HPLC method, spot urine samples) have been given for Japanese children between 3 and 14 years of age [112], Polish children and adolescents between 3 and 18 years of age [113] and Italian children and adolescents between 4 and 20 years of age [108], and for free pyridinoline and deoxypyridinoline (HPLC method, spot urine samples) for British children between 4 and 10 years of age [114].

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