ESSENTIALS IN OPHTHALMOLOGY

G. K. KRIEGLSTEIN · R. N. WEINREB
Series Editors

Glaucoma | Cataract and Refractive Surgery | Uveitis and Immunological Disorders | Vitreo-retinal Surgery | Medical Retina | Oculoplastics and Orbit | Pediatric Ophthalmology, Neuro-Ophthalmology, Genetics | **Cornea and External Eye Disease**

Cornea and External Eye Disease

Edited by
T. REINHARD
F. LARKIN

Springer

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With 138 Figures, Mostly in Color, and 20 Tables

Springer

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Foreword

Essentials in Ophthalmology is a new review series covering all of ophthalmology categorized in eight subspecialties. It will be published quarterly; thus each subspecialty will be reviewed biannually.

Given the multiplicity of medical publications already available, why is a new series needed? Consider that the half-life of medical knowledge is estimated to be around 5 years. Moreover, it can be as long as 8 years between the description of a medical innovation in a peer-reviewed scientific journal and publication in a medical textbook. A series that narrows this time span between journal and textbook would provide a more rapid and efficient transfer of medical knowledge into clinical practice, and enhance care of our patients.

For the series, each subspecialty volume comprises 10–20 chapters selected by two distinguished editors and written by internationally renowned specialists. The selection of these contributions is based more on recent and noteworthy advances in the subspecialty than on systematic completeness. Each article is structured in a standardized format and length, with citations for additional reading and an appropriate number of illustrations to enhance important points. Since every subspecialty volume is issued in a recurring sequence during the 2-year cycle, the reader has the opportunity to focus on the progress in a particular subspecialty or to be updated on the whole field. The clinical relevance of all material presented will be well established, so application to clinical practice can be made with confidence.

This new series will earn space on the bookshelves of those ophthalmologists who seek to maintain the timeliness and relevance of their clinical practice.

G. K. Krieglstein
R. N. Weinreb
Series Editors

Preface

This volume of the series *Essentials in Ophthalmology* aims to present recent developments regarding the cornea, with a discussion of diagnostic measures and particular emphasis being placed on treatment.

The therapeutic repertoire for surface disorders has increased considerably within the past decade. The chapter by Geerling and Hartwig reviews the application of autologous serum eye drops for this indication. Dua and coworkers in their chapters first help us to understand the limitations of amniotic membrane transplantation and then give us an overview regarding the various possibilities of limbal stem cell transplantation. Güell and coworkers provide an illustration of the potential of limbal stem cell transplantation following ex-vivo expansion.

Anterior lamellar keratoplasty is presented by Melles as a promising technique for patients with low grade keratoconus or opaque corneas with healthy endothelium. Endothelial immune reactions may be avoided using this procedure. In penetrating keratoplasty, immune reactions and astigmatism in patients still represent the major postoperative problems. Slegers and coworkers illustrate immunopathological phenomena, clinical features and risk factors of graft rejection. Antiangiogenic procedures are discussed by Cursiefen and Kruse which might contribute to minimizing the immunological problem in the future. Böhringer and coworkers outline modern matching techniques (major – triplet, minor matching) as a prophylactic immunological measure for patients with normal-risk as well as those with high-risk keratoplasty. Modern strategies of systemic immunosuppression following high-risk penetrating keratoplasty are presented by Reis and coworkers. An overview is given by Seitz of how best to trephine the cornea in penetrating keratoplasty in order to minimize postoperative astigmatism. Watson and Daya provide an expert opinion on a serious postoperative complication following LASIK, i. e. infection.

Adenoviral corneal opacities are still a therapeutic challenge. Hillenkamp and coworkers provide an update on the different treatment regimes. Guthoff and coworkers present confocal microscopy of the cornea as a valuable tool for the in-vivo description of corneal structures on a cellular basis. Finally, a overview of ocular allergic disease is given by Manzouri and coworkers.

All the topics covered by the book have a direct clinical relevance, and we hope they will make a significant contribution to the development of optimal diagnostic and therapeutic procedures for patients with disease of the cornea.

T. Reinhard
D. F. P. Larkin

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Autologous Serum Eyedrops for Ocular Surface Disorders

Gerd Geerling, Dirk Hartwig

Core Messages

- The viability and function of the corneal and conjunctival epithelium is supported by the antimicrobial, nourishing, mechanical and optical properties of tears, since these contain factors which promote proliferation, migration and differentiation of epithelial cells
- A lack of these epitheliotrophic factors, e.g. in aqueous tear deficiency, can compromise the ocular surface and result in serious disorders such as persistent epithelial defects
- Pharmaceutical lubricants usually replace the aqueous component of tears alone and may have little efficacy in improving surface disorders
- Serum has biomechanical and biochemical properties similar to normal tears
- In vitro cell culture experiments have shown that the morphology, migration and differentiation of ocular surface epithelia are better supported by serum than by pharmaceutical lubricants
- A number of protocols for the production of serum eyedrops have been published, which vary considerably and can influence the biochemical properties of this autologous blood product
- Under EU legislation production of serum eyedrops requires a license to produce blood products by the appropriate national body, which means that an extensive evaluation and documentation process must be successfully completed
- Alternatively the use of serum eyedrops is allowed on an intention to treat basis if production and application are all performed by the physician, i.e. in ophthalmic departments that have the necessary laboratory facilities and are able to admit the patient for the duration of the treatment
- The production of serum eyedrops should be well documented and measures for appropriate quality control (i.e. serological and microbiological tests) should be established
- The results of a considerable number of clinical cohort studies have reported beneficial effects of its use for persistent epithelial defects, severe dry eyes and other indications
- The use of serum eyedrops implies the risk of transmission of infectious diseases, from the donor to other individuals involved in the production and application of the product
- In addition contamination of the dropper bottle and subsequent microbial keratitis can occur
- Such complications can be largely avoided by testing the patient for HIV, syphilis and hepatitis B and C prior to the blood donation and testing every serum eyedrop batch for bacterial contamination. In addition, the serum therapy can be combined with topical antibiotics
- Due to these risks and the lack of randomised controlled trials, the use of serum eyedrops should still be considered experimental and informed consent should be obtained from every patient treated
- Alternative blood products with epitheliotrophic potential such as plasma, platelet concentrates or serum albumin should be evaluated since they are readily available as quality controlled blood derivatives from blood banks on a routine basis

1.1 Introduction

In recent years the use of eyedrops produced from autologous serum has gained wide acceptance for the treatment of ocular surface disorders intractable to conventional medical therapy. Such conditions include persistent epithelial defects or severe dry eyes. Autologous serum was first evaluated in 1984 by Fox et al. [9] in search of an unpreserved lubricant, which was not available from pharmaceutical providers at that time. However, it was Tsubota who repopularised their use when he described the epitheliotrophic potential of serum for the ocular surface due to its content of growth factors and vitamins [46].

1.1.1 The Rationale for Using Serum in Ocular Surface Disorders

The tear film supplies the ocular surface with many nutrient and wound healing modulating factors such as fibronectin, vitamins or growth factors, which support and modulate proliferation, migration and differentiation of the conjunctival and corneal epithelium. These epitheliotrophic factors are predominantly released into the aqueous component of the tear film by the main and accessory lacrimal glands as well as conjunctival vessels, while glucose, electrolytes and amino acids are provided by the aqueous humour.

For example fibronectin, a disulphide glycoprotein that influences cell adhesion and migration of the healing epithelium, predominantly originates from plasma when conjunctival blood vessels become more permeable during an inflammatory reaction and the lacrimal gland itself secretes vitamins, neuropeptides and growth factors such as substance P and epidermal growth factor (EGF) [36]. However, proteins of the aqueous tear film not only act as essential nutrients for the ocular surface epithelia, but also determine the biomechanical properties of the tear lipid layer. Tear-lipocalin is an example for this, which by acting as a transport protein for retinol, supports goblet cell differentiation, but also reduces the surface tension of tears. As part of inflammatory processes, additional proteins, such as lactoferrin, serum-IgA and complement factors, are released into the tears and support opsonisation and phagocytosis of microbes by macrophages and lymphocytes. Tears thus not only have a lubricating and mechanical clearance function, but also epitheliotrophic and antimicrobial properties.

If the carrier, i.e. the aqueous phase, or the epitheliotrophic factors of the tear film are diminished, the integrity of the surface epithelia can become disrupted and epithelial defects evolve, which may persist and progress. Surgical attempts to rehabilitate the ocular surface in severe dry eyes also fail frequently [2, 22] unless sufficient substitute lubrication is provided.

- **The tear film has lubricant and nutrient properties**
- **In severe dry eye it is not only the increased biomechanical stress, but also the lack of epitheliotrophic factors that promotes damage of the ocular surface**

The ideal tear substitute would possess lubricant and nutrient properties. However – with few exceptions – currently available products are optimised for their biomechanical properties only [49, 51]. Vitamin A, EGF and fibronectin have been used in vitro and in vivo to encourage epithelial wound healing, but due to stability concerns and limited clinical success, these single compounds have not become part of clinical routine management [15, 25, 29]. Autologous blood offers a number of characteristic advantages:

1. It contains a large number of substances also present in tears, such as vitamin A, epitheliotrophic and neurotrophic growth factors, immunoglobulins and fibronectin. Some of these factors are found in serum in higher concentrations than in natural tears (Table 1.1) [20, 27, 29, 45].
2. Serum can be prepared as an autologous product and thus lacks antigenicity.

Table 1.1. Biochemical and biophysical properties of undiluted serum and normal, unstimulated human tears (EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin like growth factor; NGF, neurotrophic growth factor; PDGF, platelet derived growth factor; SP, substance P; TGF, transforming growth factor) [11, 15, 20, 48–52]

	Tears	Serum
pH	7.4	7.4
Osmolality	298±10	296
EGF (ng/ml)	0.2–3.0	0.5
TGF-β	2–10	6–33
NGF (pg/ml)	468.3±317.4	54.0
SP (pg/ml)	157.0±73.9	70.9±34.8
IGF-1 (ng/ml)	0.031±0.015	105
PGDF	0–1.33	15.5
Vitamin A (mg/ml)	0.02	46
Albumin (mg/ml)	0.023±0.016	35–53
Fibronectin (µg/ml)	21	205
Lactoferrin (ng/ml)	1,650±150	266
Lysozyme (mg/ml)	2.07±0.24	0.001
SIgA (µg/ml)	1,190±904	2,500

3. Eyedrops from serum can be produced without preservatives and hence toxicity due to additives is not an issue.

A wide range of quality controlled products can be derived from full blood. These include not only serum, but also, e.g. plasma, platelet rich suspensions or various protein fractions such as albumin. *Serum* is the fluid component of full blood that remains after clotting. It should not be confused with *plasma*, which is obtained when clotting is prevented by mixing a full blood donation with an anticoagulant and removing all corpuscular elements by centrifugation (see Sect. 1.5.3). Plasma thus does not contain the significant amount of platelet derived growth factors such as EGF, PDGF and TGF-β, which are released into serum upon activation of the platelets during clotting. *Platelet rich suspensions* (platelet concentrates) are available as a standard blood product from blood banks, while *human serum albumin* fractions can be purchased from pharmaceutical companies. While all of these blood derived products are potentially available as growth factor containing solutions for topical application to the ocular surface, serum has been predominantly used in clinical studies.

The growth and migration promoting effect of serum on cell cultures in general and on corneal epithelial cells is well documented [13, 46]. In dose- and time-response experiments in vitro, we found that serum maintained the morphology and supported proliferation of primary human corneal epithelial cells better than unpreserved or preserved pharmaceutical tear substitutes [13]. It is also known that serum induces mucin-1 expression in immortalised, conjunctival epithelial cells as a sign of higher differentiation [45] and that it increases transcription of RNA for NGF as well as TGF-β receptors in human keratocytes [7]. The epitheliotrophic properties are – again according to in vitro tests – not reduced in patients suffering from systemic autoimmune disease requiring systemic immunosuppression [17].

Summary for the Clinician

- **Plasma, platelet suspensions and albumin are available as blood derived products from blood banks**
- **Serum is not a standard blood product, but can easily be produced on special request and currently is by far the most often used product in clinical studies**
- **Serum contains many epitheliotrophic factors also present in tears and can be prepared as an autologous unpreserved tear substitute that offers both lubricant and nutrient properties**

1.1.2 Legal Aspects

Autologous serum eyedrops are a blood product. The distribution of pharmaceuticals is regulated by governmental laws in most countries. Although in the European Union the manufacturing and distribution of pharmaceuticals is regulated by the individual country, several

directives have been issued (1965/65, 1975/139, 1975/318) which have been implemented in the laws of each member state of the EU. Today every pharmaceutical product requires a marketing authorisation to be issued by a competent authority of each state. Authorisation depends on the proof of efficacy in clinical trials, implementation of quality controls, reports of adverse effects, proof of expert knowledge and other regulatory aspects. These criteria can probably only be fulfilled by professional pharmaceutical manufacturers.

An exemption from the need to obtain marketing authorisation is granted if a physician manufactures a specific medical product by himself or under his supervision with responsibility to treat his own patient on a named basis. This product has to be prepared according to the doctor's specifications and autologous serum eyedrops can therefore be produced only by the physician himself or by his staff. However, it remains the physician's responsibility that manufacturing and application are performed correctly. Since even stricter regulations may especially exist for blood products in individual states, every physician producing autologous serum eyedrops needs to inform himself about specific national regulations.

In the United States, producers of drugs and medical devices have to be registered with the Food and Drug Administration (FDA). Similar to EU regulations, registration is not necessary "for practitioners licensed by law to prescribe or administer drugs or devices and who manufacture, prepare, propagate, compound, or process drugs or devices solely for use in the course of their professional practice". Again, special regulations on testing and approval of drugs by the FDA or for using blood products need to be evaluated by the practitioner.

Summary for the Clinician

- **Serum eyedrops are a blood product that can be produced on a named patient basis according to the doctor's specifications**
- **It remains the physician's responsibility that manufacturing and application are performed correctly**
- **Every patient should give their informed consent before the production of autologous serum eyedrops is initiated**
- **Quality control measures must be implemented for the production as well as the application**
- **Serum should only be applied under the supervision of the prescribing doctor**

1.2 Production and Application

1.2.1 Important Parameters of the Production Process

Although the complex composition of full blood will certainly vary between individuals, it is also known that a number of production parameters can significantly influence the biochemical composition of blood derived products. These critical steps in the production of serum eyedrops should therefore be standardised.

These include:

- Clotting phase: duration and temperature
- Centrifugation: centrifugal force and duration
- Dilution: dilution factor and diluent
- Storage: container, temperature, duration

In the absence of any controlled clinical trial evaluating the impact of such differences in the production, in vitro models have been helpful in assessing a large number of protocol variations. The published clinical studies often fail to mention important parameters such as for how long the blood was allowed to clot before centrifugation. If the full blood sample is centrifuged before the clotting process is completed, the release of platelet derived factors is reduced. We have established that the concentration of EGF, HGF, and TGF-β are higher after a 2-h clotting time when compared with paired full blood samples that were allowed to clot only for 15 min and this was associated with a trend towards better corneal epithelial cell proliferation [24]. Thus we allow the full blood donation to clot for at least 2 h at room temperature. However, a

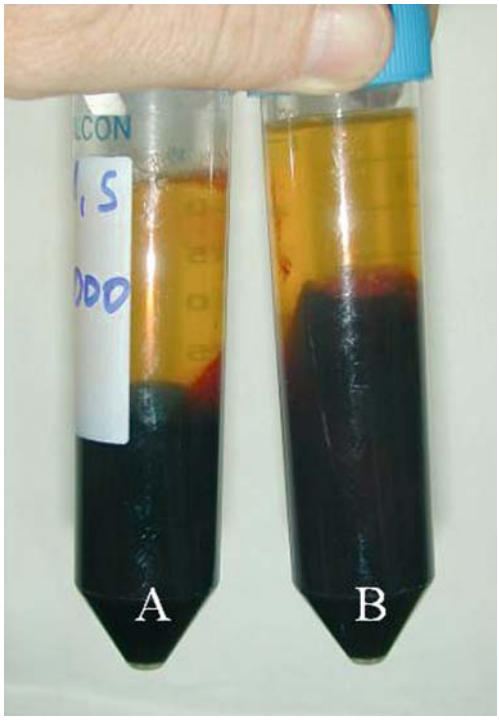

Fig. 1.1. Demonstration of the influence of the *g*-force on the volume of serum obtained from 50 ml of full blood centrifuged 2 h after donation: **A** at 3,000 *g* for 15 min; **B** at 500 *g* for 5 min

48-h period of storage at 4 °C, to allow transportation of full blood donations from peripheral ophthalmic departments to a centralised production unit, seems an equally acceptable procedure [53].

The volume retrieved from a given blood donation as well as its biochemical composition are also influenced by the centrifugation, which is determined by the centrifugal *g*-force and the time used to spin a sample. The *g*-force itself depends upon the revolutions of the rotor per minute (rpm) as well as on the diameter of the rotor. Thus *g*-force and not rpm is the parameter that should be stated in a protocol. The *g*-force in the studies published so far – if mentioned at all – probably varies by at least 1 log. A higher *g*-force not only helps to yield a larger volume of serum from a full blood sample (Fig. 1.1), but also reduces membranous platelet remnants in the supernatant, which in high concentrations have been shown to induce apoptosis [5]. Also a higher *g*-force can result in a lower concentration of TGF-β_1. As TGF-β is able to slow down epithelial wound healing, Tsubota suggested diluting the serum 1:5 with saline, which, however, reduces the concentration of other growth factors, such as EGF, that are proven to support proliferation of corneal epithelial cells. Combinations of 1,500 rpm – in an average size centrifuge equal to about 300 *g* – to 4,000 *g* (ca. 5,000 rpm) for 5–20 min have been used. A 15-min centrifugation at 3,000 *g* results in good separation of serum and blood clot, without inducing haemolysis [41].

It is obvious that the dilution of the obtained serum sample to the final concentration in the eyedrops determines the concentration of epitheliotrophic factors. In clinical studies, 20 %, 33 %, 50 % or 100 % have been used. Since the protocols for the production of serum eyedrops also varied in other parameters, there is no clear clinical evidence to favour any specific concentration. However, in vitro experiments show that cell proliferation is best supported at a 20 % concentration of serum. It was also shown that the type of diluent has an impact and that BSS rather than saline should be used [24].

In some indications, e. g. dry eye, autologous serum eyedrops are applied for many months. They are also usually produced without preservatives or stabilising additives to minimise the risk of drug induced toxicity. Since the production is labour intensive, a large number of aliquot samples is prepared from a single blood donation to keep the number of donation and processing efforts limited. To preserve the activity of the biological substances thought to be beneficial for the ocular surface, the drops can be refrigerated or stored frozen. The concentration of growth factors, vitamin A and fibronectin in pure and diluted serum was found to remain stable for at least 3 months if stored at –20 °C and for 1 month if stored at 4 °C. However, it is known that many protein concentrations in tears are reduced if stored for several weeks at 4 °C. While in developed countries access to freezers is rarely a problem, it seems preferable to store unused daily dosage vials of serum frozen [45, 38]. From this stock one container is then removed every morning and kept

refrigerated at 4 °C until it is discarded at the end of the day. Alternatively dilution of the serum with chloramphenicol 0.5 %, which has few toxic side effects, has also been advocated to allow the use of dropper bottles for up to 1 week.

Summary for the Clinician

- **The list of protocol variations for the production of autologous serum eyedrops is long**
- **The biochemical composition of serum depends on the time and conditions of clotting, the centrifugation, dilution and storage**
- **No controlled clinical trial has determined so far which of the protocols published offers the best epitheliotrophic support**
- **In the absence of such trials, the production protocol has been optimised in vitro**
- **The stock of serum eyedrops can be stored frozen for several months to preserve the biologically active components of the product**

1.2.2 Current Standard Operating Procedures Used at the University of Lübeck

Following the principles of Good Manufacturing Practice and based on extensive evaluation in vitro, the following standard operating procedures are currently used at the University of Lübeck (Fig. 1.2) [24, 19].

Patients are assessed for their suitability to donate according to the guidelines of the Bundesärztekammer and Paul-Ehrlich Institute for blood donation and use of blood products. This requires them to be in reasonably good health, with no significant cardiovascular or cerebrovascular disease, and free of bacterial infection. Anaemia (Hb<11 g/dl) is a relative contraindication. If only a small amount of blood (50–100 ml) is taken, mild anaemia or circulatory disorders need not be considered contraindications. To minimise the danger of bacterial contamination, no blood should be taken from patients suffering from suspected septicaemia. To exclude transmission of infection, patients must be tested for hepatitis-B/-C, syphilis and HIV serology (HbsAg; antibodies to HCV, HIV-I/-II, HIV-NAT, syphilis; HCV-NAT) before blood is donated for the production of eyedrops. A positive serology excludes the patient from the donation of autologous blood for serum eyedrop production. Prior to venisection, the patient must be informed in writing about the planned therapy, its experimental nature, the risks involved (e.g. bacterial contamination) and alternative methods of treatment. The patient's consent should be obtained and kept with the notes.

Venipuncture is performed at the antecubital fossa under aseptic conditions. Depending on the expected duration of treatment, 100–200 ml of whole blood is collected into sterile containers. For larger volumes a sterile blood pack without anticoagulant can be used to collect up to 470 ml. A 100-ml donation of whole blood will yield 30–35 ml of serum, which diluted to 20 % is sufficient for at least 3 months of serum eyedrops 8 times daily. Larger volumes are recommended in patients who require long-term treatment, in order to minimise labour intensive production. The containers are left standing upright for 2 h at room temperature to ensure complete clotting before they are centrifuged at 3,000 *g* for 15 min. The supernatant serum is removed under sterile conditions in a laminar air flow hood with sterile 50-ml disposable syringes. The volume retrieved is determined and diluted 1:5 with sterile BSS. Gentle shaking ensures homogenisation before portions of 2 ml are aliquoted through a 0.2-µm filter into sterile dropper bottles. The effect of filtration has not been evaluated, but Fox recommends filtration to remove fibrin strands, suspected to reduce the effect of serum eyedrops. The bottles are sealed and labelled with the name, date of birth of the patient, the date of production and the instruction "Autologous blood serum for topical use in the eye. To be stored frozen and used within 3 months after date of production. To be discarded 24 hours after opening." Two millilitres of the solution is – as required by the European Pharmacopoeia Addendum 2000 – sent for microbiological evaluation.

The product is available approximately 6 h after venesection, but is only dispatched once negative serology and microbiology of donor

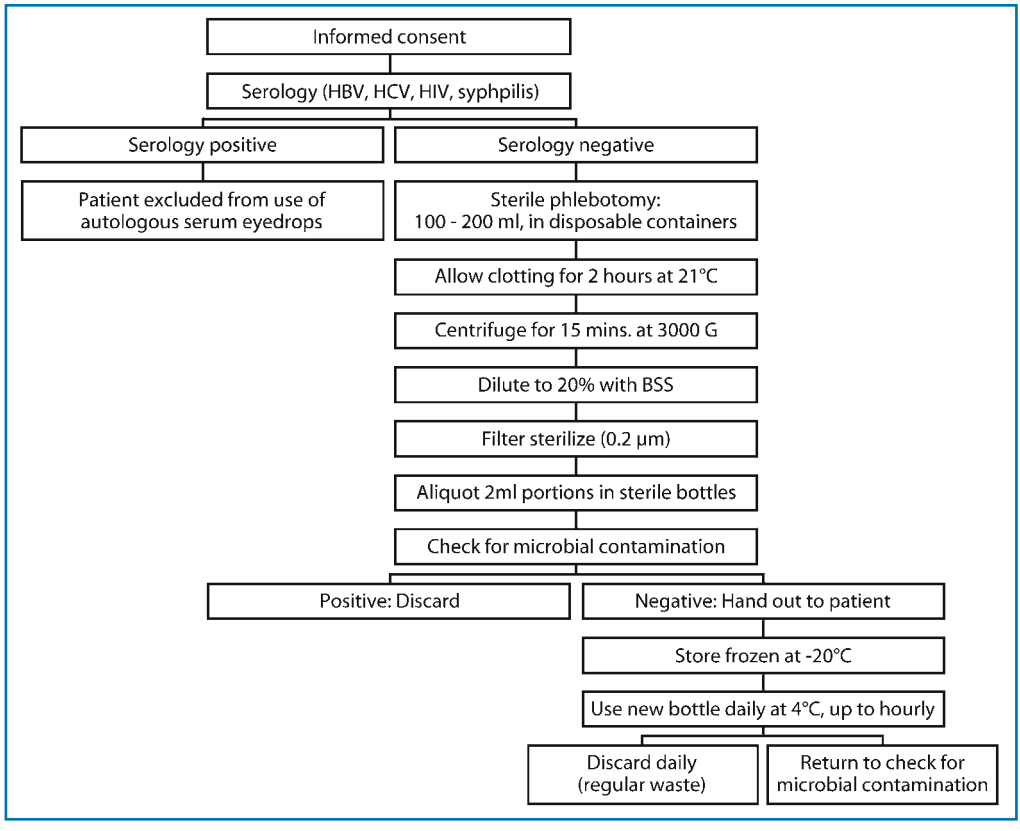

Fig. 1.2. Standard manufacturing protocol for the preparation, storage and use of serum eyedrops. Parameters which influence the biochemical character of the product are also shown

and product are confirmed. Usually the drops are applied 8 times daily. A new bottle is opened everyday. It is recommended to be stored at +4 °C and to be discarded after 16 h of use with regular household waste. The remaining bottles are stored frozen (ideally at −20 °C) for up to 3 months. If the domestic freezer has no thermometer, it is recommended to place one inside and control the temperature when taking a new vial out every day. If the temperature cannot be adjusted to about −20 °C, the dispensing doctor may consider recommendation of shorter storage episodes.

The costs of production – i.e. labour and consumables alone – for a day's dosage of serum eyedrops following this protocol are well below 5 €. This is the approximate equivalent of one bottle of preserved pharmaceutical lubricant and thus should be acceptable for the rare occasion where the ocular surface disease is so severe that the use of topical autologous serum is justified [14].

Summary for the Clinician

- **Every physician producing or prescribing autologous serum eyedrops should inform himself about specific national regulations**
- **Any production protocol should follow the principles of Good Manufacturing Practice**
- **The dropper bottles must be carefully labelled with the patient's details and instructions for storage and use**
- **If produced without preservatives the drops should be kept frozen at −20 °C until the day of use**

1.2.3 Quality Control

In order to guarantee quality, control measures should be initiated and if strictly interpreted serum eyedrops should be produced only by personnel supervised by the doctor directly in charge of the patient treated. Bacterial contamination of the product during the production as well as from the application of serum eyedrops is a potential risk. Sterile manufacturing conditions, beginning with thorough skin disinfection, are of the utmost importance. It is preferable that further processing is performed in a closed system. To minimise the risk of infection to third parties (e.g. production or nursing staff), it is strongly recommended that the donor is tested for HIV, HBV, HCV and syphilis before donation of larger amounts of blood are collected for the production process itself. For quality control purposes bacterial contamination resulting from the production process needs to be ruled out by a microbiological examination of the product prior to initiation of the clinical application and a control system must be implemented to ensure that the product is only used when the microbiological and serological tests are clear.

Prior to venipuncture and application, the identity of the patient must be confirmed. The packaging and dropper bottles must be clearly labelled with:

- The patient's name and date of birth
- The name and address of the manufacturer
- The date of manufacture and the date of expiry
- The instructions on how to store and use the drops
- A comment that the material is an autologous blood product, which is solely for application with the named patient

To minimise the variability of the product and to maximise the safety of its use, a written version of the standard operating procedures (SOP) should be established. Conscientious documentation is indispensable for good medical and manufacturing practice. Each step relevant to manufacture as well as application (including the dates of application and any unwanted effect) should be recorded. From this it becomes obvious that strict guidelines for good manufacturing, quality control and documentation must be established and maintained prior and throughout the therapeutic use of autologous serum eyedrops. All steps of the production should be documented on a form which – together with the patient's consent – is kept with the patient's notes.

- **A written protocol of the standard operating procedures should be established**
- **All production steps must be documented on a SOP form**
- **Serum eyedrops should only be produced and released once the negative serology of the donor for hepatitis, syphilis and HIV and the negative microbiology of the product have been confirmed**

1.3 Clinical Results

Serum eyedrops have predominantly been used for persistent epithelial defects and severe dry eye, but also as a supportive measure in ocular surface reconstruction and for a number of other indications.

1.3.1 Persistent Epithelial Defects

A persistent epithelial defect (PED) is defined as a defect of the corneal epithelium that – in the absence of microbial keratitis – fails to heal within the expected time course (e.g. 2 weeks) despite topical lubricants [26, 50]. A PED can occur as a result of many different pathologies, including rheumatoid arthritis, neurotrophic keratopathy or dry eye [8]. "Success" of treatment is best defined as percentage of defects healed in a given time or as total time to complete epithelial wound closure.

Table 1.2. Clinical studies using serum eyedrops to treat persistent epithelial defects. The production parameters and results are given. Success is defined as percentage of eyes/patients with complete epithelialisation. Note that the scale used to measure these changes as well as the baseline level varied between the studies (NA, not applicable; NR, not reported; rpm, revelations per minute)

Author	Concentration	Diluent	Centrifugation (g force)	Duration	Clotting time	Frequency of application	Eyes (patients)	Success objective
Alvarado	20%	0.9% NaCl	5000 RPM	10 min.	NR	NR	17 (14)	83%
De Souza	100%	NA	NR	NR	NR	Hourly	70 (63)	81%
Garcia	20%	0.9% NaCl	5000 RPM	10 min.	NR	10×	11 (11)	55%
Matsumoto	20%	0.9% NaCl	3000 RPM	10 min.	NR	5–10×	14 (11)	100%
Poon	50–100%	0.5% chloramphenicol	4000 RPM (2200 G)	10 min.	2 h	8×	15 (13)	60%
Tsubota	20%	0.9% NaCl	1500 RPM	5 min.	NR	6–10×	16 (15)	63%
Young	20%	0.9% NaCl	1500 RPM	5 min.	NR	6–14×	10 (10)	75%

1.3.1.1 Currently Available Published Data

In five prospective case series, 20% serum diluted in 0.9% saline has been used 5–14 times daily for this indication (Table 1.2). In 1999 Tsubota was the first to report a series of 16 eyes (15 patients) in whom the PEDs had persisted despite medical treatment with lubricants or bandage contact lenses for a mean of 7.2±9.4 months. Ten out of these 16 defects healed completely within 4 weeks after initiation of therapy [11, 46]. Garcia-Jimenez reported complete epithelial wound healing in 6 of 11 eyes with persistent epithelial defects with healing beginning within 3–4 weeks of treatment with serum eyedrops [1, 52]. In a group of 9 patients with predominantly diabetic or postherpetic neurotrophic keratitis, all 12 epithelial defects healed within 15.8±7.9 days and this was associated in 9 eyes with an improvement of corneal sensitivity [27] (Fig. 1.3).

A different concentration of serum was used in two other studies. Poon et al. substituted unpreserved pharmaceutical lubricants with 50–100% serum eyedrops and observed closure of a PED with a mean duration of 7.5±5.8 (1–24) weeks in 9 of 15 eyes after 3.6±2.5 weeks (3 days–8 weeks) [33]. De Souza et al. treated 70 epithelial defects with undiluted serum hourly in addition to routine medication, 45 of which had occurred early after penetrating keratoplasty [8] and had persisted for a mean of 15±17 days. Eighty-one percent of these defects with a relative short history healed within 14±12 days.

Healing generally starts within 2 weeks after initiation of the serum therapy [33]. So far no study has been able to show a correlation between size or localisation of the defect with success or failure, but the older and deeper stromal defects tended to heal less successfully. Also, when serum eyedrops are changed back to pharmaceutical lubricants the epithelial defects may recur, as happened in 6 out of the 9 eyes in Poon's and 9 out of 70 eyes in De Souza's group. These figures are difficult to compare since none of the studies was placebo controlled and the study population seems to differ significantly in terms of underlying pathogenesis and duration of the PED.

Summary for the Clinician

- **Pathogenic factors that can be avoided or treated, such as toxicity due to preserved eyedrops, steroids or active herpetic keratitis, should be ruled out before a PED is treated with serum eyedrops**

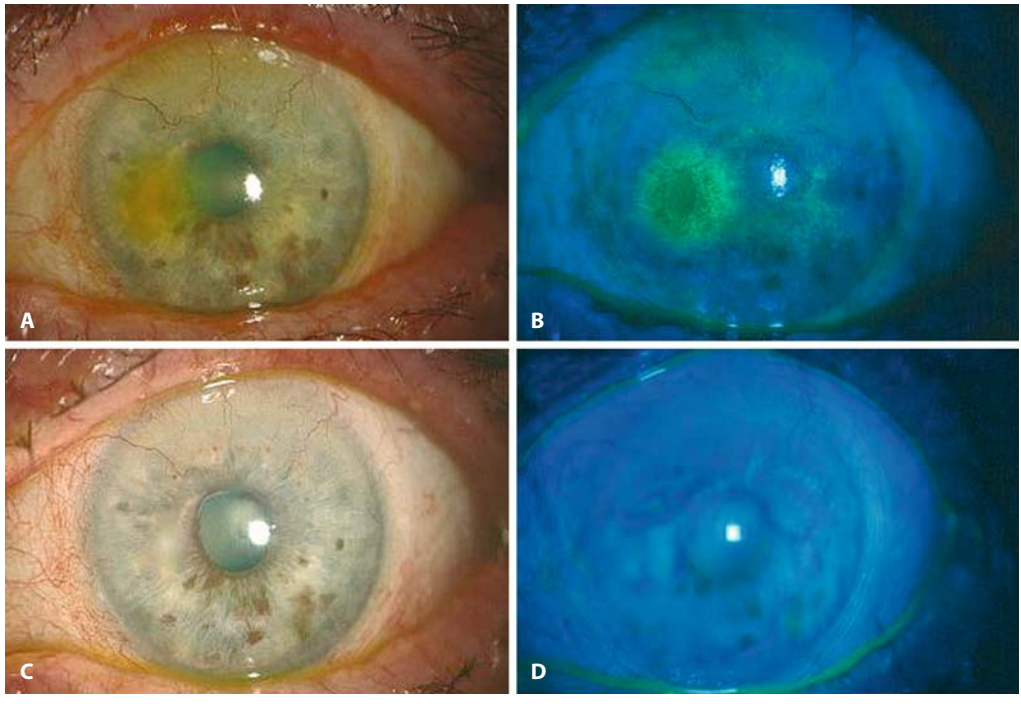

Fig. 1.3. Epithelial defect persisting unaltered for 2 weeks in the left eye of a female patient with severe aqueous tear deficiency due to secondary Sjögren's syndrome before (**A, B**) and 1 week after (**C, D**) treatment with 20 % autologous serum eyedrops 8 times daily. The defect started to heal within 2 days

- **No signs of microbial keratitis should be present if serum application is considered for an epithelial defect, since the effect of serum in this situation is unknown and may support bacterial growth**
- **Twenty percent serum eyedrops are applied approximately every 2 h until the defect is healed**
- **PEDs begin to heal generally within 2 weeks after initiation of serum eyedrops**
- **Older and deeper stromal defects tend to heal less successfully**
- **If 20 % serum fails, a higher concentration of serum may help to achieve epithelialisation**
- **When serum eyedrops are changed back to pharmaceutical lubricants the epithelial defect may recur**

1.3.2 Dry Eye

Dry eye is a group of disorders, of diverse pathogenesis, that share as common manifestations signs and symptoms due to the interaction of both an abnormal tear film and an abnormal ocular surface. It is subdivided into aqueous deficient and evaporative, i. e. lipid or mucin deficient dry eyes. Although it is believed to be one of the most common ocular problems in the Western world with an incidence of symptoms of dry eye in up to 14.6 %, ocular surface changes are observed clinically in only 0.5 %. Severe aqueous tear deficiency, however, can lead to blindness and serum eyedrops have been used in this situation.

In the dry eye "success of treatment" is more difficult to define than in PEDs, and this can be done either as subjective or objective improvement compared to baseline. "Subjective" success is determined as reduction of a score of symptoms in a questionnaire of variable length. "Objective" success is either determined by reduction of fluorescein or rose bengal positive staining of the ocular surface or improvement of histologic parameters in impression cytology, although this is usually scored more or less according to the *subjective* impression of an examiner.

1.3.2.1 Currently Available Published Data

Following the initial report of Fox in 1984, it took 15 years until Tsubota in 1999 and subsequently a number of studies reported on the use of serum eyedrops for dry eyes (Table 1.3). Fox treated 30 eyes of 15 patients with 50 % serum in 0.9 % NaCl and found that signs and symptoms improved in all of the patients, until this medication was replaced by 0.5 % serum or pure diluent. Tsubota focussed on dry eyes due to Sjögren's syndrome. When treated with 20 % serum 6–10 times daily for 4 weeks symptoms improved by only 34 %; however, fluorescein and rose bengal staining decreased by 55 % and 68 % of baseline, while tear break-up time remained unchanged [45]. Two groups of authors described similar findings in patients with dry eye due to graft-versus-host disease [32, 34] with symptoms improving within days, but punctate epithelial staining improving only after months. Ogawa also reported that – although the aqueous deficiency in his patients was less severe (≤10 mm, Schirmer test) – in 50 % symptoms recurred while the patient continued to apply serum eyedrops and 43 % required additional punctal occlusion. In a placebo-controlled prospective study of severe dry eyes with a mean Schirmer test score of less than 1 mm, 20 % serum 6 times daily was not found to be significantly more effective in improving symptoms and signs than 0.9 % saline, which had been used as diluent [40], although a trend towards reduced fluorescein and rose bengal staining was observed after 2 months of treatment.

Two studies have reported the use of higher concentrations of serum. Poon et al. found an improvement of subjective and objective criteria of severe dry eyes (Schirmer test <5 mm) in only three out of eight eyes receiving 50 % serum but all of three eyes receiving 100 % serum [33]. Noble et al. compared the efficacy of 3 months of autologous serum 50 % diluted in 0.9 % saline in a prospective clinical crossover trial against the previously used commercial lubricant and reported that 10 out 16 patients had improved symptoms [31], and that there were impression cytological findings in 6, no change in 10 and improvement in 9 of 25 treated eyes.

The efficacy seems to be dose dependent since 94 % of patients receiving eight applications daily reported reduced symptoms compared to only 58 % of those receiving four drops [39]. Overall the efficacy of serum eyedrops in dry eyes varied between 30 % and 100 % for symptomatic relief, between 39 % and 61 % for reduction of fluorescein and between 33 % and 68 % for rose bengal positive staining. However, the variation in study population, production and treatment protocol are again significant. In some studies, serum was used as an additive rather than a substitute for lubricants and in others therapeutic contact lenses or punctal occlusion were applied in addition to the serum eyedrop therapy. Comparison of the published data is therefore difficult and it has to be concluded that no definite evidence supporting the use of serum eyedrops in dry eyes is available so far.

Summary for the Clinician

- **Serum eyedrops should be reserved for the most severe cases of dry eye**
- **Punctal occlusion should be performed first**
- **Symptoms usually improve within days, but punctate epithelial staining may decrease only after months of treatment**
- **The use of serum in dry eyes is not evidence based. Using an optimised protocol for the production of serum eyedrops, a randomised controlled trial should be performed**

Table 1.3. Clinical studies using serum eyedrops to treat severe dry eyes. Success was defined either as number/percentage of all eyes/patients with improved/reduced mean baseline of objective [fluorescein (Fl) or rose bengal (RB) positive epitheliopathy or impression cytology (IPC)] or subjective (symptoms) scores. Note that the scale used to measure these changes as well as the baseline level varied between the studies (NR, not reported; NS, not significant; rpm, revelations per minute; replacement, frequency equivalent to previously applied pharmaceutical tear substitute)

Author	Concentration	Diluent	Centrifugation	Duration	Clotting time	Frequency of application	Eyes (patients)	Success objective	Success subjective
Fox	33%	0.9% NaCl	500 *g*	10 min	NR	2-hourly	30 (15)	RB 41%	51%; 100%
Noble	50%	0.9% NaCl	NR	NR	48–72 h	Replacement	32 (16)	IPC 36%	63%
Ogawa	20%	0.9% NaCl	1,500 rpm	5 min	NR	×10	28 (14)	Fl: 61%; RB: 40%	30%
Poon	50–100%	0.5% chloramphenicol	4,000 rpm (2,200 *g*)	10 min	2 h	×8	11 (9)	Fl: 55%; RB: 45%	55%
Rocha	33%	0.9% NaCl	500 *g*	10 min	NR	Hourly	4 (2)	100%	100%
Takamura	20%	0.9% NaCl	3,000 rpm	10 min	NR	×4–8	NR (26)	"Improved"	77%
Tananuvat	20%	0.9% NaCl	4,200 rpm	15 min	NR	×6	12 (12)	Fl: 39%; RB: 33%; IPC 44%	36% (NS)
Tsubota	20%	NaCl	1,500 rpm	5 min	NR	×6–10	24 (12)	Fl: 55%; RB: 68%	34%

1.3.3 Other Indications

Other indications which reportedly have been treated with autologous serum include recurrent erosion syndrome, superior limbic keratoconjunctivitis and as adjunctive therapy in surgical ocular surface reconstruction (Tables 1.4, 1.5).

1.3.3.1 Adjunctive Use in Ocular Surface Reconstruction

Absolute aqueous deficiency prevents a successful surgical ocular surface reconstruction. Tsubota used 20 % autologous serum as adjunctive treatment in a prospective cohort study on 14 eyes of 11 patients, in which due to Stevens-Johnson syndrome or ocular cicatricial pemphigoid, the Schirmer test result with nasal stimulation was 0 mm. Surface reconstruction included a limbal stem cell graft, amniotic membrane and/or penetrating keratoplasty. Within the short follow-up of 20 weeks, a stable corneal epithelium was observed in 12 of the 14 eyes [44] and these findings were confirmed by Lagnado et al. [23]. Poon used 50 % of serum in two eyes undergoing keratoplasty for PEDs. Again a stable epithelium resulted. However, epitheliopathy recurred when the serum treatment was discontinued in these patients. In another study, Tsubota also observed that in four children (mean age 9 years) with severe OSD and absolute dry eye due to Stevens-Johnson syndrome surface reconstruction failed despite the use of autologous serum eyedrops [47].

1.3.3.2 Recurrent Erosion Syndrome

Insufficient adhesion of the basal epithelial layers to the underlying basement membrane is observed following trauma or in corneal basement membrane dystrophy. This can lead to recurrent erosion syndrome (RES), which includes repeated episodes of irritation, pain, epiphora and conjunctival hyperaemia. Del Castillo treated 11 patients with unilateral post-traumatic RES with a mean of 2.2 recurrences/month in a prospective cohort study with unspecified, unpreserved lubricants and 20 % serum eyedrops TDS for 3 months in a tapered fashion. During a mean follow-up of 9.4±3.7 months the recurrence rate decreased to 0.028/month. No information is given whether previous treatment modalities were suspended for the time of the serum application. Unfortunately the authors also do not state the duration of the history of RES, which may have been rather short. Given the self-healing nature of the post-traumatic variant of the condition, these data have to be taken with caution [4].

1.3.3.3 Superior Limbic Keratoconjunctivitis

This is a rare, chronic, inflammatory disease thought to result from a localised reduction of goblet cells and tear film deficiency at the 12:00 limbus with subsequently reduced wettability and positive rose bengal staining of the corneal and conjunctival epithelium. Goto used 20 % serum eyedrops as additional therapy 10 times daily for bilateral superior limbic keratoconjunctivitis (SLK) in a prospective cohort study on 22 eyes. Within 4 weeks symptoms were improved in 9 of 11 and epitheliopathy in all patients. Also, tear break-up time increased significantly and conjunctival squamous metaplasia was reduced. When the serum application was discontinued, discomfort recurred [16].

- **Autologous serum can be used in other ocular surface disorders such as superior limbic keratoconjunctivitis or if ocular surface disease – e.g. due to dry eye or PED – becomes so severe that a surgical intervention is required**
- **However, the surface disease is likely to recur if the serum treatment is stopped**

Table 1.4. Clinical studies using serum eyedrops for ocular surface reconstruction. Success was defined as number of all eyes with a stable postoperative ocular surface score (h, hours; NR, not reported; rpm, revelations per minute)

Author	Concentration	Diluent	Centrifugation	Duration	Clotting time	Frequency of application	Eyes (patients)	Success objective
Lagnado	20%	0.9%	4,500 rpm	15 min	10–12 h	1–2 hourly	14 (14)	100%
Poon	50–100%	0.5% chloramphenicol	4,000 rpm (2,200 *g*)	10 min	2 h	×8	2 (2)	100%
Tsubota	20%	0.9% NaCl	1,500 rpm	5 min	NR	1/4 hourly	14 (11)	86%

Table 1.5. Clinical studies using serum eyedrops to treat other indications. Success is defined either as number/percentage of eyes/patients with improved/reduced mean baseline of objective [fluorescein (Fl) or rose bengal (RB) positive epitheliopathy or impression cytology (IPC)] or subjective (symptoms) score (NR, not reported; RoR, rate of recurrence)

Author	Indication	Concentration	Diluent	Centrifugation	Duration	Clotting time	Frequency of application	Eyes (patients)	Success objective	Success subjective
Del Castillo	Superior limbic keratoconjunctivitis	20%	0.9% NaCl	1,500 rpm	5 min	NR	×3	11 (11)	RoR: 99%	NR
Goto	Recurrent corneal erosion syndrome	20%	0.9% NaCl	1,500 rpm	5 min	NR	×10	22 (11)	Fl: 88%; BR: 91%; IPC 100%	21%; 82%

1.4 Complications

Potential unwanted effects of the use of autologous serum as eyedrops include worsening of the initial problem, infection, immunological reactions and contact lens deposits. Most authors mention no complications at all, but in five patients with dry eyes, discomfort or epitheliopathy increased and eyelid eczema was reported in two cases [34, 40].

1.4.1.1 Risk of Infection

An infection in the context of topical serum application can occur either locally or systemically. The risk for systemic transmission of an infectious disease arises only if serum of a donor affected by a systemic infection, such as HIV, hepatitis or syphilis, is applied to a person other than the donor. This can occur during the production or application of serum eyedrops, and a systemic as well as a topical route of entry of the infectious agent is possible, since transmission of HIV by a single serum droplet into one eye has been reported at least in one case [6]. Although the risk is small, the potentially fatal consequences make the tight quality control as described under Sect. 1.2.3 mandatory.

Although it is known that serum has antimicrobial properties, this has not been quantified for diluted, cryopreserved serum so far. Sauer et al. observed that contamination of dropper bottles with *Staphylococcus epidermidis* occurred in 3 out of 40 bottles on the 7th day of use of undiluted serum stored refrigerated and applied from week dosage containers by trained personnel in a hospital setting [35]. Lagnado et al., however, observed contamination of 18% of containers at the end of the first day of application, and 43% of inpatients treated with 20% serum diluted with sterile saline (0.9%) had at one stage received serum from a day usage dropper bottle that at the end of the day was found to be contaminated. Again most of these contaminations were *Staphylococcus epidermidis,* but one case of *S. aureus* was also reported [23]. Since the indication for the use of serum was either an epithelial defect or ocular surface reconstruction surgery, all patients had received topical antibiotics at the same time. This is likely to have reduced the rate of contamination of the serum containers and prevent cases of microbial keratitis.

The incidence of dropper bottle contamination and the risk of microbial keratitis is likely to increase when the drops are used in a domestic setting by the patients themselves and if no topical antibiotic is applied, e.g. in patients with other indications than epithelial defects. Even if 0.5% chloramphenicol was added as a preservative to the dropper bottles, microbial keratitis evolved in 3 out of 13 eyes with PEDs treated with 50% or 100% serum [33]. Since laboratory evidence suggests that dilution with an antibiotic may reduce the epitheliotrophic capacity of serum eyedrops and since ocular surface disease often requires long-term treatment, hospitalisation of patients for this purpose is not a suitable option and storage of the serum product in day dosage vials seems preferable.

1.4.1.2 Immunological Complications

In the first report by Fox in 1984 the authors mentioned [9] that some users of serum – not Fox himself – had encountered scleral vasculitis and melting in patients with rheumatoid arthritis, although the indication for the use of serum in these patients remains unknown. McDonnell reported one case of an immune complex deposition after hourly application of 100% serum. Poon observed the onset of one peripheral corneal infiltrate and ulceration within 24 h after initiation of serum drops [28, 33] and hypothesised that this could have been a result of circulating antibodies which must also be present in serum eyedrops and could have reacted with corneal antigens with a subsequent inflammatory response.

1.4.1.3 Contact Lens Contamination

We have used serum eyedrops in combination with a soft, class IV hydrogel contact lens containing 45% ocufilcon D and 55% water (Bio-

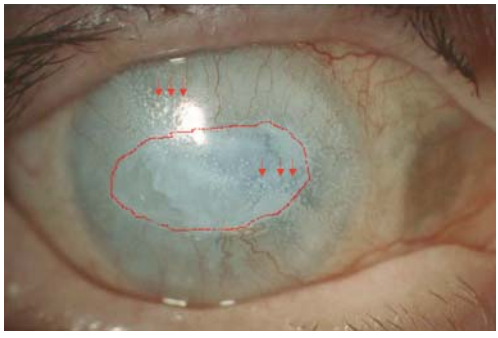

Fig. 1.4. Hydrogel bandage contact lens (45% ocufilcon D, 55% water) with contaminations in the right eye of an 88-year-old female patient after 78 days of treatment with 20% serum 8 times daily. The patient previously had developed a recurrent corneal epithelial defect due to neurotrophic keratopathy secondary to herpes zoster keratitis and severe aqueous tear deficiency due to secondary Sjögren's syndrome which persisted, perforated and recurred despite repeated multilayer amniotic membrane grafts

medics 55) in six eyes (six patients) with persistent or recurrent epithelial defects. A contact lens was applied if an epithelial defect recurred or progressed while the eye was being treated with autologous serum. In five eyes the contact lens was applied immediately after transplantation of an amniotic membrane. Three lenses in two eyes showed substantial numbers of large deposits on the anterior surface of the lens after application of serum drops 8 times a day for 18–78 days (Fig. 1.4). In addition all eyes received unpreserved ofloxacin (3 mg/ml) 4 times daily topically. All defects healed without signs of microbial keratitis or conjunctival hyperaemia. However, since protein deposition may induce ocular surface inflammation, it is recommended to use silicone hydrogel lenses, if a combination with serum drops is necessary, because this type of lens is less prone to accumulate protein on the surface [21, 42, 54].

Summary for the Clinician

- **Serum eyedrops are generally well tolerated with little or no side effects**
- **However, all possible measures should be taken to exclude transmission of systemic disease during the production or application of serum eyedrops**
- **Frequent monitoring of patients is recommended since in rare cases serious inflammatory complications can result from the use of autologous serum drops**
- **Serum eyedrops can be combined with other forms of wound healing supporting therapy, without any serious adverse effect being reported so far**
- **If a contact lens is applied, a material with little tendency to accumulate surface deposits is recommended**

1.5 Alternative Blood Products for the Treatment of Ocular Surface Disease

Serum has to be prepared from an autologous blood donation for each patient individually. This is time and labour intensive and in many countries no standard operating protocol has yet been evaluated or approved by the licensing authority. Other blood derived extracts, such as albumin, plasma or platelet concentrates, are readily available from pharmaceutical companies or blood banks. They are quality controlled and might therefore be considered for treatment of ocular surface disorders.

1.5.1 Umbilical Chord Serum

Recently umbilical chord serum has been prepared like autologous serum (5 min centrifugation at 1,500 rpm), diluted to a 20% concentration in 0.9% saline and used as an alternative treatment for promoting corneal epithelial wound healing. In a prospective randomised controlled clinical trial on 60 eyes, this led to a higher rate of healing of persistent epithelial defects than autologous serum. However, the defects treated with autologous serum healed faster than those treated with umbilical cord serum. This product is not autologous but allogeneic and hence not only immunological problems but also a higher risk of infection for the recipient may be expected. In many countries it is not supplied by a centralised blood service and thus it is more difficult to obtain. All con-

cerns regarding quality control and microbiological testing are applicable [50].

1.5.2 Albumin

One of the most abundant proteins in tears is albumin is one of the most abundant (Table 1.1). It often acts as a carrier for other factors, such as hormones, including steroids, although no physiological role for its presence in tears has been described to date. Albumin is also used in medicine for treating severe albumin deficiency, e.g. due to protein loss in extensive skin burns or liver dysfunction. Tsubota found in an animal experiment that the topical application of albumin reduces enzymes involved in apoptosis and improves epithelial cell viability and re-epithelialisation. When a 5% solution of recombinant albumin was applied 6 times daily, mean fluorescein and rose bengal, as well as break-up time and symptom scores, improved significantly from baseline, but no comments are made as to which solvent was used and whether any non-responders were found with this treatment. Although no complications were observed – as with all blood derived products – a minute risk of transmission of viral or prion mediated disease cannot be ruled out if the albumin preparation was derived from blood [37].

1.5.3 Plasma and Platelets

Plasma is the cell free supernatant after centrifugation of full blood mixed with an anticoagulant. Plasma therefore contains only small amounts of the growth factors present in platelets since these are – due to the anticoagulant – not activated by blood clotting.

Platelets are a major source of growth factors in serum. They can be obtained by means of apheresis and stimulated with thrombin to release their content. Following centrifugation the clear supernatant, which is free of any cellular remnants, can be resuspended with a buffer to a concentration of choice and stored frozen for further use. We have termed this product "platelet releasate" (PR). This blood product contains large amounts of EGF and other growth supporting mediators, but little of the extracorpuscular factors such as fibronectin or vitamins.

Both products have so far only been tested in vitro, but from these experiments it is clear that plasma is not suitable to support proliferation and migration of epithelial cells. Although platelet releasate also does not support migration, it has a substantially stronger proliferative effect than serum and thus may be promising for the treatment of ocular surface disorders. However, this still has to be evaluated in a clinical trial [18, 19].

- **Alternative blood products are routinely produced and quality controlled by blood banks. They are currently under investigation**
- **Umbilical chord serum was found to be more effective than autologous serum to heal epithelial defects, but it is more difficult to obtain and supply is limited. Since evidence about this allogeneic treatment modality is even more scarce, it should currently not be used to substitute autologous serum, unless the latter has failed to establish a stable ocular surface**
- **Albumin may be one of the components of serum that support epithelial wound healing and can be used as a single compound product without the need for an autologous blood donation. It has been reported to improve findings but not symptoms of ocular surface disease in severe dry eyes**
- **Platelet releasate, but not plasma, may be suitable as an additional treatment modality for ocular surface disease, but no clinical data are available yet**
- **Similar if not enforced legal guidelines and quality controls need to be applied to these alternative blood products, since the risk of transmission of infection is due to their allogeneic nature being theoretically increased**

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Controversies and Limitations of Amniotic Membrane in Ophthalmic Surgery

2

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Core Messages

- The amniotic membrane is a useful adjunct in the management of many ocular conditions
- Several mechanisms of action have been attributed to the membrane based on its structure and biochemical composition. Not all mechanisms are scientifically substantiated
- Many inter and intra donor variations in the structure and function of the membrane have been demonstrated. Location in relation to the placenta, duration of pregnancy, parity, gravidity, onset of labour and even age and race of the donor are all variables
- Processing and preservation of the membrane can be accomplished by different methods. Different methods affect the membrane differently and can substantially alter the membrane. Mandatory quarantine of the membrane to rule out HIV contamination does not allow for use of fresh membranes. The potential risk of transmission of serious infections from one donor to a number of recipients remains a concern
- Despite the vast literature on the use of the membrane, randomised controlled studies are virtually none
- Lack of defined criteria of success makes evaluation of outcomes difficult. This is rendered more difficult due to improper characterisation of disease severity making comparisons between studies next to impossible
- Although the beneficial effects of the membrane are emphasised in several consecutive case series, these studies often lack proper and adequate controls and it is important to bear in mind that existing, at times simpler options do exist with equivalent or better efficacy
- Standardisation of the membrane supplied for widespread clinical use is an important challenge that lies ahead. Perhaps the generation of a 'synthetic membrane' with known quantities of desired ingredients, tailored to the intended clinical use, will be possible in the future

2.1 Introduction

The first documented ophthalmologic application of the amniotic membrane was in the 1940's when it was used in the treatment of ocular burns [9, 54, 55]. Following initial reports, its use in ocular surgery, as indicated by reports in the scientific literature, abated until recently. It appears that during these 'silent years' it was being used extensively in the Soviet Union and latterly in South America [13]. Its introduction to North America in the early 1990s heralded a massive surge in the ophthalmic applications of this membrane. The amniotic membrane is now increasingly being used in ocular surface surgery for a wide range of indications. There are over 500 publications in the scientific literature with most of them reporting success. However,

the enthusiasm to extend its clinical applications and indications is not matched by the scientific rigor that should be applied to any new product or technique that is being used so extensively. There are thus several limitations of the amniotic membrane as applied to its ophthalmic usage, which are not widely known. It is therefore important that these limitations are carefully considered and all applications of the membrane be interpreted in the context of these limitations. These limitations apply to the following areas:

1. Proposed mechanisms of action of the membrane
2. Intra and inter donor variations of the membrane
3. Processing and preservation of the membrane
4. Clinical studies and outcomes [definitions of success and grading of disease severity]
5. Efficacy of membrane in relation to other established techniques and options

Summary for the Clinician

- **The amniotic membrane has been used in ophthalmic surgery since the mid 1940s**
- **Many ophthalmic applications are proposed but not are supported by scientific evidence**
- **The major controversies and limitations of the membrane are in relation to its proposed mechanisms of action, inter and intra donor variations, its methods of processing and preservations, the evaluation of its outcomes against defined criteria and in comparison to existing techniques**

2.2 Proposed Mechanisms of Action of the Amniotic Membrane

Several mechanisms of action are attributed to the membrane. These include: (a) promotes epithelialisation, (b) inhibits scarring, (c) inhibits vascularisation, (d) reduces inflammation, (e) provides a substrate for cell growth, (f) antimicrobial effects and (g) as a biological bandage [3, 13]. Most of these are inferred from the structural and biochemical composition of the membrane often without any direct evidence.

To understand the basis of some of the proposed mechanisms of action of the AM it is useful to understand the structure and composition of the membrane.

2.2.1 Amnion Structure

The AM consists of five layers from within outward: (a) a single layer of highly metabolically active, columnar to cuboidal epithelium; (b) a thin basement membrane; (c) a compact layer made of reticular fibres virtually devoid of cells; (d) a loose network of reticulum containing fibroblasts, called the fibroblast layer; and (e) a spongy layer of wavy bundles of reticulum bathed in mucin, which forms the interface with the chorion [4].

Matrix. Amniotic basal lamina contains large quantities of proteoglycans rich in heparin sulphate. Amnion contains a large amount of collagen, hyaluronan and predominantly smaller proteoglycans such as biglycan and decorin, with decorin being more prominent of the two, and is located in close connection with the collagen fibrils [36]. Collagen types I, III, IV, V and VII [2, 25, 39, 62], laminin [2] and fibronectin [33] have been identified in amniotic basement membrane and stromal amnion. Similarities between the lamini-1, laminin-5, fibronectin and type VII collagen components of the basement membranes of conjunctiva, cornea and amniotic membrane have been demonstrated [20]. The α-subchain components of collagen IV have been shown to be similar between amniotic membrane and conjunctiva but different between amniotic membrane and cornea [20].

2.2.2 Amnion Composition

Some components of the membrane that are relevant in the context of its mechanism of action, and that help understand its limitations, are mentioned below:

Enzymes. Important amongst these are enzymes involved in prostaglandin synthesis such as phospholipases, prostaglandin synthase and cyclo-oxygenase [53, 57]. Prostaglandin dehydrogenase, a prostaglandin-inactivating enzyme, has also been demonstrated [7]. Secretory leukocyte protease inhibitor, a potent inhibitor of human leukocyte elastase, has been demonstrated in human amniotic fluid and in the amniotic membrane. Its concentrations can be upregulated by exposing amniotic cells to IL-1α, IL-1β, and TNFα [63].

Cytokines. Interleukins-6 and -8 are the predominant cytokines associated with amnion cells [24, 47]. Expression of these cytokines was increased in the presence of IL-1β, TNFa and bacterial lipopolysaccharide. IL-10 and IL-1RA (receptor antagonist), both anti-inflammatory cytokines, have been shown in amnion epithelial and mesenchymal cells [22].

Growth Factors. Studies on human amniotic membrane have revealed the presence of EGF, TGFα, KGF, HGF, bFGF, TGF-β1, and -β2 by RT-PCR for the mRNA and by ELISA for the protein products [29]. TGF-β3 and growth factor receptors KGFR and HGFR were also detected by RT-PCR. A higher level of various growth factors was found in amniotic membrane with epithelium than without epithelium, indicating an epithelial origin for these growth factors [29, 45]. Neurotrophic factors like NGF (nerve growth factor) have also been demonstrated in the amniotic membrane and amniotic fluid [51, 60].

Metalloproteases and Inhibitors of Metalloproteases. Tissue inhibitors of metalloproteases (TIMPS) have been shown to be produced by both amniotic epithelial cells and mesenchymal cells [22, 50]. The presence of tissue metalloproteases (TMPS) has also been demonstrated in amniotic fluid and amniotic membrane, where they may play a role in the mechanisms of human parturition and in the regulation of host response to intrauterine infection [17, 41].

Controversies and Limitations. From the above it is obvious that there are several contradictory components in the membrane. This is not surprising as in any biologically active tissue such as the AM, balances and counterbalances for action of various molecules would be expected. However, when applied surgically to the ocular surface or elsewhere only the presence of the desirable ones for a particular set of action(s) is quoted with no regard to the opposing molecules. For example, the presence of prostaglandins in the membrane would promote inflammation but the presence of prostaglandin inactivating enzyme and of secretory leukocyte protease inhibitor would suppress inflammation. The presence of anti-inflammatory cytokines such IL-1Ra and IL-10 would suppress inflammation but the presence of IL-6 and IL-8 would promote inflammation. In eyes that are inflamed due to injury, other proinflammatory cytokines such as IL-1α, IL-1β and TNFα could also promote both pro- and anti-inflammatory cytokines and enzymes.

Similarly, the presence of various growth factors like EGF would support epithelial growth and TGF would support wound healing. However, TGF would itself promote scar tissue formation and be contradictory to the 'anti-adhesive or scar suppressing' action proposed for the membrane in preventing corneal and conjunctival cicatrisation. Likewise, the presence of TIMPS would disfavour vascularisation but the presence of TMPS would have the opposing effect. AM has been used with success in some cases of ocular surface burns with limbal ischaemia. One of the main concerns in such patients is the 'limbal ischaemia' and procedures such as tenoplasty and conjunctival flaps where possible have been advocated in an attempt to restore limbal vascularisation. Would not application of the membrane, with its 'inhibitor of vascularisation effect', have a contradictory effect on limbal ischaemia?

The mere presence of one or the other 'factor' thus cannot be presented as evidence in support of a particular mode of action for the membrane. How these various factors interplay, if at all, in the transplanted membrane to bring about some of its attributed effects remains to be elucidated.

Of the proposed mechanisms of action of the membrane the most likely manner in which it

affects its beneficial effect is perhaps as a substrate or basement membrane transplant. It provides a favourable substrate by virtue of its basement membrane, for new epithelial cells to migrate on, expand and adhere. Use of the membrane as a bandage to cover inflamed or exposed areas, due to injury or surgery, not only favourably influences the healing process but also has a dramatic favourable effect on the symptom of pain and discomfort. It is our clinical experience that when denuded areas of the ocular surface, particularly the cornea, are covered by amniotic membrane, the levels of pain and discomfort experienced by the patient are significantly reduced. This too could be as a result of the mechanical or physical presence of the membrane. One study has shown that amniotic fluid application to the corneal surface of rabbits following excimer laser photokeratectomy actually enhanced corneal sensitivity and nerve regeneration [32].

Summary for the Clinician

- **The amniotic membrane is composed of a single layer of epithelial cells, basement membrane and avascular stroma**
- **Several growth factors, cytokines, proteases and their inhibitors, antimicrobials, antiangiogenic factors and enzymes have been identified in these layers**
- **The mechanisms of action of the membrane are attributed to and inferred from its physical structure and its molecular constituents**
- **The fate of these substances after transplantation is unknown and the mere presence of a factor or molecule does not imply that it is available in its active form after surgery**
- **Often both pro and anti factors are present and a beneficial effect cannot be ascribed to any one of them without taking into account the effect of the other**
- **The most uncontroversial mechanism of action is by its physical presence as a 'substrate' transplant**

2.3 Intra and Inter Donor Variations of the Membrane

The general functional description of the amnion is that of an epithelial lining which contributes to the homeostasis of amniotic fluid. It is natural therefore that its physiological role and some corresponding morphology will change depending on the stage of gestation. This in fact has been amply demonstrated. Many changes in the biochemical composition of the membrane are known to occur nearer term and to be induced by labour, for example increased apoptosis of amnion epithelial cells occurs just before commencement of labour and IL-6 and IL-8 are found in increased concentrations in the amniotic fluid towards the end of pregnancy [24].

The amnion varies in histological appearance from conception to maturity and several different patterns are often noted even at term. There is an increase in prostaglandin synthase in the amnion at term and during labour [53]. The epithelial morphology can change to that of large flat cells and some show distinct intercellular channels. Also, the amniotic epithelial cells are columnar towards the placenta and cuboidal away from it [43]. The apical surfaces of the amniotic epithelial cells are covered by microvilli [8, 43, 61], the density of which varies during pregnancy. An amorphous material of unknown substance is seen on the surface of these microvilli at term [43]. In a recent elaborate study, using TGFβ as a test molecule to illustrate inter donor variations such differences were clearly demonstrated (see next section below). In a recent study, Fortunato et al. [16] have demonstrated a racial disparity in the ability of the membrane to respond to infectious stimuli, suggesting that race may yet be another variable to contend with.

With increasing use of the membrane it is our experience that the thickness and transparency of the membrane varies at different sites of the membrane. Generally, the membrane closer to the umbilical cord appears thicker. This also affects the transparency or translucency of the membrane. The variation

between donors can be more pronounced. The amnion can vary in thickness from 0.02 to 0.5 mm [4, 8].

Controversies and Limitations. Many differences, some yet unknown, can exist between amniotic membranes obtained from different donors. Racial variations too may exist. There are many other important variables such as the duration of gestation, trial of labour before caesarean section and perhaps parity and gravidity of the donor. Increased prostaglandin and proinflammatory cytokines nearer term could influence the effect of the membrane when applied on the ocular surface. Even for the same donor, it is the general practice to obtain numerous pieces of amnion for use in multiple operations. Clearly therefore some pieces would be from locations closer to the placenta and others distant to it. The thickness of these locations can vary as can the morphology of the amniotic epithelium. The thickness could affect the integration of the membrane with the ocular surface tissues and perhaps influence the ease with which the membrane 'comes off' at some point after surgery. The transparency would naturally affect potential vision when applied on the corneal surface. Current practice in procurement and supply of the membrane does not indicate these variables that could be important to the outcome of its usage. Age, race, parity, gravidity, duration of gestation, whether trial of labour was given or not and location of a particular piece of membrane supplied need to be considered in evaluating outcomes and eventually in bringing some semblance of standardisation in the practice of amniotic membrane transplantation. This may not be practically possible but only by recording these variables can we begin to understand whether and what effect they may have on the outcome of transplantation. One point that comes out loud and clear from the above is that membranes used in transplantation are far from standardised across donors and even within the same donor.

Summary for the Clinician

- **The amniotic epithelial morphology varies from flat to cuboidal to columnar**
- **The thickness and transparency of the membrane is different at different parts of the membrane – thinner and clearer away from the placenta, and in different donors**
- **The membrane undergoes considerable physiological changes near term and during labour**
- **Racial variations between donors can exist**
- **Duration of pregnancy, trial of labour, gravidity and parity can all influence the composition of the membrane**
- **Different pieces of the membrane from the same donor can potentially have different effects**

2.4 Processing and Preservation of the Membrane

Many methods of preserving and storing amniotic membrane for ocular and other uses have been described. Some of these are historical and others popular and currently in vogue. Methods such as lyophilisation [6, 56], air drying [35, 46], glutaraldehyde and polytetrafluoroethylene treatment [40] and irradiation [35, 46, 59] have been described but are not among the ones used commonly for ophthalmic use. Preservation by freezing is the commonest mode of preservation of the membrane before use. This involves use of two types of solutions, either DMSO in phosphate buffered saline [3, 52] or Eagle's minimum essential medium (MEM) and glycerol [26, 27]. Recently a freeze dried preparation of the membrane has been commercially introduced (Ambiodry) but not much is known about its usage. Furthermore different antibiotic cocktails, 0.5% silver nitrate [21] and 0.025% sodium hypochlorite solution [48, 49], have been used to render the membrane sterile. In many parts of the world 'fresh membrane' (within days or weeks of donation) is still used. However, in most Western countries the use of one or the other method of preservation is mandatory because of legislation requiring that the membrane be adequately screened for HIV contamination. To this end the donor is tested at the time of delivery and 6 months later (to cover the window period of infection). All

processed membranes are stored in quarantine until the second test is performed and reported negative.

Controversies and Limitations. Several differences can therefore occur between different membranes depending on whether they are used fresh or preserved and, in case of the latter, on the mode and duration of preservation. Most methods employed in the preservation of the membrane affect it in some manner.

Kruse et al. [30] demonstrated that cryopreservation significantly impaired the viability and proliferative capacity of amniotic membrane and its cells. They concluded that amniotic membrane grafts function primarily as a matrix and not by virtue of transplanted functional cells. Kubo et al. [31] have shown that after 2 months of freezing, at least 50 % of amniotic cells are viable and capable of proliferation. After 18 months of cryopreservation, they were not able to demonstrate a significant amount of cell survival. Our own studies show that cell viability is minimal, if at all, after 6 months of preservation at –80 °C; however, at this time point the membrane continues to demonstrate many growth factors and cytokines (Hopkinson A et al., submitted for publication). Fujisato et al. [18] cross-linked amniotic membrane with chemical means (glutaraldehyde) and with gamma-ray and electron beam. They showed that radiation cross-linked membranes degraded rapidly in vitro compared to chemically cross-linked membranes. Hao et al. [22], who demonstrated the presence of mRNA for both antiangiogenic and anti-inflammatory factors in amniotic membrane, have suggested that amniotic membrane should be applied epithelial cell surface down in order to deliver a high concentration of these factors to the damaged ocular surface. This would be applicable more to fresh rather than to preserved membranes if one were to accept (despite lack of any evidence in support) that the amniotic epithelial cells continue to produce the desired 'factors' in biologically active forms after transplantation onto the ocular surface.

Hopkinson A et al. (submitted for publication) studied extensively the growth factor TGF-β1 to determine intra- and intermembrane variations. They showed that at both the gene and protein level TGF-β1 is the highest expressed isoform of TGF-β, and that expression is lower in AM than in chorion. In addition, they demonstrated considerable variations in TGF-β1 gene expression between membranes, with expression at different locations within a single membrane also appearing to vary. Another important observation was that maximal presence of TGF-β1 was in the acellular spongy layer, which suggests that the spongy layer most likely acts as a depot for chorion-derived TGF-β1. It is possible that the spongy layer acts as a physical barrier, preventing chorionic-TGF-β1 from diffusing into the AM during gestation. They also showed that alterations in the method of handling the membrane could drastically alter the concentration of TGF-β1. Any method of processing and storage of the membrane, therefore, that did not get rid of this layer would yield a product that would enhance wound healing and scarring and vice versa. This study has conclusively demonstrated that, not only for this one factor but for other proteins as well, considerable variations exist between membranes, at different locations within the same membrane and can be profoundly affected by the method of processing and storage of the membrane (Fig. 2.1). Clinically, such variation between membranes is not considered prior to surgery, and therefore the effect on clinical efficacy is unknown. To determine the clinical significance of such variables, further studies are required.

Koizumi et al. [28] cultivated rabbit limbal and corneal epithelial cells on denuded human amniotic tissue. They demonstrated significantly improved growth of cells on membrane denuded of epithelial cells compared with intact membrane. To the contrary, most of the membrane supplied in the USA is with the amnioitic epithelium in situ. It is claimed that ocular surface epithelial cells grow better on this surface. This controversy remains unresolved. Clinically, it is our experience that both membranes, with and without the amniotic epithelium in situ, seem to support growth of ocular surface cells.

The weight of the evidence available supports the notion that viability of the tissue components of the amniotic membrane is not essential for its biological effectiveness. However, the extent of the effectiveness could vary and result

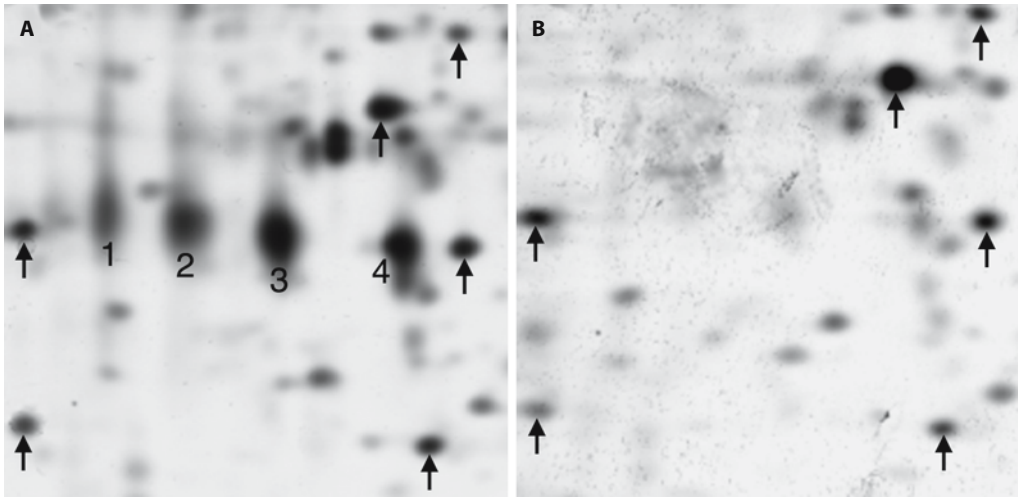

Fig. 2.1 A, B. Two-dimensional gel electrophoresis of solubilised protein from transplant ready amniotic membrane. Sixty micrograms of protein from two different donor membranes (**A, B**) was separated on 18-cm pH 3–11 IPG strips (Amersham Biosciences) and then on a 8–19 % gradient polyacrylamide gel followed by silver staining of protein spots. Comparable spots of similar intensity representing similarities (reference markers) between membranes are indicated (*arrows*). Variation between membranes, represented by spots detected in some membranes but not in others, is also indicated (**A** *1–4*). Examples shown are of comparable zoomed areas of the whole gel, and representative experiments of a total of 24 performed are shown

in inconsistent results. Until the effect of these different methods of preservation and storage on the membrane has been evaluated and standardised, success or failure of the membrane should be qualified by the method of preservation employed.

Despite the prolonged quarantine, the potential risk of transmission of serious infections from one donor to a number of recipients remains a concern. This concern is heightened in countries where fresh membrane is used though mitigated to some extent by the fact that only a limited number of surgeries (recipients) are performed with a given donor.

Summary for the Clinician

- **Many different methods of preservation and storage of the membrane exist**
- **Wet freezing in phosphate buffered saline or in minimum essential medium are currently the two most popular methods**
- **A freeze-dried membrane has recently been introduced in the market and initial usage seems to suggest clinical efficacy**
- **The effect of these different methods on the structural and biochemical composition of the membrane is not fully understood. Such effects could have a direct consequence on the proposed mechanisms of action**
- **Studies have shown that preservation and processing can have profound effects on the membrane constituents**
- **Fresh, and to some extent even preserved, membranes that have been tested twice carry a potential risk of spread of serious infections**

2.5 Clinical Studies and Outcomes (Definitions of Success and Grading of Disease Severity)

Most published reports on use of the amniotic membrane in ophthalmology have been consecutive case series or retrospective studies. Randomised controlled studies are practically non-existent bar one or two [5]. This must be

considered a serious limitation of the use of the amniotic membrane. Another significant shortcoming has been the lack of clear definitions of success or failure and the evaluation of the outcomes against such definitions. This limitation is to some extent matched by a similar lack of consistency in gradation and classification of severity of the disease that make up the major indications for use of the membrane, for example ocular surface burns. These two factors combine to make evaluation of the effects of the membrane difficult and in some instances rendering it even impossible to compare outcomes between different groups [12, 15, 23, 37].

We have used a pre-determined protocol to define outcomes and propose this as one model that may be considered in evaluating the efficacy of the membrane (Maharajan et al., submitted for publication). When the membrane was used with the intention of it becoming incorporated into the recipients tissue it was termed a graft and when the intention was for it to come away or be removed at a certain point following surgery, it was termed a patch. In our group of patients it was used primarily as a graft or a patch with four objectives: (a) to establish epithelial cover in an area where none existed, (b) to prevent corneal perforation in eyes at risk due to stromal melting, (c) to limit scarring where the clinical likelihood was high or where scarring (symblepharon/adhesions) previously existed, and (d) to limit inflammation and neovascularisation. The outcome was evaluated against both the intended purpose of the membrane, patch or graft, and whether the objective was achieved or not. Three outcomes were thus defined, success, partial success and failure.

Criteria for Success or Failure (Maharajan et al., submitted for publication). The purpose of AMT was to act as a patch, graft or both. AMT was carried out to fulfil one or more of the following objectives: (a) to establish epithelial cover in an area where none existed, (b) to prevent corneal perforation in eyes at risk due to stromal melting, (c) to limit scarring where the clinical likelihood was high or where scarring (symblepharon/adhesions) previously existed, and (d) to limit inflammation and neovascularisation. Three outcome measures were applied: (1) success: when the membrane served the purpose that was intended, i.e., acted as a patch or graft and the objective was achieved; (2) partial success: (a) when the membrane did not serve the purpose that was intended, i.e. acted as a patch when intended as a graft or vice versa but the objective was achieved, for example re-epithelialisation of a persistent epithelial defect (PED) occurred, (b) when the membrane (as a patch) did not persist long enough but the objective was nevertheless achieved, for example epithelialisation continued till the defect was closed, (c) when multiple objectives were set and not all were realised; (3) failure: when the objective was not achieved even though the purpose may have been achieved, for example if the

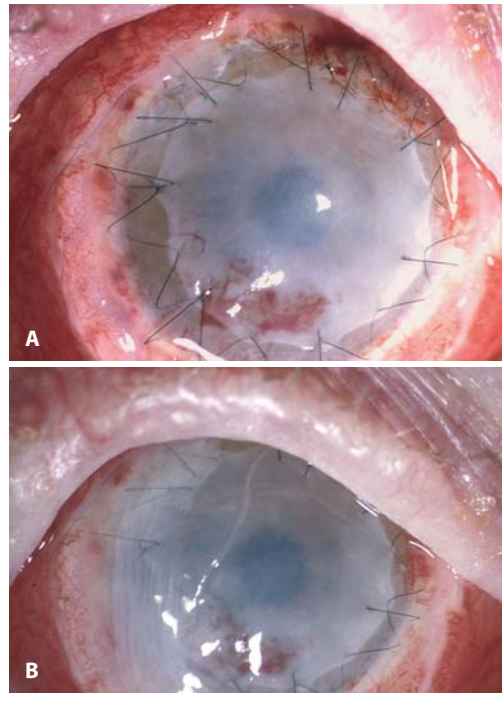

Fig. 2.2. **A** Ocular surface reconstruction with allolimbal transplantation and use of two membranes. The inner 9-mm disc acts as a graft and the outer larger membrane as a patch. The outer membrane prevents conjunctival epithelial cells mixing with the transplanted limbus-derived cells growing on the inner membrane (author's technique: H.S. Dua). **B** The outer membrane has cut through sutures and retracted, exposing the inner membrane. This was considered as a partial success

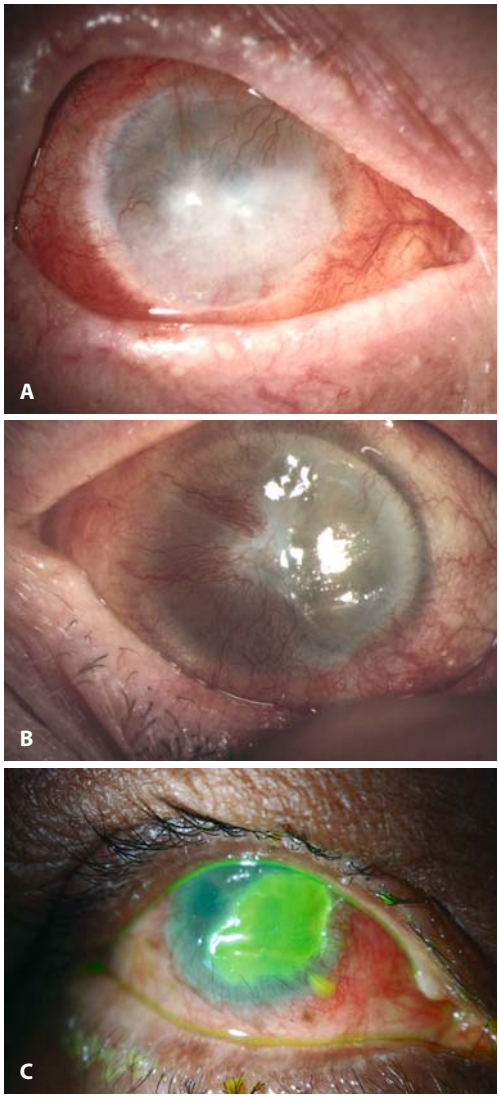

Fig. 2.3. **A** Corneal scarring and vascularisation despite allolimbal transplantation and use of amniotic membrane in a case of stem cell deficiency. This patient was considered a failure. **B** Failed amniotic membrane transplant in a patient of acute ocular surface burn. **C** Amniotic membrane and corneal stromal melting in a patient of bullous keratopathy treated with amniotic membrane transplantation. The picture was taken on day 7 after surgery

membrane was intended as a patch and acted as such for the expected duration but the PED did not heal.

When the above criteria were applied to 74 procedures involving use of the amniotic membrane, failure of the procedure was observed in 44 % of patients where the membrane was used in the presence of stem cell deficiency, in 33 % of procedures where the membrane was used in the absence of stem cell deficiency and in 44 % of patients where the membrane was used for conjunctival reconstruction. This clearly illustrates that a significant proportion of failures can occur and these should be appropriately recognised and documented in order to refine and temper the vastly expanding indications for use of the membrane (Figs. 2.2, 2.3).

Controversies and Limitations. Lack of randomised controlled trials, lack of clearly defined criteria of success and failure against intended use of the membrane and inadequate gradation or classification of disease severity all contribute significantly to highlight a serious limitation surrounding the clinical use of the membrane, ascertaining its efficacy for different indications and comparing and evaluating outcomes of use.

Summary for the Clinician

- **The membrane can be used to serve either as a patch, when the membrane is removed after some time or is expected to fall off, or as a graft (including carrier of ex vivo expanded cells) when the membrane is incorporated into the host tissue**
- **Important objectives of use of the membrane are to provide epithelial cover, arrest melting, limit scarring, limit inflammation and neovascularisation**
- **Outcomes should be clearly defined, for example as success, partial success or failure**
- **Proper gradation or classification of clinical conditions is necessary for proper evaluation of the membrane**
- **Randomised controlled studies are required to scientifically evaluate the efficacy of the membrane**

2.6 Efficacy of Membrane in Relation to Other Established Techniques and Options

Another interesting point of note is that many reports of the success of the use of the membrane for one indication or the other do not compare it with valid controls or even against standard existing techniques for the same indication. This leads to the erroneous message that the membrane should be used for a particular indication without flagging that an existing procedure or alternative to the membrane may be equally effective or in some cases better. The following illustrations emphasise this point:

When the advocated use of the membrane to repair leaking trabeculectomy blebs was studied against the standard conjunctival advancement, the latter was found to be more reliable [5]. In some studies where the membrane has been used to repair failing blebs, antimetabolites too have been used as adjuncts. In the absence of any controls it is impossible to assess the contribution of the membrane to any success, which may equally have been due to the antimetabolite [13, 19].

Its use in pterygium surgery too is not fully clarified. It may be a useful alternative but autologous conjunctival grafts seem to have a better success rate than amniotic membrane grafts. Prabhasawat et al. [44] found that in pterygium surgery the recurrence rate was higher with amniotic membrane compared to autologous conjunctival grafts. Ma et al. [34] found no difference between the amniotic membrane, mitomycin C or autologous conjunctival grafts in the management of pterygium, but recommend use of the membrane.

An important case in point is the reported success of the membrane in treating patients with partial stem cell deficiency. The membrane can offer help in instances where a fibrovascular pannus has to be excised, but where abnormal conjunctiva derived epithelium has encroached onto the corneal surface the membrane is often not required though reportedly used and advocated. Tseng have shown good results with use of the membrane following superficial debridement of abnormal cells from the surface of the cornea in cases of partial stem cell deficiency [1, 38, 58]. In a technique now established as sequential sector conjunctival epitheliectomy (SSCE), Dua has shown [10, 11, 14] that the same effect can be achieved with simple debridement without the use of the membrane. The studies that advocated the use of the membrane for this indication did not include any controls where simple debridement was undertaken without amniotic membrane transplant. The SSCE technique can be taken as akin to controls for these studies and illustrates that proposed indications of the membrane are not always substantiated scientifically.

Similarly, the use of the membrane for the indication of painful bullous keratopathy [42] needs to be evaluated against the alternative of anterior stromal puncture and temporary placement of a bandage contact lens. The latter is a simple outpatient procedure with comparatively little expense. In an ongoing stratified (according to pain score) randomized controlled study we have performed the two different procedures in 25 patients and thus far no significant difference between amniotic membrane transplantation and anterior stromal puncture is emerging.

Controversies and Limitations. Many proposed indications of the membrane are not founded on hard evidence. In some instances use of the membrane may be an option but not necessarily a better option. In some cases it has distinct disadvantages over existing techniques. Longer follow-up studies are needed to ascertain duration of its efficacy.

- **Alternative options exist for many of the clinical indications for use of the membrane**
- **In most instances the membrane has not been compared directly with these options to evaluate its superiority or otherwise**
- **Clear examples of the failure of the membrane exist where other options have succeeded**

- **In some cases where the membrane may be just as good as an existing technique, the associated disadvantages of an interventional procedure requiring use of an operative room should be considered**

This chapter has been written to put in perspective the widespread use of the membrane and highlight areas which need to be addressed by further studies and continued critical analysis. That is not to say that the membrane does not have its uses. It is certainly a useful option in many clinical situations and in this context is the subject of another chapter in this text. Standardisation of the transplant ready amniotic membrane (TRAM) in relation to donor variables and processing and preservation variables; proper categorisation of the extent and severity of the diseases for which it is used; and defining criteria of success and failure to evaluate outcomes will go a long way in putting the amniotic membrane on a sound scientific footing. Perhaps the generation of a 'synthetic membrane' with known quantities of desired ingredients, tailored to the intended clinical use, will be possible in the future.

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Transplantation of Limbal Stem Cells

Harminder S. Dua

Core Messages

- Most self renewing tissues are served by a population of stem cells
- Potency and plasticity are two important characteristics of stem cells. They may also have the potential to transdifferentiate
- Stem cells usually reside in a defined 'niche'. The corneal epithelial stem cells are believed to be located in the limbal palisades
- Clinical and laboratory evidence strongly supports the notion that corneal epithelial stem cells are located at the limbus but no marker yet exists that can positively identify a limbal stem cell
- Limbal stem cell deficiency can be congenital or acquired. Ocular surface burns, immune mediated ocular surface diseases and chronic inflammation are important causes of limbal stem cell deficiency
- The effects of limbal stem cell deficiency can range from mild, such as loss of limbal anatomy or conjunctivalisation of the peripheral cornea, to severe, such as corneal invasion by a thick fibrovascular pannus or persistent epithelial defects with stromal melts
- The diagnosis of limbal stem cell deficiency is essentially clinical, but impression cytology may help. Presence of goblet cells on the cornea is diagnostic
- Limbal stem cell deficiency can be unilateral or bilateral, partial or total
- Mild cases of partial deficiency can be treated by sequential sector conjunctival epitheliectomy
- Total unilateral cases can be treated with auto-limbal transplantation
- Bilateral cases often require allo-limbal transplantation from living related or cadaver donors
- Auto-limbal and living related donor transplantation should be avoided in the presence of active inflammation. Auto-limbal transplantation should be avoided in unilateral manifestation of a systemic disease
- Amniotic membrane transplant can be combined with any of the limbal transplant procedures
- Allografts usually require long-term systemic (and/or topical) immunosuppression
- All associated pathology such as lid malpositions, trichiasis, secondary glaucoma and cataracts should ideally be managed prior to considering limbal transplantation, as far as clinically possible
- Buccal mucosa grafts help restore some moisture to a dry ocular surface. Living tissue transplants usually do not survive in a dry environment
- Long-term outcomes of auto-limbal transplants are far better than those of allo-limbal transplants
- Ex vivo expansion of limbus derived epithelial cells as a sheet on different substrates can also be used in ocular surface reconstruction with good results but is also subject to immune rejection

3.1 Introduction

In this chapter the general characteristics of stem cells (SC) and their niche are first described. The evidence supporting the existence of SC at the corneoscleral limbus, both clinical and scientific, is then explored providing the scientific basis for the transplantation of the limbus in the management of limbal SC deficiency. A brief account of the causes and effects of SC deficiency is provided as background to the indications and different techniques of limbal SC transplantation. The surgical techniques are elaborated together with postoperative management and any adjunctive procedures that may complement limbal transplantation.

Ex vivo expanded limbal stem cells on amniotic membrane or other substrates can also be used in ocular surface reconstruction. This constitutes a distinct method of putative limbal stem cell transplantation and is the subject of another chapter in this book. This technique of preparing the tissue construct and stem cell transplantation is therefore omitted from this chapter.

3.2 Stem Cells

3.2.1 Definition

Stem cells are progenitor cells that are responsible for cellular replacement and tissue regeneration. They are the ultimate source cells from which arise almost all other cells that constitute a given organ served by the SC. SC can be found in both embryonic and adult tissues and represent only a very small proportion (0.01–10 %) of the total cell mass [1, 32, 44].

3.2.2 Characteristics of Stem Cells

Stem cells are poorly differentiated or undifferentiated, long-lived, slow cycling but highly clonogenic cells that have a high capacity for self renewal and an increased potential for error-free division [65–67]. They have the ability to proliferate indefinitely [32] and generally live for the duration of the organ(ism) in which they reside. A constant pool is maintained by different strategies of cell division. The most accepted strategy is that of asymmetric cell division whereby one daughter cell stays back in the stem cell niche and the other follows the path of proliferation and differentiation, acquiring functional characteristics of the tissue or organ. The same balance can be maintained if the two daughter cells from one SC proceed down the path of differentiation and the two daughter cells of another SC stay back in the niche as stem cells [57, 61].

The daughter cell(s) that step outside the stem cell pool are destined to divide and differentiate with the acquisition of features that characterise the specific tissue. Such a cell is called a 'transient amplifying cell' (basal corneal epithelial cells) and is less primitive than its parent stem cell. It is believed in some quarters that there exists a window of opportunity during which some of these cells ('transient cells') [54, 55] can revert to the SC pool as SC. Transient amplifying cells divide more frequently than stem cells but have a limited proliferative potential and are considered the initial step of a pathway that results in terminal differentiation. They differentiate into 'postmitotic cells' (wing cells) and finally to 'terminally differentiated cells' (superficial squamous cells). Both postmitotic and terminally differentiated cells are incapable of cell division. All cells except stem cells have a limited life span and are destined to die [45, 66, 84].

Potency and plasticity are two key attributes of SC. SC have the potential to give rise to different cell lineages. This potency is, however, not uniform and there exists amongst SC a hierarchy of potential. SC can be totipotent, pluripotent, multipotent, or unipotent. The zygote that can form the entire embryo and part of the placenta is an example of a totipotent cell. Cells of the inner cell mass from which most tissues that arise from the three germ layers can be derived, but not components of the placenta, are considered pluripotent. Most tissue specific SC are multipotent, capable of producing lineages that

can differentiate in two, three or more different cell types with functional attributes of the organ in which they reside. Some SC, in their natural environment, may have only limited potential with the ability to generate only one specific cell type. These SC are labelled unipotent SC or committed progenitors. SC of the epidermis and the corneoscleral limbus are considered to be examples of this category [1, 3, 68].

The 'plasticity' of SC refers to their ability to transdifferentiate. Some SC when relocated to a different site (tissue) can assume the role that supports the structure and function of the new site, thus aiding in regeneration, repair and maintenance of the cell population at the new site. Stem cell potential and plasticity are both more pronounced in embryonic SC compared to adult SC. Embryonic SC have virtually an unlimited potential for self-renewal and differentiation. Given the right microenvironment and the right signals, adult and embryonic SC can (theoretically) be made to follow a desired path of differentiation or propagated indefinitely, in an undifferentiated state. Herein lies the immense therapeutic potential of SC [3].

3.2.3 The Stem Cell 'Niche'

The microenvironment in which the SC reside is referred to as their 'niche' [60]. SC are usually confined to their 'niche' where the microenvironment supports and maintains the stemness of SC and affords a degree of protection. In solid organs, where cell migration commences at one point and progresses until the cells are shed at a distant point(s), the SC niche is usually located at the point of commencement. The 'niche' represents the collective influence of other local matrix cells, the extracellular matrix, its vascularity, basement membrane characteristics and prevalent growth factors and other cytokines. In the intestinal mucosa, for example, it is believed that the pericryptal fibroblasts/subepithelial myofibroblasts may serve as niche cells [60, 79] and in the epidermis, beta 1 integrin mediated adhesion to its ligand, type IV collagen, is shown to influence behaviour of epidermal SC [44]. The niche also affords protection to the all-important SC [79, 95].

- **Stem cells:**
  - **Undifferentiated**
  - **Long lived**
  - **Slow cycling**
  - **Clonogenic**
  - **Asymmetric division**
  - **Potency: usually pluripotent or multipotent**
  - **Plasticity: transdifferentiation**
  - **Niche: SC microenvironment**
- **SC progeny:**
  - **'Transient cells'**
  - **Transient amplifying cells – basal epithelium**
  - **Postmitotic cells – wing cells**
  - **Terminally differentiated cells – superficial squamous cells**

3.3 Limbal Stem Cells

3.3.1 The Clinical Evidence

During corneal epithelial wound healing and normal epithelial turnover, cell migration and migration of sheets of epithelium [20] have been shown to occur in a centripetal manner from the corneoscleral limbus towards the centre of the cornea [4, 5, 51]. Large corneal epithelial wounds, where the wound edge is closer to the limbus, heal at a faster rate than smaller wounds [59]. Repeated denudation of the central corneal epithelium shows that the healing rate of the second wound is more rapid than that of the first. This suggests that rapidly dividing younger cells of the periphery have moved to more central areas after the first trauma and respond readily to the second [80].

Human corneal epithelial defects with partial limbal involvement demonstrate a preferential circumferential migration of a population of cells along the limbus, from both ends of the remaining intact limbus [21] (Fig. 3.1 A). Complete epithelial cover for the corneal surface is not established until limbal re-epithelialization is first complete, suggesting that the circumfer-

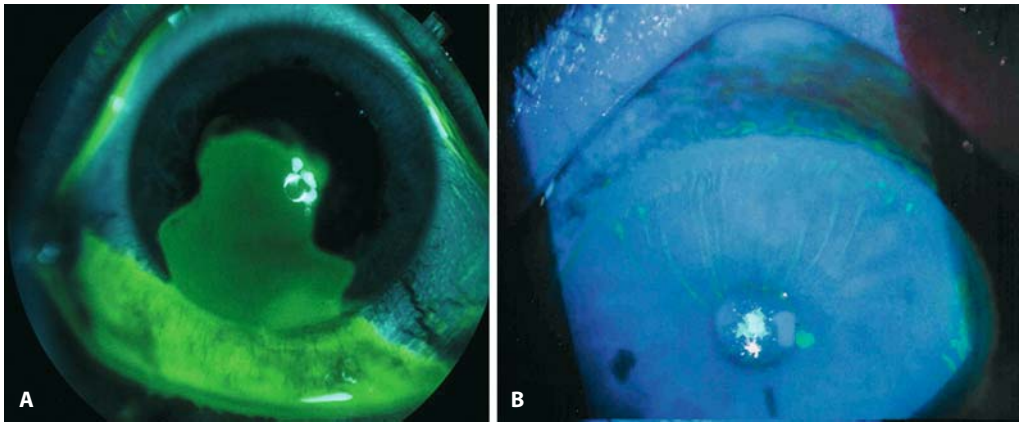

Fig. 3.1. A Healing of corneal epithelial wound involving the limbus showing a preferential circumferential migration of tongue-shaped sheets of limbal epithelial cells arising from either end of the remaining intact epithelium. (Slit lamp anterior segment photograph with fluorescein dye) (with permission from *Br J Ophthalmol:* Dua et al. 2001; 85:1379–1383). **B** 'Columnar keratopathy' is the name given by the author to this presentation of alternating columns of fluorescein stained epithelium and normal corneal epithelium. These correspond to the limbal palisades and represent an early sign of limbal stem cell deficiency

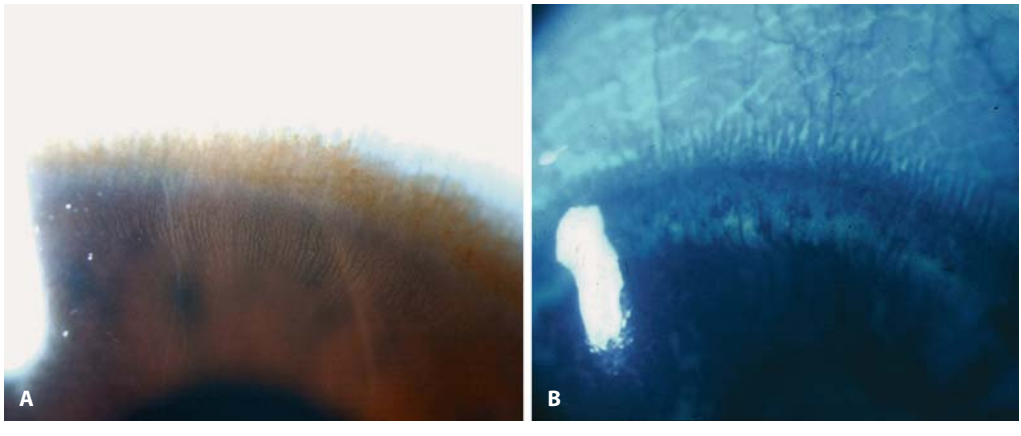

Fig. 3.2. Slit lamp photograph of the limbus showing the palisade (of Vogt) structure with: **A** pigment columns migrating into peripheral cornea and **B** fluorescein staining of columnar migration in response to a central abrasion

entially migrating population of cells probably represents in part the healing response of limbal stem cells. In patients with limbal abnormalities, alternating columns of normal and fluorescein staining cells often corresponding to limbal palisades – columnar keratopathy – have been noted to extend from the limbus towards the centre in radial or curvilinear rows [22] (Fig. 3.1B). The palisades of Vogt and the interpalisade rete ridges provide a unique structure to the limbus (Fig. 3.2). The structure of the palisades and the rete ridges, their vascularity and pigmentation are all analogous to repositories of stem cells in the monkey palm epidermis [9, 86, 88]. It has also been reported that hemidesmosomes of peripheral cells of normal and healing mouse corneas are arranged in radial rows, leading to the interpretation that this

orientation represents centripetal migration of epithelial cells [5]. Very recently, a unique anatomical structure, termed the limbal epithelial crypt [27], has been discovered at the peripheral end of the interpalisade rete ridges, numbering approximately five to seven per human cornea. This has features consistent with those of a SC repository or 'niche'.

3.3.2 The Scientific Evidence

Basic research has identified a number of characteristics that are unique to the limbal basal epithelial cells and set them apart from the rest: Mitosis rates are highest at the limbus, both in the normal physiological state and following stimulation [31, 37, 8]. Limbal epithelial cells have the greatest proliferative potential in vitro, compared to any other part of the cornea [28–30]. Limbal basal cells lack the epithelial cell differentiation cytokeratin CK3 [18, 56, 73, 101]. Impression cytology examination of the human limbus shows that, morphologically, the limbal cells are smaller, more densely packed and have a greater nucleus to cytoplasm ratio compared to adjacent corneal and conjunctival cells (H.S. Dua, unpublished observations, Fig. 3.3A, B).

Several other attributes that are unique to the limbal epithelium (Table 3.1) have also been described. These include the presence of alpha-enolase [101, 102], EGF receptors [100, 103], pigment [9], cytokeratin profile (CK3/12 negative) [7, 73], presence of vimentin [53–55], CK19 and specific basement membrane characteristics [34, 35, 85]. Vimentin and CK19 positive, CK3 negative clusters of cells with unique electron microscopic morphology have been demonstrated [54, 55]. Connexin 43 (Cx43), a gap junction protein, has been noted in human corneal but not limbal basal epithelium [12, 58, 96]. It has been proposed that absence of Cx43 segregates cells from adverse events generated in neighbouring cells and helps preservation of SC in their microenvironmental niche [96].

Zhao et al. [98] have recently reported that limbal epithelial cells cultured in the presence of mitogens express neural progenitor markers, specifically nestin. A transcriptional factor, p63 involved in morphogenesis, has been proposed to identify keratinocyte stem cells at the limbus [63], but its role as a marker of limbal SC is controversial [25, 46]. Similarly, well defined markers of haematopoietic SC, namely CD34 and

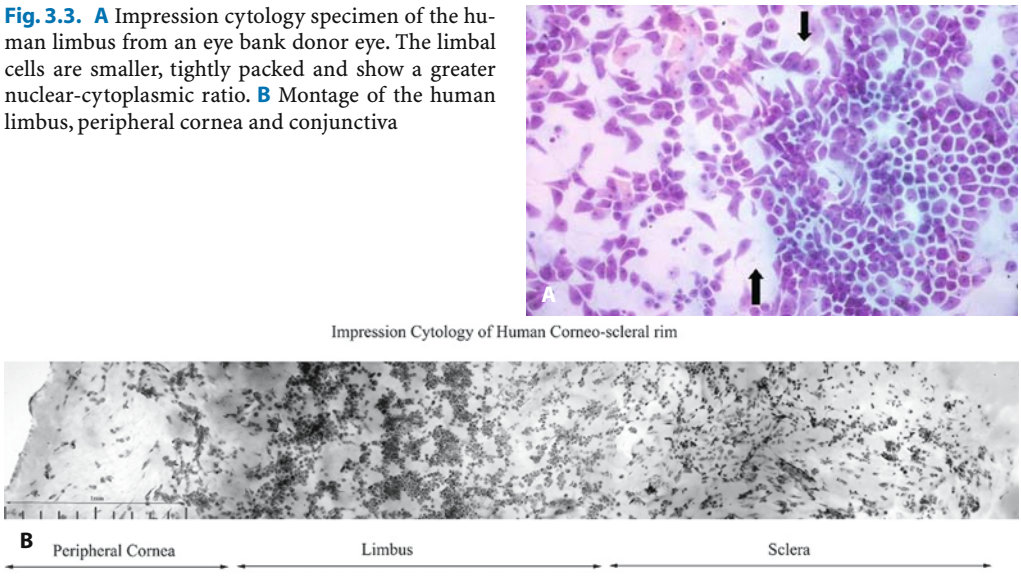

Fig. 3.3. **A** Impression cytology specimen of the human limbus from an eye bank donor eye. The limbal cells are smaller, tightly packed and show a greater nuclear-cytoplasmic ratio. **B** Montage of the human limbus, peripheral cornea and conjunctiva

Table 3.1. Differences between epithelial cells of the limbus and central cornea (CK, cytokeratin; CX, connexin; EGFR, epithelial growth factor receptor)

Limbus	Central cornea
CK 5/14+ve	CK 5/14–ve
CK 3/12–ve	CK 3/12+ve
CK 19+ve	CK 19–ve
P63+ve	P63–ve but see results in this paper
CX 43–ve	CX 43+
Vimentin+ve	Vimentin–ve
Intrinsic melanogenesis	
Cytochrome oxidase and ATpase+ve	
Alpha-enolase+ve	Alpha-enolase+ve
Beta-1-integrin+ve	Beta-1-integrin+ve
EGFR+ve (strong)	EGFR+ve
ABCG2+ve	ABCG2–ve

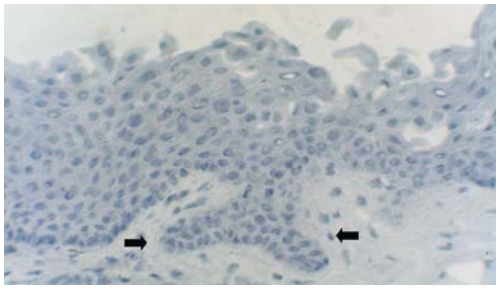

Fig. 3.4. Limbal epithelial crypt: representing a solid cord of cells extending from the undersurface of a limbal palisade. These cells are positive for the putative stem cell marker ABCG2. Haematoxylin stained cryo section, ×100

CD133, have failed to demonstrate any unique subpopulation of cells at the limbus [25, 47]. An ATP-binding cassette transporter protein, ABCG2, is believed to be a marker of a side population of cells that have the ability to efflux Hoechst 3342 dye [99]. Side population cells that contain this transporter protein are believed to be stem cells [36]. Limbus epithelial cells have been shown to express ABCG2 [94] and these may represent the subpopulation that contain the stem cells. The limbal epithelial crypt recently demonstrated by Dua et al. [27] contains cells that predominantly stain positive for ABCG2, indicating that the crypt may provide the niche for corneal epithelial SC (Fig. 3.4).

The above data strongly supports the notion that progenitor cells exist at the corneoscleral limbus. Whether these are truly SC as defined in other organ systems remains to be established. There is evidence to suggest that SC or progenitor cells for the conjunctival epithelium reside maximally in the fornices and for goblet cells and perhaps for conjunctival epithelium may also be scattered throughout the epithelial surface.

Summary for the Clinician

- **Evidence for corneal epithelial (limbal) stem cells:**
- **Clinical:**
  - **Unique palisade architecture**
  - **Centripetal migration from limbus**
  - **Circumferential migration along limbus**
  - **Pigment and other deposits migrating in columnar manner from limbus**
  - **Larger corneal epithelial wounds (closer to limbus) heal faster**
  - **Second wounds heal faster**
  - **Relative resistance of limbus epithelium to denudation**
  - **Columnar keratopathy**
  - **Limbal deficiency allows conjunctivalisation of cornea and persistent epithelial defects**
- **Scientific:**
  - **Different morphology of limbal cells**
  - **Increased hemidesmosomes at limbus basal epithelium**

- Increased mitosis rates at limbus
- Increased proliferative potential of limbal basal cells
- Absence of cytokeratin 12 in limbal basal cells
- Absence of gap junctions in limbal basal cells
- Presence of certain enzymes such as alpha-enolase and ABCG2
- Different basement characteristics at limbus compared to central cornea
- Presence of limbal epithelial crypts (niche)

3.4 Limbal Stem Cell Deficiency

3.4.1 Causes of Limbal Stem Cell Deficiency

Stem-cell deficiency can be congenital or acquired. Congenital SC deficiency occurs as a result of hereditary aplasia of limbal stem cells as occurs in aniridia and congenital erythrokeratodermia. More often though, stem cell deficiency is acquired as a result of extraneous insults that acutely or chronically destroy limbal stem cells. These include chemical or thermal injuries, ultraviolet and ionising radiation, Stevens-Johnson syndrome, advanced ocular cicatricial pemphigoid, multiple surgery or cryotherapy, contact lens wear, or extensive/chronic microbial infection such as trachoma. Keratitis associated with multiple endocrine deficiencies, neurotrophic (neural and ischaemic) keratopathy and chronic limbitis also lead eventually to SC deficiency but are less common [13, 18, 24, 33, 41, 42].

- Causes of limbal stem cell deficiency:
- Congenital: aniridia, erythrokeratodermia Acquired:
  - Chemical and thermal burns
  - Chronic inflammatory disorders
  - Progressive cicatrisation conditions – OCP, SJS
  - Prolonged contact lens wear
  - Multiple ocular surface surgery
  - Medicamentosa including preservatives
  - Idiopathic

3.4.2 Effects of Limbal Stem Cell Deficiency (Modified from Dua et al. [25])

The hallmark of limbal stem cell deficiency is 'conjunctivalisation' of the cornea and the most significant clinical manifestation is a persistent corneal epithelial defect.

The clinical symptoms of limbal deficiency may include decreased vision, photophobia, tearing, blepharospasm, and recurrent episodes of pain (epithelial breakdown), as well as a history of chronic inflammation with redness.

Depending on the extent of limbal involvement, SC deficiency can be partial or total. Partial SC deficiency may vary in extent to involve the pupillary area, when intervention is usually required, or exclude the visual axis when none or minimal intervention with topical medication may be required. Further, partial SC deficiency may vary in severity from mild, when only an abnormal epithelial sheet covers a variable area of the cornea, to severe when a part of the cornea, usually including the pupillary area, is covered by a thick fibrovascular pannus.

The clinical features of SC deficiency, from mild to severe, include the following [13, 14, 18, 21, 22, 24, 43, 64, 88]: (a) loss of limbal anatomy, (b) irregular, thin epithelium, (c) stippled fluorescein staining of the area covered by abnormal epithelium, (d) unstable tear film, (e) filaments and erosions, (f) superficial and deep vascularisation, (g) persistent epithelial defects leading to ulceration, melting and perforation, (h) fibrovascular pannus, and (i) scarring, keratinisation and calcification.

1. Loss of limbal anatomy: The normal limbal architecture with rows of palisades and the perilimbal vascular arcade is usually best defined at the superior and inferior limbus. The architecture may vary depending on age of the individual. With increasing age the definition of palisades becomes less distinct nasally and temporally. Pigmentation of the limbal palisades is a feature in some races. Alterations in limbal anatomy include con-

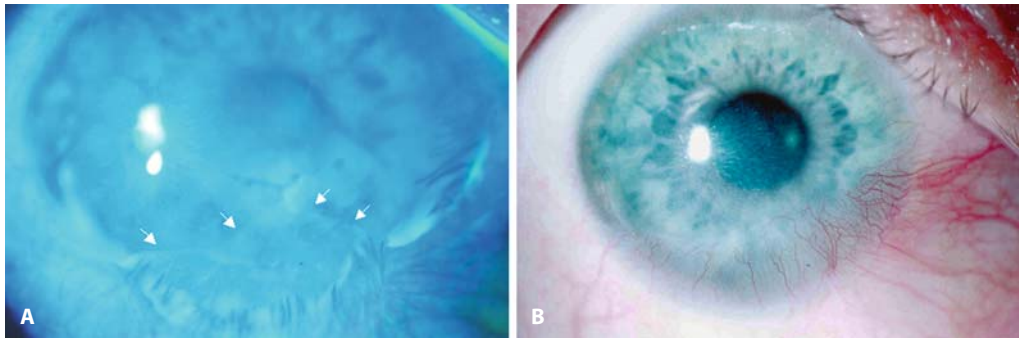

Fig. 3.5. A Signs of mild limbal stem cell deficiency – peripheral conjunctivalisation highlighted with fluorescein staining. The junction of corneal and conjunctival phenotypes of epithelia is marked with arrows. **B** Peripheral vascularisation with loss of limbal architecture

tiguous or patchy fluorescein staining of conjunctiva derived cells at the limbus and extending onto the peripheral cornea, segmental limbal hyperaemia indicating chronic inflammation, thickening of limbal epithelium, vascularisation of peripheral cornea and scarring (Figs. 3.1B, 3.5A, B).

2. Irregular, thin epithelium: When the initial injury is mild and superficial or the disease process leading to stem cell deficiency is slowly progressive, loss of a segment of limbal epithelium may occur without significant damage to the substratum. A sheet of conjunctival/metaplastic epithelium consequently covers the cornea without any notable vascularisation. This epithelium is usually thin and irregular as can be seen by the pooling of fluorescein dye at the junction of the abnormal and remaining normal epithelium (Fig. 3.6, see also Fig. 3.11A) [14].
3. Stippled fluorescein staining of the area covered by abnormal epithelium: The abnormal conjunctival/metaplastic epithelium readily takes up fluorescein dye [43], allowing easy visualisation of the abnormal cells and their pattern of distribution. The abnormal fluorescein-staining 'conjunctivalised' epithelium may take on the pattern of columns, whorls or wedges with the broad base towards the limbus and the narrow curving apex toward the corneal centre (Figs. 3.5A, 3.6) [22].
4. Unstable tear film: The abnormal epithelium demonstrates a rapid tear film break up time

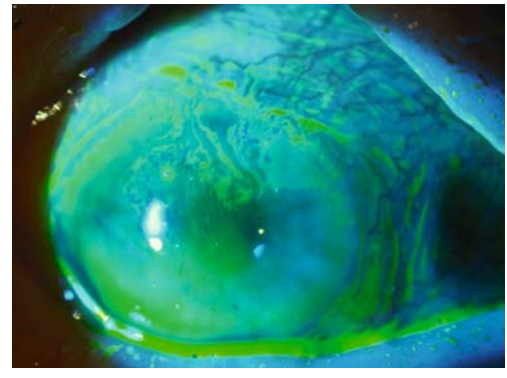

Fig. 3.6. Peripheral conjunctivalisation with pooling of dye and stippled staining of the abnormal epithelium, between 12 and 3 o'clock

over it and areas of negative and positive fluorescein staining.

5. Tags of loose epithelium, filaments with mucus and recurrent erosions are other features associated with the abnormal epithelial cover on the cornea.
6. Superficial and deep vascularisation: In moderate to severe cases of stem cell deficiency, superficial and/or deep vascularisation of the cornea occurs. It is largely restricted to the area of stem cell deficiency and may affect a segment of the limbus or the entire circumference may become involved (Fig. 3.7).
7. Persistent epithelial defects (Fig. 3.8): Chronic non-healing ulceration of the corneal epithelium or cycles of repeated breakdown

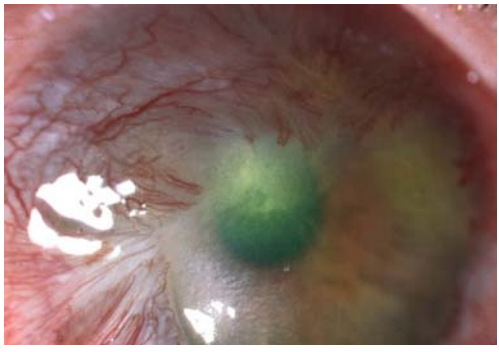

Fig. 3.7. Superficial and deep vascularisation with a fibrovascular pannus encroaching on the cornea following chemical burn in which 9.5 clock hours of the limbus and 60% of the conjunctiva were involved (clinical grade – 9.5/60%) (with permission from *Br J Ophthalmol:* Dua et al. 2001; 85:1379–1383)

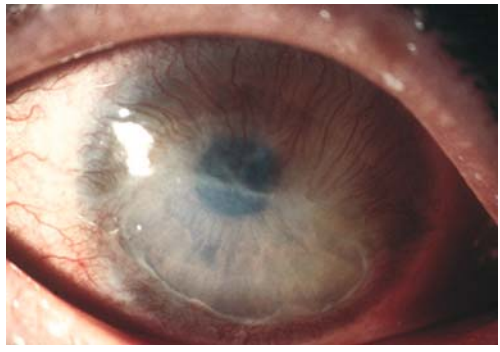

Fig. 3.8. Persistent epithelial defect and fibrovascular pannus on cornea related to total stem cell deficiency following unilateral alkali (cement) burn (clinical grade 12/65%) (with permission from *Br J Ophthalmol:* Dua HS and Azuara-Blanco A 2000; 84:273–278)

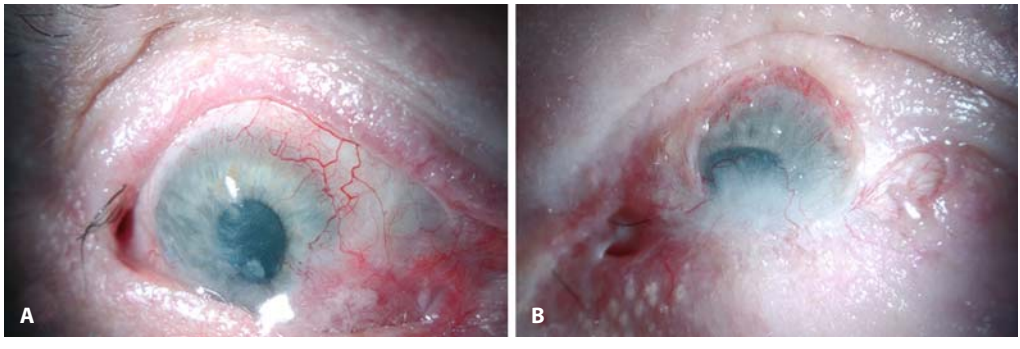

Fig. 3.9. **A** Right eye of patient with 10 clock hours of limbus and 70% conjunctival involvement following a chemical (alkali) burn. **B** Left eye of same patient with 12 clock hours of limbs and 90% conjunctival involvement (clinical grade 10/70% RE, 12/90% LE). Scarring, vascularisation, adhesions and some keratinisation are present. The lids on both sides were also severely damaged

followed by healing, associated with a chronic low grade inflammation, is a feature of limbal stem cell deficiency. These defects are liable to lead to deep stromal infiltrates that may or may not be related to infection. The edges of the epithelial defect have a distinct rolled-up or heaped appearance. Over time, progressive melting of the corneal stroma with perforation can occur.

8. Fibrovascular pannus: In moderate to severe cases of stem cell deficiency, epithelial cover of the denuded cornea is associated with encroachment of fibrovascular tissue of varying thickness (Figs. 3.7, 3.8) [49]. This tissue supports the thickened multilayered conjunctiva derived epithelium.
9. Scarring, keratinisation and calcification: The end stage of the aftermath of limbal stem cell deficiency, whatever the cause, is scarring and eventually calcification of the affected tissue. Usually by this stage the inflammation has subsided and the eye is comparatively comfortable. In patients who have associated severe dry eyes the covering epithelium becomes totally or partially keratinised (Fig. 3.9 A, B).

Summary for the Clinician

- Effects of limbal stem cell deficiency
  - Mild → severe
  - Loss of limbal anatomy
  - Conjunctival epithelial ingress onto cornea – stippled fluorescein staining
  - Columnar keratopathy
  - Unstable tear film over affected area
  - Frank conjunctivalisation
  - Corneal vascularisation – superficial and deep
  - Fibrovascular pannus covering corneal surface
  - Persistent epithelial defect
  - Stromal melting
  - Perforation, scarring, calcification
  - Keratinisation

3.4.3 Diagnosis of Stem Cell Deficiency

The diagnosis of stem cell deficiency remains essentially clinical. On slit lamp biomicroscopic examination, the conjunctivalised cornea presents a dull and irregular reflex. The epithelium is of variable thickness and translucent to opaque. Conjunctival epithelium on the cornea appears to be more permeable than corneal epithelium and takes up fluorescein stain in a stippled or punctate manner. In cases of partial conjunctivalisation of the cornea, fluorescein dye tends to pool along the junction of the sheets of corneal and conjunctival epithelial cell phenotypes. At this junction, the corneal epithelial sheet shows tiny processes or undulations that give the junction its characteristic appearance.

Loss of architecture of the limbal palisades of Vogt and vascularisation are other common features. When damage is extensive, vascularisation occurs in the form of fibrovascular pannus, which increases the thickness of the affected area of the cornea. However, the underlying corneal stroma may be considerably thinned by the initial insult of disease process.

The presence of goblet cells on impression cytology specimens taken from the corneal surface or in biopsy specimens of the fibrovascular pannus covering the cornea is pathognomonic of conjunctivalisation of the cornea (Fig. 3.10 A) [25, 69]. Biopsy specimens also demonstrate a multilayered, at times keratinised epithelium overlying dense fibrous and vascular tissue (Fig. 3.10 B). Intraepithelial lymphocytes, which are a feature of conjunctival epithelium, are also seen on conjunctivalised corneal epithelium. These are predominantly CD8+/*HML-1 + cells (cytotoxic T lymphocytes expressing the human mucosal lymphocyte antigen) [23, 25]. Features of squamous metaplasia or loss of cornea specific cytokeratins (CK 3/12) on immunohistology are other effects noted on biopsy specimens.

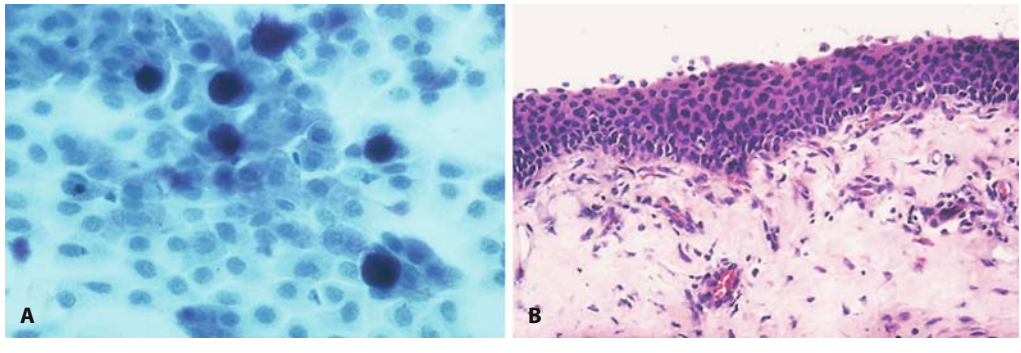

Fig. 3.10. **A** Impression cytology from surface of cornea with stem cell deficiency and a fibrovascular pannus showing goblet cells. PAS stain, ×400. **B** Biopsy of fibrovascular pannus showing multilayered epithelium, vascularisation and intraepithelial lymphocytes along the basal layers

Summary for the Clinician

- Diagnosis of limbal stem cell deficiency
  - Essentially clinical
  - Impression cytology – goblet cells on cornea pathognomic
  - Biopsy – multilayered epithelium, intraepithelial lymphocytes, vessels
  - Vimentin and CK 19 positive cells in central cornea (normally present in peripheral cornea and limbus)

3.5 Limbal Transplant Surgery

3.5.1 Principles

Management of stem cell deficiency can be considered in the following steps:

After Acute Injury. When a patient presents after an acute insult it should be ascertained whether the involvement of the limbus is partial or total. This can be done by use of fluorescein stain and slit lamp examination. If partial, appropriate medication required for the underlying cause and to control inflammation should be initiated. The eye should be examined at 24- or 48-h intervals and the process of re-epithelialisation observed. If this is occurring from the remaining intact limbal epithelium [21], this should be encouraged and any attempt at re-epithelialisation from the conjunctival epithelium should be discouraged by sequential sectoral conjunctival epitheliectomy (SSCE, see below) [14, 15]. If total, allow the cornea to be covered by conjunctival epithelium, if possible, before contemplating surgical intervention. This may take several days. The guiding principle should be that corneal epithelial cover for cornea and conjunctival epithelial cover for conjunctiva is the ideal end result but conjunctival epithelial cover for cornea is preferable to no epithelial cover to cornea.

In Established Cases. The principles underlying surgical procedures involving limbal stem cells are firstly to expand the corneal epithelial sheet derived from any existing sector of limbus in the affected eye. This can be achieved by SSCE (see below) [14, 15], especially if the cornea is partially covered by a layer of thin, metaplastic, conjunctivalised epithelium. If no healthy sector of limbus is available in the affected eye and if the other eye is normal with a positively documented absence of involvement in the original injury, autologous limbal transplantation should be considered. If the other eye is also affected or the underlying condition is a systemic illness such as Stevens-Johnson syndrome, allografts from a living related donor or from a cadaver donor should be considered. In the acute stage of limbal stem cell deficiency, for example acute chemical burns, auto-limbal or living related donor limbal transplants should be avoided at all costs. The chances of the transplanted material becoming caught up in the inflammatory and scarring process are high with loss of a valuable resource for future reconstruction. Use of auto or living related donor tissue, if available, should be attempted in quiet eyes. All the above procedures can be complemented with amniotic membrane transplantation. Penetrating keratoplasty may be combined with or following any of these procedures.

Limbal transplantation involves taking a lamellar strip of limbal tissue, usually with some adjacent peripheral cornea and/or conjunctiva and transplanting it to a suitably prepared bed in the host eye. Sutures are usually required to keep the donor graft in place.

3.5.2 Preoperative Considerations

All associated lid abnormalities, intraocular pressure problems and presence of cataract should ideally be dealt with prior to undertaking ocular surface restorative surgery. Symblepharon correction with amniotic membrane or buccal mucosa graft should also precede stem cell grafting. At times, if a corneal graft procedure is being contemplated at the time of stem cell grafting, it can be combined with cataract extraction and lens implantation. When an intumescent cataract is associated with raised pressure, corneal grafting may become a necessity if

a dense fibrovascular pannus or corneal scar precludes visualisation of the interior of the eye.

Patients with limbal SC deficiency and conjunctivalised corneal surface tend to manifest persistent chronic inflammation. Stem cell grafts do not perform well in the presence of inflammation and can be destroyed by the inflammatory and scarring processes. Ideally inflammation should be controlled and the eye rendered as quiet as possible with the use of topical and systemic steroids or other immunosuppressants which may become necessary in some conditions such as Stevens-Johnson syndrome and ocular cicatricial pemphigoid.

Most stem cell grafts do not survive in a dry (eye) environment. At times the injurious insult resulting in stem cell deficiency also results in a severe dry eye state. In such situations, if topical lubricants including autologous serum drops, punctal occlusion and buccal mucosa grafts do not restore adequate moisture to the ocular surface, a keratoprothesis procedure should be considered.

Summary for the Clinician

- Treatment algorithm
- General principles:
  - Manage underlying factors, e.g., chronic inflammation, contact lens wear, topical medications
  - Topical lubrication
  - All associated problems, e.g., raised pressure, conjunctival adhesions, lid malpositions, should be addressed before undertaking ocular surface reconstruction
  - Limbal transplants do not perform well in dry eyes
- In acute limbus injury:
  - If partial, i.e. some limbus is surviving – allow corneal epithelialisation to occur from limbus derived cells – SSCE
  - If total:
    a) Allow conjunctival epithelium to grow onto cornea
    b) Transplant sheet of ex vivo expanded limbal epithelial cells
    c) Avoid use of autologous or living related donor tissue until acute inflammation is well under control
- In established cases:
  - Treat eye lid problems, glaucoma and conjunctival adhesions first
  - Partial or total
  - Partial:
    a) Visual axis not involved: symptomatic, lubricants of SSCE
    b) Visual axis involved: SSCE
    c) Dense fibrovascular pannus: sector limbal transplant

    Total:
    a) Unilateral: auto-limbal transplant
    b) Ex vivo expansion of autologous limbal cells
    c) Bilateral: allo-limbal transplant
    d) Ex vivo expansion of cells (living related, living non-related, cadaver)
    e) Amniotic membrane and autologous serum drops as adjuncts
    f) Allo-transplants require systemic immunosuppression

3.6 Surgical Techniques

3.6.1 Sequential Sector Conjunctival Epitheliectomy (SSCE) [14, 15]

(Figs. 3.11, 3.12)

In cases with partial, mild to moderate conjunctivalisation of the cornea, without significant fibrovascular pannus, removal of the conjunctivalised epithelium is all that is required. This can be achieved at the slit lamp under topical anaesthesia, using a crescent blade or a surgical knife. It is important to remove all conjunctival epithelium, especially along its line of contact with the remaining corneal epithelium. Following removal of conjunctival epithelium from the corneal surface, it is important to closely monitor the patient to ensure that the denuded surface is re-epithelialised by cells derived from the remaining corneal epithelial sheet, i.e. limbal derived cells and not by conjunctival cells. This can be effected by repeatedly debriding (sequential epitheliectomy) any conjunctival epithelium that encroaches upon the limbus until

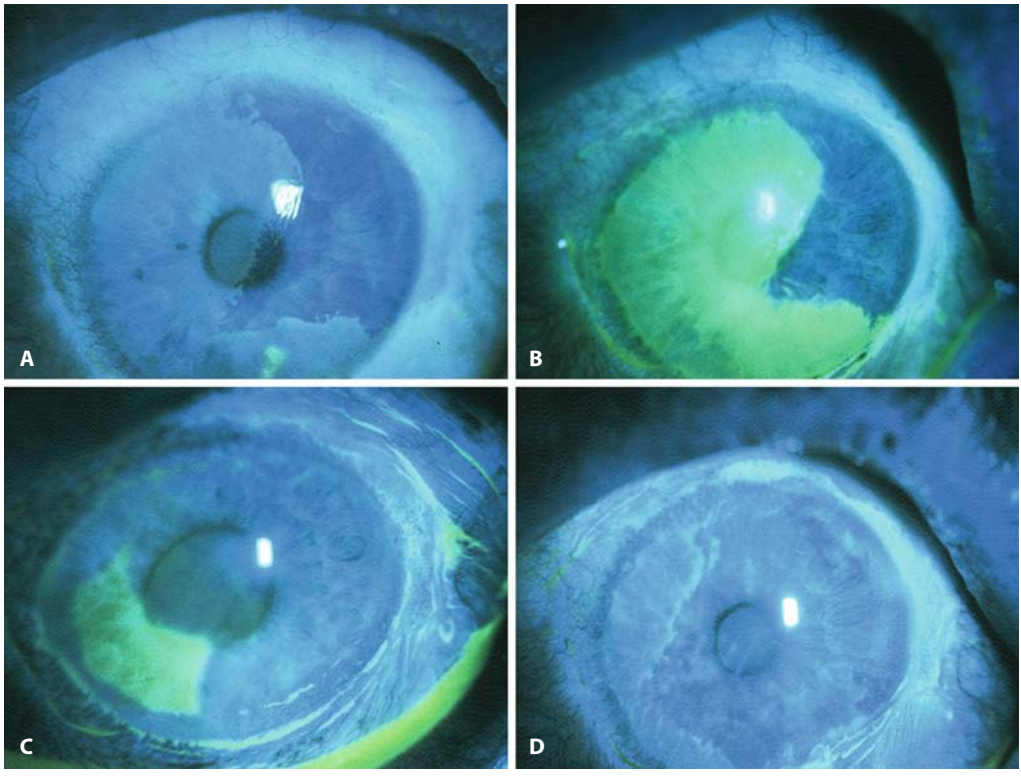

Fig. 3.11 A–D. Sequential sector conjunctival epitheliectomy (SSCE, H.S. Dua). **A** Conjunctivalisation of the cornea involving the visual axis following chemical injury. The demarcation between the two phenotypes of cells is clearly visible. **B** Appearance immediately after removing the abnormal epithelium (epitheliectomy). **C** The corneal epithelial sheet is migrating across the surface but the conjunctival epithelium too has started to re-encroach on the cornea. **D** After complete healing, the visual axis is now covered by healthy corneal epithelium. A new line of contact between conjunctival and corneal epithelium is established (fluorescein stained anterior segment photographs). The patient's vision improved from 3/18 to 6/9 (with permission from *Br J Ophthalmol:* Dua HS 1998; 82:1407–1411)

the limbus and corneal surface is re-populated by limbal epithelium derived cells.

In cases where only 1 or 2 clock hours of limbal epithelium is surviving, it may be appropriate to attempt re-epithelialisation of the visual axis only, with limbal derived cells. An area corresponding to the visual axis is debrided off its conjunctival epithelial cover and re-epithelialisation with limbal derived cells is achieved. This has the theoretical advantage of not overstressing the small remaining sector of limbal 'stem' cells. This technique of SSCE can also be usefully combined with limbal transplant to allow cells derived from transplanted limbal tissue (auto or allo) to re-populate the host corneal surface without 'contamination' from conjunctival epithelium (see below).

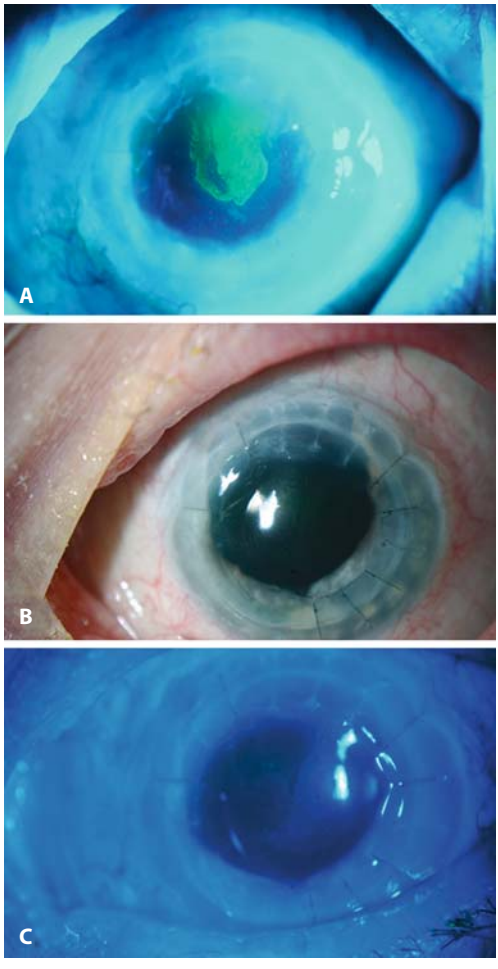

Fig. 3.12. A Conjunctivalisation of the superior cornea involving the visual axis. **B, C** After SSCE without and with fluorescein stain, respectively. The visual axis is now clear

3.6.2 Auto-limbal Transplantation

(Figs. 3.13, 3.14)

In patients where total stem cell deficiency affects only one eye, an auto-limbal transplant procedure is the ideal option [6, 19, 42, 48, 49, 88, 89]. It is important, however, to be absolutely certain that the donor eye was not involved at the time of the initial injury. In unilateral manifestations of systemic diseases, harvesting tissue from the apparently normal eye is not recommended.

The surgical technique consists of the following steps (the author's [19] modified technique is described): (a) a 16-mm Flieringa ring is sutured in place when the procedure is to be combined with a corneal graft (and lens extraction with implant). A 360° peritomy is first performed in the recipient eye. (b) The fibrovascular pannus covering the corneal surface is dissected off at a suitable plane. Any bleeding points are individually cauterised with light diathermy. (c) The donor tissue consisting of corneal-limbal-conjunctival explants is harvested from the contralateral normal eye. Two explants, corresponding to 2 clock hours (11–1 o'clock and 5–7 o'clock) and consisting of a very narrow strip (1 mm or less) of peripheral cornea, limbus and 3 mm of bulbar conjunctiva, are harvested. The conjunctival area to be removed is marked with a surgical marker pen. The conjunctiva is incised superficially with a pair of scissors and dissected in a superficial plane up to the limbus. An angled bevelled blade

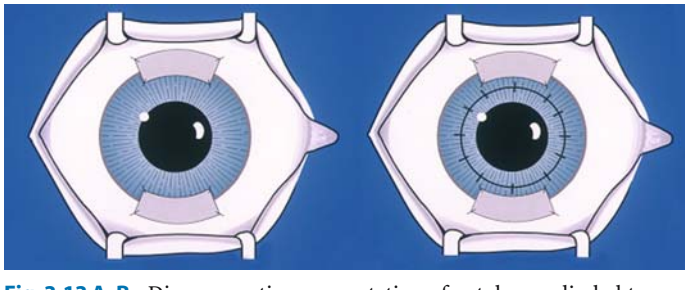

Fig. 3.13 A, B. Diagrammatic representation of autologous limbal transplantation. **A** Positioning of explants on recipient limbus at the 12 and 6 o'clock positions without or with (**B**) a corneal graft (with permission from *Br J Ophthalmol:* Dua HS, Azuara-Blanco A 2000; 84:273–278)

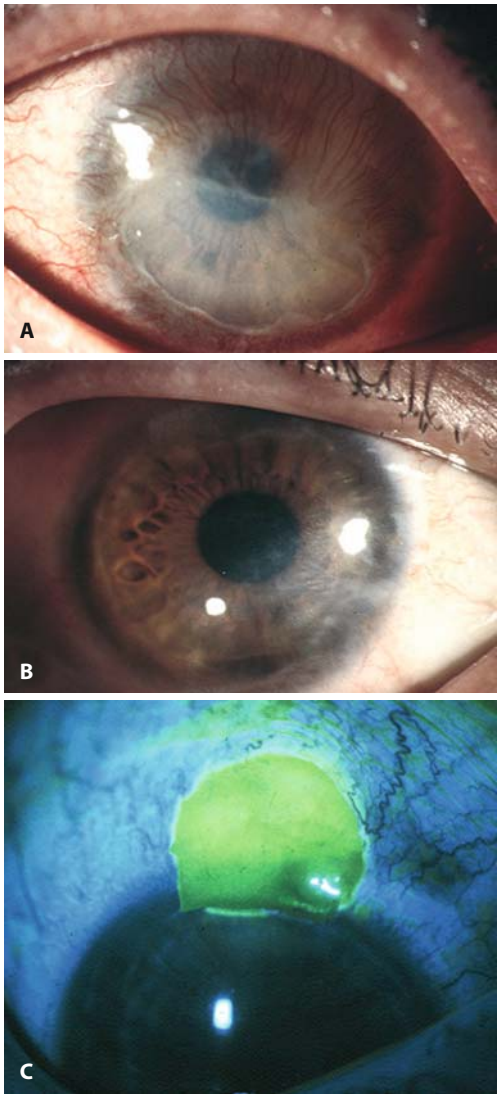

Fig. 3.14 A–C. Auto-limbal transplantation. **A** Preoperative persistent epithelial defect following an alkali (cement) burn as shown in Fig. 3.8. **B** Postoperative status after auto-limbal transplants at the 6 and 12 o'clock positions. The patient's eye is stable over 5 years postoperatively (with permission from *Br J Ophthalmol:* Dua HS, Azuara Blanco A 2000; 84:273–278. **C** Donor site for autologous limbal transplant, stained with fluorescein. Note that the central edge of the removed tissue needs to extend just central to the limbal vascular arcade

is used to (lamellar) dissect the corresponding limbal area extending into peripheral cornea to just inside (central) to the vascular arcade. (d) Suitable beds may be prepared at the superior and inferior limbus of the recipient eye by using the excised explants as templates to mark the area to be prepared. This is not always essential. (e) The donor tissue is then sutured onto the recipient eye with two interrupted 10-0 nylon sutures at the corneal margin and two along the scleral edge of the explant. Care should be taken not to bury the knots in the explant tissue as this could strip the explants off when attempting to remove the sutures in the postoperative period. At times the knots may be left unburied to facilitate removal. The conjunctiva of the recipient eye is then approximated to the donor conjunctiva with interrupted 8-0 Vicryl sutures (absorbable), taking a bite into episclera. (f) When a penetrating keratoplasty is also required, this is performed after the limbal explants are first sutured in place. (g) A bandage contact lens is placed on the cornea and subconjunctival antibiotics and corticosteroids are injected at the end of the procedure.

Adjunctive use of amniotic membrane can be made either as a graft to provide a suitable bed for limbal explant derived epithelial cells to grow on the cornea and/or as a patch to prevent conjunctival epithelial cells from extending onto the cornea and admixing with the limbal explant derived cells (see below).

3.6.3 Allo-limbal Transplantation

3.6.3.1 Living Related Donor

When a living related donor, who is tissue matched to the recipient, is available, tissue is harvested from one donor eye and used on the recipient eye exactly as described above for auto-limbal transplantation [10, 70].

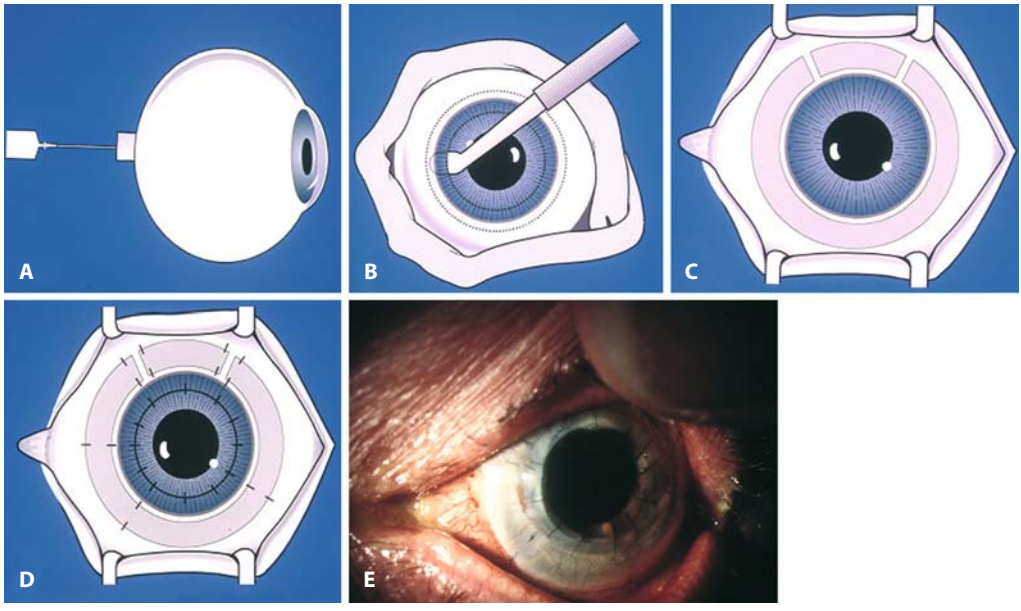

Fig. 3.15 A–E. Diagrammatic representation of allo-limbal transplantation. **A** Injection of air to firm the donor globe. **B** Harvesting the limbal circumference from the donor globe. **C** Positioning of explants on recipient limbus without **C** or with **D** a corneal graft. As the donor explant is placed slightly peripheral to the recipient limbus, more than one donor may be required as shown. **E** Combined allo-limbal transplant and corneal graft (with permission from *Br J Ophthalmol:* Dua HS, Azuara-Blanco A 1999; 83:414–419

3.6.3.2 Cadaver Donor

In most instances, limbal tissue is obtained from cadaver donor eyes [16, 41, 42, 48, 81–83, 88, 89, 91]. In such an event, tissue matching is not usually practical. In the author's protocol, a pair of 'fresh' donor eyes is used within 48 h of death. Donor eye retrieval should be done within 24 h of death and surgery within the next 24 h. Donor age of less than 50 years is preferred. 'Fresh' and 'young' donor eyes are preferred because the success of the procedure depends on the transplantation of healthy limbal stem cells.

The surgical technique consists of the following steps (the author's technique [16] is described) (Fig. 3.15): Donor limbus tissue is prepared before the patient is anaesthetised. (a) The donor eyeball is inflated with air (1–2 ml), injected through the stump of the optic nerve, to make the globe firm. (b) The globe is fixed on a Tudor Thomas stand. A vacuum (or manual) trephine with a diameter 3 mm smaller than the corneal diameter (i.e., average of vertical and horizontal corneal diameter) is used to trephine the central donor cornea to one-fourth to one-fifth of the stromal depth (approximately 150 µm). Proper centration is important to ensure that a uniform width of peripheral cornea is obtained. (c) Superficial lamellar dissection of the peripheral cornea is then carried out using an angle bevelled blade, and extended into the sclerocorneal junction and 1 mm beyond, into sclera. Approximately 1–2 mm of donor conjunctiva, if present, is maintained. The dissected tissue is divided at one point and excision completed with a curved scissors, by cutting along the outer circumference of the dissected tissue. The limbal tissue to be grafted thus consists of an open ring of peripheral corneal and limbal epithelium (and conjunctival epithelium at places), and superficial corneal, limbal and scleral stroma. (d) Preparation of the recipient eye is similar to that described for auto-limbal transplantation except that a 'bed' is not prepared to receive the limbal

ring explant. The 'open ring' of donor tissue is placed on the host limbus and sutured with interrupted 10-0 nylon sutures at the corneal and scleral margin. Six to eight sutures are first passed along the inner (corneal) edge of the donor tissue and partial thickness of host corneal stroma. A similar number of sutures are then passed directly opposite to the inner sutures, along the outer (scleral) edge of the donor tissue. These are anchored to the superficial sclera of the host. The tension on these sutures determines the final tension on the inner sutures. The knots are trimmed and buried. (e) This method invariably leaves a small gap (approximately 5–8 mm) between the two ends of the donor tissue ring (superiorly). This is filled with a piece of donor limbal tissue, cut to size, harvested from the other eye of the same donor. This piece usually requires a couple of additional sutures along either edge. (f) The host conjunctiva is approximated to the scleral edge of the transplanted limbal ring with interrupted 8-0 Vicryl sutures (absorbable). (g) A penetrating keratoplasty if required at the time of surgery is performed after the limbal ring is sutured in place. The donor graft for penetrating keratoplasty (usually 7–7.5 mm) is obtained from the central cornea of the donor whole globe. (h) A bandage contact lens is placed on the cornea and subconjunctival antibiotics and corticosteroids are injected at the end of the procedure.

Adjunctive use of amniotic membrane can be made either as a graft to provide a suitable bed for limbal explant derived epithelial cells to grow on the cornea and/or as a patch to prevent conjunctival epithelial cells from extending onto the cornea and admixing with the limbal explant derived cells (see below).

3.6.4 Adjunctive Surgery

3.6.4.1 Amniotic Membrane Grafts

The amniotic membrane serves as a useful adjunct to stem cell grafting [2, 17, 26, 50, 77, 90]. It is commonly deployed to provide a suitable substratum for the transplanted limbal graft derived epithelial cells to migrate on and form adhesion complexes. After excision of the fibrovascular tissue, if the underlying host bed is found to be irregular and scarred, use of a 9- or 10-mm disc of amniotic membrane, epithelial side up, can provide a suitable substratum for the transplanted limbal derived epithelial cells to migrate upon. The amniotic membrane can also be deployed as a biological bandage to the

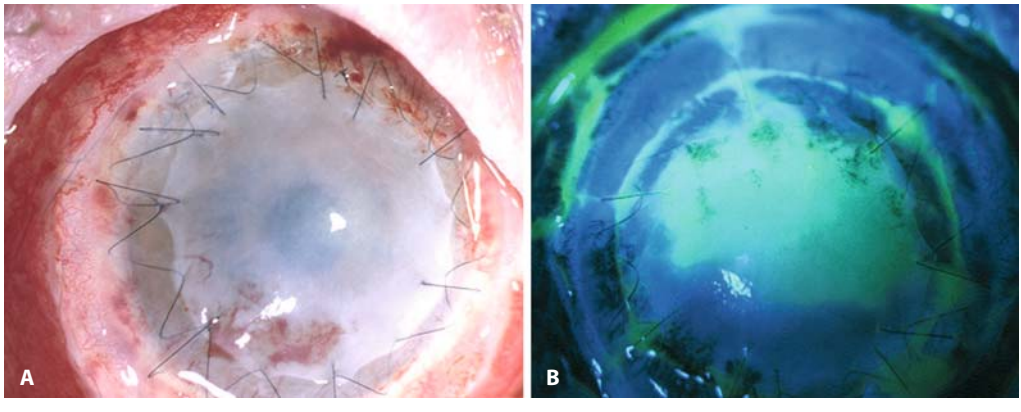

Fig. 3.16 A, B. Use of double amniotic membrane to prevent admixture of conjunctival and corneal epithelium on the corneal surface. **A** The inner membrane disc is sutured with the epithelial surface up to act as a graft and substrate for the cells to grow on, and the outer membrane, epithelial side down, acts as a patch. **B** Regenerating cells from the peritomised conjunctiva are seen growing on the outer membrane. In the absence of the outer membrane (patch) these would have encroached on the corneal surface

denuded corneal stroma, allowing epithelialisation to occur beneath it whilst trapping inflammatory cells and downregulating inflammation and scarring at the same time. Two amniotic membranes, one inner one serving as a graft and one outer membrane serving as a patch, can be simultaneously applied. The outer membrane is sutured such that its edges are tucked under the peritomised conjunctiva. Conjunctiva derived epithelium then grows on the outer membrane and is prevented from admixing with limbus-derived epithelium that is spreading onto the corneal surface. This technique was developed by the author and is regularly employed [26] (Fig. 3.16). It avoids the need for SSCE postoperatively. The outer membrane falls off or can be removed in 10–14 days. For further details on amniotic membrane, see Chap. 2 on amniotic membrane transplantation.

3.6.4.2 Ex Vivo Expansion of Limbal Cells

Limbal 'stem cell' transplantation can also be carried out by ex vivo expansion of limbal epithelial cells, either directly or on a substrate of fibrin, collagen or amniotic membrane [26, 62, 74, 75, 87].

3.6.4.3 Corneal Grafts

Lamellar or full thickness corneal grafts can be combined with auto- or allo-limbal transplantation. This may be necessary when the cornea damage is severe and when it is considered that the host corneal bed will not support a healthy epithelium despite use of an amniotic membrane. In general terms, a definitive corneal graft for visual purposes should be deferred until ocular surface epithelial integrity has been restored by limbal transplantation. However, in a recent study using limbal tissue remaining after keratoplasty from organ cultured corneoscleral discs, we have shown that such tissue (where the death to enucleation time and the time lapse between enucleation to placement in organ culture is short and where the donor is relatively young) retains good proliferative capacity for up 30 days in storage (V. Shanmuganathan, submitted to Br J Ophthalmol 2005). This offers the opportunity to carry HLA typing and matching and also allows for depletion of antigen presenting Langerhans cells. It should therefore be possible to use organ culture preserved corneoscleral discs for simultaneous allo-limbal transplant and keratoplasty with reduced risk of immune mediated rejection.

3.6.5 Postoperative Treatment

Topical preservative-free antibiotic drops such as chloramphenicol 0.5 % are used four times a day for the first month. Topical preservative-free steroid drops such as prednisolone acetate 1 % are used four times a day for the first 8–12 weeks, and slowly tapered during the ensuing weeks. A low dose of topical corticosteroids (one drop per day) is maintained unless elevation of intraocular pressure occurs. Autologous serum eyedrops (20 %) [52, 92, 93] are given hourly until the epithelialsation is complete, usually in 7–10 days. Preservative free artificial tears are then instituted. It is important to closely monitor the re-epithelialisation process until completed. Any attempt by conjunctiva derived cells to encroach onto the corneal surface should be thwarted by SSCE, until the periphery (limbus) of the host cornea is re-epithelialised by limbus derived cells.

All patients undergoing allo-limbal transplantation also need systemic immunosuppression. Besides steroids, azathioprine, cyclosporin A, rapamycin, mycophenolate mofetil and tacrolimus (FK506, Prograf) have been used [11, 72, 78, 97]. Theoretically, immunosuppression should be continued almost indefinitely. The author has used cyclosporin A and of late FK506 up to 18 months postoperatively [78]. Attempts to reduce or stop the drug have resulted in limbal and/or corneal graft rejection episodes. Fortunately, the dose required to prevent or control rejection episodes is very low (2–8 mg/day, maintaining a blood trough level of 1–12 µg/l). Serious side effects, though they occur, are not very common, but require constant monitoring of patients and measures of kidney and liver functions. It is good practice to involve a clinical

immunologist or a physician versed in immunosuppressive therapies in the management and monitoring of these patients.

Successful stem cell grafting is a team effort involving the corneal and oculoplastic surgeons in close cooperation with the clinical immunologist. Often multiple surgical procedures are required and visual outcome, though useful from the patients' viewpoint, may be limited. The threat of limbal graft rejection is real and considerable. The all-important question of the duration of systemic immunosuppression remains to be answered. Not all long-term DNA tracking studies on recipient eyes have been able to show presence of donor derived cells even in the presence of a healthy corneal surface [38–40, 71, 76]. This would suggest that restoration of a normal surface and 'microenvironment' may allow host stem cells, either surviving limbal stem cells or bone marrow derived stem cells, to repopulate the surface. In this situation long-term immunosuppression would not be a necessity. On the other hand, long-term follow-up studies have also demonstrated that the outcome of allo-limbal transplant is not as good as that of auto-limbal transplant. This may reflect chronic 'immune mediated' damage and attrition of the transplanted limbal stem cells or the relative 'freshness' of auto grafts, conferring upon them a survival advantage.

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Limbal Stem Cell Culture

4

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Core Messages

- The stem cells of the corneal epithelium seem to be located in the limbal basal layer and are the ultimate source of constant epithelial renewal
- Ex vivo expansion of limbal epithelial stem cells has been developed mainly to circumvent potential complications, for both the recipient and the living donor, arising from standard conjunctival limbal transplantation
- With the appropriate procurement and preservation, human amniotic membrane can be used as a biological substrate without viable and proliferative active cells but with the advantages of basement membrane and as a source of beneficial biological factors
- To examine the epithelial phenotype, immunostaining techniques are used with a panel of monoclonal antibodies to mucins and keratins
- The preparation of human amniotic membrane and the culture of explanted tissue must be performed by specially trained personnel in a stem-cell management laboratory
- Preliminary clinical experience is highly encouraging, not only regarding final clinical outcome but also because of the flexibility of the technique, the increase in possible cases to be treated and the reduced need for systemic immunosuppression
- Ocular surface reconstruction is becoming a much more staged than artistic approach, and we are of the opinion that its final clinical results will improve strongly in the near future especially with the help of the new cell bioengineering technique

4.1 Introduction

The stem cells of the corneal epithelium seem to be located in the limbal basal layer and are the ultimate source of constant corneal epithelial renewal. A new strategy of treating limbal stem cell deficiency is to transplant a bioengineered graft by expanding limbal epithelial stem cells ex vivo on amniotic membrane.

Ex vivo expansion of limbal epithelial stem cells has been developed to circumvent potential complications both for the recipient and the donor arising from conjunctival limbal autograft transplantation. This technique expands limbal epithelial progenitor cells from a small biopsy using a 3T3 fibroblast feeder layer [13] or amniotic membrane. Using the amniotic membrane to constitute such a composite graft, successful reconstruction of the normal corneal surface has been achieved in several human studies with partial or total limbal stem cell deficiencies [8, 9].

As previously described, amniotic membrane, the innermost layer of the fetal or placental membrane, consists of an epithelial monolayer, a thick basement membrane, and an avascular stroma (Fig. 4.1). With appropriate procurement and preservation, amniotic membrane can be used as a biological substrate without viable and proliferative active cells [8]. It is thus non-immunogenic and therefore does not require immunosuppression when used for transplantation. The amniotic membrane stroma also contains quantities of growth factors, various antiangiogenic and anti-inflammatory proteins and natural inhibitors to various proteases.

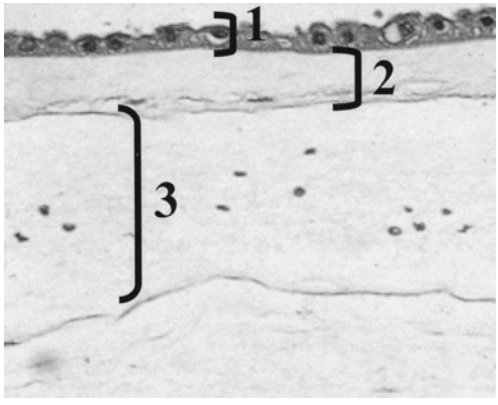

Fig. 4.1. Human amniotic membrane. Cross-section reveals a cuboidal epithelial monolayer (*1*), a thick basement membrane (*2*) and an avascular stroma (*3*)

Recently, Grueterich et al. identified the culture environment that will favor the maintenance of the stem cell containing the limbal epithelial phenotype [9]. This is achieved by culturing limbal explants on an intact human amniotic membrane, which retains the devitalized amniotic epithelium, without the use of a 3T3 fibroblast feeder layer. The expanded epithelium on intact amniotic membrane adopts a limbal epithelial phenotype whereas that on denuded amniotic membrane reveals a corneal epithelial phenotype.

4.2 Epithelial Phenotype

To examine the epithelial phenotype, immunostaining techniques are used with a panel of monoclonal antibodies to mucins and keratins. In normal ocular surface epithelia, AE5 antibody, which recognizes K3 keratin, stains the suprabasal limbal epithelium and the full thickness of the central corneal epithelium, but not the conjunctival epithelium. AE5 antibody stains suprabasal human limbal epithelial cells (HLEC) cultured on amniotic membrane for 13–21 days. Immunostaining for K12 keratin by AK2 antibody is also positive for limbal suprabasal epithelial cells and for the full thickness of the corneal epithelium, but negative for the conjunctival epithelium in vivo. HLEC on amniotic membrane are negative for AK2. K14 keratin is expressed in the basal and suprabasal cell layers of the conjunctival limbal and peripheral corneal epithelium, but is found predominantly in the basal epithelial cells of the central corneal epithelium. HLEC cultured on amniotic membrane showed full thickness staining to K14 keratin after 13–21 days of culturing. MUC5AC (Mucina 5AC) recognizes conjunctival goblet cell secreting mucins and stains conjunctival goblet cells in vivo. MUC5AC do not stain any cells cultured on amniotic membrane. Collectively, these results indicate that the resultant phenotype of HLEC grown on amniotic membrane retains a limbal origin, is predominantly basal epithelial cells, and remains undifferentiated (Table 4.1) [9].

4.3 Preparation of Human Amniotic Membrane

Amniotic membrane tissue can be obtained, processed, and preserved frozen as reported by Lee and Tseng [9] at the Eye Bank. The amniotic membrane measuring 5×5 cm, after thawing and washing, is tightened on a 3-cm culture plate with the basement membrane side up.

Table 4.1. Immunostaining expression of keratins K3, K12 and K14, and MUC5AC of corneal epithelium, conjunctival epithelium, limbal epithelium and HLEC grown on amniotic membrane cultures

Immunostaining	Corneal epithelium	Conjunctival epithelium	Limbal epithelium	HLEC
K3 keratin	Positive	Negative	Positive suprabasal	Positive suprabasal
K12 keratin	Positive	Negative	Positive suprabasal	Negative
K14 keratin	Positive basal	Positive	Positive	Positive
MUC5AC	Negative	Positive	Negative	Negative

4.4 Culture of Explanted Tissue

A piece of limbal tissue measuring 1×2 mm containing epithelial cells and part of the corneal stroma is obtained for ex vivo culture. The tissue source is the contralateral eye in autologous transplantation or a living donor in related allotransplantation. Cadaveric donor is also a source of limbal tissue for conditions in which both eyes are affected and no living donor is available.

The obtained tissue is placed with Ham's F12 medium containing 50 µg/ml gentamicin and 1.25 µg/ml amphotericin B until it is processed. Limbal tissue is exposed for 5 min to Dispase II (1.2 U/ml in Mg^{2+} and Ca^{2+} free Hank's balanced salt solution, HBSS) at 37 °C under humidified 5% CO_2. The explants are then cultured in DMEM medium, which is a 1:1 mixture of DMEM and Ham's F12 medium containing 5 ng/ml epithelial growth factor (EGF), 5 mg/ml insulin, 5 mg/ml transferrin, 5 ng/ml sodium selenite, 0.5 mg/ml hydrocortisone, 30 ng/ml cholera toxin A, 0.5% dimethylsulfoxide (DMSO), 50 mg/ml gentamicin, 1.25 mg/ml amphotericin B and 5% autologous serum, at 37 °C under 5% CO_2 and 95% humidity. The medium is renewed every 2–3 days [2]. For allogeneic related transplantation, donor serum is used and for allogeneic non-related transplantation AB tested blood bank serum is employed. The limbal epithelial cell explants are plated onto the basement-membrane side of the amniotic membrane, placed in the center. The extent of each outgrowth is monitored with a phase contrast microscope. During the expansion phase the limbal epithelial outgrowth exhibits a compact and uniform cell layer (Fig. 4.2).

The culture is maintained for 2–3 weeks, by which time the epithelial cells have grown and spread to form a cell layer that covers an area 2–3 cm in diameter. Every week bacteriological testing is performed to assess microorganism contamination. The mycoplasma content test and Gram's test are performed 24 h before transplantation.

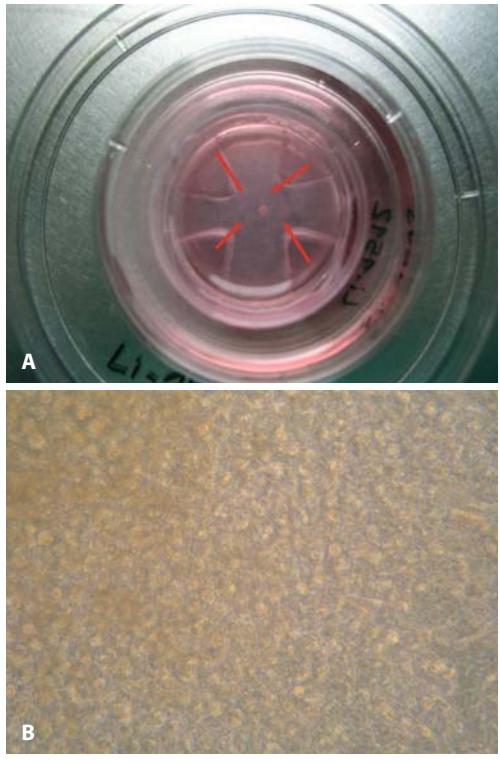

Fig. 4.2 A, B. Ex vivo expansion of limbal stem cells from limbal biopsy. **A** Culture plate with the tightened amniotic membrane and a limbal biopsy placed in the center (*arrows*). **B** Phase-contrast microscopy of the expanded cells reveals a monolayer of epithelial cells of small and uniform size, ×400

4.5 Tissue Procurement

The safety of biological medicinal products relies on rigorous control of each of their components. Human tissue should be handled according to European Commission Directive 2003/63/EC [8]. Corneoscleral tissue from human donor eyes is obtained after proper informed consent in the case of a living donor or from an authorized Eye Bank for cadaveric donors. Human amniotic membrane should be obtained after elective cesarean delivery when blood-borne microorganisms such as human immunodeficiency virus, hepatitis virus type B and C, and syphilis have been excluded by sero-

logic tests. Hepatitis virus type C and human immunodeficiency virus should additionally be excluded by means of PCR.

Summary for the Clinician

- **Immature corneal cells are located in the epithelial basal layer of the limbus**
- **Ex vivo expansion of limbal epithelial cells is a technique that avoids potential complications to the donor and the recipient eye**
- **Immunohistochemical features allow us to distinguish immature from mature cells on the cultured cells**
- **Human amniotic membrane provides the limbal epithelium with an adapted microenvironment and a solid basal layer**
- **Amniotic membrane epithelium favors limbal stem cell growth**
- **Biological medicinal products rely on rigorous control of each of their components and should be handled according to the specific laws of each country**

4.6 Preliminary Clinical Experience

In standard ocular surface reconstruction, amniotic membrane transplantation in the cornea and conjunctiva provides a basement membrane, especially when the ocular surface is highly irregular, and a source of biological factors to enhance and improve reepithelialization [3–7]. Once the amniotic membrane is sutured, we may proceed with our standard limbal transplantation over it.

Because sclerocorneal limbal tissue is highly vascularized with a high antigenic weight, we need to prescribe long-term systemic immunosuppression in most limbal transplantation cases (cadaver or relative donor).

Limbal stem cell culture is a new technique with obvious advantages over the other techniques used to restore ocular surface. The aim is to cause as little damage to the ocular surface as possible. Ex vivo expansion of corneal stem cells is a technique described previously [7, 8, 11, 13], and has been developed by several groups. It basically consists of taking a small piece of the donor eye limbus, expanding the cells in the laboratory over a piece of amniotic membrane and using them in a pathologic eye to restore the corneal epithelium. We prefer amniotic membrane because its basal membrane is very similar to corneal epithelial basal membrane [7, 11], and the epithelium of the amniotic membrane contains several growth factors that promote cellular proliferation [7]. The main advantage is that in nearly all cases we avoid immunosuppression, and cause minimal injury to the donor eye, so there is no risk of iatrogenous limbal deficiency in that eye.

Limbal deficiency must have been confirmed previously by clinical examination, impression cytology and, if possible, immunofluorescence on the affected eye.

The process starts with, after informed consent, the extraction of a limbal biopsy from the donor eye (the healthy eye of the same patient or a relative or cadaver donor eye). It must be done in the operating room, under sterile conditions and with great care being taken to avoid the conjunctival tissue, because we could expand other cells than corneal epithelial stem cells. The size of the biopsy must be as small as possible, but large enough to ensure cellular growth in culture (2 mm^2), with a depth of 100 µm, without blood vessels or conjunctival tissue. Once we have the biopsy, it must be taken to the laboratory as soon as possible, where it is treated as mentioned above, and then we need to wait for 2 or 3 weeks, depending on the cellular growth observed, before we can implant the amniotic membrane with the expanded cells on the eye. Every process which involves any manipulation of the tissue must be done under sterile conditions.

4.6.1 Principles for Taking the Biopsy

1. Avoid or eliminate the conjunctiva. Conjunctival epithelial cells grow easily in culture and interfere with corneal epithelium proliferation.
2. Perform the biopsy under sterile conditions.
3. Take a biopsy as small as 1–2 mm^2 and 100 µm in depth.

4. Take a biopsy from the superior limbus if possible, to ensure immature cells are included on it (the superior limbus contains more stem cells) [13]

We usually implant the tissue after a lamellar keratectomy is performed to eliminate any remains of conjunctival tissue over the cornea, suturing the graft, with the small biopsy included, over the corneal surface, then placing a therapeutic contact lens over the graft to preserve it and avoid blinking-trauma during the first 2 weeks. Postoperative treatment consists of topical steroids and antibiotics, autologous serum drops to promote epithelial growth, and immunosuppressive agents if the biopsy is performed on a relative or unknown donor.

In our series of seven patients transplanted, the only complication detected in the immediate postoperative period was corneal melting with perforation, which was solved with penetrating keratoplasty (PKP).

The most important purpose of this technique is to expand immature cells from the proliferative compartment of the limbus and keep them active for as long as possible, which ensures a healthy ocular surface, and allows us to perform other procedures to restore visual acuity such as PKP or lamellar keratoplasty

4.6.2 Advantages of Limbal Stem Cell Culture

1. In many cases our patient has enough healthy limbal area in at least one of the eyes for a small biopsy to be taken but not for a large (90°) standard biopsy to be done for standard transplantation.
2. Many close relatives (HLA matched if possible) will be amenable to a small biopsy but not to a large one with the known associated risks. In both (1) and (2), we might eliminate or strongly reduce systemic immunosuppression
3. In those cases where the donor tissue is from a cadaver, the main advantage of the culture is the density of viable cells at the time of transplantation compared with the fresh original piece of tissue.

On the other hand, one limitation is preoperative cell type determination. To assess limbal stem cell transplant success we do not manipulate the culture. Rather we implant the central area, where the graft shows the best growth, and we verify the particular biological and immunohistochemical characteristics of the cultured cells with the remaining piece of tissue after the surgery. In all cases limbal stem cell immunophenotyping is demonstrated.

- **Amniotic membrane transplantation on the cornea and conjunctiva contributes, in standard ocular surface reconstruction, as a basement membrane and as a source of biological factors**
- **In most standard limbal transplantation cases, we all need to prescribe long-term systemic immunosuppression**
- **The use of amniotic membrane as our "cell transporter" was decided upon because of our wide and long-term experience of using it in our standard ocular surface reconstructive techniques**
- **The most important purpose of our technique is to expand immature cells from the proliferative compartment of the limbus and keep them active for as long as possible**
- **We must keep in mind the main conceptual advantages of limbal stem cell culture in clinical practice**

4.7 Case Report

A 31-year-old Caucasian male with a story of bilateral caustication came to us in July 2002. At the first exploration of the eyes in early September 2002, the right eye showed visual acuity (VA), light perception, diffuse corneal conjunctivalization with central perforation and extensive superior symblepharon; in the left eye the VA was count fingers 10 cm, and diffuse corneal conjunctivalization with extensive inferior symblepharon was seen. Our case notes then read as follows:

12.09.02 Emergency PKP plus amniotic membrane as a patch OD

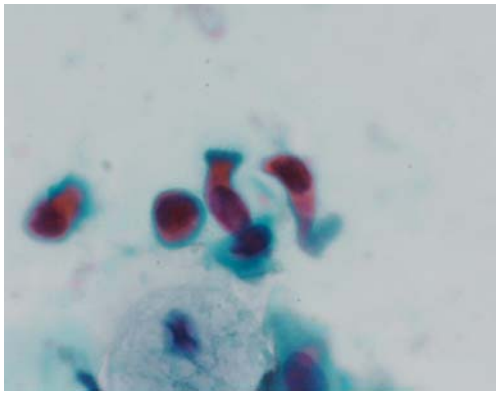

Fig. 4.3. Specimen from a limbal deficiency eye. Preoperative image, Papanicolaou's stain

10.12.02 Inferior symblepharon surgery OD
25.02.03 Superior symblepharon surgery OD
26.08.03 Inferior symblepharon surgery OS
08.03.04 (Fig. 4.3) Impression cytology OS (Fig. 4.5): only 2 h (10–12 o'clock) with healthy limbal tissue; healthy limbal area biopsy for culture on amniotic membrane
25.03.04 Lamellar corneal scar dissection and cultured cells on amniotic membrane sutured on the cornea – conjunctival surface OS
29.04.04 (Fig. 4.6) PKP + standard amniotic membrane transplantation + lateral temporary tarsorrhaphy
19.10.04 (Fig. 4.7) Sectorial conjunctival epitheliectomy as described by Dua [7] (area from 6 o'clock to 8 o'clock) and selective suture removal

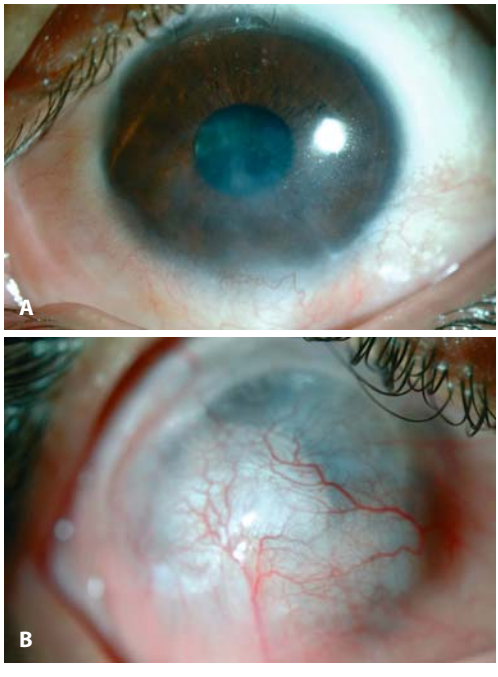

Fig. 4.4 A, B. Slit-lamp appearance of both eyes in March 2004. **A** left eye; **B** white cornea with vessels, right eye

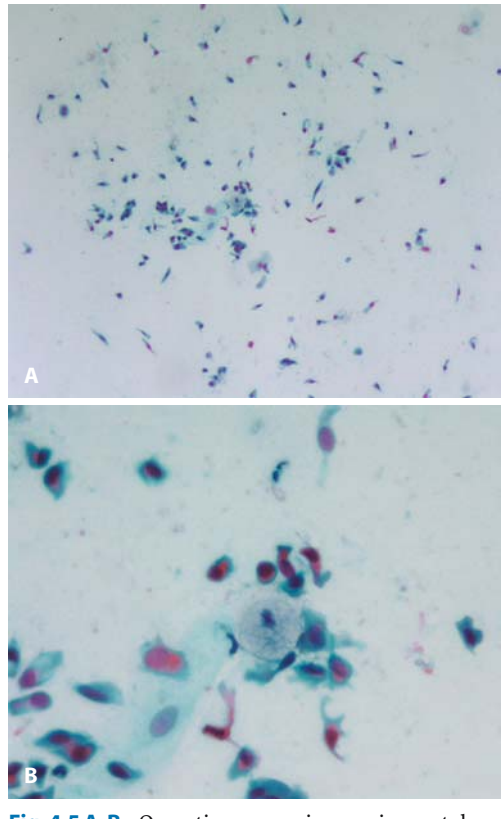

Fig. 4.5 A, B. Operating room impression cytology of the left eye. Note the presence in all the specimens of goblet cells characteristic of conjunctival epithelium

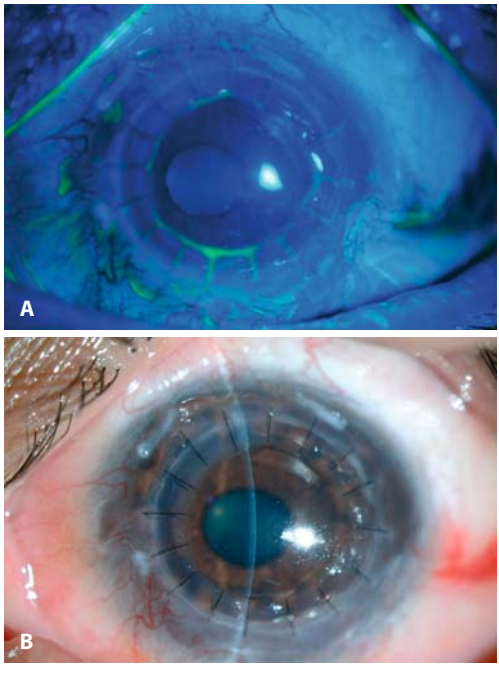

Fig. 4.6 A, B. Slit-lamp appearance of the left eye in March 2004

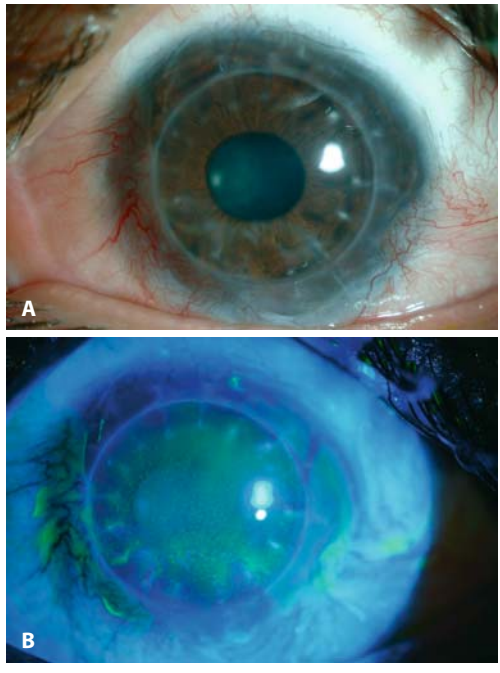

Fig. 4.7 A, B. Slit-lamp appearance of the left eye in September 2004. Note the diffuse epithelial toxicity (late fluorescein staining)

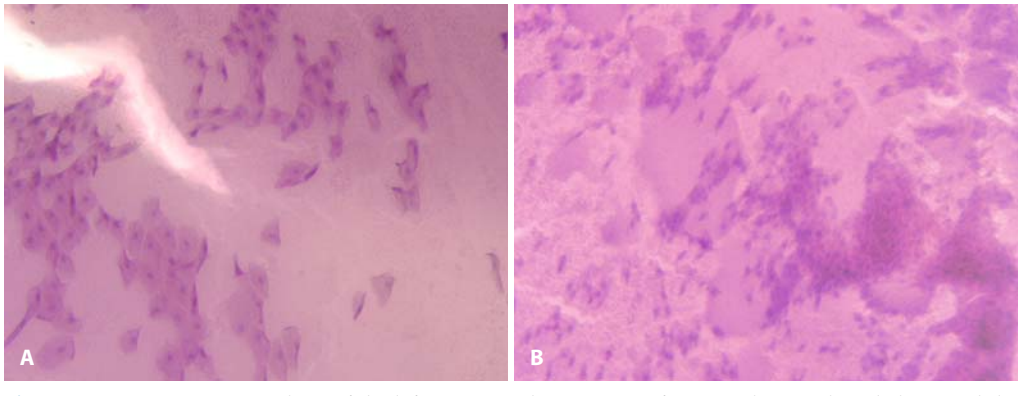

Fig. 4.8 A, B. Impression cytology of the left eye. Note the presence of a normal corneal epithelium and the absence of globet cells

16.11.04 Best corrected visual acuity (BCVA) OS 20/40 posterior subcapsular cataract and healthy ocular surface (Fig. 4.8)

The patient will receive cataract surgery on OS and right eye ocular surface reconstruction with a limbal biopsy of his left eye.

4.8 Future Standard Staging Approach for Ocular Surface Reconstruction

1. IOP control: shunt tube if necessary
2. Lids and conjunctival "cul de sac" reconstructive surgery
3. Limbal reconstruction: limbal stem cell culture will definitely improve the actual results of corneal reconstruction. We must remember that the presence of new conjunctival cells is also necessary in some cases such as pemphigoid or Stevens-Johnson syndrome
4. PKP or deep anterior lamellar keratoplasty if necessary
5. Keratoprothesis in those cases where stage 3 and 4 fails

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Deep Anterior Lamellar Keratoplasty

5

Gerrit R.J. Melles

Core Messages

- With current techniques, the clinical outcome of a lamellar keratoplasty may be similar to that after penetrating keratoplasty
- Compared to conventional penetrating keratoplasty, new lamellar keratoplasty techniques provide safer "closed-system" surgeries with less morbidity and better clinical outcomes
- The scope of surgical tools for lamellar keratoplasty has been expanded to provide feasible and adjustable techniques
- Since postoperative treatment may be the most challenging aspect in keratoplasty surgery, careful patient selection and psychologic preparation are important in achieving good results
- Especially in the patient population eligible for lamellar keratoplasty, the procedure may have major advantages for both the surgeon and the patient, such as longer graft survival, less aftercare and less dependency on the health care system
- Lamellar keratoplasty surgery may be slowly tending towards the use of custom-made transplants

5.1 Introduction

In the past few years, deep anterior lamellar keratoplasty (DALK) has seen renewed interest as an alternative to conventional penetrating keratoplasty. The introduction of several new dissection techniques, the optical visualisation of dissection depth during surgery, and the availability of various lasers may provide new possibilities for the management of anterior corneal disorders. In fact, we may be currently witnessing the most dramatic change in the concept of keratoplasty from being a conventional penetrating procedure towards that of custom-made corneal tissue replacements. Below, the most recent developments are summarised and the most important issues concerning DALK are discussed.

5.2 Main Drawbacks of Conventional DALK

Since the early 1900s, penetrating keratoplasty has been the procedure preferred by most surgeons for the treatment of corneal disorders. On average, the overall clinical result of penetrating keratoplasty seems rather poor and is often complicated by a high degree of astigmatism, suture related complications, and incomplete wound healing. Despite its advantages, i.e. less risk of intraocular complications and allograft rejection, a lamellar procedure is often considered troublesome, mainly because of the risk of perforation during surgery, the development of interface haze, and the time-consuming, tedious surgical technique.

5.3 Different Concepts

To overcome these problems, several techniques are now available with which to perform a controlled deep anterior lamellar keratoplasty pro-

cedure at a planned corneal depth with minimal risk of perforation and interface haze development.

- The creation of an optical interface by filling the anterior chamber with air allows the surgeon to visually control the dissection depth during the entire surgery. This allows the surgeon to choose the desired dissection depth. In cases in which a penetrating keratoplasty may have a bad prognosis, for example a patient with Down syndrome or recurrent herpes simplex virus keratitis, the surgeon may aim for a relatively shallow dissection depth to complete the procedure with a minimal risk of perforation.
- Instead of removing the anterior corneal tissue layer by layer, the cornea may be dissected to the desired depth at the first go. This saves time, and if a perforation occurs, it is in the very early phase of the surgery, so the procedure can be quickly converted to a penetrating keratoplasty.
- Instead of performing a blunt dissection, several instruments and medical devices as well as various lasers are currently available with which to obtain a regular and smooth dissection plane.

Summary for the Clinician

- **New lamellar keratoplasty techniques allow for a single stromal dissection at an optically controlled depth**
- **Improved instrument designs and various lasers allow the creation of a smooth recipient stromal bed**

5.4 Important Preoperative Considerations

Careful patient selection and patient preparation may in part determine whether the outcome of a deep anterior lamellar keratoplasty procedure is considered succesful both by the surgeon and the patient. The main parameters for patient selection are:

- Endothelial cell density. The good condition of the recipient endothelium is a prerequisite for a lamellar procedure. Although relatively infrequent, corneas with an anterior corneal disorder may also have a compromised endothelium, due to combined disease, the disease itself or prolonged medication (for example, combined dystrophies or herpes simplex virus keratitis/endotheliitis).
- Penetrating keratoplasty bad prognosis. In patients in whom a conventional penetrating procedure is contraindicated, complete visual rehabilitation is often not the primary goal. In these cases, a relatively shallow dissection depth (70–80 %) may be considered, which greatly reduces the risk of perforation during surgery, for example, extreme keratoconus in Down syndrome or long-standing recurrent herpes keratitis.
- Patient age. Young patients with an isolated corneal disorder like keratoconus may benefit the most from a lamellar procedure. The endothelial cell loss after lamellar keratoplasty has been found to show a similar pattern to that of the physiological cell loss in a virgin cornea, which may significantly improve the long-term expectation for graft survival. Since the integrity of the globe is better preserved in a lamellar procedure and the risk of wound rupture or dehiscence may be relatively low, a lamellar keratoplasty may give fewer restrictions to sports and other daily activities that are relatively important to young people. With a lamellar procedure, the risk of allograft rejection may also be greatly reduced, which may give fewer restrictions to people travelling to or living in countries with a less sophisticated health care system.
- Atopic constitution. The long-term results of any type of keratoplasty procedure may be relatively poor in patients with atopic disease or a concurrent facial skin disorder. In our series, the occurrence of relatively serious and long-standing complications such as persistent epithelial defects, suture infiltrates, and partial melts proved relatively frequent in these cases and difficult to manage.
- Alternative treatments. Several procedures are currently available that may allow for a visual outcome similar to a deep anterior lamellar keratoplasty, but that are more patient friendly. For example, amniotic membrane transplantation with subsequent con-

tact lens fitting often provides a useful visual acuity. Phototherapeutic keratectomy can be effective in the treatment of epithelial dystrophies. Intrastromal ring segments may give fairly good results in corneal thinning disorders. The femtosecond laser may also broaden the possibilities for replacement of specific corneal layers.

Summary for the Clinician

- **Important considerations prior to lamellar keratoplasty include assessment of the condition of the recipient endothelium, overall prognosis, patient age, presence of an atopic constitution, and less invasive treatments**

5.5 Psychological Preparation of the Patient

Once the decision to perform a deep anterior lamellar procedure has been taken, it is recommended that the expectations of the patient are appropriately modified. Since most patients are familiar with the outcome of cataract surgery, the surgeon may explain to the patient that current keratoplasty surgical techniques on average do not provide similar visual results.

Since a lamellar procedure always bears the risk of perforation and the need for conversion to a penetrating procedure, it should be explained to the patient that a lamellar keratoplasty will be attempted but that in the surgery has to be completed as a conventional penetrating procedure. Although the risk of perforation with some techniques may be as low as 5%, the patient then is less likely to perceive the surgery as 'a failure' in the event of a perforation, i.e. conversion to a penetrating procedure. The patient should also be informed that secondary surgery may be necessary shortly after the keratoplasty to position the donor properly. If the anterior chamber needs to be filled with air to manage a perforation, the patient anticipates on surgical aftercare, whereas such a minor secondary treatment may otherwise alarm the patient as it is quickly perceived as emergency surgery to save the eye.

In the Netherlands, keratoconus is the indication in approximately half of the patients eligible for deep anterior lamellar keratoplasty. A fairly large number of these patients may have a long history of uncomplicated contact lens wear up to the moment that the steepening corneal contour causes unacceptable contact lens discomfort. Since none of the currently available DALK procedures allows for a controlled restoration of the corneal contour, and postoperative contact lens fitting often greatly improves the final visual outcome, it may be recommended to inform the patient that the goal of the surgery is to flatten the cornea and enable contact lens wear rather than to restore the corneal surface contour.

- **Patient preparation for lamellar keratoplasty may include a downgrading of expectations and a definition of the intended goal of surgery**

5.6 Choice of DALK Surgical Technique

Given the indication, the presence of a contraindication for penetrating keratoplasty, and the anatomy of the individual patient, the surgeon may first want to consider whether intraoperative perforation is unacceptable and what dissection depth is desired. In most cases, a dissection depth of >90% or a separation of Descemet's membrane from the recipient posterior stroma will give the best postoperative result. The surgeon may also want to consider what perforation might occur and/or how it should be dealt with during the surgery.

- Air- or viscodissection of Descemet's membrane. Separation of Descemet's membrane from the recipient posterior stroma by using air dissection (big bubble technique) or viscodissection has the advantage that a near anatomical donor to host interface can be obtained with a minimal risk of interface haze development. The dissection method is indirect (not manually controlled but dependent on the pressure built by air or viscoelastic at the cleavage plane above Descemet's membrane) and, as a result, if a perforation occurs, the perforation tends to

be paracentral and large, often requiring conversion to a penetrating graft. For that reason, the surgeon may choose to have donor tissue available with good quality endothelium.

- Manual corneal dissection at a visually controlled depth. To monitor the dissection depth during surgery, the anterior chamber should be filled with air to create an optical reference plane. Using an optical reflex, the surgeon can perform a dissection of up to 90–95% depth, removing most of the diseased corneal stroma. Visualisation of the depth of the dissection instruments greatly reduces the risk of perforation to less than 5%. If a perforation occurs, the perforation is usually small and located at the 12 o'clock surgical position or in the far periphery. If necessary, the perforation can be sealed by dissecting slightly shallower 'over' the perforation site, so that the hole is closed by a self-sealing stromal flap. The number of cases requiring conversion to a penetrating procedure may therefore be low, so that the availability of a donor cornea with good quality endothelium is not mandatory.
- Laser dissections. Several groups have evaluated the use of the excimer laser to create a host bed for a deep lamellar graft. The current equipment and software allows for the input of topography and pachymetry values, so that recipient corneas with an irregular surface or thinned stroma can be managed with a topo-linked and pachy-linked deep excimer ablation. With the introduction of the femtosecond laser, the precision of the ablation/dissection plane created may further improve, but not all software currently allows dissections over 400 µm in depth.

Summary for the Clinician

- **The lamellar keratoplasty surgical technique should be chosen taking into consideration the desired dissection depth, management of inadvertent perforations and availability of donor tissues**

5.7 Clinical Results (Figs. 5.1–5.3)

In conventional anterior lamellar keratoplasty, the recipient anterior corneal tissue was removed 'layer by layer'. The most common technique was that of lifting the tissue, stretching the fibres at the dissection plane and cutting the fibres with a crescent knife. The donor tissue was usually dissected using a blunt spatula to avoid perforation. Although the approach appeared effective, the dissection methods may have been the major cause for the development of interface haze, the major drawback of a lamellar procedure, since it affected the clinical outcome significantly.

Physically the normal cornea is milky white, but within the spectrum of visible light it appears transparent through 'constructive interference', i. e. the incoming light rays will bounce between the layered collagen fibres until they have crossed the cornea. As a result, any disorganisation of the layered fibrous structure may be expected to degrade the ability of the light to pass the cornea. The scattered light is clinically observed as opaque areas at the donor-to-host interface, or interface haze.

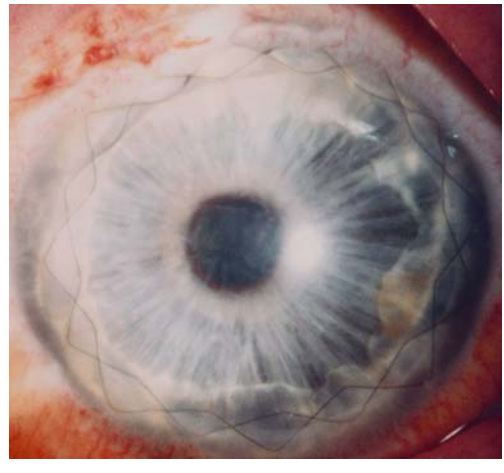

Fig. 5.1. Slit-lamp photograph of a deep anterior lamellar keratoplasty on the first postoperative day. Note that the organ cultured full-thickness donor button positioned onto the lamellar bed is still swollen

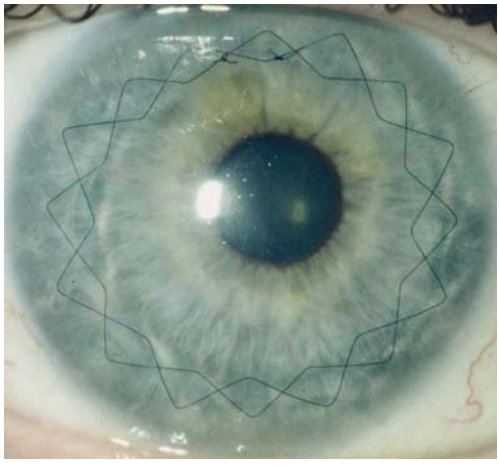

Fig. 5.2. Slit-lamp photograph of a deep anterior lamellar keratoplasty performed with manual dissection at an optically controlled corneal depth

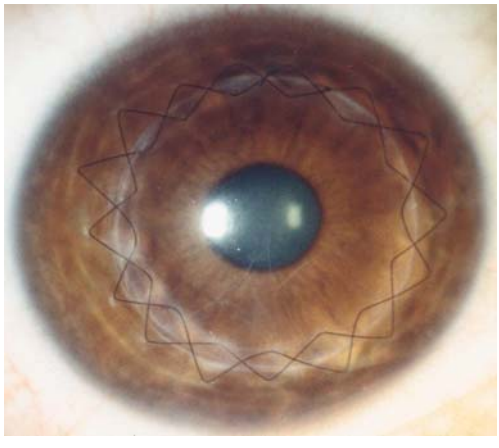

Fig. 5.3. Slit-lamp photograph of a deep anterior lamellar keratoplasty performed using viscodissection of Descemet's membrane. Note the remnant recipient stromal fibres at the level of Descemet's membrane that are typical for the procedure

From LASIK treatments we learned that corneal dissections do not per se induce interface haze. Since histologically interface haze can be correlated with interface scarring, it seems essential to obtain a smooth interface. With all the approaches mentioned above a smooth host bed can be obtained. With air or viscodissection of Descemet's membrane the anterior Descemet's will be exposed, excimer laser ablation will provide a smooth host bed, and if the dissection is made manually it may be recommended to use sharp instruments rather than a blunt dissection knife. When the host bed is sufficiently deep, a full-thickness donor button can be positioned onto the host bed. Descemet's membrane may be stripped off the donor cornea, which leaves a perfectly smooth surface of posterior corneal stroma.

As a result, current techniques for a deep anterior lamellar keratoplasty are not associated with significant interface haze development, and multiple investigators have found the final visual performance to be similar in eyes with a lamellar or penetrating keratoplasty. The final astigmatic error may be lower with lamellar procedures, especially when a large graft diameter is used. The visual outcome does vary with the indication for surgery. For example, eyes grafted for keratoconus on average achieve a much better visual acuity than those grafted for recurrent herpes simplex.

Thus, with the main drawback in performing a lamellar transplant eliminated, there may be few arguments to prefer a penetrating to a lamellar procedure. First, because the endothelial cell density after lamellar keratoplasty may show a pattern of cell loss as in virgin corneas, the long-term survival of a lamellar graft may be expected to be much better than after. Second, the risk of allograft rejection is minimised. Although stromal rejections with secondary keratic precipitates onto the recipient endothelium do occur, such rejections are most often less severe and easily managed with a topical steroid pulse therapy. Third, a lamellar procedure may provide the patient with much more social freedom. The integrity of the eye is better preserved, sutures can be removed much sooner, medication can be tapered quicker, and the overall aftercare necessary to maintain a functional graft is greatly reduced. Since deep anterior lamellar keratoplasty indications are limited to anterior corneal disorders, and these disorders are usually found in young people, long-term graft survival as well as less dependency on the health care system is important in this patient population.

Summary for the Clinician

- The clinical outcome of a lamellar keratoplasty with current techniques may be similar to that after penetrating keratoplasty
- Especially in the patient population eligible for lamellar keratoplasty, the procedure may have major advantages for both the surgeon and the patient, such as longer graft survival, the need for less aftercare and a lower dependence on the health care system

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Corneal Transplant Rejection

T.P.A.M. Slegers, M.K. Daly, D.F.P. Larkin

Core Messages

- Allograft rejection is the commonest single cause of corneal graft failure
- Corneal transplant antigen recognition in most cases is almost exclusively mediated by recipient antigen-presenting cells. $CD4^+$ T-lymphocytes have the central role in the alloreactive cell population
- Endothelial rejection episodes can be reversed by intensive topical steroid in most patients. Poor outcomes result from delay in presentation and/or initiation in treatment
- Patients with recipient corneal vascularisation, a previously rejected ipsilateral transplant and inflammation at the time of transplantation, are at highest risk of rejection and have very poor graft survival
- Little information is available from randomised trials on prophylaxis by transplantation antigen matching or immunosuppression in this patient group

6.1 Introduction

Despite the relative immune privilege of the cornea as a transplant tissue and both the recipient corneal bed and anterior chamber being an immune privileged site [35, 49], the most common cause of corneal graft failure in all reports is allogeneic rejection. In first graft recipients with no vascularisation of the recipient corneal bed, 2-year survival rates exceed 90 %; this decreases to 35–70 % in recipients with high risk factors for rejection [18, 53]. In one-third of all corneal grafts that are deemed failed, signs of a destructive attack by the immune system have been observed [61].

A rejection episode results in loss of donor endothelial cells, critical for maintenance of corneal transparency. As human endothelial cells do not repair by mitosis to any meaningful extent, the consequence is that donor corneal transparency is lost if cell density falls below the threshold necessary for prevention of stromal swelling. Endothelial decompensation results either (1) from the time of an irreversible episode of acute graft rejection or (2) at an interval following one or more episodes of rejection which have been reversed by therapy. Endothelial cells are thus the critical target in the allogeneic response.

While, on the one hand, reversal of acute graft rejection episodes does not present such challenges in cornea as in other transplanted tissues, effective prophylaxis in corneal graft recipients identified at high risk of rejection is much less evidence-based. Thus the impact of graft rejection continues to justify high priority in corneal research. Although the first successful penetrating corneal graft was reported in 1906, it took another half a century before the first description of opacification of a previously clear corneal graft was published. Paufique named this event "*maladie du greffon*" (disease of the graft) and suggested that this clinical finding was caused by sensitisation of the donor by the recipient [37]. This description followed the experiments reported by Medawar a few years previously, in which differences were observed between rabbit skin grafts of donor and recipient origin, giving rise to the term "histo-

compatibility" [32]. Maumenee subsequently confirmed this suggestion in a rabbit model of corneal transplantation in which he showed that donor corneas could induce an immune reaction [31]. The development of corneal transplantation models in rat [59] and mouse [46] facilitated study of rejection in inbred donor and recipient animals with a wide range of investigative immunological reagents.

6.2 Incidence

In reports from large cohorts of corneal graft recipients, the proportion undergoing a rejection episode at some stage post-transplant ranges from 18% to 21% [12, 22, 60]. In those graft recipients in whom rejection occurs, reported rates of successful reversal of the rejection episode range from 50% to 90% [21, 34]. Allograft rejection occurs most commonly in the second 6 months postgrafting, and it has been reported that more than 10% of the observed reactions can take place as late as at least 4 years after surgery [23, 34, 39]. This indicates that all corneal grafts need long-term surveillance and are at risk practically indefinitely.

6.3 Factors Predisposing to Corneal Graft Rejection

Preoperative characteristics of the graft recipient eye can be clearly identified in many patients to indicate significantly high risk of graft failure. Proposed graft recipient corneas (1) with two or more quadrants of deep vascularisation, (2) bearing a previously rejected graft (Fig. 6.1) and (3) that are inflamed at the time of transplantation are at significantly higher risk of rejection [2, 6, 30, 57, 63, 62]. There is less robust evidence in the published literature that grafts in children, large diameter donor corneas and proximity of donor cornea to the recipient limbus are at higher risk (Table 6.1) [30, 41, 54, 56]. Clearly more than one of these factors may be operational in one patient. There may also be association of one or more of the above factors predisposing to failure due to rejection with additional clinical features that confer significant risk of graft failure due to other complications, such as glaucoma or ocular surface disease [26, 41]. These preoperative clinical features must be evaluated carefully in the decision whether to proceed with corneal transplantation.

Once transplantation is successfully completed, care must be taken to prevent postoperative events which predispose to rejection, such as vascularisation of recipient cornea (Fig. 6.2) or graft wound, suture loosening, or graft infec-

Fig. 6.1. Subepithelial infiltrates in a penetrating corneal allograft. The appearance is similar to that seen in adenovirus keratitis, involving the donor cornea only

Table 6.1. Risk factors for rejection

Preoperative	Deep vascularisation of two or more quadrants Previously rejected ipsilateral graft Active corneal inflammation at time of graft Paediatric graft recipient Large diameter graft Graft proximity to the limbus
Postoperative	Loosening of sutures Removal of sutures, wound dehiscence Graft inflammation HSV infection recurrence Non-viral graft infection

tion by bacteria or recurrent herpes simplex virus (HSV).

6.4 Clinical Features

Epithelial rejection, diagnosed by a linear opacity which stains with fluorescein, comprised up to 10 % of all rejection episodes in one series and occurs on average 3 months after grafting [1]. Although dead donor epithelial cells are rapidly replaced by recipient epithelial cells and no scarring occurs, the presence of this type of rejection reflects that the recipient is now sensitized to the donor and can progress to stromal and/or endothelial rejection. Stromal rejection is characterised by nummular subepithelial infiltrates (Fig. 6.1), identical to those found in adenovirus keratitis. Patients with both epithelial and stromal types of rejection may be asymptomatic or have mild ocular discomfort only. In contrast, patients with endothelial rejection will usually present with visual disturbance and iritis symptoms. If examined early after rejection symptom onset, anterior chamber cell infiltration without flare or graft abnormality will be seen. At later times after symptom onset, the signs in succession are (1) aggregated alloreactive cells adherent to graft endothelium evident as keratic precipitates, (2) an endothelial line with precipitates and (3) localised oedema corresponding to a rejection line or total graft oedema (Fig. 6.2). Visible graft precipitates on slit-lamp biomicroscopy imply focal and variable but irreversible endothelial cell loss, compromising endothelial pump function and resulting in stroma oedema in those grafts with severe inflammation or low endothelial cell density prior to rejection onset. Pachymetry is helpful in detecting an increase in oedema and also deturgescence following the start of steroid treatment. In one study it was found that next to the preoperative diagnosis, graft thickness during rejection, as objectively measured by pachymetry, is a prognostic sign for reversibility of a rejection episode [34]. Risk factors for significant endothelial cell loss are delay in initiating anti-rejection treatment more than 1 day and recipient age greater than 60 years [13].

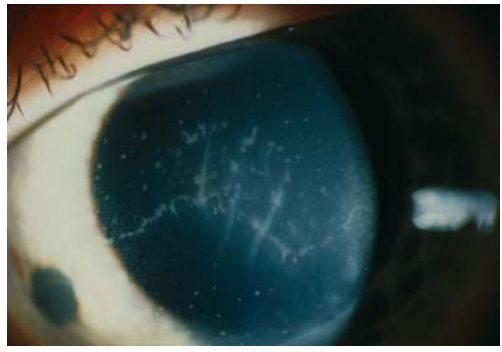

Fig. 6.2. Endothelial rejection line, keratic precipitates and folds in Descemet's membrane in rejection

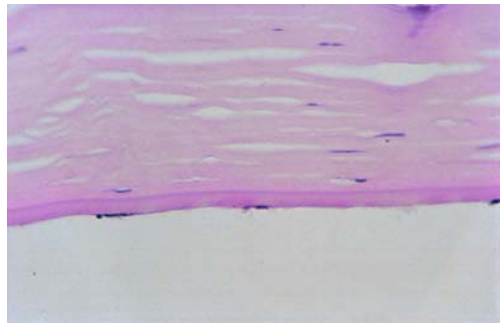

Fig. 6.3. Almost total loss of endothelial cells in corneal graft specimen removed at graft replacement 6 months following rejection onset

6.5 Histopathology

Descriptions of the pathological features of corneal transplant rejection result from examination of grafts replaced following irreversible failure. Therefore these specimens illustrate late changes in end-stage corneal opacification, usually some months at least following treatment of rejection. Characteristic findings in stroma are vascularisation with mononuclear cell infiltration and keratocyte loss; few if any endothelial cells remain (Fig. 6.3) [28]. Several studies have shown increased numbers of HLA class II positive cells infiltrating stroma in sections of rejected grafts [38, 58].

6.6 Immunopathological Mechanisms

6.6.1 Immune Privilege and Its Breakdown

Immune privilege is a dynamic phenomenon in which the destructive effect of a "normal" immune response to particular antigens is either altered or absent in order to protect the microanatomy of highly organised tissues in the eye. In corneal transplantation, both (1) the recipient corneal bed and anterior chamber and (2) the transplanted tissue have features of immune privilege.

Several features of the anterior chamber contribute to immune privilege. There are *mechanical barriers* that impair immune cell access to the anterior chamber and transplanted cornea. One barrier is the lack of blood and lymphatic vessels in a normal cornea. While experimental and clinical studies have clearly shown that transplants are much more likely to be rejected in vascularised corneas, the stimuli to vascularisation are likely also to induce lymph vessel growth. Following transplantation, it is in lymph vessels that antigen-presenting cells (APC) migrate from the graft to lymphoid organs for presentation of graft antigens to T lymphocytes. Another route for alloreactive cells to reach the anterior chamber and donor corneal endothelium is closed by the tight junction barrier formed between non-pigmented epithelial cells and non-fenestrated iris vessels [10].

In the event that leukocytes enter the anterior chamber, mechanisms are available to either deviate or blunt a potentially harmful immune response. For example the aqueous humour contains *immunosuppressive molecules* as transforming growth factor (TGF)-β, vasoactive intestinal polypeptide (VIP), α-melanocyte stimulating hormone (MSH), and calcitonin gene related protein (CGRP), which contribute to induction by an allograft of deviated systemic delayed-type hypersensitivity [35, 52].

In addition to lack of blood and lymphatic vessels, cornea allografts have been shown in laboratory studies to have additional features which contribute to immune privilege. These include the paucity of donor-derived major histocompatibility complex (MHC) class II^+ APC, and corneal epithelial and endothelial expression of Fas ligand [7, 16], interaction of which with Fas on alloreactive effector cells leads to death of the infiltrating leukocyte.

Corneal grafts at high risk of rejection are identified by several risk factors, most of which reflect breakdown of facets of immune privilege. Prospective clinical outcome studies identify the most significant of these to be recipient corneal vascularisation, corneal inflammation at the time of transplantation, which induces APC infiltration in the recipient cornea prior to surgery, and a previously rejected ipsilateral graft.

6.6.2 Afferent Arm of the Allogeneic Response

In circumstances where the immune privileged features of the cornea are bypassed by the immune system, the first stage in rejection is recognition of the presence of non-self tissue. There are two routes of allorecognition. By the *indirect* pathway, recipient APC enter the graft to capture and process donor antigens, migrating to the lymphoid system to present the antigen in context with self MHC class II molecules to T cells. Most experimental evidence points to the neck lymph glands as the location for antigen presentation [40, 45, 65].

Recent identification in the central cornea of a population of dendritic cells, which can become MHC II^+ and migrate to the draining lymph nodes [11, 17, 29], and MHC class II^+ macrophages [9] indicates that *direct* allorecognition of the corneal graft antigens is possible. By this pathway, donor APC bearing alloantigens migrate from the graft and activate T lymphocytes via their own non-self MHC class II molecules. Direct allorecognition would be more prominent in the occasional clinical circumstance in which a donor cornea is transplanted which has an increased population of APCs, such as after viral infection. However, in most circumstances it is assumed that corneal allorecognition is predominantly by the indirect pathway.

Evidence from cell kinetic studies in murine corneal grafts demonstrates that within several hours of transplantation the graft is infiltrated by granulocytes and macrophages [27]. From macrophage depleting studies evidence has been provided that these cells play a crucial role in the afferent phase of graft rejection [47].

6.6.3 Efferent Arm of the Allogeneic Response

When T-helper cells have identified the presented antigen as non-self, effector mechanisms are generated against donor tissue. Cytokines including particularly tumour necrosis factor [42] and interferon-γ [25] have been clearly identified in aqueous humour and the cornea prior to observed endothelial rejection onset. After corneal transplantation it has been shown that alloantibody, cytotoxic T lymphocytes and delayed type hypersensitivity responses are components of the effector response. Experimental studies, using CD4+ knockout mice and monoclonal antibodies directed against CD4+ T cells, have pointed to the central role of this lymphocyte subpopulation [3, 64]. The mechanism by which corneal endothelial cells are killed is not yet clear. At time of graft destruction increasing levels of natural killer (NK) cells, known to be able to lyse corneal endothelial cells, are detected in the aqueous humour of grafted rats [14]. There is additional evidence that nitric oxide could mediate in destruction of donor endothelial cells [8, 44, 51].

6.7 Treatment of Rejection

The objective of treatment is to reverse the rejection episode at the earliest possible time, in order to minimise donor endothelial cell loss and preserve graft function. With the anatomical advantage that corneal transplants are superficial, intensive administration of topical corticosteroid, such as dexamethasone 0.1%, treatment is successful in reversing most endothelial rejection episodes. In most cases in which topical steroid fails to reverse rejection, it is likely to be due to delay in recognition and initiation of treatment, with resulting significant donor endothelial cell loss [13]. In others, failure to reverse rejection may be due to failure of topical steroid to reverse effector components of the allogeneic response. In respect of additional systemic steroid, a single dose of intravenous methylprednisolone was found to be more effective than oral steroid in patients with endothelial rejection who presented within 8 days of onset [20]. A second pulse of intravenous methylprednisolone at 24 or 48 h gave no benefit when compared to a single dose at initial diagnosis [19]. However, a subsequent randomised trial demonstrated no significant benefit of intravenous methylprednisolone in addition to topical steroid, in respect of graft survival or interval to a subsequent rejection episode within a 2-year follow-up period [21]. In the same study, endothelial rejection was reversed in 33 of 36 patients treated, indicating that steroid-resistant rejection is uncommon. Other studies examining the efficacy of topical or oral cyclosporin administered in combination with intravenous steroid have reported similar outcomes, with irreversible rejection in a small proportion of patients [66, 67].

6.8 Prevention of Rejection

6.8.1 Immunosuppression

In patients without risk factors for graft rejection identified prior to surgery, typical postoperative immunosuppression comprises steroid drops such as dexamethasone 0.1% four times daily for the first 2–3 months, reducing gradually to zero by 6 months post-transplant. Regimes vary from centre to centre. There is much less consensus on which additional measures to take as prophylaxis in patients at high risk of rejection (Table 6.1), in whom topical steroid alone is insufficient to prevent rejection. The result of a continuing shortage of large comparative prospective studies is that immunosuppression protocols in current use result from individual clinical experience, with some influence from

experimental evidence and small uncontrolled and/or retrospective clinical studies. However, ophthalmologists are cautious about administering potentially toxic systemic immunosuppressive agents, even in those patients in whom a surviving graft would allow vision in the only eye. The subject of immunosuppression in prevention of corneal graft rejection is discussed in another chapter in this text.

6.8.2 HLA Matching

In vascularised organ allotransplantation there is robust evidence supporting HLA matching of donor and recipient, with the data of Opelz and others demonstrating stratification of the risk of rejection according the number of class I and especially class II mismatches [36]. HLA matching is in routine use internationally in cadaveric renal and other organ transplantation. In corneal transplantation by contrast, for recipients at high risk of rejection HLA class I and class II -DR matching is routinely done in some countries, whereas in other countries no matching takes place at all. Roelen suggested a benefit for HLA-A and -B matching in high-risk corneal allograft recipients based on his findings that primed, donor-specific cytotoxic T cells were present in rejected corneas but absent in donors with good graft function [43]. However, the benefit of histocompatibility matching in corneal transplantation has been disputed and is certainly less clear than for solid organ grafts, even in corneal recipients at perceived high risk of graft rejection. Two large prospective studies on HLA-A, -B, or HLA-DR antigen matching high-risk recipients have reported divergent findings. The Collaborative Corneal Transplant Studies Research Group reported that matching of these antigens did not decrease the risk of corneal graft failure secondary to rejection [53]. In contrast the Corneal Transplant Follow-up Study found there was increased risk of graft rejection with mismatch of HLA class I antigens (relative risk 1.27 per mismatch), but decreasing risk of rejection with -DR mismatches (relative risk 0.58 per mismatch) in high risk patients [55]. This study therefore supported matching at HLA-A, and -B but not HLA-DR. The possible benefit of planned -DR mismatching in a setting of known class I histocompatibility is at present being investigated in an ongoing prospective trial, the outcome of which is awaited with interest. In 1996, a randomised although retrospective study reported a beneficial effect of DRB1 matching in recipients at high risk on account of vascularisation and/or retransplantation [4]. Subsequently a beneficial effect of HLA-DPB1 matching in high-risk corneal transplantation with a significantly higher rate of 1-year rejection-free graft survival compared to those without matching was shown [33].

In corneal transplantation therefore the effect of HLA matching is less than clear and the data are most ambiguous for class II matching. Resolution of this clinically important issue is not simple. In contrast to solid organs, results of matching for cornea are likely to be influenced by the facts that: (1) allorecognition is predominantly by the indirect pathway in most patients [5], and (2) minor transplantation antigens, shown to have a significant effect on graft survival in untreated rodent recipients [24, 48, 50] and presented by the indirect pathway, remain unmatched in HLA-matched recipients. It is also worth noting here that the effects of HLA matching on corneal graft outcome have not yet been investigated in the setting of systemic immunosuppression prophylaxis. Studies in solid organ transplantation have shown that more effective rejection prophylaxis can override an HLA matching effect in unsensitised recipients.

6.9 Future Prospects

Reducing the impact of allograft rejection is a major challenge in corneal disease. It can be expected that following developments in techniques of lamellar keratoplasty, wider use of this type of surgical procedure, particularly for stromal corneal pathology, will reduce the impact of endothelial rejection. However, the presence of a group of patients with no alternative to penetrating keratoplasty, a high risk of rejection and no alternative prophylactic intervention justifies clinical trials of novel treatment strategies

[15]. These developments are likely to derive from a better understanding of the immune mechanisms of rejection and from importation of refinements in systemic immunosuppression strategies validated in solid organ transplantation. There remains one opportunity in corneal grafting which is unique in transplantation: the possibility of storage ex vivo of donor cornea for a period of weeks prior to elective surgery. Modification of donor tissue by pharmacological or gene-based approaches may be of additional benefit in attenuating donor injury by the allogeneic response.

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New Aspects of Angiogenesis in the Cornea

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Core Messages

- Corneal angiogenesis is associated with the most common forms of corneal blindness both worldwide as well as in industrialized countries
- Corneal angiogenesis is primarily caused by inflammatory diseases of the cornea (most commonly keratitis), corneal hypoxia (contact lens wear) and limbal antiangiogenic barrier defects (most commonly aniridia, chemical burns)
- In corneal inflammation, (hem)angiogenesis (i.e., outgrowth of pathologic blood vessels into the cornea) is usually accompanied by lymphangiogenesis (outgrowth of lymphatic vessels)
- Pathologic corneal lymphatic vessels are invisible at the slit-lamp, but can be visualized using specific immunohistochemical markers in explanted vascularized corneas
- Preexisting blood and lymphatic vessels are strong risk factors for immune rejections after keratoplasty
- In addition, about 50 % of patients undergoing low-risk keratoplasty also develop corneal angiogenesis *post*operatively. In animal models of corneal transplantation, this postoperative mild combined hem- and lymphangiogenesis significantly increases the risk for immune rejections
- Novel antiangiogenic and antilymphangiogenic therapies can improve graft survival after both low-risk and high-risk keratoplasty by reducing the incidence of immune rejections (novel therapeutic concept)
- Novel, directly antiangiogenic drugs for application against corneal angiogenesis will be available medium term as a spin-off of antiangiogenic cancer treatments

7.1 Introduction

Corneal avascularity is of paramount importance in maintaining corneal transparency, the latter being essential for good visual acuity. Therefore, in all higher animals depending on good vision, the cornea normally is devoid of blood and lymphatic vessels [12, 13, 21, 30]. Nevertheless, several diseases and surgical manipulations can lead to corneal (hem)angiogenesis (i.e., ingrowths of blood vessels from the limbal vascular arcade into the cornea) and lymphangiogenesis (i.e., ingrowths of lymphatic vessels from the limbal vascular arcade into the cornea [12, 13, 30]). Corneal hem- and lymphangiogenesis can cause a significant reduction in visual acuity and blindness as well as render these corneas high risk in the case of a subsequent penetrating keratoplasty [12, 13, 30]. In fact, corneal angiogenesis is associated with the most common cause of corneal blindness worldwide (trachoma) as well as the most common form of infectious blindness in western countries (herpetic keratitis [12, 13, 30]). Whereas the animal cornea has been used as in vivo model to study the mechanisms of angiogenesis for decades, the molecular pathways responsible for maintaining normal avascularity of the human cornea ("angiogenic privilege") have only started to evolve in recent years [15]. The same is true for the role of lymphatic vessels growing into the cornea in inflammatory corneal diseases (lymphangiogenesis [3, 4, 11, 16]). Corneal lymphangiogenesis has recently been shown to be of essential importance in the induction of immune responses after corneal transplantation, so that novel antihem- and antilymphan-

giogenic therapies are starting to emerge as new tools to improve graft survival in both the low-risk and the high-risk setting of corneal transplantation [3, 17].

7.2 "Angiogenic Privilege of the Cornea" or "How Does the Normal Cornea Maintain Its Avascularity?"

Although the cornea – due to its anatomically exposed position – is in constant contact with numerous minor inflammatory and thereby angiogenic stimuli, the normal cornea remains avascular [12, 13, 15]. Even after more severe trauma – such as refractive surgery – the cornea in contrast to other tissues does not respond with angiogenesis. This active maintenance of corneal avascularity has been termed "*corneal angiogenic privilege*" [12, 13, 15]. Corneal angiogenic privilege is not only essential for good visual acuity but is also responsible for the excellent survival of corneal grafts placed into avascular low-risk recipient beds, since in these cases the graft is physically separated from both the *afferent* (lymphatic) and the *efferent* (blood vascular) arm of a so-called *immune-reflex arc*, leading to immune rejection after keratoplasty (Fig. 7.1A [3, 13, 17, 30]). Whereas the cornea has served as *the* in vivo model system for the study of the mechanisms of angiogenesis for decades [in fact, as early as the 1940s Michaelson hypothesized the existence of a soluble angiogenic factor mediating corneal angiogenesis (which later turned out to be primarily the angiogenic

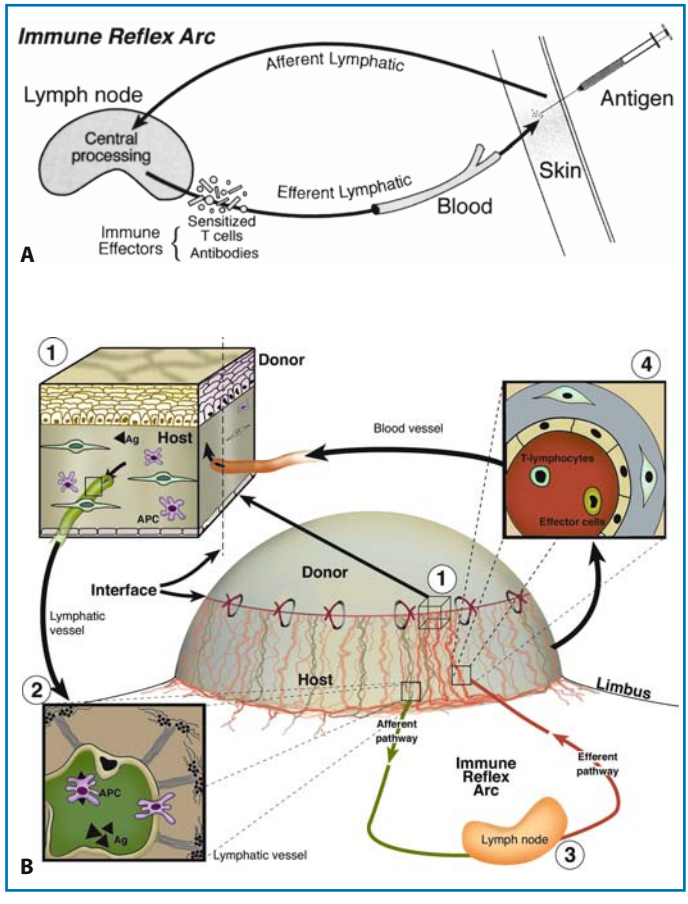

Fig. 7.1. A Schematic diagram of the so-called "immune reflex arc" leading to immune rejections after keratoplasty. The *afferent* arm consists of lymphatic vessels allowing exit of antigen presenting cells from the donor cornea, the *central processing unit* is the regional cervical lymph nodes [initiation of production of immune effector cells], and the *efferent* arm consists of blood vessels allowing entry of immune effector cells to the graft [with kind permission from Streilein JW (1999) Immune responses and the eye. Karger, New York, p 17]. **B** Immune reflex arc in a vascularized high-risk cornea (with kind permission from [13]). The donor cornea has direct access both to the *afferent* lymphatic arm (*1, 2*) and thereby to the regional lymph node (*3*) as well as to the *efferent* blood vascular arm (*4*) of an immune-reflex arc. This explains the much higher rate of immune rejections occurring in vascularized high-risk eyes

Table 7.1. Angiogenic and antiangiogenic factors (selection)

Angiogenic growth factors	Antiangiogenic factors
VEGF (VEGF-A, VEGF-C, VEGF-D)	Thrombospondins (TSP 1 and TSP 2)
FGF (bFGF, aFGF)	PEDF
Interleukin-1	Angiostatin
TGF (alpha, beta)	Endostatin

growth factor VEGF)], the strategies used by the cornea for the normal maintenance of its avascularity are only partly understood [15, 19]. In general, angiogenesis or inhibition of angiogenesis depend on a balance between proangiogenic factors (such as the VEGF family growth factors VEGF-A, -C and -D) and antiangiogenic factors (such as the thrombospondins; Table 7.1 [1, 28]). If the balance tips more to proangiogenic factors, angiogenesis starts [1, 28]. In the cornea, normally the balance is shifted towards antiangiogenic factors to maintain avascularity. Indeed, several antiangiogenic factors (such as PEDF, thrombospondins 1 and 2, antiangiogenic matrix cleavage products such as angiostatin and endostatin, IL1RA) have already been identified in the cornea [2, 12, 13]. It seems that these antiangiogenic factors are strategically located at the inner and outer linings of the cornea (Descemet's membrane and epithelial basement membrane) to counteract angiogenic stimuli both from inside (e.g., high concentrations of angiogenic growth factors in the aqueous humor during proliferative diabetic retinopathy) and from outside (e.g., against angiogenic growth factors from the tear film [2, 12, 13]). Animal experiments using mice deficient in one or more antiangiogenic factors (such as thrombospondins 1 and 2) have shown that the corneal angiogenic privilege is *redundantly organized* so that the absence of one or two factors does not cause spontaneous ingrowths of limbal blood vessels [15]. This is in contrast to other intraocular tissues such as the iris, where the absence of these factors causes increased vascularity [15]. This demonstrates that evolutionarily the cornea has acquired a *robust and redundant antiangiogenic system* normally maintaining avascularity unless it is overrun by overwhelmingly strong (usually inflammatory/infectious) stimuli for angiogenesis which threaten the integrity of the whole eye or even the whole body [15].

Summary for the Clinician

- **Cornea and cartilage are the only avascular tissues of the human body**
- **Corneal avascularity is actively maintained, e.g., after refractive surgery ("*corneal angiogenic privilege*")**
- **Corneal angiogenesis is associated with and potentially causative of the most common causes of corneal blindness worldwide (trachoma) and the most common form of infectious corneal blindness in industrialized countries (herpetic keratitis)**

7.3 Corneal (Hem)angiogenesis

7.3.1 General Mechanisms of Corneal (Hem)angiogenesis

According to Folkman, a balance between angiogenic and antiangiogenic factors in each tissue and situation determines whether angiogenesis occurs or not. If the balance is tipped towards angiogenic growth factors, vessel outgrowth starts ("*angiogenic switch*"), whereas if inhibitors prevail, angiogenesis is prohibited. Several angiogenic growth factors [primarily growth factors from the VEGF family (VEGF-A, VEGF-C, VEGF-D), FGF, IL-1, etc.] as well as inhibitors of angiogenesis have been identified in recent years (PEDF, thrombospondins, angiostatin, endostatin, etc.; see Table 7.1 [1, 28]). Pathologic angiogenesis (to clearly separate this process from *lymph*angiogenesis, we will subse-

Table 7.2. Common causes of corneal angiogenesis

Pathomechanism	Diseases/conditions
1. Corneal inflammation/infection	Keratitis (most commonly herpetic keratitis, but also bacterial and fungal) Graft rejection Autoimmune diseases affecting the cornea/sclera
2. Corneal hypoxia	Contact lenses with low Dk values (especially extended wear)
3. Defects of the limbal antiangiogenic barrier	Inherited defects (e.g., aniridia) Acquired limbal defects (e.g., after chemical cautery)
4. Secondary/iatrogenic	After keratoplasty After corneal wound repair

quently refer to it as "*hem*angiogenesis") and lymphangiogenesis into the cornea mainly occur in settings of an inflammatory "insult" to the cornea, corneal hypoxia or limbal barrier defects, all overriding the *angiogenic privilege* of the cornea, which is actively maintained [2, 12, 13, 15]. Clinical conditions most commonly associated with corneal neovascularization include keratitis (herpetic and bacterial in nature), contact lens wear as well as inherited or acquired limbal deficiency states (primarily chemical burns [2, 12, 13, 15]). Growth factors of the VEGF family have been identified as key players in both inflammation-driven hem- and lymphangiogenesis into the normally avascular cornea [7, 12, 13].

Release of angiogenic growth factors generally is induced primarily by two factors: (1) inflammation and inflammatory cytokines (at the cornea, e.g., keratitis) and (2) hypoxia (at the cornea, e.g., contact-lens induced). The general process of sprouting angiogenesis follows the following steps: (1) vasodilatation, (2) degradation of extracellular matrix, (3) mitotic activation of endothelial cells and (4) chemotactic migration of endothelial cells out of the preexisting vessels towards an angiogenic stimulus.

The precise mechanisms whereby the sharp limbal border between hem- and lymphvascularized conjunctiva and avascular cornea is maintained are unclear. It is possible that limbal stem cells located at niches in that area contribute to the antiangiogenic barrier of the limbus. Clinically the fact that destruction of limbal stem cells, e.g., by cautery, causes destruction also of the limbal antiangiogenic barrier supports this concept.

7.3.2 Common Causes of Corneal (Hem)angiogenesis

The most common causes for corneal angiogenesis are enlisted in Table 7.2 [6, 24]. According to the mechanisms leading to angiogenesis outlined above, these corneal diseases fall into three general categories: (1) diseases leading to strong inflammation within the cornea (autoimmune or infectious; most commonly herpetic keratitis); (2) diseases leading to hypoxia within the cornea (most commonly contact lenses with low Dk values) and (3) diseases with inherited (e.g., aniridia) or acquired (e.g., after chemical cautery) defects of the limbal "antiangiogenic" barrier [2]. In addition, "secondary" corneal angiogenesis can occur after surgical manipulations at the cornea, which primarily involve placement of corneal sutures (e.g., after corneal wound repair, after corneal transplantation, after block excision [9]).

7.3.3 Clinical Consequences of Corneal Hemangiogenesis

Corneal angiogenesis can lead to reduced visual acuity not only by the physical presence of blood vessels itself, but also due to leakage of

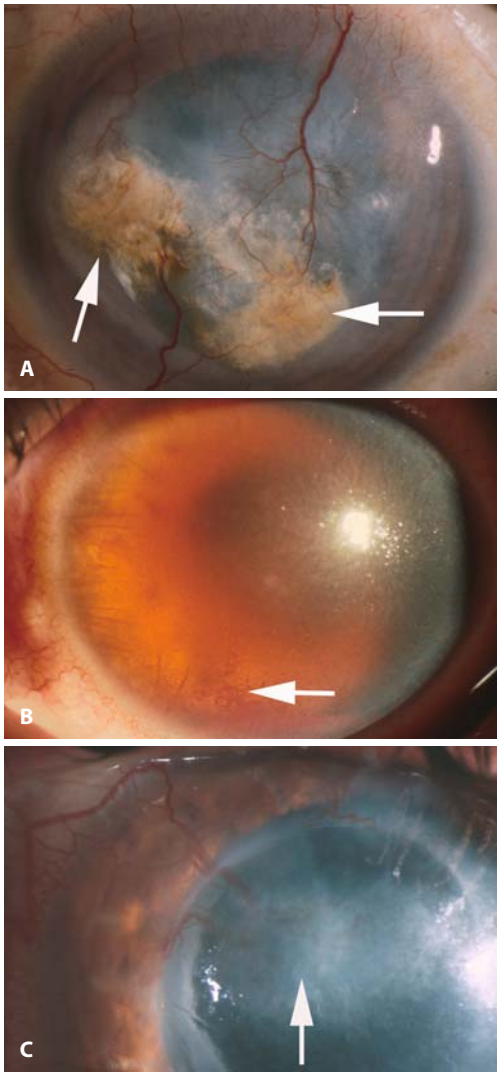

Fig. 7.2 A–C. Complications of corneal angiogenesis leading to reduced visual acuity: **A** lipid keratopathy (*arrows*); **B** intrastromal hemorrhage; **C** secondary stromal edema due to leakage from immature blood vessels. In addition, corneal angiogenesis makes such a cornea a high-risk recipient bed in the case of subsequent keratoplasty (with kind permission from [12])

products from immature corneal blood vessels (Fig. 7.2). This includes corneal edema due to water leakage, corneal lipid keratopathy due to lipid leakage and intrastromal or subepithelial hemorrhage (e.g., in contact lens patients). Furthermore, as outlined in Sects. 7.3.4 and 7.4.2, corneal angiogenesis impairs the prognosis of corneal grafts placed into vascularized high-risk corneas. In fact, the Collaborative Corneal Transplantation Study [24] (and numerous other clinical and experimental studies [30]) revealed preexisting corneal blood vessels as the strong(est) risk factor for subsequent immune rejections.

Summary for the Clinician

- **Corneal angiogenesis starts when the balance between proangiogenic and antiangiogenic factors in the cornea is shifted towards angiogenic growth factors**
- **The most common clinical conditions associated with corneal angiogenesis are corneal inflammation (keratitis), hypoxia (contact lens) and limbal barrier defects (chemical burns)**
- **Corneal angiogenesis leads to reduced visual acuity by the physical presence of vessels itself, but also by leakage of water, lipids and erythrocytes**
- **Preexisting corneal blood vessels are a strong risk factor for subsequent immune rejections after keratoplasty**

7.3.4 Corneal Hemangiogenesis *After* Keratoplasty

Preexisting corneal blood vessels – as mentioned above – have long been identified as strong risk factors for immune rejection after keratoplasty [24]. But, until very recently the role of the mild angiogenesis (and – as we will discuss in Sect. 7.4.2 – parallel lymphangiogenesis) occurring *after* keratoplasty in preoperatively avascular recipient beds was unclear [8–10].

7.3.4.1 Corneal Hemangiogenesis *After* Low-Risk Keratoplasty

The exact pathomechanism for postkeratoplasty neovascularization is unknown. The interaction of suture material with corneal epithelium/

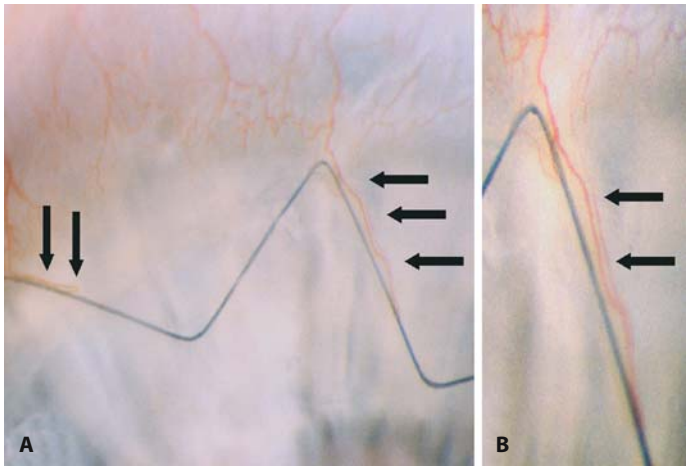

Fig. 7.3 A, B. Secondary corneal angiogenesis after low-risk keratoplasty (**A** with kind permission from [12]). New capillaries develop in every second patient, are usually oriented towards the outer suture turning point and then grow centripetally along the suture track (*arrows*). In 10 % of patients they reach the donor-host junction or grow further into donor tissue. The most common location of postkeratoplasty angiogenesis is at the 6 o'clock and 12 o'clock positions

stroma as well as wound healing processes in the interface seem to be important. Indeed, comparing corneal neovascularization within the first postoperative year between patients having undergone mechanical (more intense wound healing) versus nonmechanical (excimer laser: less wound healing) keratoplasty showed that the incidence of corneal angiogenesis was lower in the nonmechanical (48 %) compared to the mechanical trephination group (75 %; $p<0.01$ [10]). This indicates that in the low-risk setting, development of postoperative corneal neovascularization seems to be affected by the trephination technique and subsequent wound-healing response. Support for this concept comes from experimental data where postoperative corneal hem- and lymphangiogenesis within the first week do not differ between allogeneic and syngeneic grafts in the mouse model of corneal transplantation [17]. Since in the syngeneic model by definition there is no immune response possible because donor and host are immunologically identical, postoperative hem- and lymphangiogenesis really seem to be triggered by surgery and wound healing responses [17]. This establishes surgery itself and the degree of wound healing after keratoplasty as novel risk factors for the induction of immune responses after keratoplasty. Conventional steroid therapy is not able to stop or prevent this postoperative angiogenesis sufficiently, further underlining the need for more specific antiangiogenic therapies (see Sect. 7.5.2 [9, 10]).

A retrospective semiquantitative analysis of 136 patients having undergone low-risk keratoplasty (primarily patients with keratokonus and Fuchs' dystrophy) revealed that more than 50 % of patients *after* low-risk keratoplasty (with *pre*operatively avascular corneas) *post*operatively develop corneal angiogenesis within the first year [8, 9]. New vessels are primarily located in the 6 o'clock and 12 o'clock positions and tend to grow towards the outer suture turning points (Fig. 7.3). Thereafter, capillaries usually follow the suture track towards the interface [9]. In about 10 % of patients these new vessels actually reach donor tissue. Risk factors for postoperative neovascularization include: embedding of the suture knots in the host stroma, active blepharitis, and a large recipient bed. Experiments in the mouse model of low-risk keratoplasty have recently shown that these capillaries are always accompanied by biomicroscopically invisible lymph vessels (lymphangiogenesis; Fig. 7.4; see Sect. 7.4.2 [16, 17]). Therefore, even if it clinically appears only mild, this combined angiogenesis and lymphangiogenesis *after* low-risk keratoplasty provides access for both arms (*afferent* lymphatic as well as *efferent* blood vascular) of an immune reflex arc towards the graft (Fig. 7.1). Indeed, experiments in the mouse

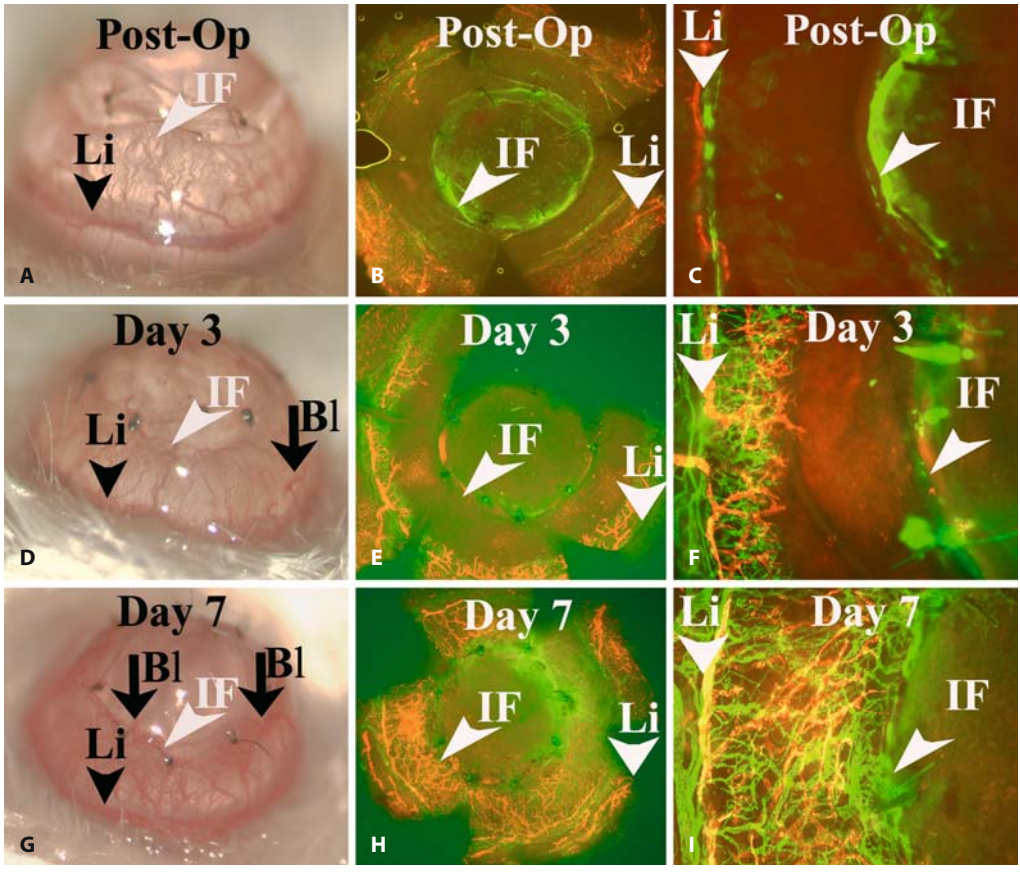

Fig. 7.4 A–I. The mouse model of low-risk keratoplasty demonstrates early and parallel outgrowths of both blood and lymphatic vessels after low-risk keratoplasty (*left column* slit-lamp aspect, *middle column* corneal whole mounts, *right column* detail showing limbus *at left* and interface *at right*; blood vessels stained in *green*, lymphatic vessels stained in *red*; with kind permission from [17]). Whereas there are no blood or lymphatic vessels immediately postoperatively in the low-risk recipient corneal bed, after 3 days both vessel types clearly grow out from the limbal arcade and at day 7 in this model reach the host-graft interface

model of low-risk keratoplasty recently identified postkeratoplasty neovascularization as a risk factor for subsequent immune rejections [16, 17]. An antihem- and antilymphangiogenic therapy significantly improved graft survival after low-risk keratoplasty (Fig. 7.5). Studies are under way to evaluate whether this also holds true for the human low-risk keratoplasty setting.

7.3.4.2 Corneal Hemangiogenesis *After* High-Risk Keratoplasty

Even after high-risk keratoplasty, preexisting blood vessels tend to increase (Cursiefen et al., unpublished observation, 2004). Only after keratoplasty for herpetic keratitis does anecdotal evidence suggest that removal of the angio-

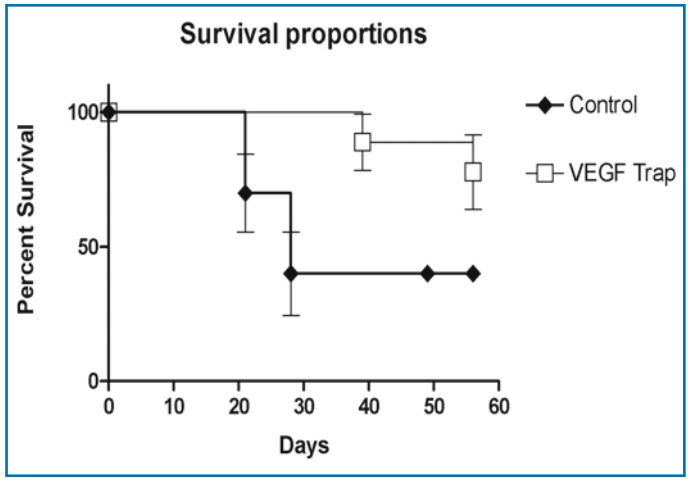

Fig. 7.5. Inhibition of hemangiogenesis and lymphangiogenesis after low-risk keratoplasty using a VEGF-A specific cytokine trap (VEGF Trap) in the mouse model of corneal transplantation significantly reduces immunological graft rejections ($p<0.05$; with kind permission from [17])

genic stimulus leads to a reduction in corneal angiogenesis [33]. Animal experiments recently clearly demonstrated that even after high-risk keratoplasty there is a significant further increase in both hem- and lymphangiogenesis. In addition, inhibition of these processes even *after* high-risk keratoplasty (in the mouse model) could improve subsequent graft survival (Cursiefen et al., submitted).

Summary for the Clinician

- **Corneal angiogenesis postoperatively occurs in about 50 % of patients *after* low-risk keratoplasty in preoperatively avascular recipient beds**
- **Postoperative angiogenesis reaches donor tissue in more than 10 % of patients**
- **Animal experiments suggest that angiogenesis after keratoplasty is accompanied by clinically invisible lymphangiogenesis**
- **Postoperative hem- and lymphangiogenesis have been identified as risk factors for immune rejection after keratoplasty (mouse model)**
- **Inhibition of postkeratoplasty angiogenesis and lymphangiogenesis seem to improve graft survival both in the low-risk and high-risk setting (mouse experiments)**
- **Surgery itself and the degree of wound healing after keratoplasty are novel risk factors for the induction of immune responses after keratoplasty**

7.3.5 Corneal Angiogenesis Due to Contact Lens Wear

Prevalence of contact lens-associated corneal angiogenesis varies widely in the literature, but is generally estimated to be within a range of 11–23 % of contact lens wearers. The intensity also can vary from some small capillaries usually at the 6 o'clock and 12 o'clock positions to deep stromal mature blood vessels with secondary scar formation. The cause seems to be a reduced oxygenation of corneal epithelium and stroma, not primarily due to reduced diffusion through the contact lens, but due to a reduced exchange of the sub-lens tear film which leads to reduced oxygenation of this tear film by lid capillaries and thereby corneal hypoxia. This in turn leads to upregulation of proinflammatory cytokines, VEGF-A and then angiogenesis [5–7]. Since even the mild hem- and lymphangiogenesis occurring after low-risk keratoplasty have been identified as risk factors for immune rejections after keratoplasty – in analogy – careful attention should be given to contact lens-induced corneal angiogenesis occurring in keratoconus patients, since these patients might go on to keratoplasty and one might create a higher-risk scenario in the case of later keratoplasty [16, 17].

7.3.6 Angiogenesis as a Cause of Disease Progression, not a Sequel (Herpetic Keratitis)

Corneal angiogenesis not only can follow inflammatory and infectious diseases of the cornea, but may also be pathogenetically relevant for the induction of certain corneal diseases. Recent work by Rouse and coworkers demonstrated that inhibition of angiogenesis in animal models of herpetic keratitis could prevent or diminish the intensity of herpetic stromal keratitis [33]. This suggests that efferent blood vessels may be essential in the pathogenesis of stromal herpetic keratitis by providing CD4+ lymphocytes an entry site into the corneal stroma. Novel emerging antiangiogenic therapies (see Sect. 7.5.2) may become part of the pharmacologic armamentarium to treat or prevent herpetic stromal keratitis [33].

7.3.7 Surgery in Vascularized Corneas

Surgery in vascularized corneas necessitates special approaches. Whereas refractive surgery in heavily vascularized eyes cannot be recommended since intraoperative bleeding causes changes in the ablation profile, LASIK, e.g., can be performed in eyes with minor peripheral blood vessels in the cornea. Care should be taken not to cause bleeding and if so to carefully stop bleeding and keep the ablation zone free of erythrocytes.

For penetrating keratoplasties in heavily vascularized corneas it may be advisable to preoperatively – functionally – occlude larger vessels, e.g., using fine-needle diathermy [27]. Alternative approaches are discussed below in Sect. 1.5.2.

- **Care should be taken with contact-lens induced corneal angiogenesis in keratoconus patients, since that may compromise the success of a subsequent keratoplasty due to increased risk of immune rejections**
- **Angiogenesis seems to play a pathogenic role in herpetic stromal keratitis; antiangiogenic therapy should be helpful in these patients**
- **Fine-needle diathermy is an easy to perform, quick and cheap approach for the temporary occlusion of larger corneal vessels prior to corneal surgery**

7.4 Corneal Lymphangiogenesis

Lymphangiogenesis, i.e., the development of new lymph vessels, has recently gained wide interest for its important role in tumor metastasis and induction of alloimmunity after organ transplantation [26]. Antilymphangiogenic strategies have improved survival in animal tumor models by reducing tumor metastasis. Furthermore, antihem- and antilymphangiogenic strategies have improved graft survival after organ transplantation in the mouse model of corneal transplantation (see below in Sect. 7.4.2 [3, 17]). On the other hand, pro-lymphangiogenic treatment is desirable for patients with congenital or acquired lymphedema.

7.4.1 Mechanisms of Corneal Lymphangiogenesis

Whereas it has been known for more than 100 years that the normally avascular cornea can be invaded by blood vessels (hemangiogenesis), it was unclear until very recently whether the normally alymphatic human cornea could be invaded by lymphatic vessels from the lymphatic arcade at the limbus (lymphangiogenesis [2, 12, 13]). The main reasons for that unclarity were: (1) the fact that lymph vessels – in contrast to erythrocyte-filled blood vessels – are not detectable biomicroscopically using the normal slit-lamp magnification and (2) the lack of specific markers for lymphatic endothelium. The latter has changed in the last 5–10 years with the advent of several specific markers of lymphatic endothelium (such as LYVE-1, podoplanin and VEGF receptor 3 [26]). These novel markers

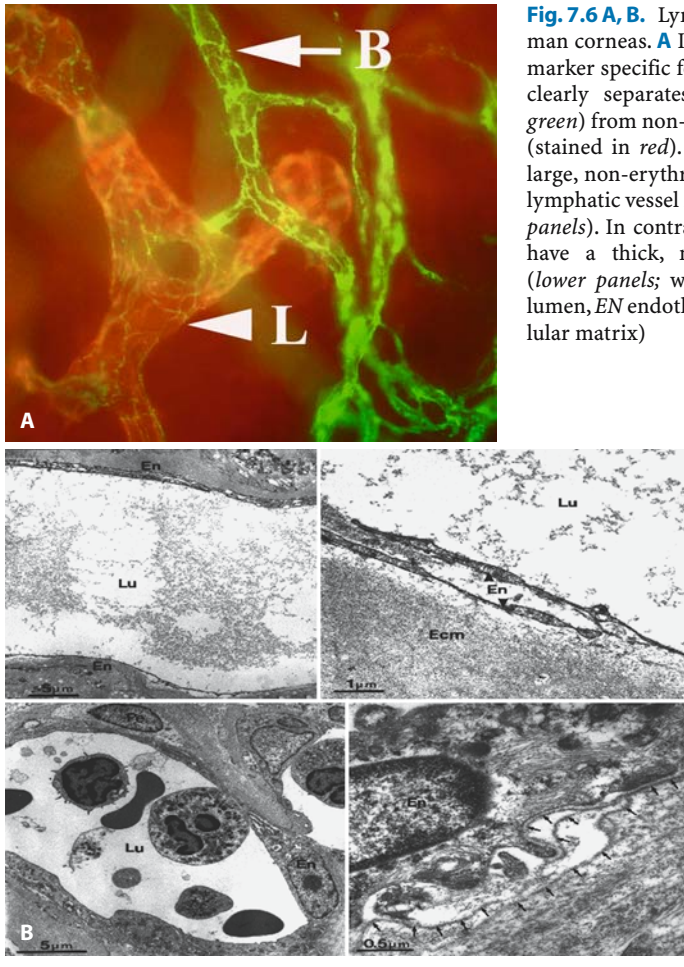

Fig. 7.6 A, B. Lymphatic vessels in vascularized human corneas. **A** Immunohistochemistry with a novel marker specific for lymphatic endothelium (LYVE-1) clearly separates blood vessels (stained here in *green*) from non-erythrocyte-filled lymphatic vessels (stained in *red*). **B** Electron microscopy reveals the large, non-erythrocyte-filled lumen of a thin-walled lymphatic vessel in a vascularized human cornea (*top panels*). In contrast, erythrocyte-filled blood vessels have a thick, multilayered basement membrane (*lower panels;* with kind permission from [11]; *Lu* lumen, *EN* endothelial cell, *Pe* pericyte, *ECM* extracellular matrix)

enabled for the first time the precise identification of lymphatic vessels in vascularized human corneas (Fig. 7.6 [11]). Lymphatic vessels were significantly more common in corneas with a short history of corneal inflammation (usually keratitis or trauma) and also were significantly more common in heavily vascularized corneas [11]. Therefore the chance of having both pathological blood and clinically invisible lymphatic vessels present is strongly correlated with the degree of corneal angiogenesis, which can be judged by slit-lamp evaluation. Furthermore, recent work suggests that it is possible to demonstrate lymphatic vessels in vivo in the cornea using confocal microscopy (HRT II using the Rostock module). Since lymphatic vessels are invisible at slit-lamp magnifications, they might not be as detrimental for corneal transparency as blood vessels are. In fact, animal experiments suggest that the "antilymphangiogenic privilege" of the cornea is not redundantly organized.

Although previous studies especially by Collin and coworkers from the 1970s suggested the existence of lymphatic vessels in vascularized animal corneas [4], only very recently has it again become possible unequivocally to identify corneal lymphangiogenesis, e.g., in the mouse model of corneal transplantation using the above-mentioned markers (Fig. 7.7 [3, 16, 17]). Using the mouse model of corneal neovascularization, we were recently able to demon-

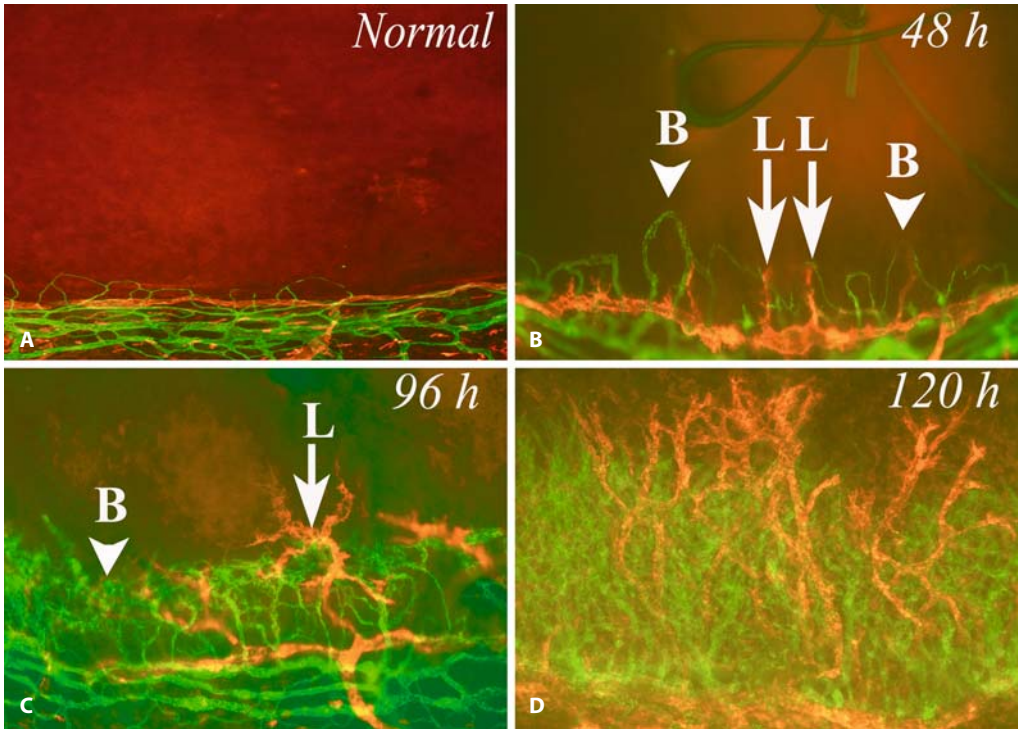

Fig. 7.7 A–D. Pathologic corneal blood vessels (stained in *green* with CD31) and lymphatic vessels (stained in *red* with LYVE-1) originate from the limbal arcade (**A**). Time course of parallel outgrowth of blood and lymphatic vessels after an inflammatory stimulus (suture) in segments from a corneal whole mount (limbus *at bottom*, central cornea with suture *at the top*; *B* blood vessel, *L* lymphatic vessel)

strate that after an inflammatory stimulus to the cornea, there is usually parallel and very early (within 48 h) outgrowth of both blood and lymphatic vessels. Both originate from the limbal vascular arcade (Fig. 7.7 [16]). The cornea therefore is also an excellent model system with which to study the mechanisms not only of angiogenesis but also lymphangiogenesis and test pharmacologic compounds for the relative inhibition of both processes in the animal model [19]. Compared to blood vessels, lymphatic vessel tend to regress much more quickly and more completely after an inflammatory challenge to the cornea [18]. For example, after a short, 2-week-long inflammatory stimulus (corneal sutures), all lymphatic vessels in the mouse cornea are completely regressed after 6 months, whereas blood vessels persist (partly as non-perfused ghost vessels) indefinitely. As outlined below, this supports the clinical practice of not performing penetrating keratoplasties in freshly inflamed eyes, but of waiting until inflammation has calmed down to improve graft survival [18]. Lymphangiogenesis is mediated by the VEGF family growth factors VEGF-A, -C and -D as well as by FGF and PDGF [26]. Stimuli for the release of the main lymphangiogenic growth factor VEGF-C are primarily inflammatory in nature, explaining the clinical observation that human corneal lymphangiogenesis is more common shortly after keratitis [11, 26].

Summary for the Clinician

- **During corneal inflammation there are parallel outgrowths of both blood and lymphatic vessels from the limbus into the cornea (combined *hem*angiogenesis and *lymph*angiogenesis)**

- **Novel immunohistochemical studies provide unequivocal evidence for lymphangiogenesis in vascularized human corneas, although lymphatic vessels are *not* visible using slit-lamp magnifications in vivo**
- **Lymphatic vessels are more common in heavily vascularized human corneas and are more common shortly after a corneal inflammation (keratoplasty, keratitis, immune rejection, etc.)**
- **In vivo confocal microscopy seems to be a technique for the visualization of lymphatic vessels in vivo in the cornea**

7.4.2 Importance of Lymphangiogenesis for Induction of Alloimmunity After Keratoplasty

Normal corneas lack both blood and lymphatic vessels. This corneal avascularity is not only essential for corneal transparency but also contributes to the enhanced prognosis of low-risk keratoplasty compared to other solid organ transplantation by suppressing both "arms" of a potential "immune reflex" that could lead to transplant rejection after keratoplasty [29, 30]. However, both secondary to a variety of diseases and after surgical manipulations, the cornea can be invaded by new blood and lymphatic vessels outgrowing from limbal blood vessels, as outlined above. This implies that, e.g., in vascularized high-risk corneas after keratoplasty the graft has *direct* contact *both* to the blood and to the lymphatic system (Fig. 7.1 B). Whereas the blood vessels provide a route of *entry* for immune effector cells ($CD4^+$ alloreactive T-lymphocytes, macrophages, etc.), corneal lymphatic vessels provide a drainage pathway for both antigenic material (cells, cellular debris) and antigen-presenting cells (APCs) from the graft to the regional lymph node. In addition, immunomodulatory cytokines, present in high-risk beds, or induced after surgical manipulation of the graft, could travel to the lymph node as could memory T cells and hyaluronic acid (HA) breakdown products that are known to activate dendritic cells [12, 13, 29]. Besides enabling the transport itself, lymphatic vessels also enhance speed and amount of antigenic material or APCs traveling to the regional lymph node. This induces alloimmunization at the lymph node and production of alloreactive effector cells, which then travel via the efferent blood vascular arm to the donor cornea and induce graft rejection (Fig. 7.1 B).

The relative importance of lymphatic vessels (representing an *exit* route for APCs) versus blood vessels (representing an *entry* route for effector cells) in vascularized corneas in relation to graft rejections is not fully understood. But since clinically detectable corneal blood vessels are neither necessary nor sufficient for immune rejection of experimental corneal grafts, an important role of the afferent *lymphatic* pathway mediated by lymphatic vessels is likely [20, 25, 30–32]. The relatively higher importance of the afferent *lymphatic* arm of the immune response in dictating the outcome of corneal graft survival has recently been demonstrated by several elegant studies: Indefinite survival of both fully mismatched orthotopic non-high-risk grafts [31] and 90 % survival of fully mismatched high-risk corneas [32] in BALB/c mice was achieved by removal of cervical lymph nodes by cervical lymphadenectomy. Furthermore, pharmacologic strategies inhibiting (angiogenesis and) lymphangiogenesis after low-risk keratoplasty (see Sect. 7.5.2) and even after high-risk keratoplasty can significantly improve corneal graft survival. In summary, interference with both the *afferent* lymphatic and the *efferent* blood vascular arm of an immune reflex arc after both low-risk and high-risk keratoplasty is a novel and interesting strategy to improve graft survival. All this supports the novel concept that antiangiogenic therapies can modulate immune responses after keratoplasty and thereby improve graft survival.

7.4.3 Non-immunological Effects of Corneal Lymphangiogenesis

Whereas the normal human cornea is devoid of HA, upregulation of HA expression in all corneal layers can consistently be observed in inflammatory corneal diseases, after trauma

and keratoplasty. Increased amounts of HA in the cornea, e.g., after refractive procedures, are associated with reduced corneal transparency ("haze"). This might suggest that HA causes local shifts in water content in the corneal wound and thereby also local shifts in transparency due to interference with the fine-tuned spacing of corneal collagen fibrils. In extraocular tissues, most of the inflammation-associated HA deposited is transported to and metabolized in regional lymph nodes and the liver. LYVE-1, one of the specific lymphendothelial markers, is an HA receptor, and is thought to mediate HA uptake into lymphatic vessels and to facilitate transport to regional lymph nodes. Since LYVE-1 is expressed on corneal lymph vessels, these could be involved in transport of pathologic corneal HA from the cornea to regional lymph nodes. Lymphangiogenesis into the cornea in this setting might be beneficial for removal of surplus HA, which would otherwise interfere with corneal transparency [12].

Summary for the Clinician

- **Since lymphatic vessels are the essential and required afferent arm of immune rejections after keratoplasty, antilymphangiogenic pharmacologic strategies can improve graft survival**
- **New concept: Antiangiogenic therapy modulates immune responses**

7.5 Antiangiogenic Therapy at the Cornea

Antiangiogenic therapeutic approaches at the cornea can be broadly divided into three categories [21–23]:

1. Angiostatic/antiangiogenic, i.e., to stop the outgrowths of new vessels (classical antiangiogenic approach)
2. Angioregressive (meaning regression of already established pathologic vessels, which is especially important, e.g., in prevascularized high-risk eyes)
3. Angio-occlusive [meaning the (temporary) functional occlusion of blood vessels, usually prior to corneal surgery]

All three approaches need to be modified according to whether only blood, only lymphatic or both vessel types are to be targeted. So far, no specific antiangiogenic therapy for application to the cornea is available. But a lot of specific antiangiogenic drugs have already entered phase II and III clinical trials in cancer and, e.g., AMD treatment, so that there is a realistic chance that as a "spin-off" of antitumor treatment, specific antihem- and antilymphangiogenic agents will be available (preferably in topical formulations) for use at the cornea medium-term.

7.5.1 Established and Novel Antiangiogenic Therapies

The drugs available so far to inhibit angiogenesis in the cornea only have indirect *antiangiogenic* effects. Topical steroids and cyclosporin A suppress the inflammatory component inducing angiogenesis. Since they have no strong direct antiangiogenic mechanism, their effect is limited [9]. But since there is so far no alternative, they are still the mainstay of topical antiangiogenic treatment at the cornea. Amniotic membrane transplantation and even amniotic membrane supernatant have been shown to exhibit antiangiogenic effects. Whether this also covers an antilymphangiogenic effect is unknown. Much more attractive for inhibiting corneal angiogenesis and lymphangiogenesis are direct antiangiogenic agents. Numerous of these have already entered phase II and III clinical trials for anticancer indications (see www.cancer.gov/clinicaltrials/developments/anti-angio-table.html). The only antiangiogenic agent with FDA approval so far is Avastin (Genentech). Other candidates in trials include the cytokine trap VEGF Trap (Regeneron), VEGF aptamers (Macugen, Eyetech), antiangiogenic steroids (anecortave acetate, Alcon) and many more. We recently demonstrated a very potent antiangiogenic effect of the VEGF-A cytokine trap (Regeneron) on both inflammation-induced corneal hemangiogenesis and surprisingly also on lymphangiogenesis (Fig. 7.8 [16]). This nearly complete inhibition shows the much higher potency of direct

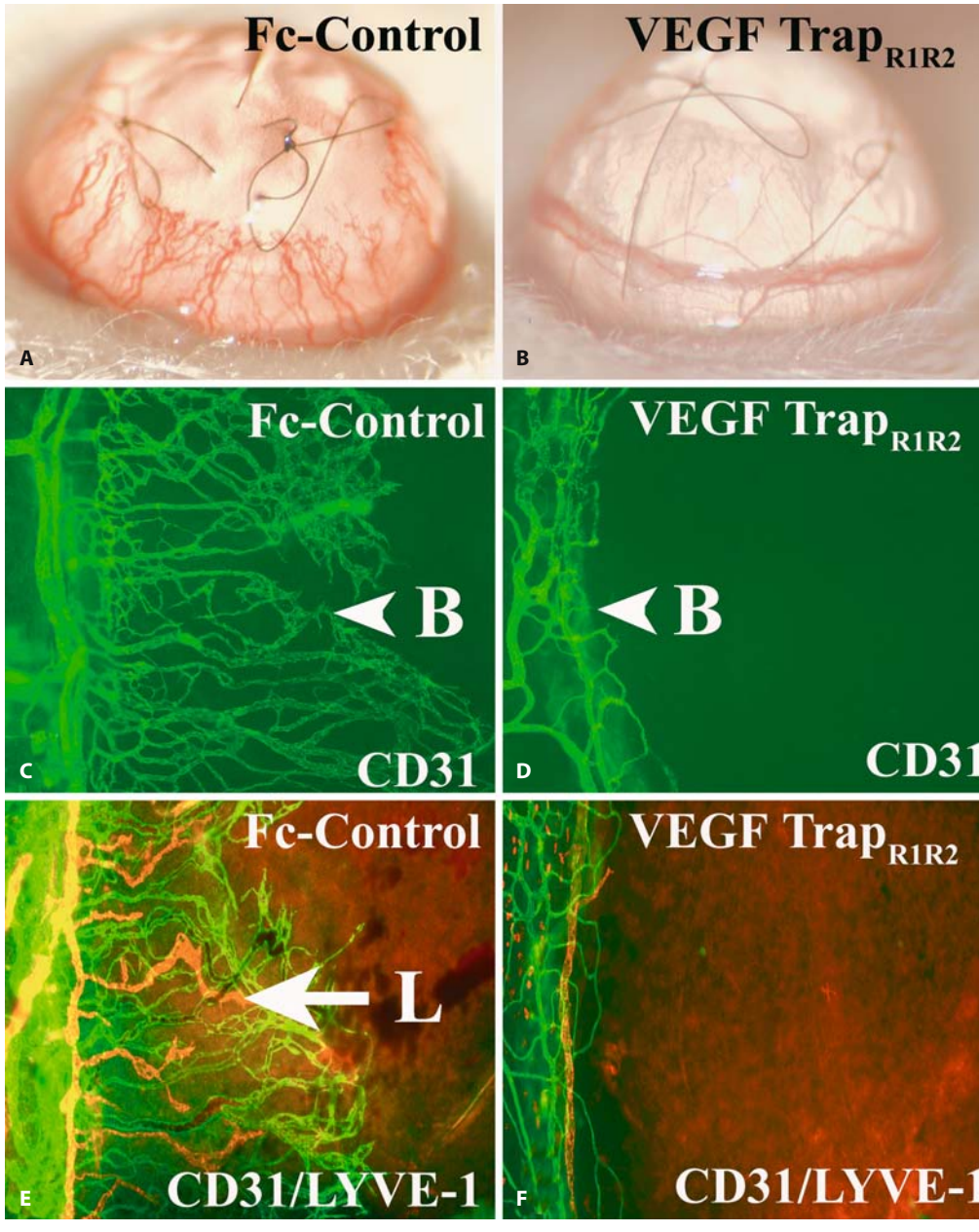

Fig. 7.8 A–G. Profound inhibitory effect of the VEGF-A cytokine trap (VEGF Trap) on both inflammation-induced corneal hemangiogenesis and lymphangiogenesis in the mouse model of suture-induced corneal neovascularization (with kind permission from [16]). *Left pictures* are controls, *right pictures* are VEGF trap-treated animals (blood vessels: *green*; lymphatic vessels: *red*). Note the nearly complete inhibition of both hem- and lymphangiogenesis

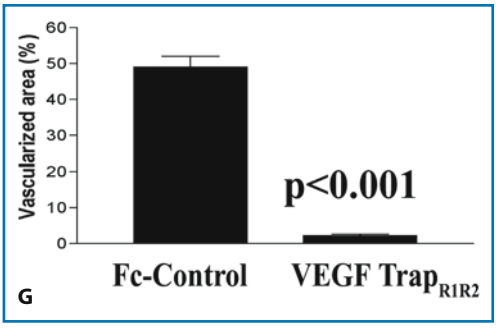

Fig. 7.8 G. (*continued*)

antiangiogenic agents compared for, e.g., steroids (although no formal comparison has been performed so far). Since all of these compounds are in trial for systemic applications in tumor and posterior eye diseases, some time will evolve until topical formulations for treatment at the cornea become available. Nevertheless, the dramatic developments in the antiangiogenic cancer field will definitively provide useful spin-off products for the anterior eye segment.

Angioregressive therapies would allow the regression of preformed pathologic corneal blood vessels. Whereas the regression of novel, newly outgrown blood vessels (in the so-called "pruning phase" [14]) can be achieved by removal of angiogenic agents such as VEGF, older and more mature and pericyte-covered vessels no longer depend on angiogenic signaling [14]. Induction of regression of these mature vessels is more complex, and would have to involve anti-VEGF strategies combined with agonists of the vascular endothelial TIE2 receptor (angiopoietin 2). The regression phase for immature vessels again is very short, so that, e.g., removal of a loose suture or a hypoxia-inducing contact lens needs to be performed very early after the onset of vessel outgrowths to cause regression of the new vessels. Since the mechanisms responsible for the maintenance of lymphatic vessels are poorly understood, so far no approach for the regression of lymphatic corneal vessels is known. Fortunately, lymphatic vessels in the cornea seem to regress spontaneously after the (inflammatory) stimulus subsides [18].

Angio-occlusive approaches are useful, e.g., to prevent intraoperative bleeding during keratoplasty in vascularized high-risk eyes or to stop leakage into the cornea out of these blood vessels. Besides the more experimental approach of corneal photodynamic therapy, fine-needle diathermy is a reliable, cost-effective and quick treatment option. Corneal vessels are either directly cautered or a suture needle is placed into/next to the vessel and the needle tip is then cautered (Fig. 7.9 [27]).

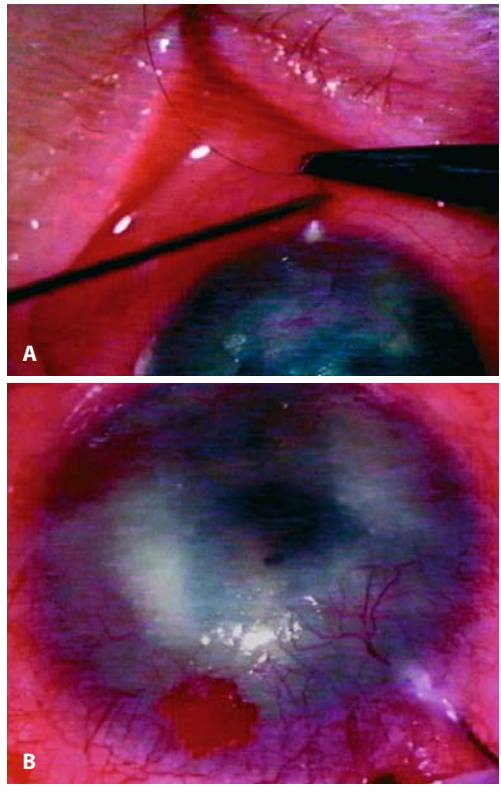

Fig. 7.9 A, B. Fine-needle diathermy is an easy and quick method for the functional occlusion of larger corneal stromal blood vessels, e.g., prior to keratoplasty (with kind permission from [27]). Either the vessel is coagulated directly or a needle is passed through/adjacent to the vessel and the cautery applied to the needle

Summary for the Clinician

- **Antiangiogenic treatments fall into three categories: angiostatic, angioregressive and angio-occlusive**
- **Mainstays so far are topical steroids and cyclosporin A eyedrops**
- **Novel (topical) antiangiogenic drugs will dramatically improve the potential to inhibit corneal angiogenesis effectively**

7.5.2 Novel Antihemangiogenic and Antilymphangiogenic Therapies to Improve Graft Survival After Keratoplasty

Using one of the novel direct antiangiogenic agents [the VEGF-A specific cytokine trap (VEGF Trap) from Regeneron], it was shown recently that inhibition of hemangiogenesis and lymphangiogenesis *after* low-risk keratoplasty improves corneal graft survival significantly (Fig. 7.5 [17]). Furthermore, pharmacologic strategies targeting the VEGF receptor 3, mediating primarily lymphangiogenesis and partly hemangiogenesis, were also able significantly to improve graft survival postkeratoplasty [3]. This establishes postkeratoplasty neovascularization in the low-risk bed as a novel risk factor for subsequent immune rejections and supports the novel concept of modulating immune responses after corneal grafting by antihem- and antilymphangiogenic therapies.

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Histocompatibility Matching in Penetrating Keratoplasty

8

Daniel Böhringer, Rainer Sundmacher, Thomas Reinhard

Core Messages

- HLA matching reduces graft rejections in normal- as well as in high-risk keratoplasty
- Most patients can be served with an HLA compatible graft within well below a year, even on a monocenter waiting list
- Waiting time for a histocompatible graft can be predicted and discussed with each patient in advance
- The *HLAMatchmaker* algorithm can balance waiting time and histocompatibility for patients with rare HLA phenotype
- The HLA-A1/H-Y minor antigen equals immunogenicity of HLA mismatches: allocating male HLA-A1 positive donors for female recipients should be avoided
- Long-term graft survival will improve upon routinely matching major and selected minor histocompatibility antigens. This strategy will outweigh the cost of HLA typing in the long run

8.1 Introduction

In this chapter, the recent evolvements in histocompatibility matching for penetrating keratoplasty are presented and a strategy for clinical practice is recommended.

8.1.1 Immune Reactions Constantly Threaten Graft Survival

Despite the anterior eye chamber immune privilege, graft rejections are a major complication of penetrating keratoplasty as they facilitate subsequent graft failure. Application of topical corticosteroids for several months has commonly been thought to be sufficiently protective. On average, 18 % of patients with normal-risk keratoplasty [13] and up to 75 % in high-risk cases [15] nevertheless experience immune reactions. Life span of affected grafts is significantly reduced from an endothelial graft reaction. Immune reactions thus increase the incidence of re-keratoplasties due to failed grafts in the long run.

Graft reactions can be reduced by means of intensified and prolonged prophylaxis with topical corticosteroids and with systemic immunosuppression [14]. The protective effect, however, ceases upon discontinuation of the immunosuppressive regimen. Any long-term dependence on immunosuppression is associated with additional costs and potentially serious side effects.

Antigen matching, that is avoiding grafts bearing antigens that are foreign to the recipient upon allocation, improves histocompatibility. Matching of human leukocyte antigens ("HLA Matching," Sect. 8.1.2) and more recently of further antigen systems ("Minor Matching," Sect. 8.1.3) has turned out to be a potent adjunct to immunosuppression in the fields of allogeneic transplantation, i. e., in penetrating keratoplasty.

Summary for the Clinician

- **Unlike immunosuppression, HLA matching can *permanently* reduce graft rejections.**

8.1.2 Major Transplantation Antigens (HLA)

Experimental transfer of tumors from one mouse strain to others led to the discovery of the major histocompatibility complex (MHC). In humans, these antigens were termed human leukocyte antigens (HLAs). Antibodies against HLAs were first discovered after a transfusion reaction of a multiparous woman despite blood group compatibility.

The HLA antigens are subdivided into three classes according to their expression pattern and their association with other molecules. Only HLA genes of class I and II are relevant to transplantation medicine as they encode for polymorphic membrane bound molecules.

- Class I molecules are expressed on almost all nucleated body cells. They comprise a heavy chain, a light chain (β_2-microglobulin) and a small peptide of nine amino acid residues. Three gene loci of major importance have been identified for this system: HLA-A, HLA-B and HLA-C.
- Class II molecules are homodimers. These molecules are exclusively expressed on cell families that share the ability to present extracellular antigens to the immune system (e.g., monocyte and lymphocyte derived strains). HLA class II loci of major immunologic importance are HLA-DR and HLA-DQ.

8.1.2.1 Methods for Typing HLA Antigens

HLA antigens were discovered by means of serological assays. In the beginning, only the macroagglutination assay was available. For this assay, patient serum was mixed with test cells bearing only the respective antigen. Any macroscopically visible agglutination demonstrated the presence of antibodies directed against the respective test cells. Depending on the availability of appropriate test cells, only a subset of all HLA antibodies could be detected at that time. Today, the serologic HLA typing assay is performed using the complement-dependent cytotoxicity assay (CDC). For HLA typing, cells are incubated against a battery of standardized antibodies as defined by the International Histocompatibility Workshop (IHW). These IHW test sera define the HLA antigens that can be detected from the assay. The assay is further incubated with rabbit complement. Lysis is induced by complement activation in cells that were recognized by a specific antibody. For detection of lysis, a dye is eventually added.

The first generation of CDC test sera was unable to detect differences of only a few amino acid residues between certain HLA alleles. Upon availability of monoclonal antibody techniques, the International Histocompatibility Workshop released a new generation set of test sera that eventually was able to split most alleles known at that time ("broads") into further HLA alleles ("splits").

As the HLA nomenclature was already established at that time, the newly discovered alleles were termed splits of the established broad antigens and sequentially assigned higher numbers than the established broad antigens.

Further improvements in HLA typing were achieved by means of molecular techniques as discovery of new alleles with these molecular methods no longer depends on isolation of viable cells. Using the polymerase chain reaction (PCR), small parts of the genome can be detected by means of specific primers and an amplification reaction. This is achieved either by sequence specific oligonucleotide priming where alleles are identified by characteristic "fingerprint" sequences or by the more expensive sequence typing of the whole allele. These molecular techniques led to further subdifferentiation of most split alleles.

For example, the broad antigen HLA DR3 is dividable into the split antigens DR17 and DR18, which in turn can be subdivided into DRB1*0302 and DRB1*0303 at the molecular level.

In corneal transplantation, blood for HLA typing is commonly collected up to 72 h after the donor's death. From autolytic changes, a sufficient amount of viable cells is often unavail-

able for serologic analysis. In this situation, molecular methods can still detect the HLA phenotype with excellent precision.

Summary for the Clinician

- **Various HLA molecules are bound to the surface of all nucleated cells. These antigens are potentially targeted by the immune system unless they are tolerated as of birth. One or two different versions (alleles) of each HLA locus can be produced by each cell. Over 20 alleles have been identified for each of the HLA loci.**
- **In penetrating keratoplasty, the alleles of donor and recipients should be typed with immunogenetic techniques due to higher accuracy and precision, especially in blood samples collected up to 72 h after clinical death of the donor.**

8.1.2.2 HLA Matching in Penetrating Keratoplasty

In penetrating keratoplasty, contrary to other fields of transplantation, the potentials of HLA matching are currently mostly unexploited as the beneficial effect was demonstrated only recently. This calls for explanation as the human cornea has been known for longer to express HLA antigens and these antigens are known targets of cytotoxic T cells in the process of graft rejection [12].

8.1.2.2.1 Methodical Problems with Older Investigations

Numerous studies performed in the past 2 decades came to contradictory results as to the usefulness of HLA matching [17]. Three shortcomings of study design in these older investigations, however, compromised the power to demonstrate any matching effect:

1. The major problem with almost all older studies is the poor quality of HLA typing at that time. The importance of highly accurate HLA typing for successful HLA matching was recently recognized. Even 5% of faulty HLA DR typing obscures the beneficial effect as demonstrated in a recent simulation analysis [18]. The CCTS for example was based on typing data that differed by 55% from retyping with modern techniques, invalidating all conclusions drawn from that particular investigation.
2. Additionally, most studies suffered from poor statistical power due to heterogeneity of the study groups. Multiple centers, lack of standardization regarding surgical experience and keratoplasty procedure as well as differences in immunosuppression regimens most likely also influenced outcome and thus confounded or obscured the HLA effect.
3. Finally, most studies were performed on high-risk patients, who not only are at increased risk of immune reactions but are also at risk of graft failure from events other than graft rejection. When correlating total graft failures with HLA matching, any statistical association might be obscured from non-immunological graft failures such as protracted elevated intraocular pressure.

8.1.2.2.2 Current Evidence

On the basis of modern and reliable HLA typing techniques, recently four monocenter studies from Europe were published. These well-designed investigations uniformly confirmed a beneficial effect of HLA compatibility in penetrating keratoplasty [1, 13, 15, 18]. Each study stresses additional aspects with respect to HLA matching as follows:

- In 1,681 consecutive transplantations from only one center, a benefit was found from matching the class II locus HLA-DR additionally to the class I loci A and B [18].
- A beneficial effect from class I matching alone was only observed when matching is based on split rather than broad (see Sect. 8.1.2.1) HLA alleles [1].
- A beneficial effect was demonstrated even for normal risk (first keratoplasty for bullous keratopathy, Fuchs' endothelial dystrophy, keratoconus with centrally sutured graft or avascular corneal scars) keratoplasty alone when matching HLA A, B and DR broad alleles (Fig. 8.1) [13].

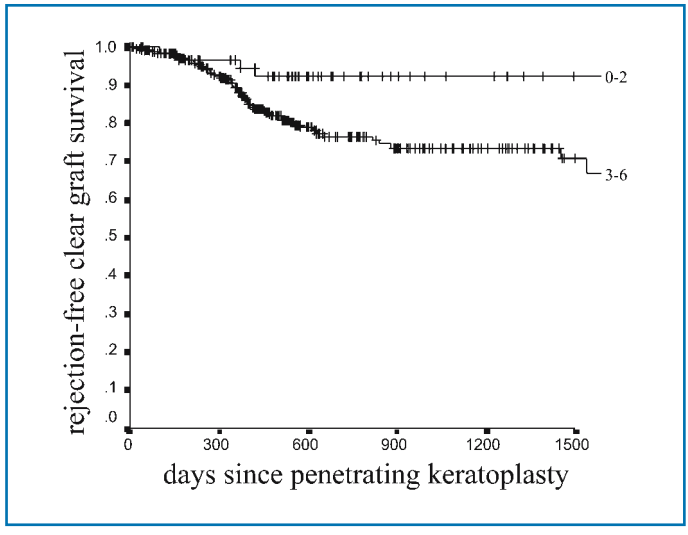

Fig. 8.1. Beneficial effect for normal risk keratoplasty alone when matching HLA A, B and DR broad alleles [13]

Summary for the Clinician

- **A mounting body of evidence supports the beneficial effects of HLA matching in normal- as well as in high-risk keratoplasty.**
- **Accuracy and precision of HLA typing are crucial to HLA matching.**

8.1.2.2.3 Variable Immunogenicity of Individual HLA Mismatches

HLA mismatches differ in strength of immunogenicity and thus in deterioration of graft survival. This has been observed for longer in kidney transplantation [6] and in keratoplasty [5]. For HLA class I loci, the structural basis of this phenomenon has recently been established [7, 8, 9, 10]. This paved the way for predicting "acceptable" mismatches on an individual basis: the *HLAMatchmaker* algorithm defines nearly 50 omnipresent epitopes within the molecular structure of all HLA class I alleles. These epitopes are thought to be particularly exposed to the immune system and partitioned into triplets of amino acid residues to account for thermodynamic characteristics of the antibody recognition reaction. All triplets are formally concatenated to form the triplet-string for a particular allele. The association of the triplet-string with a particular allele is exclusively defined for alleles at molecular typing resolution.

Degree of matching is assessed by counting all triplets of the donor's triplet-strings that are not identical to any of the corresponding triplets of the recipient's four triplet-strings (two for HLA-A and -B each).

HLA mismatches with zero to few mismatched triplets are supposed to be fully histocompatible with regard to the antibody epitopes. Additionally, they are known not to cause a deterioration of graft survival in kidney transplantation [10]. In penetrating keratoplasty, recently a beneficial effect of this algorithm was demonstrated (Fig. 8.2) [3].

Summary for the Clinician

- **The *HLAMatchmaker* algorithm can help in reducing graft rejections for penetrating keratoplasty to a similar extent as conventional HLA matching**
- **This algorithm is crucial for providing histocompatible grafts to patients with rare HLA phenotypes within a reasonable time. Alternatively, waiting time can be traded for a better match grade**

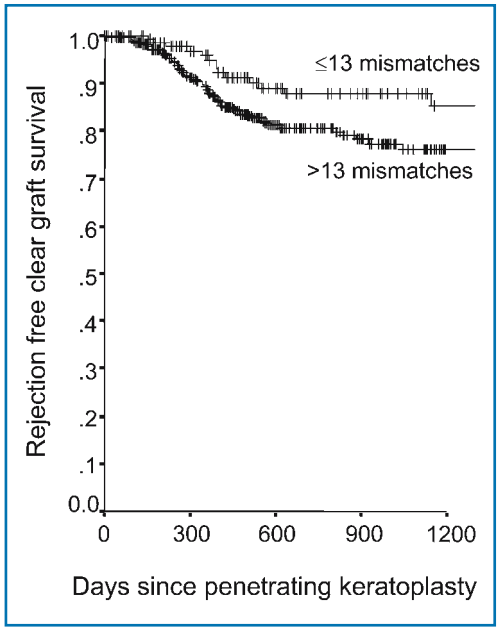

Fig. 8.2. Beneficial effect of the HLAMatchmaker algorithm in penetrating keratoplasty [3]

8.1.3 Minor Transplantation Antigens

Graft rejections are observed even in HLA identical allogeneic transplantations. These effects are ascribed to disparities in minor histocompatibility (H) antigens. A single minor H mismatch even exceeds the immunogenicity of a single MHC mismatch in a mouse-model for high-risk keratoplasty.

H antigens are peptides derived from polymorphic proteins. Their immunogenicity arises as a result of their presentation on the plasma membrane in the context of HLA class I or II, where they are recognized by alloreactive HLA restricted T cells. In animal models, antigen presenting cells (APCs) such as limbal Langerhans cells have been demonstrated to migrate from the graft to the host spleen via the cameros-splenic axis. The spleen might thus be the source of a cytotoxic specific immune response directed against foreign graft H antigens presented by graft APCs.

8.1.3.1 Selected Minor Antigens

8.1.3.1.1 H-Y

Male grafts can be subject to alloimmune reactivity in female recipients, as antigens of the H-Y group are only expressed in male individuals and not in females. H-Y antigens are supposed to occur in all tissues including the human cornea.

Epitopes of the H-Y antigen family are expressed either in the context of HLA-A1 or HLA-A2. The HLA-A1/H-Y antigen is located in the Y-chromosome-encoded DFFRY protein, The HLA-A*0201-restricted HLA-A2/H-Y antigen contains a post-translationally modified cysteine that significantly affects T-cell recognition.

As to matching the HLA-A1/H-Y epitope, a 20% reduction of graft rejections was recently demonstrated on 252 keratoplasties (manuscript in preparation), whereas matching of the HLA-A2/H-Y epitope did not affect graft survival.

From this observation, male HLA-A1 positive donors should not be allocated to female recipients. The prevalence of this setting is as high as 13% in the German keratoplasty population.

8.1.3.1.2 HA-3

Another HLA-A1 restricted H antigen expressed in all corneal layers is the HA-3 epitope. This epitope is derived from the lymphoid blast crisis (Lbc) oncoprotein. Two alleles, VTEPGTAQY (HA-3T) and VMEPGTAQY (HA-3M), have been demonstrated. T-cell immune reactivity has only been observed in the direction of HA-3T. The prevalence of the immunogenic setting is as low as 3% in the German keratoplasty population. HLA-A1/HA-3 matching can thus be thought of as being of slight importance and HA-typing is not recommended for routine use.

8.1.3.1.3 Blood Group Antigens

Blood group antigens are expressed on the cornea. In high risk situations, a significant reduction of graft reactions after penetrating keratoplasty was observed in retrospective investigations [4, 11].

Future research holds the prospect of demonstrating an additional HLA restricted influence of blood group antigens in normal risk situations as well.

Summary for the Clinician

- HLA-A1 positive grafts from male donors should not be allocated to female recipients
- Matching blood group antigens reduces graft rejections in high-risk keratoplasty
- The rapidly evolving field of minor antigens is subject to ongoing and future research in penetrating keratoplasty

8.2 Time on the Waiting List Associated with Histocompatibility Matching

8.2.1 Waiting Time Variance Has Been a Barrier to Histocompatibility Matching

Histocompatibility matching is associated with additional time on the waiting list from refusing all newly available grafts while waiting for the first that satisfies the histocompatibility requirements. Due to the social and individual costs of blindness, waiting periods exceeding 1 year are hardly reasonable.

This waiting period is highly variable, depending on the recipient's histocompatibility antigens: individuals with common HLA phenotypes can be routinely provided a matching graft within a few months as prevalence of compatible phenotypes is common in the donor population as well. On the other hand, individuals with a rare HLA phenotype commonly remain on the waiting list for years without being allocated a compatible graft. These patients, waiting in vain for an HLA match, contributed to the long-standing reluctance towards HLA matching in penetrating keratoplasty.

When these patients are identified in advance, a randomly assigned graft with appropriate immunosuppression can be opted for a priori or the stringency with respect to histocompatibility can be reduced, e.g., using the *HLAMatchmaker* algorithm (Sect. 8.1.2.2.3). An algorithm for predicting the waiting period is thus vital for informed consent on histocompatibility, which has to be discussed with each patient individually. This problem was solved recently with an algorithm that can predict the expected time on the waiting list on an individual basis.

8.2.2 Algorithm for Predicting the Time on the Waiting List

An algorithm that is based on the HLA phenotype, a database of the most common haplotype frequencies in the donor population and last but not least parameters of the local cornea bank can robustly predict the estimated waiting time for an HLA compatible graft. This algorithm has been retrospectively validated against an historical waiting list of almost 1,400 HLA typed patients (Table 8.1) [2]. The assumptions of this algorithm are summarized in the following two sections.

8.2.2.1 Percentage of HLA Compatible Grafts

Twenty-seven different HLA phenotypes match any recipient who is a heterozygote for the HLA loci A, B and DR. Only one phenotype, however, matches an individual who is completely homozygous at these loci. The total percentage of the donor population matching the HLA phenotype of any recipient is well approximated by the sum of all population frequencies (donor population) of the compatible HLA phenotypes. The frequency of any HLA phenotype is the product of both haplotype frequencies. Haplotype frequencies for the HLA loci A, B and DR can be retrieved from a common database comprising the respective donor population [16].

Table 8.1. Validation of algorithm against a historical waiting list of almost 1400 HLA typed patients [2]

	Zero mismatches	One mismatch	Two mismatches
Predicted period (only of recipients for which a match was found below)	17±159	7±49	1±6
Simulated period	15±14	5±9	1±3
	(29 %)	(71 %)	(83 %)
	R=0.28; p<0.001	R=0.36; p<0.001	R=0.45; p<0.001

8.2.2.2 Actual Estimation of the Waiting Time

The daily rate of new HLA compatible grafts equals the product of the daily rate of new HLA typed grafts and the percentage of HLA compatible donors as described in the previous section. Assuming a Poissonian distribution of donors, expected waiting period is reciprocal to the daily rate of new HLA compatible grafts. The algorithm is summarized in Eq. 8.1.

$$t = \frac{\frac{1}{365}}{2GR\sum GF} \tag{8.1}$$

where t [years] is expected waiting period, GF is total share of compatible HLA phenotypes and GR is local daily rate of new donors.

A certain percentage of grafts are unsuitable for transplantation due to quality control. This can be adjusted using a local empiric constant for each cornea bank.

Summary for the Clinician

- **Most patients can be served with an HLA compatible graft within well below a year, even on a monocenter waiting list**
- **Patients that waited in vain for an HLA match for a long time contribute to the reluctance towards HLA matching in penetrating keratoplasty**
- **Waiting time for a histocompatible graft can be predicted from the HLA phenotype and discussed with the patient in advance**

8.3 Recommended Clinical Practice

According to current knowledge, HLA matching should be performed at least for the HLA loci A, B and DR.

All corneal grafts should be typed for these HLA loci in order to increase the pool available for histocompatibility matching. Molecular typing should be preferred over serologic methods as molecular methods can still detect the HLA phenotype with excellent precision when blood for HLA typing is collected after up to 72 h postmortem. An additional benefit of molecular typing is the applicability of the *HLAMatchmaker* algorithm (Sect. 8.1.2.2.3).

As for the recipient, all patients should be typed upon the keratoplasty being indicated, again preferably with immunogenetic techniques for the *HLAMatchmaker* algorithm.

All patients awaiting normal risk keratoplasties should be told their expected time on the waiting list (Sect. 8.2). Improved prognosis from HLA compatibility should be weighed against the expected waiting time. This strategy needs to be discussed with each patient individually.

Patients awaiting high-risk keratoplasty should be provided a histocompatible graft in almost all cases. The *HLAMatchmaker* algorithm can be applied to balance time on the waiting list with the degree of histocompatibility that is realistically achievable in patients with rare HLA phenotype.

Matching of HLA and additional antigen systems (e.g., H-Y/HLA-A1 and blood group antigens), the *HLAMatchmaker* algorithm and waiting time prediction are only feasible with an integrated highly specialized software package from professionalized high-volume institutions responsible for allocation.

In summary, long-term graft survival will improve upon routinely matching major and certain minor histocompatibility antigens in all keratoplasties. This policy will outweigh the costs of HLA typing in the long run.

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Current Systemic Immunosuppressive Strategies in Penetrating Keratoplasty

9

Alexander Reis, Thomas Reinhard

Core Messages

- Immunologic rejection is the main cause of corneal graft failure
- Acute rejection is mainly mediated by T cells and can be prevented with steroids, IL-2 inhibitors (cyclosporine, tacrolimus), mycophenolate mofetil and TOR inhibitors (everolimus, rapamycin)
- Based on their risk of immunologic rejection, corneal transplants are rated as either normal-risk or high-risk transplants
- In a normal-risk situation the postoperative application of topical steroids is sufficient to prevent acute graft rejection in most cases
- In high-risk keratoplasty systemic immunosuppression with cyclosporine, mycophenolate mofetil or tacrolimus has to be used to maintain clear graft survival
- As corneal transplantation is not a life-saving procedure, the side-effect profile is a central issue when choosing immunosuppressive medication

9.1 Introduction

Immunologic graft rejection is the single most important reason for graft failure following corneal transplantation. If corneal transplantation is performed in a high-risk situation without the use of systemic immunosuppression, corneal graft failure can be expected in over 50 % of cases within the first postoperative year [15, 33].

Despite the advantage that the transplanted organ can directly (and not via the vascular system) be reached with topical steroids in extremely high concentrations, thereby interfering with the host's immune system right at the "battlefield" of graft rejection, this strategy is only sufficient in a normal-risk situation.

The clonal expansion of alloreactive T cells occurs in lymphoid organs (i.e. lymph nodes and spleen): after the recognition of the foreign tissues by T cells, these specific T cells start to proliferate and generate an immunological army against the graft. It is therefore crucial not only to work with topical steroids but to employ immunosuppressive substances systemically. *In a high-risk situation you have to fight the inhospitable host in its hinterland to achieve graft survival in the long run.*

To understand the possible targets of immunosuppression and immunomodulation we need to take a look at the underlying immunology.

9.2 Immunology

Immunological responses against the transplanted cornea remain the major cause of allograft injury and loss. The innate and adaptive immune systems are variously involved in rejection. Several factors determine the strength and nature of the immune response: (1) the nature of the grafted cornea, i.e. whether it is a clear corneal button or a limbocorneal transplant; and (2) the nature of the recipient's graft bed (i.e. whether it is clear, vascularised or has a limbal stem cell insufficiency). Additionally,

inflammatory responses (and graft rejection is a form of inflammatory response) are physiologically suppressed in the anterior chamber: on the one hand, antigens injected intraocularly elicit deviant systemic immune responses that are devoid of immunogenic inflammation (a phenomenon called anterior chamber associated immune deviation, ACAID). On the other hand, the ocular microenvironment (aqueous humor, secreted by cells that surround this chamber) suppresses intraocular expression of immunogenic inflammation [51].

These special anatomical features are responsible for the excellent results in normal-risk corneal transplantation when compared to solid organ transplantation or high-risk corneal transplantation.

The nature of the host's immune response can be determined by its histopathology and time course as acute or chronic rejection.

9.2.1 Acute Rejection

Acute rejection which may occur weeks to years after transplantation involves both humoral and cell-mediated immune reactions. T cells play a central role in acute rejection by responding to alloantigens, predominantly major histocompatibility complex (MHC) molecules, presented on endothelial, epithelial, or stromal cells. Both CD4+ and CD8+ T cells contribute to acute rejection. CD4+ T cells mediate acute rejection by secreting cytokines and inducing delayed-type hypersensitivity-like reactions in the graft. Recognition and lyses of foreign cells by cytotoxic CD8+ T cells are an important mechanism of acute rejection. T cells may be activated by two distinct mechanisms: the direct and the indirect pathway.

Based on the target, immune reactions against the transplanted cornea may be divided into endothelial, stromal or epithelial rejection. The most frequent and most severe form of immune response is against the endothelium. The reason is that the immunogenic epithelial cells are replaced within approximately 1 year by the host's epithelium and the stroma mostly consists of intracellular substance and only a small number of cells. Therefore the endothelium shows the highest immunogenicity of a corneal graft.

9.2.2 Major Histocompatibility Complex

9.2.2.1 Direct Pathway of Allorecognition

Direct recognition of foreign MHC antigens by T cells is the primary cause of acute rejection: recipient T cells recognise donor MHC class I and class II molecules, resulting in the generation and clonal proliferation of helper and cytotoxic T cells.

9.2.2.2 Indirect Pathway of Allorecognition

This occurs when the MHC molecules of the donor tissues are taken up and processed by antigen-presenting cells, which present the foreign peptides to T cells. Since MHC molecules are highly polymorphic in nature, they are mainly responsible for allograft rejection. Transplantation between individuals with identical MHC molecules may also fail in the late phase because at this time the so-called minor histocompatibility antigens come into play.

9.2.3 Chronic Rejection

The pathogenesis of chronic rejection is not clear [3, 30]. It is likely that most of the adaptive and innate immune systems are involved in this process. Chronic rejection cannot be prevented with current immunosuppressive drugs (which mainly work through their interference with T cells), so the present strategy is to limit the number of acute rejection episodes. The best prospects for overcoming late graft loss due to chronic rejection may reside in a new generation of immunosuppressive agents [28]. Many risk factors may increase the incidence of chronic rejection: MHC incompatibility, the number and severity of acute rejection episodes and the recurrence of herpetic ocular disease.

9.3 Normal-Risk Versus High-Risk Transplantation

Based on their risk of graft rejection, corneal transplants can be divided into normal-risk or high-risk transplants.

9.3.1 Normal-Risk Transplantation

In a normal-risk situation (e.g. first transplant in keratokonus or Fuchs's endothelial dystrophy), a 5-month course of topical steroids (e.g. prednisolone acetate 1%, Inflanefran forte®) 5 times a day, reduced by one drop every month) accompanied by systemic steroids (prednisolone 1 mg/kg tapered within 3 weeks) is sufficient to maintain a 5-year clear graft survival of up to 90%. Up to 20% of normal-risk corneal transplants experience an acute rejection episode which can be converted in about 50% of cases with topical and systemic steroids.

9.3.2 High-Risk Transplantation

Postoperative systemic immunosuppression is widely accepted as the treatment of choice in immunologic high-risk groups. High-risk corneal transplantation can be defined as follows:

- History of previous graft rejections
- Deep vascularisation of the recipient cornea in more than three quadrants
- Limbal stem cell deficiency, which requires a corneolimbal graft
- Severe atopic dermatitis

In addition to topical and systemic application of steroids as mentioned previously, systemic immunosuppression should be applied for at least 6 months following transplantation.

9.3.3 Rationale for Systemic Immunosuppression

The first goal of a timely limited systemic immunomodulation is the prevention of acute rejection episodes. The second goal is the interference with the initial graft-host interaction in a way that graft-protective cells and cytokines are promoted, hence enabling a clear graft survival without any further medication. We have already shown clinically that we can reach the first goal in most patients when using cyclosporine or mycophenolate mofetil systemically. Unfortunately, we still do not have convincing results with our therapeutic strategies when looking at long-term graft survival.

9.3.4 Why Is Immunomodulation with Topical Steroids Not Sufficient To Prevent Immunologic Graft Rejection in High-Risk Patients?

The cornea is a privileged place for transplantation, for both its anatomical features (see above) and the possibility of bringing medication directly to the transplanted organ, thereby reducing systemic side effects. In a high-risk situation the immunological privilege is diminished and the risk of graft loss within 1 year lies over 50% without the use of systemic immunosuppression [15, 33]. Why are topical steroids not enough?

The activation of the recipient's immune system against the transplanted cornea, i.e. the priming of naïve T cells, occurs in lymphoid tissues. This hypothesis is supported by experiments in which T-cell activation and therefore graft rejection did not occur when secondary lymphoid organs were absent [18]. These experimental data indicate that leukocytes participate in host T-cell priming by migrating from the graft to the host's lymph node and/or spleen, where they activate alloreactive host T cells in the direct and indirect pathway. Such primed T cells circulate and target MHC molecules expressed by cells of the graft.

As topical steroids do not reach the secondary lymphoid organs, and even systemic steroids do not interfere sufficiently with the clonal expansion of activated T cells, it is essential to administer systemic immunosuppressives in order to achieve clear graft survival.

Summary for the Clinician

- **If corneal transplantation is performed in a high-risk situation without the use of systemic immunosuppression, corneal graft failure can be expected in over 50 % of cases within the first postoperative year**
- **Definition of high-risk corneal transplantation:**
  - **History of previous graft rejections**
  - **Deep vascularisation of the recipient cornea in more than three quadrants**
  - **Limbal stem cell deficiency which requires a corneolimbal graft**
  - **Severe atopic dermatitis**
- **In a high-risk situation it is crucial not only to work with topical or systemic steroids but to employ immunosuppressive substances systemically**
- **T cells play a central role in rejection by responding to alloantigens, predominantly MHC molecules, presented on endothelial, epithelial, or stromal cells**
- **The activation of the recipient's immune system against the transplanted cornea, i.e. the priming of naïve T cells, occurs in lymphoid tissues**
- **As topical steroids do not reach the secondary lymphoid organs, and even systemic steroids do not interfere sufficiently with the clonal expansion of activated T cells, it is essential to administer systemic immunosuppressives in order to achieve clear graft survival**
- **The first goal of a timely limited systemic immunomodulation is the prevention of acute rejection episodes**
- **The second goal is the interference with the initial graft-host interaction in a way that graft-protective cells and cytokines are promoted, hence enabling a clear graft survival without any further medication**

9.4 Immunosuppressive Agents

9.4.1 History

Along with the increase in the number of solid organ transplants, our therapeutic armamentarium and knowledge of immunosuppressive drugs in corneal transplantation has been improved.

In the 1950s the selection of immunosuppressive drugs was limited to corticosteroids and azathioprine. In the 1960s polyclonal antilymphocyte (ALG) and antithymocyte (ATG) globulins supplemented the repertoire. In the late 1970s cyclosporine A led to a real breakthrough in clinical solid organ transplantation (Table 9.1). Motivated by the encouraging results in graft survival, the research in this immunological field then led us to a wide range of

Table 9.1. Immunosuppressives: history

1949	Cortisone was shown to alleviate rheumatoid arthritis
1959	Cyclophosphamide was demonstrated to suppress the formation of antibodies and was used for bone marrow transplantation
1960s	Azathioprine was found to delay organ graft rejection
1969	Methotrexate was shown to inhibit antibody formation and the development of delayed hypersensitivity in guinea pigs
1976	T-cell-inhibiting properties of cyclosporine were demonstrated
1982	Development of mycophenolate mofetil
1987	Tacrolimus (FK506) was shown to inhibit IL-2 production and lymphocyte proliferation
1980s	Interest in the antibiotic sirolimus was renewed when it was shown to prevent allograft rejection
1978	Leflunomide
1981	Mizoribine, deoxyspergualin, brequinar
1990s	IL-2 antagonists (daclizumab, basiliximab)

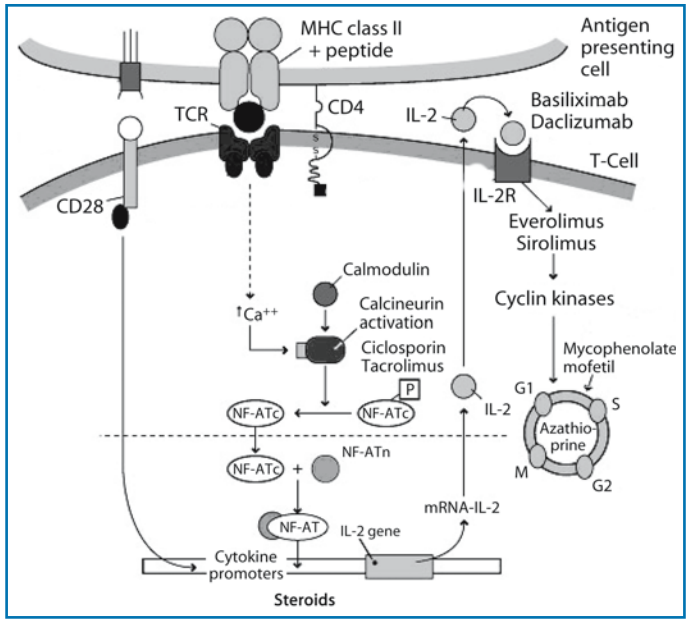

Fig. 9.1. Immunosuppressives: sites of action

Table 9.2. Immunosuppressives: mode of action

Mode of action	Substances
Regulators of gene expression	Glucocorticoids, vitamin D analogs, deoxyspergualin
Alkylating agents	Cyclophosphamide
Kinases and phosphatase inhibitors	Cyclosporine, tacrolimus, everolimus, rapamycin
Inhibitors of de novo purine synthesis	First generation: mercaptopurine, azathioprine, methotrexate
	Second generation: mizoribine and MMF
Inhibitors of de novo pyrimidine synthesis	Brequinar, leflunomide, malononitrilamides

potent immunosuppressive agents with highly specific sites of action (Fig. 9.1).

According to their mode of action these new drugs can be divided up into agents that selectively inhibit cytokine gene transcription/expression (cyclosporine, tacrolimus), antiproliferative agents (mycophenolate mofetil, azathioprine) and agents that interfere with intracellular signal transduction (rapamycin, everolimus). Immunosuppressives might also be classified as biologics which are defined as naturally occurring or genetically engineered mammalian proteins (thymoglobulin, basiliximab and daclizumab) or xenobiotics (drugs produced from microorganisms, e.g. cyclosporine, tacrolimus) (Table 9.2).

Despite the tremendous breadth of the discipline of immunosuppressive molecules, only a small number of drugs have made it as far as being used for experimental or clinical corneal transplantation. We have decided to focus on the following agents:

- Corticosteroids
- Cyclosporine (Sandimmun, Neoral, CSA)
- Tacrolimus (Prograf, FK506)
- Mycophenolate mofetil (CellCept, Myfortic, MMF)
- RAD (Everolimus, Certican)

- Rapamycin (Sirolimus, Rapamune)
- FTY720
- Biological agents (basiliximab, daclizumab)

9.4.2 Corticosteroids

Corticosteroids prevent interleukin (IL)-1 and IL-6 production by macrophages and inhibit all stages of T-cell activation. Adverse effects of systemic steroids include Cushing's disease, bone disease (e.g. osteoporosis, avascular necrosis), cataract, glucose intolerance, infections, hyperlipidaemia, and growth retardation. Adverse effects of topically applied steroids include cataract, glaucoma and in the case of epithelial defects – steroid ulcers.

9.4.3 Cyclosporine A (CSA, Sandimmun, Sandimmun Optoral, Sandimmun Neoral)

The fermentation product from the fungi *Tolypocladium inflatum* Gams was first isolated in 1970 by Thiele and Kis. Its immunosuppressive properties were discovered in 1972 by Borel. Sandimmun Neoral is a special galenic formulation based on microemulsion technology.

Cyclosporine A binds to the intracellular immunophilin cyclophilin (immunophilins are proteins which bind to immunosuppressive drugs). The CSA-cyclophilin complex blocks calcineurin-calmodulin-induced phosphorylation of NFAT (nuclear factor of activated T cells), transcription factor for IL-2 and other early T-cell specific genes (Fig. 9.1) and hence is highly T cell specific.

Clinical efficacy and safety data have mostly been acquired in solid organ transplantation, and it is still the gold standard in all forms of solid organ transplantation (except liver transplantation) mainly in combination with steroids, azathioprine or mycophenolate mofetil.

CSA in Corneal Transplantation. The first documented clinical experiences in corneal transplantation date back to the mid 1980s with the exceptional efforts undertaken by Hill and colleagues in South Africa [14, 15]. These initial positive clinical experiences with systemic CSA to prevent corneal allograft rejection in high risk keratoplasty have been confirmed by others [32, 33, 35].

Despite the significant improvement of outcome in high-risk keratoplasty, the use of CSA is limited due to its considerable toxicity and the need for costly drug monitoring. The toxicity is mostly caused by the CSA-cyclophilin-calcineurin-calmodulin complex, which interferes with tubular and endothelial cell functions: nephro- and hepatotoxicity, and alterations in glucose metabolism, hypertension, and gingival hyperplasia. To avoid the systemic toxicity, attempts have been undertaken to apply CSA in topical formulations including the use of collagen carriers [6, 16]. The encouraging results of these mostly experimental studies in preventing corneal graft rejection did not hold true clinically [29]. However, we have shown that topical CSA is efficient in the treatment of distinct immunological disorders of the cornea (e.g. Thygeson's keratitis, persistent nummular infiltrates following adenovirus infections) [34, 36].

9.4.4 Tacrolimus (FK506, Prograf)

Tacrolimus has been proven clinically superior to CSA following solid organ transplantation [5, 54]. Tacrolimus, like CSA, is a macrolide antibiotic (structurally related to erythromycin and rapamycin) derived from a fungus, *Streptomyces tsukubaensis* [17]. Its immunosuppressive properties were discovered by Ochai in 1985. In vitro studies have shown that, even in concentrations 40–200 times lower than CSA, tacrolimus possesses extremely powerful immunosuppressive effectiveness [45, 55]. Although the final step in modulating the immune system is the same for CSA and tacrolimus, i.e. interfering with the intracytoplasmatic calcineurin system and hence the interleukin IL-2 production, both drugs manage this in a different manner. Tacrolimus binds to the intracellular FKBP-12 (FK-binding protein-12). The tacrolimus-FKBP-12 complex blocks calcineurin-

calmodulin-induced phosphorylation of the cytoplasmic component of NFAT transcription factor for IL-2 and other "early" genes. Like CSA, tacrolimus is a highly specific inhibitor of lymphocyte activation. Its toxicities are similar to CSA (probably due to its calcineurin-mediated interference with tubular and endothelial cells), i.e. nephro-, neurotoxicity, arterial hypertension, diabetogenicity.

Tacrolimus in Corneal Transplantation. Up to now there have only been limited clinical data available about the efficacy of tacrolimus in corneal transplantation [49]. This might partially be explained by its relatively narrow safety margins. Whereas CSA might be given in a body weight adjusted dose (a suboptimal therapeutic approach which is practised in some centres in the United States), tacrolimus has to be closely monitored because the risk of overimmunosuppression is great. This is also the reason for the initially rather unjustified poor reputation of this drug: initially blood levels of 20–30 ng/ml were targeted (with corresponding adverse events), whereas today blood levels of 5–10 ng/ml are considered to be the optimal range.

The potency of this drug to inhibit experimental corneal allograft rejection after systemic administration has been proven [2, 42, 43]. As with CSA, much hope is pinned on finding an efficient topical administration to prevent systemic side effects. Experimentally the efficacy of topical tacrolimus has yet been proven [9, 13, 22, 23], and clinical studies of topical tacrolimus in atopic conjunctivitis are under way.

9.4.5 Mycophenolate Mofetil (MMF, CellCept, Myfortic)

Mycophenolate mofetil (MMF) is the bioavailability-enhanced morpholinoethylester of mycophenolic acid (MPA), which was originally isolated from *Penicillium* spp. MMF is rapidly converted to MPA, its active compound. Its safety and effectiveness in combination with CSA following kidney transplantation have been proven in several clinical studies [8, 11, 48, 52]. Unlike CSA or tacrolimus, MMF does not interfere with IL-2 pathways. Mycophenolic acid reversibly inhibits the de novo formation of guanosine nucleotides [1] by inhibiting the enzyme inosine monophosphate dehydrogenase (with high affinity to the isoform II, which is expressed in activated lymphocytes). As T and B cells are predominantly dependent on the de novo synthesis of guanosine nucleotides, the purine biosynthesis of these cells is selectively inhibited [24].

As MMF is not an antimetabolite and does not lead to genetic miscoding, it is not carcinogenic.

Mycophenolate Mofetil in Corneal Transplantation. We have been able to prove the potency of this drug and its synergistic effect on CSA and FK506 in delaying corneal allograft rejection in the rat keratoplasty model [41]. Following these initial positive experiences we conducted a prospective clinical trial with MMF and CSA in high-risk keratoplasty patients. The data from this study show a similar efficacy of MMF and CSA in preventing allograft rejection [44]. But due to the high therapeutic margin and favourable safety profile of MMF, costly drug monitoring is not indicated. Additionally we used this substance in immunological disorders of the eye, again with favourable results [40].

9.4.6 Rapamycin (Sirolimus, Rapamune)

Sirolimus (Rapamune) is an immunosuppressive agent previously known as rapamycin. It was under development for more than 20 years before it gained FDA approval in 1999. Sirolimus is a macrocyclic lactone produced by *Streptomyces hygroscopicus* found in the soil of Easter Island. Structurally, sirolimus resembles tacrolimus and binds to the same intracellular binding protein or immunophilin known as FKBP-12. However, sirolimus has a novel mechanism of action. Whereas tacrolimus and cyclosporine block lymphokine (e.g. IL-2) gene transcription, sirolimus acts later to block IL2-dependent T-lymphocyte proliferation and the stimulation caused by cross-linkage of CD28, possibly by

blocking activation of a kinase referred to as the mammalian target of rapamycin or "*mTOR*", a serine-threonine kinase that is important for cell cycle progression. Therefore, sirolimus is believed to act in synergy with cyclosporine (or tacrolimus) in suppressing the immune system.

Rapamycin has been shown to be highly efficient in preventing experimental solid organ [25, 53] and clinical renal transplantation [4, 26]. It is noteworthy that rapamycin is not nephrotoxic, which makes this drug especially interesting for renal transplant recipients.

Sirolimus in Corneal Transplantation. A couple of experimental studies have shown the efficacy of sirolimus in inhibiting murine corneal allograft rejection [27, 52]. We have conducted a small clinical study with sirolimus in high-risk corneal transplantation. We started Rapamune on the day of transplantation at a dose of 2 mg/day. The dose was adjusted to reach plasma levels of 4–10 ng/ml on subsequent days, trying to keep plasma levels close to 4 ng/ml. We have seen that the efficacy of Rapamune in preventing corneal allograft rejection is comparable to that of cyclosporine and MMF. But it is worth mentioning that we have seen a high incidence of side effects in this small group of patients.

9.4.7 RAD (Everolimus, Certican)

Everolimus is an oral rapamycin derivative produced by Novartis Pharma. It is chemically derived from rapamycin which has been obtained by fermentation of an *Actinomycetes* strain. It has been found that everolimus (40–0-[2-hydroxyethyl])-RPM is stable in oral formulations and that its efficacy after oral dosing is at least equivalent to that of rapamycin [7, 47]. The mode of action is equivalent to that of rapamycin, i. e. binding to FKBP, inhibiting TOR1 and 2 and hence inhibiting cell-cycle progression of activated T cells.

Everolimus and sirolimus are also called *proliferation signal inhibitors* (PSI), because they prevent proliferation of T cells.

Everolimus may have a special role in solid organ transplantation as it has been shown to reduce chronic allograft vasculopathy in such transplants [10].

Everolimus in Corneal Transplantation. We have tested this new compound in the rat model of corneal transplantation both as a single therapy and in combination with CSA and MMF. It appears that the potency of everolimus to prevent corneal allograft rejection is comparable to CSA. Additionally we have found a synergistic effect of everolimus in a double-drug regimen with CSA as well as with MMF [36, 38, 39].

There are to date no clinical data on the efficacy and safety of everolimus in corneal transplantation.

9.4.8 FTY 720

The chemical 2-amino-2[2-(4-octylphenyl) ethyl]-1, 3, propane diol is one of a class of small-molecule immunosuppressive agents. This compound was chemically synthesised in an effort to minimise the toxic in vivo properties of a structurally related and highly potent immunosuppressive agent, myriocin. The mechanism of action of FTY720, although not fully characterised, appears to be unique among immunosuppressants. In vivo, FTY720 induces a significant reduction in the number of circulating lymphocytes. It is thought to act by altering lymphocyte trafficking/homing patterns through modulation of cell surface adhesion receptors. Although much research has yet to be done to unravel the nature of the mechanism of action of FTY720, its efficacy has been sufficiently proven in numerous animal models, especially when administered in combination with cyclosporine. It has been shown that FTY720 is efficacious in a variety of transplant and autoimmune models without inducing a generalised immunosuppressed state and is effective in human kidney transplantation.

FTY720 in Corneal Transplantation. We have been able to show the efficacy of FTY720 in inhibiting murine corneal allograft rejection [20]. There are to date no data of FTY720 in clinical corneal transplantation.

9.4.9 Biologic Agents

Reports about the use of biological agents in corneal transplantation are very rare [46]. To complete this overview their mode of action is briefly outlined.

9.4.9.1 Polyclonal Antibodies (e.g. Antithymocyte Globulins)

These agents are derived by injecting animals with human lymphoid cells, then harvesting and purifying the resultant antibody. Polyclonal antibodies induce the complement lysis of lymphocytes and uptake of lymphocytes by the reticuloendothelial system and mask the lymphoid cell-surface receptors. Preparations include horse antithymocyte globulin (Atgam) and rabbit antithymocyte globulin (thymoglobulin). These agents are used for induction therapy and for the treatment of acute rejection in solid organ transplantation.

Adverse effects include fever, chills, thrombocytopenia, leukopenia, haemolysis, respiratory distress, serum sickness, and anaphylaxis.

9.4.9.2 Muromonab-CD3

Muromonab-CD3 is a murine monoclonal antibody of immunoglobulin 2A clones to the CD3 portion of the T-cell receptor. It blocks T-cell function and has limited reactions with other tissues or cells. This agent is used for induction and for the therapy of acute rejection (primary treatment or steroid resistant).

Adverse effects include cytokine release syndrome (i.e. fever, dyspnoea, wheezes, headache, hypotension) and pulmonary oedema.

9.4.9.3 Basiliximab and Daclizumab

Basiliximab (Simulect) and daclizumab (Zenapax) are humanised monoclonal antibodies that target the IL-2 receptor. Clinically, both agents are very similar, and both are used for induction therapy in solid organ transplantation. These agents have a very low prevalence of adverse effects, although hypersensitivity reactions have been reported with basiliximab (Simulect), albeit rarely.

Perioperative basiliximab has been tested in combination with cyclosporine postoperatively in a small clinical study with favourable results [46]. In 2004 we started a prospectively randomised clinical trial of basiliximab as monotherapy compared to cyclosporine.

- **To date the efficacy in preventing corneal graft rejection has only been proven for cyclosporine, mycophenolate mofetil and rapamycin in prospective clinical trials**
- **The efficacy and safety of tacrolimus in high-risk corneal transplantation has been described in a retrospective manner**
- **Especially in high-risk corneal transplantation as it is not a life-saving procedure it is important to weigh the pros and cons of any immunosuppressive regimen**
- **With respect to the profile of side effects, we prefer cyclosporine and mycophenolate mofetil over rapamycin and tacrolimus**

9.5 Guidelines for Practitioners

9.5.1 Preoperative Evaluation

As systemic immunosuppression might promote tumour growth or reactivation of a chronic infection, patients need to be checked by their internists to rule out neoplasms and infections (blood chemistry, abdominal ultrasound, chest X-ray) before immunosuppression is started. Additionally due to possible drug-specific side effects of immunosuppressive agents, renal and

hepatic functions should be controlled. The patients should undergo these examinations at the time they are put on the waiting list for transplantation. If any contraindications against systemic immunosuppression are found, these conditions need to be cleared before transplantation. If the conditions cannot be cleared, the indication for high-risk corneal transplantation should be reconsidered. In this situation the use of an optimally matched graft might be an interesting alternative to systemic immunosuppression. In the case of drug-specific contraindications, alternative drugs should be used (e.g. in the case of renal impairment, mycophenolate mofetil should be used instead of CSA).

9.5.2 How To Use Cyclosporine in High-Risk Corneal Transplantation

In addition to perioperative topical and systemic steroids, CSA is started on the day of operation in a dosage of 100 mg twice daily. Within the first postoperative week, full blood trough levels of CSA (12 h after administration) need to be checked daily, and the dose adjusted to reach serum levels of 120–150 ng/ml. We adjust the dose by using increasing or decreasing steps of 25 mg. If serum levels appear to be stable, we reduce drug-monitoring to once a week in the first months and afterwards to once a month. Additionally we check for liver and kidney functions. Depending on the risk situation we continue therapy for at least 6 or 12 months and taper therapy by reducing CSA in 25-mg steps daily.

CSA is especially helpful in high-risk patients who also suffer from atopic dermatitis. CSA should only be used with great caution in patients with renal impairment, diabetes mellitus and arterial hypertension.

9.5.3 How To Use MMF in High-Risk Corneal Transplantation

The application of MMF following high-risk corneal transplantation is easier than the use of CSA. In addition to perioperative topical and systemic steroids, MMF is started on the day of operation in a dosage of 1 g twice daily. Drug-monitoring is not mandatory due to the drug's broad safety margins. We perform blood chemistry once a month as MMF might be myelosuppressive and might lead to a rise in liver enzymes. If side effects occur, we reduce MMF to 0.5 g twice daily. In the case of drug-specific side effects or graft rejection drug-monitoring is indicated to rule out inadequate dosing.

MMF is especially valuable in herpetic ocular disease when combined with acyclovir due to its synergistic antiherpetic effect [20].

In cases of:

- Arterial hypertension
- Diabetes mellitus
- Chronic renal disease
- Low compliance

MMF is favoured above CSA or tacrolimus because of its safety margins and the profile of possible side effects. After at least 6 or 12 months following transplantation, MMF is tapered and discontinued within 1 week. In the case of drug-specific side effects we monitor blood levels.

9.5.4 How To Use Rapamycin in High-Risk Corneal Transplantation

In our pilot study we have seen that Rapamune effectively prevents acute allograft rejection. But due to the rather broad range of side effects, we do not recommend Rapamune in high-risk keratoplasty at this time point. Rapamune should especially not be given to patients with metabolic disorders (i.e. hypercholesterolaemia and hypertriglyceridaemia) as it aggravates these conditions in more than 50 % of patients.

9.5.5 How To Use Tacrolimus in High-Risk Corneal Transplantation

In addition to perioperative topical and systemic steroids, tacrolimus is started on the day of transplantation. As with CSA, blood levels should be controlled daily in the 1st week. Plas-

ma levels of 5 ng/ml should be aimed for. If plasma levels appear to be stable, drug monitoring can be reduced to once a week in the first months and once a month thereafter. Therapy should be applied for at least 6–12 months depending on the clinical course and tapered out within 2 weeks. Probably due to its calcineurin mediated interference with tubular and endothelial cells, the profile of side effects is similar to that of CSA. Tacrolimus should not be used in patients with diabetes mellitus, arterial hypertension or renal impairment. Due to the drug's narrow safety margins, special care must be taken in cases where compliance is not guaranteed. MMF should be used in these patients instead.

9.5.6 Combination Therapies

In special situations, i.e. high-risk keratoplasty in an oculus ultimus patient or in limbal stem cell deficiency, the use of a highly effective immunosuppressive therapy might be indicated. Immunosuppressive potency might be enhanced by combining two immunosuppressants, thereby minimising a drug-specific toxic side effect. We have demonstrated the efficacy and safety of a double-drug regimen with cyclosporine and MMF in patients following limbokeratoplasty [31].

9.6 Conclusion

It is now 60 years since we learned about immunologic graft failure thanks to the pioneering work of Medawar and Maumenee in the 1940s. Since that time impressive developments have been undertaken in the field of pharmaceutical immunosuppression. CSA has for many years been the gold standard in organ and high-risk corneal transplantation, but it is now accompanied by mycophenolate mofetil and tacrolimus.

The new compounds now give us the possibility not just to maximise immunosuppressive potency but to apply a more patient-oriented immunosuppressive regimen. This means firstly that the immunosuppressants may be chosen according to the underlying diseases of the patient (i.e. CSA or tacrolimus in keratoplasty in a patient with atopic dermatitis, MMF in keratoplasty in patients with herpetic eye disease or patients with impaired renal function). Secondly the efficacy of immunosuppression may now be adjusted to the clinical situation by adding another immunosuppressant to a baseline medication, thereby minimising a drug-specific toxic side effect. Especially in high risk corneal transplantation since it is not a life-saving procedure, it is important to weigh the pros and cons of any immunosuppressive regimen.

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Trephination in Penetrating Keratoplasty

10

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Core Messages

- Donor and host trephination should be performed with the same system from the *epithelial side*
- A horizontal position of the limbal plane is essential
- The graft size should be adjusted individually ("as large as possible, and as small as necessary")
- Limbal centration is to be preferred over pupil centration (especially in keratoconus!)
- Avoid excessive graft over- or undersize
- Intraoperative adjustment is required of double running suture
- Nonmechanical excimer laser trephination results in:
  - Lower astigmatism
  - Higher regularity of topography
  - Better visual acuity – especially in young patients with keratoconus
- In unstable corneas (e.g., after RK, iatrogenic keratectasia after LASIK, descemetocele, perforated ulcer), laser application makes trephination feasible
- New nut-and-bolt type variants for potentially self-sealing donor/host appositions are on the horizon ("no-stitch keratoplasty")
- Femtosecond laser application may be the "excitement of tomorrow" in microsurgery of the cornea

10.1 Introduction

Zirm in 1905 was the first surgeon to perform a successful homologous penetrating keratoplasty (PKP) in a human patient [84]. The operation became more successful with the development of more delicate instruments, use of the operating microscope, and the availability of antibiotics, antivirals and corticosteroids. Today, still unsolved problems include: (1) high/irregular astigmatism, (2) trephination of unstable cornea, (3) surface pathologies, (4) immunologic graft rejection, (5) secondary glaucomas, (6) chronic endothelial cell loss of the transplant, (7) recurrences of the disease, and (8) a lack of donor tissue.

With the improved understanding and management of immunologic problems during past few decades, the microsurgeon's main attention in corneal transplantation has shifted from preserving a "clear graft" towards achieving a good refractive outcome. Thus, PKP today is no longer just a "curative" but has also become a sort of "refractive" procedure. Today, a crystal clear corneal graft after PKP with high and/or irregular astigmatism – especially if in association with high anisometropia – can no longer be considered "successful" in normal-risk keratoplasties. Deluded by advertisements of refractive surgery, patients expect an optimal visual acuity preferably without spectacles. Many patients consider the necessity of wearing contact lens as representing a partial failure of the intervention. Especially older PKP patients cannot cope with contact lenses manually and/or mentally. Additional "dysfunctional tear syndrome" and blepharitis further promote contact lens

intolerance in this age group. Persisting corneal hypesthesia after PKP for many years can delay recognition of contact lens induced damage to the cornea.

It has been debated whether cutting or suturing is more important for the regularity of the transplant curvature. We have always stressed that: (1) early postoperative astigmatism *with sutures in place* should be differentiated from (2) late persisting postoperative astigmatism *without sutures* [59].

Summary for the Clinician

Two major types of post-PKP astigmatism need to be distinguished:

1. **Early postoperatively with sutures in place predominantly depending on:**
   - **Symmetry of suture positions**
   - **Depth of suture track in graft and recipient**
   - **Homogeneity of suture tension**
   - **Microsurgeon's "hand writing"**
2. **Late postoperatively persisting without sutures predominantly depending on:**
   - **Cut quality**
   - **Wound configuration (horizontal/vertical)**
   - **Symmetry of graft placement**
   - **Wound healing**

10.2 Astigmatism and Keratoplasty

10.2.1 Definition of Post-keratoplasty Astigmatism

The cornea contributes about two-thirds of the refractive power of a human eye. Surgical procedures on the cornea may therefore influence the state of refraction considerably. Corneal astigmatism is an optical aberration, resulting from unequal refraction of entering light in different meridians of the corneal surface. Astigmatism after PKP is often *irregular*, i.e., two or more meridians are separated from each other by an angle not equal to 90°. Two or more steep hemimeridians are not located opposite to each other. The same may be true for the flat hemimeridians. In addition, the refractive power of corresponding hemimeridians may differ. Especially with sutures in place, patients accept much less subjective cylinder than indicated by objective measures such as keratometry or topography analysis [20]. In cases of highly irregular astigmatism, good visual acuity can only be achieved by hard contact lenses (Table 10.1).

Table 10.1. Assessment of astigmatism and visual acuity after keratoplasty (SRI, surface regularity index; SAI, surface asymmetry index; PVA, potential visual acuity)

1. Uncorrected visual acuity
2. Keratometry
   a) Absolute values
   b) Angle of steep and flat meridian separately ($\Delta \neq 90°$)
   c) Classification of irregularity [59, 62]
3. Topography analysis
   a) Meridians
   b) Hemimeridians
   c) Irregularity (SRI, SAI)
   d) Semiquantitative classification [29]
4. Objective refractometry/retinoscopy
5. Subjective refractometry and spectacle-corrected visual acuity
6. Pinhole
7. Diagnostic contact lens

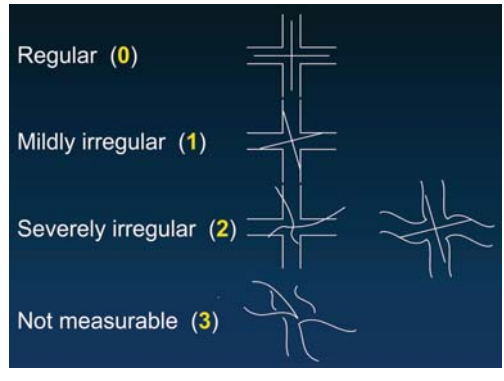

Fig. 10.1. Semiquantitative classification of regularity of keratometry mires (ophthalmometer, type H, 190071, Zeiss, Jena, Germany) (0, regular; 1, mildly irregular; 2, severely irregular; 3, not measurable) [59, 62, 83]

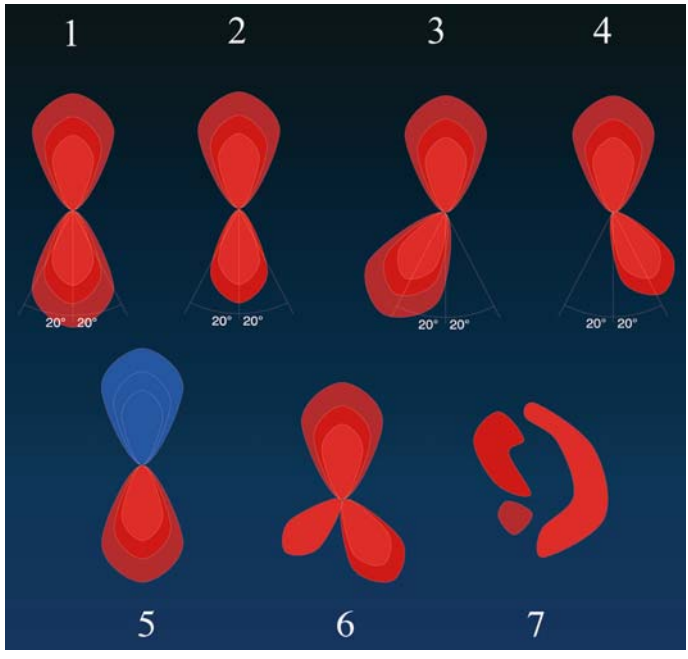

Fig. 10.2. Semiquantitative classification of corneal topography after PKP [29]: *1*, orthogonal symmetric (i.e., difference of maximal powers of opposing hemimeridians is less than 2 diopters and deviation of axis of opposing hemimeridians is less than 20°); *2*, orthogonal non-symmetric; *3*, non-orthogonal symmetric; *4*, non-orthogonal non-symmetric; *5*, keratoconus-like (a steep sector is opposing a flat sector at the apex, difference between steep and flat hemimeridian at least 2 diopters); *6*, polyaxigonal (at least three steep/flat sectors can be recognized, at least 2 diopters of power difference between steep and flat hemimeridians); *7*, irregular

After PKP we recommend documenting the keratometric refractive power separately in the steep and in the flat meridian with individual axis notation and assessment of the degree of "keratometric irregularity" (Fig. 10.1). Instead of "42.0+4.5/0°," we suggest writing "42.0/0° (irreg. 1); 46.5/70° (irreg. 2)" [62].

Besides keratometry, topography analysis is indispensable for mapping the corneal power over the entire graft. Refractive powers and individual axes of the four hemimeridians are complemented by system specific indices, e.g., SRI (surface regularity index) and SAI (surface asymmetry index) of the TMS-1 topography system. In addition, we suggest a semiquantitative classification of post-keratoplasty topography in seven groups (Fig. 10.2).

Summary for the Clinician

Studies intending to compare the corneal curvatures after different trephination or suturing techniques for PKP should include the following:

- **Subjective cylinder *and* keratometric/topographic astigmatism**
- **Portion of irregular/not measurable astigmatism**
- **Astigmatism with "all-sutures-out" and vector-corrected astigmatism**

10.2.2 Reasons for Astigmatism After Keratoplasty (Table 10.2)

Each of the multiple steps from donor selection, intraoperative trephination and suturing technique to type and quality of postoperative care can determine not only the clarity of the graft but also its final refractive result.

Besides intrinsic factors of donor and recipient, the *short-term astigmatism with sutures in place* seems to depend more on the symmetry of the sutures including methods of intra- and postoperative suture adjustments. After suture removal corneal curvature typically becomes more regular [35, 62], but the amount of net astigmatism may increase considerably [36, 38].

Thus, it has been concluded that factors directly or indirectly related to the quality of the wound geometry have a predominant influence on the *long-term residual astigmatism after suture removal* [59].

Table 10.2. Potential causative factors of high and/or irregular astigmatism after keratoplasty [59]

1. Preoperative factors
   a) Age of donor (infant!)
   b) Size of recipient cornea
      i) Keratoconus >Fuchs' dystrophy [60]
      ii) Microcornea
   c) Topography of donor
   d) Topography of recipient
   e) Disharmony between donor and recipient topography
   f) Pathologic properties of recipient
      i) Peripheral thinning or ectasia
      ii) Focal edema/focal scar
      iii) Defects in Bowman's layer
      iv) Vascularization
      v) Preceding keratoplasty (especially decentered)
   g) Aphakia
2. Intraoperative factors
   a) Decentration of donor excision and/or recipient bed
   b) "Vertical tilt" due to discrepancies of wound configuration [42]
      i) Application of different trephine systems for donor and recipient
      ii) Trephine tilt (i.e., not parallel to optical axis)
      iii) Limbal plane not horizontal
      iv) "Shifting" of trephine during cutting
      v) Too high/low intraocular pressure
   c) "Horizontal torsion" [42]
      i) Asymmetric placement of second cardinal suture ($\Delta \neq 180°$)
      ii) Mismatch of donor and recipient due to form incongruence
      iii) Focal overlap or dehiscence of donor button in recipient bed
   d) Excessive over-/undersize of donor
   e) Distortion and squeezing of cornea (e.g., due to dull trephine)
   f) Traumatizing the cornea with instruments
   g) Suture-related factors
      i) Suture material
      ii) Suture technique (interrupted, single running, double running, combinations)
      iii) Length of stitch
      iv) Depth of stitch
      v) Angle of stitch towards graft-host apposition
      vi) Suture tension
      vii) "Depth disparity"
   h) Simultaneous intraocular surgery (e.g., triple procedure, IOL exchange)
   i) Fixation rings and lid specula
   j) **Surgeon's experience**
3. Postoperative factors
   a) Suture-related factors
      i) "Cheese wiring" of sutures
      ii) Suture loosening
      iii) Suture adjustment/selective suture removal
      iv) Time point of suture removal
   b) Wound healing processes
      i) Wound dehiscence
      ii) Retrocorneal membrane
      iii) Incarceration of overlapping tissue
      iv) Focal vascularization
   c) Medication (e.g., corticosteroids)
   d) Postoperative trauma

10.2.2.1 Preoperative Determinants

Infant corneas have high refractive power (>50 diopters) and tend to steepen further after transplantation due to the biomechanical instability of the tissue. Thus, Pfister and Breaud suggested using infant corneas to compensate for aphakia. However, the refractive outcome varied considerably and was not predictable [49]. Thus, we do not recommend the use of infant donor corneas for grafting.

Today, donor topography is still rarely performed. The higher the immanent preoperative astigmatism of donor and recipient, the more probable it is that dysharmony between donor and recipient topography results in high astigmatism after suture removal [10, 15, 56]. Especially high congenital astigmatism, keratoconus and previous corneal refractive surgery must be ruled out in potential donors.

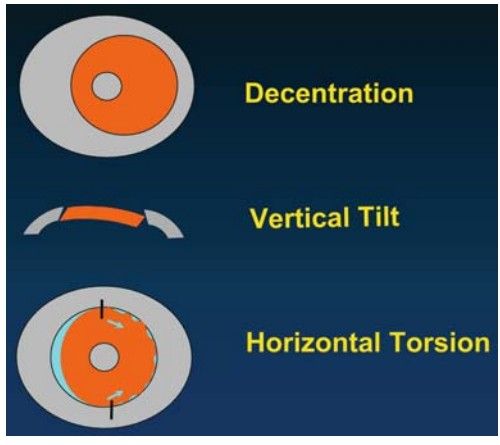

Fig. 10.3. Main reasons for high post-keratoplasty astigmatism: *top* decentration of donor and/or recipient trephination; *middle* "vertical tilt" due to incongruent cut angles; *bottom* "horizontal torsion" due to asymmetric suturing (modified from [42])

10.2.2.2 Intraoperative Determinants (Fig. 10.3)

Asymmetrically placed fixation rings (e.g., Flieringa or McNeill-Goldmann) may induce an astigmatism of up to 10 diopters [45]. Thus, post-PKP astigmatism is typically higher in aphakic than in phakic or pseudophakic PKP [48]. Even simple lid specula may be responsible for 3 diopters of with-the-rule astigmatism [45].

Decentration. Besides a higher incidence of immunologic graft reactions due to proximity to the limbal vessels, decentration of host trephination (>1 mm) may result in higher astigmatism. The flat axis of astigmatism points towards the direction of decentration [30, 75]. Due to the thickness gradient from the center to the periphery, donor decentration may also have a minor impact on post-PKP astigmatism [61].

"Vertical Tilt." The amount of persisting post-PKP astigmatism after suture removal depends significantly on the incongruences ("mismatches") of shape and cut angles of donor and recipient wounds [50, 74, 75]. Theoretically, a trephine tilt of 5° (10°) can induce 1.6 (5.9) diopters of astigmatism with an 8-mm-diameter graft [22]. Especially tilted hand-held trephines and neglecting the *horizontal position of the limbal plane* are reasons for the "vertical tilt" phenomenon. In addition, application of different trephine systems and different trephination directions (e.g., punching the donor from the endothelial side) in donor and host are crucial factors.

"Horizontal Torsion." One of the major predispositions for regular all-suture-out curvature after PKP is the 360° symmetric apposition of the donor button in the recipient bed. Especially the correct positioning of the *second cardinal suture* opposite to the first one is crucial. Asymmetric placement of the second cardinal suture results in a tissue deficit on one side which needs to be compensated by forced suture adaptation. In the case of long shallow suture bites, a regional flattening may result. In the case of short and deep suture bites, a central steepening may result, in analogy to sutured wedge resections. On the other side a tissue surplus may result in peripheral donor tissue compression with peripheral steepening and consecutive central flattening [74].

An analogous situation arises when the recipient bed is cut asymmetrically elliptical [34, 46, 78]. This may result from asymmetric bulging of the unstable cornea into the trephine opening or even by using an obturator in the case of keratoconus [21]. Mechanical trephines, such as hand-held or motor trephines, may result in oval-shaped host beds even if a circular round excision was intended [9].

Likewise, in donor trephination a trephine tilt of 20° may induce a difference of about 0.5 mm between the maximal and minimal diameter, resulting in an elliptical donor button [45]. Suturing of such an elliptical donor button in a round bed will result in a peripheral steepening in the major axis due to tissue compression and – consequently – a central flattening in this (hemi-)meridian [8]. A wound disparity of 0.1 mm is supposed to create an astigmatism of about 1 diopter [45, 74].

Undoubtedly, the technique for adequate graft-host adaptation by means of four to eight cardinal sutures is determined – at least in part – by the experience of the microsurgeon. The same holds true for the correct performance, interpretation and consequences of intraoperative keratoscopy. However, even if adequate suture distribution and tension as well as intra-/postoperative suture adjustments compensate for the fundamental intraoperative determinants of post-PKP astigmatism in the early stage, suture removal – even after years – may result in major changes of topography and a dramatic increase in astigmatism [36, 38].

Summary for the Clinician

Major intraoperative determinants for high/irregular astigmatism after suture removal include [42]:

- **Decentration (donor and/or recipient trephination)**
- **"Vertical tilt" (incongruent cut angles between donor and host)**
- **"Horizontal torsion" (horizontal discrepancy of donor and host shape or asymmetric suturing – second cardinal suture!)**

10.2.2.3 Postoperative Determinants

Postoperative suture adjustment or selective removal of single sutures may have a favorable impact on the early post-PKP astigmatism. However, changes of corneal curvature are unpredictable after suture removal [36, 38]. At this time there is still no reliable indicator available to the microsurgeon instructing him about the amount and direction of impending astigmatism changes of the graft after suture removal. There is some evidence that a high coincidence of the axes of refractive, keratometric and topographic astigmatism with the suture in place speaks in favor of decreasing astigmatism to be expected after suture removal [54]. Thus, in the case of intact sutures, lack of vascularization, a low amount of astigmatism, and high topographic regularity resulting in good spectacle-corrected visual acuity, microsurgeons will tend to leave the suture in place for a longer period of time under regular controls and adequate counseling of a compliant patient. However, it must be considered an illusion that keeping the sutures in place for a longer time would help to preserve a favorable topography after final suture removal [11, 14, 36, 38, 70]. Especially step formations after suture removal – often after inadequate trauma – will result in a flat hemimeridian and irregular high astigmatism. For this reason, such steps at the graft-host junction need immediate surgical repair to preserve a good long-term refractive result even if the anterior chamber is not opened [18].

Summary for the Clinician

The pathomechanism of astigmatism increase after suture removal may be as follows:

- **A low quality of trephination wound and geometric incongruences (horizontal and vertical) require a higher suture tension to guarantee:**
  - **Watertight wound closure**
  - **A pseudo-optimal topography early postoperatively**
- **Asymmetric regional forces between donor and host may cause inhomogeneous wound healing**

- **Removal of sutures liberates forces due to: (1) geometric incongruences and (2) inhomogeneous wound healing**
- **Thus: horizontal, vertical and topographic discrepancies between donor and host *intraoperatively* are responsible for an increase in astigmatism *after* suture removal**

10.2.3 Prevention/Prophylaxis of Astigmatism After Keratoplasty

The large number of treatment options for astigmatism after PKP leads to the conclusion that none of the methods is really convincing. Therefore, prophylaxis of high and/or irregular astigmatism is preferred over treatment [59].

10.2.3.1 Alternatives "Without Sutures"

Alternatives "without sutures" include *phototherapeutic keratectomy* (PTK) in the case of superficial corneal diseases. PTK yields good results especially with recurrences of corneal dystrophies after PKP. In order to avoid sutures involving Bowman's layer, potentially self-sealing nut-bolt variants of donor-recipient apposition have been investigated. One approach is *divergent cut angles* that may be created using lasers [57]. The increased contact area reduces the probability of wound dehiscence, the smaller diameter at the level of Bowman's layer increases the distance from the limbal vessels with favorable effects concerning immunologic graft reactions, and the larger diameter at the level of Descemet's membrane increases the amount of transplanted endothelial cells with favorable effects in Fuchs' dystrophy and aphakic/pseudophakic bullous keratopathy. It has been shown that the stability of the graft in the recipient bed increases with increasing divergence of the cut angles [57]. Additional application of tissue glue, a temporary therapeutic contact lens or an intrastromal suture may further increase the stability of the graft-host junction.

An analogous approach was followed by introducing an *inverse mushroom-shaped trephination* with the larger diameter of the graft at the level of Descemet's membrane [7, 67].

In order to leave the architecture of the central cornea untouched, endothelial cell transplantation has been investigated and *posterior lamellar keratoplasty* (PLKP) has been introduced into clinical routine by Melles [37] in Europe in 1998 and later modified by Terry in the United States [71] in cases of sole endothelial failure.

10.2.3.2 Ten Precautions During Surgery

1. Donor topography should be attempted for exclusion of previous refractive surgery, keratoconus/high astigmatism, and "harmonization" of donor and recipient topography [16, 56, 59].
2. Donor and recipient trephination should be performed from the epithelial side with the same system, which – from our point of view – predisposes to congruent cut surfaces and angles in donor and recipient. For this purpose an artificial anterior chamber is used for donor trephination although the whole globe would yield even better results [27].
3. Orientation structures in donor and host facilitate the correct placement of the first four cardinal sutures to avoid horizontal torsion [2].
4. A measurable improvement seems possible using the Krumeich guided trephine system (GTS) [4], the second generation Hanna trephine [81] and our technique of nonmechanical trephination with the excimer laser [58, 66].
5. Horizontal positioning of head and limbal plane is indispensable for state-of-the-art PKP surgery in order to avoid decentration, vertical tilt and horizontal torsion [59].
6. Graft size should be adjusted individually ("as large as possible, as small as necessary") [60, 62].

7. Limbal centration should be preferred over pupil centration (especially in keratoconus – "optical displacement of pupil") [31].
8. Excessive graft over- or undersize should be avoided to prevent stretching or compression of peripheral donor tissue [19, 47, 82].
9. As long as Bowman's layer is intact, a double running cross-stitch suture (according to Hoffmann [17]) is preferred since it results in greater topographic regularity, earlier visual rehabilitation and less loosening of sutures, with suture replacement only rarely required.
10. Intraoperative keratoscopy should be applied *after* removal of lid specula and fixation sutures. Unstable donor epithelium would be better removed to allow for reproducible results. Adjustment of double running sutures or replacement of single sutures may be indicated [3].

Summary for the Clinician

Requirements for *"the optimal trephination"* include:

- **Full visual control**
- **No contact**
- **Optimal donor and host centration**
- **Identical shape of donor and host (typically circular)**
- **Congruent cut angles**
- **360° symmetric donor host alignment**
- **No necessity to complete trephination by scissors**
- **No damage to intraocular tissues**
- **Future: self-sealing donor/host apposition**

10.3 Trephination Techniques

The principal indications for keratoplasty include optical, curative and tectonic factors (Table 10.3). Overlaps between the different categories may occur. But corneal transplants may also be classified according to the type of donor material, the vertical shape of the graft, the horizontal shape of the graft and the location of the graft within the host (Table 10.4) [40].

Table 10.3. Principal indications for keratoplasty (modified from [40])

1. Optical
   a) Opacities
   b) Pathologic curvature
2. Curative
   a) Deep keratitis (e.g., herpetic keratitis with granulomatous reaction to Descemet's membrane or *Acanthamoeba* keratitis)
   b) Endothelial diseases (primary or secondary)
   c) Perforated corneal ulcer
3. Tectonic
   a) Traumatic corneal defects
   b) Infectious corneal defects
   c) Postoperative fistula after cataract extraction or antiglaucomatous surgery
   d) After "block excision" [44]
      i) Uveal tumors
      ii) Localized epithelial downgrowth (cysts)
   e) Reconstruction of the anterior segment

A few general technical details concerning PKP need to be mentioned [40, 42]:

1. *General anesthesia* has advantages over local anesthesia. The arterial blood pressure should be kept low as the eye is opened ("controlled arterial hypotension").
2. To protect the crystalline lens in phakic keratoplasty, usually the *pupil is constricted*.
3. Before recipient trephination, a stab-like *paracentesis at the limbus* is performed.
4. The *limbal plane* must be *horizontal* during trephination.
5. An *iridotomy* prevents pupillary block and acute angle closure glaucoma (so-called Urrets-Zavalia syndrome in the case of dilated pupil with iris sphincter necrosis [43]).
6. The *second cardinal suture* is crucial for graft alignment.

Table 10.4. Terminology of various types of keratoplasty (modified from [40])

Donor cornea	Vertical shape of graft	Horizontal shape of graft	Location within the host
Autologous (autograft) Homologous (allograft) Heterologous (xenograft) Alloplastic (keratoprosthesis)	Lamellar (anterior vs. posterior) Penetrating Mushroom Inverse mushroom [67]	Circular Elliptical Semilunar Rectangular Triangular Ring-shaped	Central Eccentric Marginal

10.3.1 Principal Considerations

10.3.1.1 Donor Trephination

From a 16-mm corneoscleral button as provided by the Eye Bank, the transplant can be created in two principal ways:

1. The original method used is for the donor button to be punched *from the endothelial side* against a firm surface (such as a paraffin or Teflon block) using special trephines (*Lochpfeifentrepan*) [6, 80]. Care must be taken to ensure a proper alignment when cutting since a beveled cut will result if the blade is not perpendicular to the cutting block. This risk may be decreased by the use of "guided donor trephine" systems (e.g., "guillotines") (Fig. 10.4).
   On histological evaluation, the cut surfaces without consideration of the cut angles seem to be almost "perfect." However, deviation of the cut direction outwards results in *convergent cut angles* due to a smaller diameter at the level of Descemet's membrane and a larger diameter at the level of Bowman's layer ("*undercut*") (Fig. 10.4 D) [76].
2. Since the development of "*artificial anterior chambers*" [23], microsurgeons have had the opportunity to perform donor trephination from the epithelial side, which is the same direction as in the host. If pressure in the artificial anterior chamber is kept normal (e.g., 22 mmHg), the advantages with respect to cut angles are obvious [55]. However, fixing the corneoscleral button in an artificial anterior chamber may induce a considerable amount of astigmatism. This problem can be overcome by using an artificial anterior chamber with a larger central opening, leaving the limbus untouched during fixation for trephination from the epithelial side. In this setting the corneoscleral limbus seems to have a protective effect concerning the central corneal topography of the fixated cornea [27].

- **Trephination of the donor button should preferably be performed *from the epithelial side* using an artificial anterior chamber with a large central opening**
- **Punching the donor *from the endothelial side* results in an undercut at the level of Descemet's membrane with convergent cut angles**

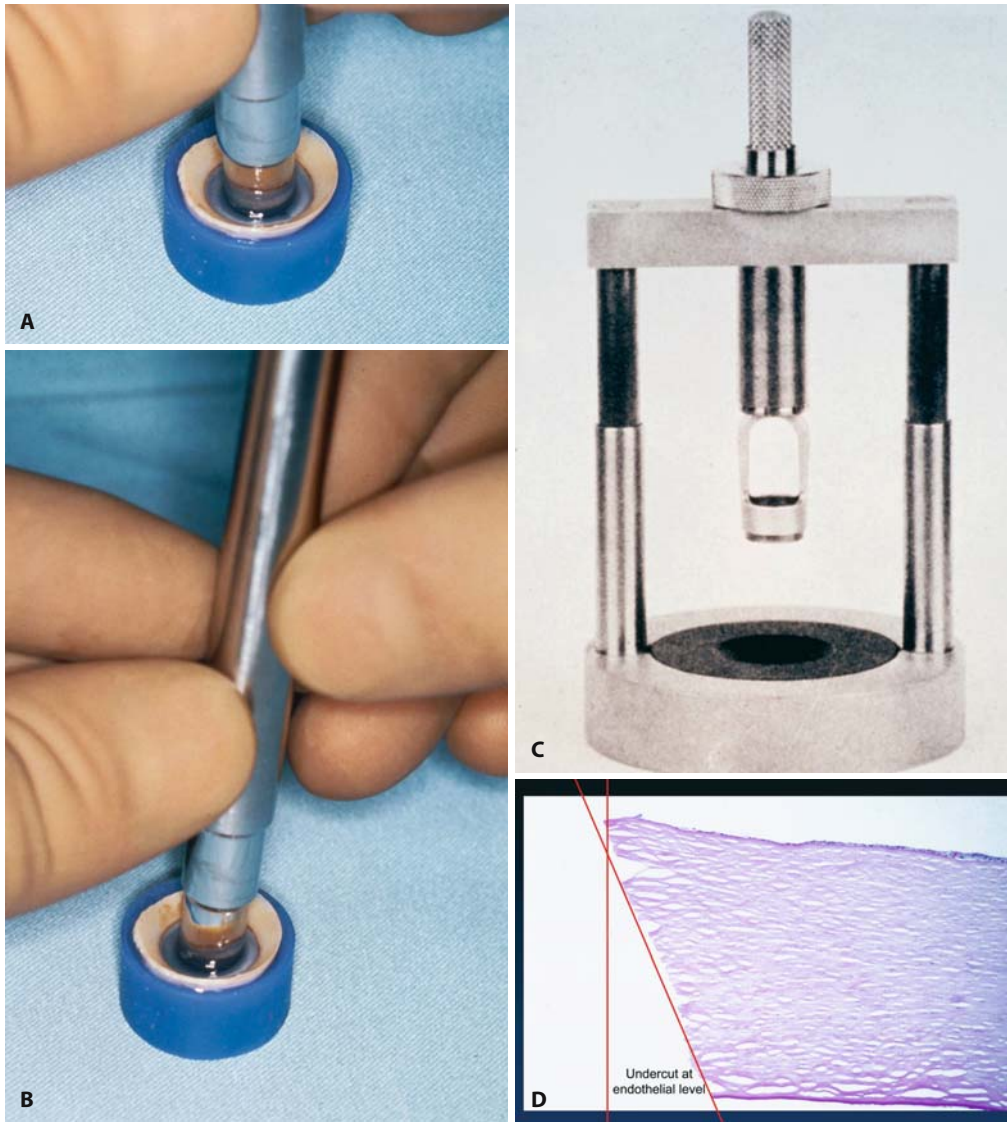

Fig. 10.4 A–D. Donor trephination *from the endothelial side.* **A** Correct position of hand-held trephine; **B** tilted trephine; **C** "guillotine" to avoid trephine tilt; **D** smooth cut surface but "undercut" at the level of Descemet's membrane

10.3.1.2 Recipient Trephination

For recipient trephination, the horizontal position of the head and especially the limbal plane is indispensable. To increase the overview and reduce *vis à tergo*, the Lieberman speculum is preferred. Any viscoelastic agent may be used to stabilize the anterior chamber during trephination. A Flieringa ring is not necessary for PKP or the triple procedure, but is helpful in cases of aphakic eyes, especially if a secondary sclera-fixated IOL is inserted. The ring can be sutured temporarily onto the globe using 6-0 Vicryl sutures through the conjunctiva and episclera.

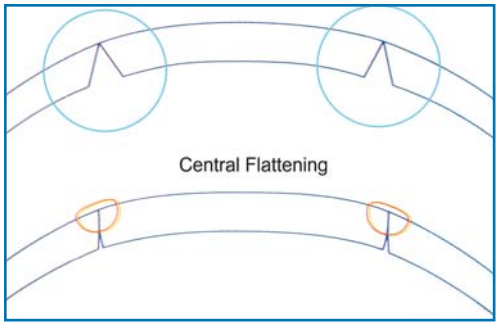

Fig. 10.5. Combination of donor trephined *from the endothelial side* (convergent cut angle) and mechanically trephined recipient (divergent cut angle) results in a triangular-shaped tissue deficit at the level of Descemet's membrane which has to be compensated by suture tension resulting in central flattening and vertical tilt

Investigations by Van Rij and Waring demonstrated that in recipient trephination all trephine systems result in an opening larger than the trephine size. In addition, the diameter is larger at the level of Descemet's membrane, resulting in *divergent cut angles* [76]. This can be explained by the "ballooning" of the cornea to be excised into the trephine opening due to the pressure executed. The higher the intraocular pressure, the more divergent the angles to be expected [55]. This phenomenon of "ballooning" is one of the major drawbacks of a mechanical trephine and can be prohibited – at least in part – by the use of an "obturator." However, Kaufman stresses that the use of an obturator in keratoconus may result in other than round host openings such as pear-shaped holes [21].

The combination of a donor punched from the endothelial side with convergent cut angles and a host opening with divergent cut angles will result in a triangular-shaped tissue defect at the level of Descemet's membrane that has to be compensated for with increased suture tension and – consequently – vertical tilt (Fig. 10.5).

Summary for the Clinician

- **Horizontal positioning of limbal plane is indispensable**
- **Flieringa ring is only necessary in aphakic eyes**
- **The higher the intraocular pressure (iatrogenic!) the more divergent are the cut angles to be expected [55]**

10.3.1.3 Graft Size and "Oversize"

Graft Size. In a quantitative study we found that the corneal diameter of keratoconus patients was larger than that of Fuchs' patients (mean horizontal diameter of 11.8 mm in keratoconus patients and 11.3 mm in Fuchs' patients) [60]. *In general, a good optical performance requires a larger graft, whereas a low rate of immunologic graft reactions tends to be seen with smaller grafts.* Therefore, the graft should be "as large as possible, but as small as necessary." For many eyes with keratoconus an 8.0-mm diameter and in many eyes with Fuchs' dystrophy a 7.5-mm diameter prove to be good options as a prerequisite for obtaining tissue from the Eye Bank. Today, graft diameters of 5.5–7.0 mm are only rarely required and justified.

It has been supposed that smaller grafts might be associated with a higher post-keratoplasty astigmatism. In a recent study we found [62]:

1. A flatter curvature with smaller grafts
2. A higher topographic irregularity with smaller grafts
3. A higher proportion of unmeasurable keratometry mires with smaller grafts
4. A tendency towards regularization of topography after suture removal
5. No difference concerning the amount of net astigmatism between different graft sizes either with or without sutures

The major reason for the flatter and more irregular graft with smaller diameters seems to be the closer position of the proximal suture ends in relation to the optical center of the graft. This will be pronounced in particular with wider suture bites. After suture removal the potentially topography disturbing circular scar at the graft-host junction is located closer to the line of sight with smaller grafts. This may explain that overall the regularity of graft topography increases

with suture removal but that major differences between various graft sizes do persist.

Larger sizes may be considered for eccentric tectonic corneoscleral grafts (e.g., after the block excision of tumors of the anterior uvea or cystic epithelial downgrowth [44]) and in buphthalmos [73]. But we do not recommend graft sizes over 8.5 mm in buphthalmos for immunologic reasons [52].

Recent studies indicate that the rate of chronic endothelial cell loss after PKP depends on the initial diagnosis [32, 53]. Endothelial migration from donor to recipient in pseudophakic bullous keratopathy along a density gradient is thought to be the reason for this phenomenon. Therefore, eyes with bullous keratopathy may require a larger graft not just to improve the optical performance but rather to transplant as many endothelial cells as possible. Nevertheless, *graft size has to be judged by the surgeon individually in every single case before recipient trephination* to achieve the best compromise between immunologic purposes and optical quality [59, 60]. A slit lamp with a measuring device (scale), e.g., a Haag-Streit slit lamp, or calipers for intraoperative application may be helpful. Prior removal of vascularized pannus (in contrast to vascularized stromal scars) may render a larger "individual optimal graft size" possible for transplantation of more endothelial cells and better graft topography.

Graft "Oversize." In mechanical trephination, the diameter of the recipient bed tends to be larger and the diameter of the donor button, punched from the endothelial side, tends to be smaller than the trephine diameter, which may affect the resulting spherical equivalent [76]. Thus, "oversizing" the donor button by 0.25–0.50 mm is commonly done to compensate for refractive effects and to reduce crowding of the chamber angle and therefore postoperative "glaucoma" [47]. An oversize of 0.25 mm compared to one of 0 mm or 0.5 mm may account for a difference in keratometric readings of 1.5 diopters after suture removal. Javadi et al. found no difference in astigmatism in comparing 0.25 mm and 0.50 mm graft oversize [19]. However, Perl et al. stressed that oversizing the graft by 0.5 mm (punched from the endothelial side) may result in significantly increased corneal astigmatism [47]. In keratoconus, same size donors were found to reduce resulting myopia. We do not recommend undersizing of a graft!

In contrast, with guided trephines and laser trephination (donor from the epithelial side), attempted diameters are indeed achieved with congruent cut angles. Thus, donor oversize is not necessary.

Summary for the Clinician

- **Typically, keratoconus corneas are larger than Fuchs' dystrophy corneas**
- **Graft size has to be judged by the microsurgeon individually in every single case *before* recipient trephination to achieve the best compromise between immunologic purposes and optical quality**
- **Donor trephination from the endothelial side results in a smaller donor button than trephine size and convergent cut angles ("undercut")**
- **Recipient trephination results in larger openings than trephine size and divergent cut angles**
- **This discrepancy makes a donor "oversize" of ≥0.25 mm necessary**
- **Same size grafts are feasible if the donor is created by means of an artificial anterior chamber from the epithelial side**
- **Undersizing the graft for simultaneous correction of myopia in keratoconus is *not* recommended (watertight wound! irregular astigmatism!)**

10.3.1.4 Pupil Versus Limbal Centration

Centration is crucial with respect to immunologic graft reaction and post-PKP astigmatism. Typically a compromise between limbal and pupil centration is attempted in the case of non-traumatized pupils. However, limbal centration is preferred especially in keratoconus, scars after trauma or irregular astigmatism of other origins. In such eyes the center of the visible ("entrance") pupil may be dislocated from that of the real anatomic pupil [31].

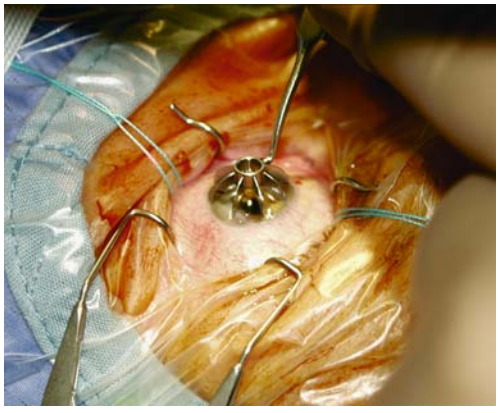

Fig. 10.6. An eight-line radial keratotomy marker (colored with methylene blue) may be used to facilitate limbal centration

An eight-line radial keratotomy marker may be used to ensure centration (Fig. 10.6). An additional central dot-like mark may be helpful for certain trephine systems (e.g., Hessburg-Baron).

If the broadening of the superior limbus due to a vascularized pannus is neglected intraoperatively, an inferior decentration may be recognized on the next day at the slit-lamp.

Summary for the Clinician

- **In doubt, limbal centration is preferred over pupil centration**

10.3.1.5 "Harmonization" of Donor and Patient Corneal Topography

Keratometric readings of the donor cornea are still usually neglected. However, it might be better to consider them to improve predictability of the final refractive outcome after PKP [10, 16, 56]. This may help to avoid transplantation of corneas with unusual or abnormal curvatures. In addition, it may allow a more accurate selection of intraocular lens power in triple procedures.

The vertical difference at the graft-host junction due to the different curvatures of donor and recipient must be compensated intraoperatively by suture tension to avoid a step formation. The resulting forces may be co-responsible for the amount of relative change in curvature after suture removal. Therefore, "*harmonization*" of donor and recipient topography should allow for minimization of the residual astigmatism for a given pair of donor and recipient [56]. The use of an artificial anterior chamber enables donor topography analysis and allows the "contour line" of the trephination edges in both donor and recipient to be calculated. A computerized simulation of graft rotation in the recipient bed may help to find an angle of graft rotation at which topographical misalignment is minimal.

Grütters et al. have proposed "astigmatism-oriented perforating keratoplasty", i.e., matching the flat axis of the donor with the steep axis of the host cornea [16].

Summary for the Clinician

Consideration of donor topography may:

- **Eliminate the use of donors with abnormal or unusual curvatures (such as high astigmatism, keratoconus, previous refractive surgery)**
- **Allow for "harmonization" of donor and recipient topography**

10.3.1.6 The Vascularized Cornea

Excessive bleeding after trephination of vascularized corneas with blood clots left in the anterior chamber may result in increased risk of immunologic graft reaction and peripheral anterior synechiae due to contraction. Thus, the following precautions should be taken:

Before trephination the microsurgeon should differentiate between vascularized pannus tissue ("plus") and vascularized scars ("minus"). Vascularized fibrous tissue between the epithelium and Bowman's layer or the superficial stroma in the case of defective Bowman's layer can be removed easily with a hockey knife. Typically, bleeding stops after a few minutes without additional measures. In contrast, distinct "feeder vessels" of vascularized scars may be incised with a pointed scalpel at the limbus. Pillai et al. have proposed sophisticated kauterization techniques for coagulation of afferent and efferent vessels [51]. In the case of diffusely

capillarized scars, ice-cold balanced salt solution (BSS) or topical alpha-mimetic vasoconstringent drops (such as naphazoline nitrate) may help to reduce bleeding during trephination.

Summary for the Clinician

- **Removal of vascularized pannus tissue may help to increase the "individually optimal graft size"**
- **Incision or kauterization of distinct "feeder vessels" of scars at the limbus may reduce bleeding during trephination**

10.3.1.7 Keratoconus and Disabling High Astigmatism of a Graft

Keratoconus. In keratoconus, a central round PKP is indicated as soon as hard contact lenses are no longer tolerated. Excessively steep corneas before surgery do not have less favorable outcome than less deformed corneas after PKP using the excimer laser for nonmechanical trephination [83].

Keratoconus eyes have larger corneas than normal eyes and other dystrophies allowing for larger graft diameters (typically 8.0 mm) [60]. A larger graft diameter in keratoconus patients may help to preserve a sufficiently thick cornea at the trephination margin in the patient since the "cone" can be excised almost completely. Kauterization of the cone has been suggested to avoid divergent cut angles, but its effect may not be reproducible. Thus, we do *not* advocate kauterization of the cone. Kaufman has suggested not using obturators in the case of keratoconus to prevent unintended creation of elliptical or pear-shaped openings [21].

We do *not* advocate centering the trephination on the cone, thereby typically decentering the trephination with respect to the limbus. In addition, pupil centration may be misleading due to "optical displacement" of the visible pupil because of irregular refraction of incoming rays of light by the irregularly curved corneal surface in keratoconus [31]. We do *not* advocate undersizing of the donor to reduce myopia, since irregular astigmatism is to be expected.

Due to inhomogeneous corneal thickness, an early perforation at the site of the thinned cornea is to be expected. This has to be taken into account with conventional trephines to avoid inadvertent injury of the iris or even the lens.

Peripheral thinning of the host cornea, e.g., with keratotorus (= pellucid marginal degeneration) or Fuchs-Terrien marginal degeneration, is very rare but difficult to treat. Treatment options include an eccentric semilunar lamellar/penetrating graft or an overdimensioned preferably elliptical eccentric through-and-through graft.

Disabling High Astigmatism of a Graft. Eyes with high disabling astigmatism after PKP are often – but not always – associated with small and/or decentered grafts. The re-graft should be well centered and large enough to cut out the previous graft entirely. However, in some cases the previous graft-host junction cannot be excised in toto (cf. Sect. 10.3.1.3, "Graft Size" and "Oversize"), leaving a "wedge" of the first donor tissue in situ.

After second suture removal, astigmatism may increase again and may no longer be significantly different in comparison to the preoperative values [70].

Our own results suggest a potentially important role of the remaining second running suture in keeping corneal astigmatism values low and topographic regularity high after repeat PKP in patients with high and/or irregular post-keratoplasty astigmatism. After removal of the last suture, the curvature may change in an unpredictable and often unfavorable manner. The presumed original instability of the host rim, which on final suture removal may be transferred to the center of the graft ("memory effect"), is probably responsible for the increase in astigmatism and the increase in irregularity of the corneal surface. In addition, the host rim instability may be exacerbated by incomplete excision of the previous graft-host junction in severely decentered first grafts. However, the exact role of any such residual tissue has yet to be clarified.

The long-term value of so-called "intracorneal rings" inside the graft-host junction with respect to stabilization of the topography in such eyes has yet to be determined [13, 24].

Summary for the Clinician

- **With keratoconus a large excision should be centered at the limbus (not the "cone") and non-contact laser trephination is preferred to prevent "other-than-round" recipient openings**
- **Where repeat PKP is performed in eyes with high and/or irregular astigmatism in clear grafts, visual rehabilitation may be limited by an increase in astigmatism and topographic surface irregularity after removal of the last running suture**
- **In such eyes it may be advantageous to postpone final suture removal for as long as possible**

10.3.1.8 The Unstable Cornea

Unstable corneas include:

1. Corneal perforations or descemtoceles typically arising from ulcerative necrotizing stromal keratitis of herpetic or bacterial origin
2. Eyes after unfavorable keratorefractive surgery such as after radial keratotomy and iatrogenic keratectasia after laser in-situ keratomileusis (LASIK)

In the "open eye" situation mechanical trephines may lead to compression and distortion of the cornea although a high-viscosity viscoelastic agent is used to stabilize the anterior chamber. Especially with large perforations the trephine can only be used to mark the excision, the keratotomy has to be deepened with a diamond knife and the excision is completed with scissors. Nonmechanical laser trephination has been advocated since it may allow non-contact round and elliptical trephinations (Fig. 10.7) [26]. One suggestion has been to insert a trimmed part of a soft contact lens via large paracentesis, unrolling it inside the anterior chamber and thus achieving a stable eye for trephination after pressurizing the globe by insertion of viscoelastic agent via paracentesis ("valve"). A larger than usual graft oversize (e.g., 0.5 mm) is recommended to avoid peripheral synechiae in eccentric or even peripheral grafts.

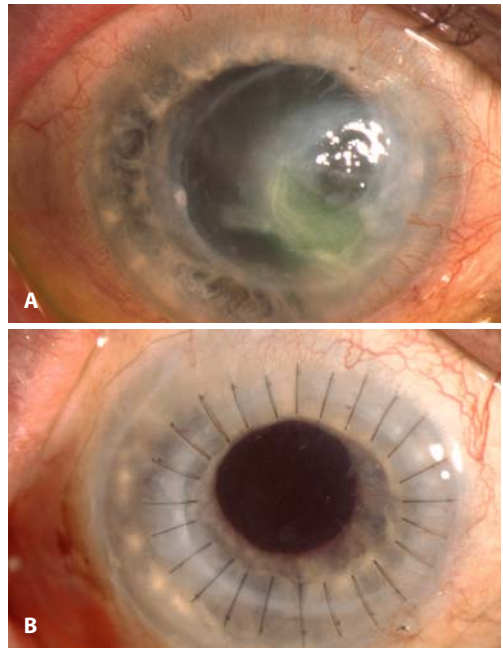

Fig. 10.7. A Descemetocele after ipsilateral autologous keratoplasty for localized central herpetic scar; **B** eccentric elliptical triple procedure *à chaud* (7.0×8.0 mm/7.1/8.1 mm, excimer laser trephination)

In the case of excisions involving the limbus, the *scleral spur* has to be preserved during (partly lamellar) trephination. In the case of peripheral small perforations, an eccentric minikeratoplasty may have immunologic advantages. Wide limbus-parallel perforations – typical of rheumatoid origin – may best be treated with a crescent graft. For this partly "freehand" procedure, an outer segmental trephination with a smaller diameter (e.g., 10 mm) is combined with an inner segmental trephination with a larger diameter (e.g., 16 mm). Adequate preparation of the slightly oversized graft is best achieved from an intact donor globe but is quite difficult using a corneoscleral button from the Eye Bank (protection of endothelium!).

After excessive *radial keratotomies* resulting in irregular astigmatism and glare/halos due to scars in the optical field, deep epithelial plugs are typically present inside the original radial cuts for years. Instability leads to opening of these plugs during mechanical trephination. Certain types of circular sutures have been proposed before trephination. However, non-contact laser trephination seems to be the method of choice for such eyes. In analogy, iatrogenic keratectasia after LASIK is prone to opening of the lamellar interface between the stromal bed and flap during conventional contact trephination. This may result in oval host wounds and different sizes of the excised button at the flap and bed levels [64]. Again, non-contact laser trephination seems to be the method of choice for such eyes, the incidence of which is supposed to increase over the next few decades.

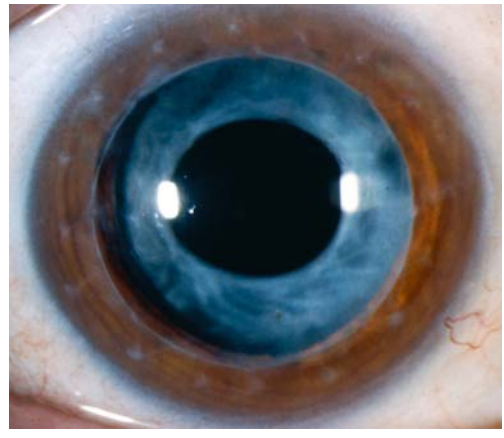

Fig. 10.8. Well centered (1) trephination, (2) capsulorhexis, and (3) posterior chamber lens inside the capsular bag after triple procedure in Fuchs' dystrophy (7.5/7.6 mm, excimer laser trephination with eight "orientation teeth/notches")

Summary for the Clinician

- **In the "open eye" situation conventional trephines typically only mark the host excision which has to be completed freehand with diamond knife and scissors**
- **With unstable corneas non-contact non-mechanical laser trephination has major advantages over conventional mechanical trephination**

10.3.1.9 The Triple Procedure

Since the introduction of the triple procedure [= simultaneous penetrating keratoplasty (PKP), extracapsular cataract extraction and implantation of a posterior chamber intraocular lens (PCIOL)] in the mid-1970s, there has been an ongoing discussion among corneal microsurgeons concerning the best approach (*simultaneous* or *sequential*) for combined corneal disease and cataract [65]. For the refractive results after the triple procedure, some intraoperative details are crucial: trephination of recipient and donor from the epithelial side without major oversize (guided trephine system or non-mechanical excimer laser trephination) should preserve the preoperative corneal curvature. Graft and the PCIOL placed in the bag after large continuous curvilinear capsulorhexis should be centered along the optical axis (Fig. 10.8). If possible, performing the capsulorhexis under controlled intraocular pressure conditions prior to trephination may help to minimize the risk of capsular ruptures. In the case of excessive corneal clouding, a capsulorhexis forceps is used via the "open sky" approach. Delivery of the nucleus is achieved via the "open sky" approach by means of manual irrigation, and removal of the lens cortex by automated irrigation-aspiration.

The major advantage of the triple procedure is the faster visual rehabilitation achieved and less effort required for the mostly elderly patients. In contrast, sequential cataract surgery has the potential for a simultaneous reduction of corneal astigmatism (appropriate location of the incision, simultaneous refractive keratotomies or implantation of a toric PCIOL). Disadvantages may include the loss of graft endothelial cells and the theoretically increased risk of immunologic allograft reactions. After the triple procedure, major deviations from target refraction have been reported. However, individual multiple regression analysis may help to minimize this problem with appropriate methods of trephination [77]. Since suture removal after PKP may result in major individual changes of the corneal curvature, IOL power

calculation for the sequential approach requires all sutures to be removed at the time of cataract surgery. However, even after complete suture removal the abnormal proportions between anterior and posterior curvatures and/or the irregular topographies after PKP may be responsible for marked IOL power miscalculations in the individual eye [65].

Summary for the Clinician

- **The postulated better prediction of refraction after sequential keratoplasty and cataract surgery is opposed by a markedly delayed visual rehabilitation**
- **We consider the triple procedure including cataract extraction via "open sky" in general anesthesia as the method of choice for combined corneal and lens opacities**

10.3.1.10 Impact of Trephination on Suturing

The trephination modality may have a major impact on the correct placement of the first four or eight cardinal sutures. The predominant purpose of the *cardinal sutures* is: (1) symmetric horizontal distribution of donor tissue in the recipient bed, (2) good adaptation of graft and host on Bowman's level (external steps are to be avoided, internal steps may be tolerated in the case of thin recipient corneas such as in pellucid marginal degeneration or herpetic scars), and (3) stabilization of the anterior chamber for further homogeneous suturing.

Unintentionally other than round host opening may create a challenge even for the experienced PKP surgeon concerning the correct placement of the *second cardinal suture*. After removal of the cardinal sutures the quality of the trephination and graft positioning are major determinants for watertight wound closure. The better the trephination, the smaller the final suture tension required for watertight wound closure after removal of the cardinal sutures. The smaller the final suture tension, the better the visual acuity as long as the sutures are in place. Generally, in cases where Bowman's layer is intact, a 16-bite double-running diagonal cross-stitch suture (10-0 nylon) according to Hoffmann (Fig. 10.9) is preferred. The more rapid visual rehabilitation with these sutures in place in contrast to single sutures is due to a more regular corneal topography avoiding cornea plana.

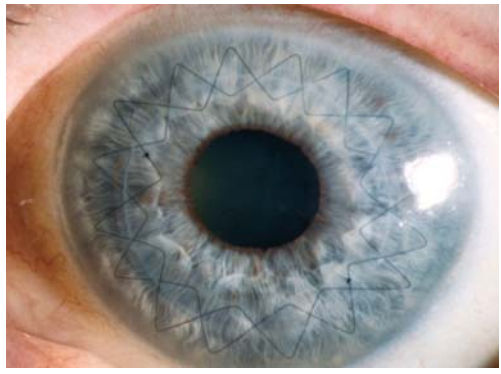

Fig. 10.9. Typical double running 10-0 nylon cross-stitch suture with 8 bites each (according to Hoffmann [17]) in keratoconus (8.0/8.1 mm, excimer laser trephination with eight "orientation teeth/notches")

Summary for the Clinician

- **The better the trephination the easier watertight wound closure is achieved**
- **Inadequately high suture tension to achieve watertight wound closure may deteriorate the regularity of the topography after PKP and delay visual recovery**

10.3.2 Conventional Mechanical Trephines

(Table 10.5)

In 1886 Arthur von Hippel was the first to use a mechanical clock-watch driven trephine (Fig. 10.10) for transplantation of a lamellar corneal graft from a rabbit to a human [79]. The same trephine was used by Eduard Zirm for his first successful PKP in a patient in 1905 [84].

Conventional mechanical trephination is associated with *deformation* of corneal tissue including a distortion of the cut margin with rough-cut edges as a consequence of *axial and radial forces* induced by the trephine. The cut angle deviates from the perpendicular and it may be different in donor and recipient, especially if the donor trephination is undertaken from the endothelial side. The fitting of the

Table 10.5. Characteristics of mechanical trephines

Type	Geuder Micro-Keratron (discontinued)	Moria (Hanna)	GTS (Krumeich)	Hessburg-Barron	Asmotom (Gliem & Franke)
Motorized cutter	Yes	No	No	No	Yes
Vacuum fixation for recipient	No	Yes (limbus)	Yes (limbus)	Yes (cornea)	Double
Cutter feed	No	No	No	No	Yes
Depth adjustment	No	Yes	Yes	Limited	Yes
Auto-retract	No	No	No	No	Yes
Anterior chamber maintainer required for donor	Yes	Yes	Yes	Possible	No
Automation	No	No	No	No	Yes

Table 10.6. Trephines used in Germany in the year 2002 for 4583 penetrating keratoplasties (German Keratoplasty Registry Erlangen) (122 institutions contributed) [5]

	GTS	Manual	Barron	Motor trephine	Asmotom	Excimer laser	Unknown
Donor	1555	1040	716	415	393	313	151
%	33.9	22.7	15.6	9.1	8.6	6.8	3.3
Recipient	1570	818	745	640	346	313	151
%	34.3	17.8	16.3	13.9	7.6	6.8	3.3

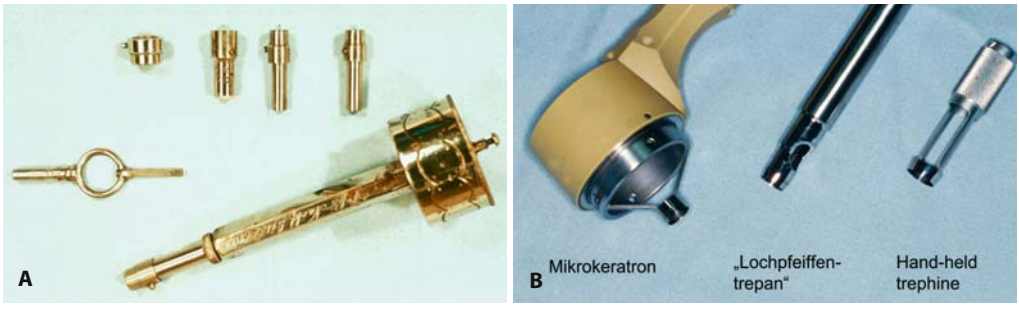

Fig. 10.10 A, B. Mechanical trephines. **A** Arthur von Hippel's clock-watch driven trephine. **B** "Modern" mechanical trephines (motor trephine, *Lochpfeiffentrepan*, hand-held trephine [39])

donor tissue into the malleable recipient cornea is extremely difficult to achieve in a perfectly symmetric fashion. After suturing the incongruent cut edges in order to achieve watertight wound closure, wound healing may cause marked distortion of the surface topography after suture removal due to this "vertical tilt." In addition, asymmetric cardinal suture placement may result in unequal donor tissue distribution in the host wound, particularly if the second cardinal suture is not placed exactly opposite to the first ("horizontal torsion") [42].

A questionnaire was sent to all German keratoplasty surgeons in 2002 asking for their preferred technique of trephination. As outlined in Table 10.6 for recipient trephination, most surgeons use the GTS (34.3%), the hand-held trephine (17.8%) or the Hessburg-Barron trephine (16.3%). Motor trephines are used more rarely and the laser trephination has still not entered many operating theaters because it is bulky and expensive. As many as 12% of all procedures were performed with different trephine systems for donor and recipient [5]!

10.3.2.1 Freestanding Blade/Hand-Held Trephines

Hand-held trephines are available in a wide range of diameters from very small (e.g., 1.5 mm) to very large (e.g., 16.0 mm). Hand-held trephines may be dull with reduced visual control under the operating microscope despite recent improvements [39]. Thus, centration may be a problem. Typically, the donor is punched from the endothelial side (*Lochpfeiffentrepan*). Francheschetti-type freestanding blades (Fig. 10.11) seem to create more reproducible cuts than other hand-held trephines [72, 76].

Fig. 10.11. Francheschetti-type freestanding blades are available in a wide range of diameters

10.3.2.2 Motor Trephines (Mikro-Keratron, Asmotom)

Mikro-Keratron. The *Geuder Micro-Keratron* trephine is a non-automated motor-driven trephine system for PKP. The depth of the cut is not preadjustable, so that this trephine system has no impact on lamellar keratoplasty. Rotation (variable speed) may be started and stopped by pressing down and releasing a foot pedal. Different blades mounted on the unit allow for a wide range of trephination diameters. To trephine the donor cornea from the epithelial side, the tissue has to be mounted into an artificial anterior chamber maintainer. Motor trephine rotation may lead to "shifting" of the trephine within the corneal stroma.

Asmotom. The *Asmotom ATS* is an automated trephine system for PKP. The trephination of patient and donor eyes as well as corneoscleral disks is performed with separate instrumentation sets. For non-perforating cuts the cutting depth is preadjustable with offset rings for the patient. The cutter sets provided by the distributors include five different diameters (6.0–8.2 mm). The ATS uses an innovative double fixation design. Vacuum is applied to both the central and the peripheral section of the cornea. The trephine rotates between the two concentric areas of fixation, using an automatic feed. Once the pre-set depth is reached, the cutter retracts back into its initial position, holding on to the separated central portion, until vacuum is released. The ATS marker facilitates the centering of the trephination cut to the cornea. The system does not require an artificial chamber maintainer for graft trephination.

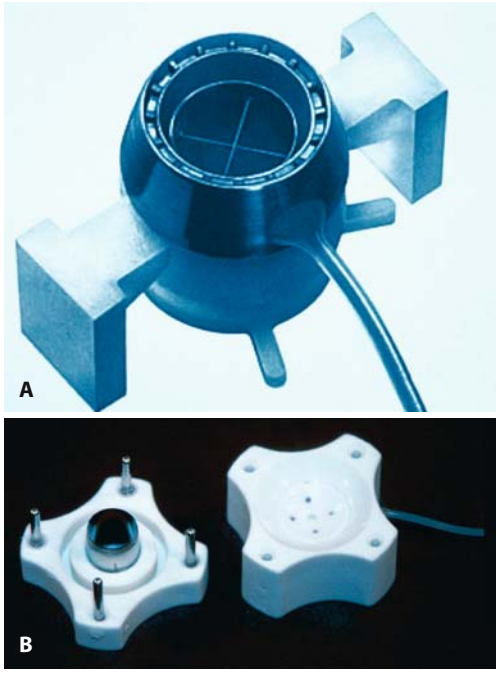

Fig. 10.12 A, B. Hessburg-Barron suction trephine. **A** Recipient trephine with cross-hairs for centration; **B** Donor trephination is performed from the endothelial side

10.3.2.3 Suction Trephines (Hessburg-Barron)

The classical *Hessburg-Barron trephine* (HBT) has been on the market for over 25 years. The HBT vacuum trephine is an easy to handle single-use product. The suction is applied to the peripheral cornea. The depth of the lamellar trephination can be predicted to a certain degree. One full rotation is presumed to achieve 250 µm of corneal depth. Perforation is typically limited to one-third to one-half of the circumference of the excision. The recipient trephine has cross-hairs for centration. No obturator is applied (Fig. 10.12 A). The Hessburg-Barron trephine leads to divergent cut angles and a larger diameter of the hole at the level of Descemet's membrane [72, 76].

In the classic version the donor is punched from the endothelial side with the aid of a suction device for fixating the donor epithelial side down. Tilt is avoided by four metal rods in the periphery of the blade-containing part and four corresponding peripheral holes in the suction-containing part (Fig. 10.12 B). In addition, four small holes inside the cut area which are colored before the corneoscleral button is placed inside give a reference with respect to the first four cardinal sutures. The donor is typically oversized by 0.25 mm [12].

Recently, a single-use artificial anterior chamber has been available, to create donor trephination from the epithelial side using the recipient trephine for donor trephination first.

10.3.2.4 Guided Trephines (GTS, Hanna)

The guided trephines result in the best cut qualities possible with mechanical trephines [72, 76]. These new generation suction trephines such as the Hanna trephine [80] and the Krumeich trephine ("guided trephine system," GTS) [4, 23] are preferred over the Hessburg-Barron trephine because they stabilize the globe by suction at the limbus – not the peripheral cornea. Thus – at least theoretically – the cut angles should be parallel to the optical axis, the dimensions for donor and recipient should be equal and, therefore, no graft oversize is required [50]. Overall, handling of both trephines requires a *special introduction* to the microsurgeon and the staff before application in patients.

GTS (Fig. 10.13). The *Krumeich guided trephine system* (GTS) is designed for PKP, lamellar keratoplasty, and circular keratotomy. The GTS can be used with and without an obturator preventing ballooning of the excised tissue into the trephine opening.

Advantages of the GTS include: (1) trephination of donor and recipient from the epithelial side using an artificial anterior chamber, (2) pre-defined depth of trephination, e.g., for lamellar procedures, and (3) in experienced hands through-and-through trephination without the necessity of cut completion with scissors can be achieved.

Potential *disadvantages* of the GTS include: (1) it is difficult to apply in patients with narrow lid fissure or deeply set eyes with prominent or-

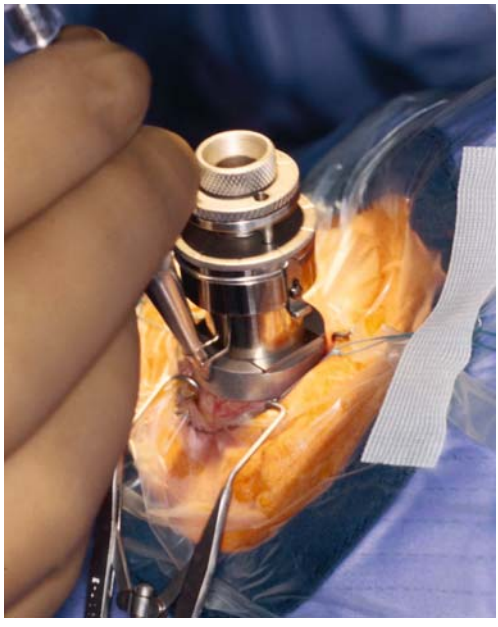

Fig. 10.13. The Krumeich guided trephine system (GTS) is designed for PKP, lamellar keratoplasty, and circular keratotomy. In patients, the GTS can be used with and without an obturator preventing ballooning of the excised tissue into the trephine opening

bital bones (which is not an uncommon issue in keratoconus), preexisting filtering blebs or conjunctival chemosis, (2) centration is difficult due to the limited view, (3) injury if the iris and lens are not securely prohibited, and (4) eccentric mini-keratoplasty with a small diameter (e.g., 4 mm) cannot be accomplished.

Hanna Trephine (Fig. 10.14). The *Hanna (Moria) trephine system* is one of the most advanced trephines which is designed to create a proper donor/recipient match. The Hanna trephine attaches firmly to the eye through suction applied to the limbal conjunctiva. Uniform support over the whole cornea during trephination prevents corneal vaulting. From a fully retracted position, the blade rotates while descending to a preset depth, after which the blade rotates without further descent, cutting the displaced tissue and creating a uniform incision. The Hanna trephine in combination with the artificial anterior chamber allows the surgeon to trephine

Fig. 10.14. The Hanna (Moria) trephine system. In patients this trephine attaches firmly to the eye through suction applied to the limbal conjunctiva. The Hanna trephine in combination with the artificial anterior chamber allows the surgeon to trephine both the recipient and the donor cornea from the epithelial side

both the recipient and the donor cornea from the epithelial side, thus reducing shape disparity. In the original version the donor trephination was performed from the endothelial side [81].

Summary for the Clinician

- **If conventional trephines are used it is recommended to use at least the same system with trephination of the donor from the epithelial side using an artificial anterior chamber for placement of the corneoscleral button from the Eye Bank**
- **The trephine should be as sharp as possible**

10.3.3 Nonmechanical Laser Trephination

Hypothesizing that the properties of the wound bed are much more important for the final "all-suture-out" astigmatism and the final optical performance of the graft than various types of suture techniques or methods of suture adjustment, we have developed and optimized the technique of *nonmechanical* corneal trephination since 1986.

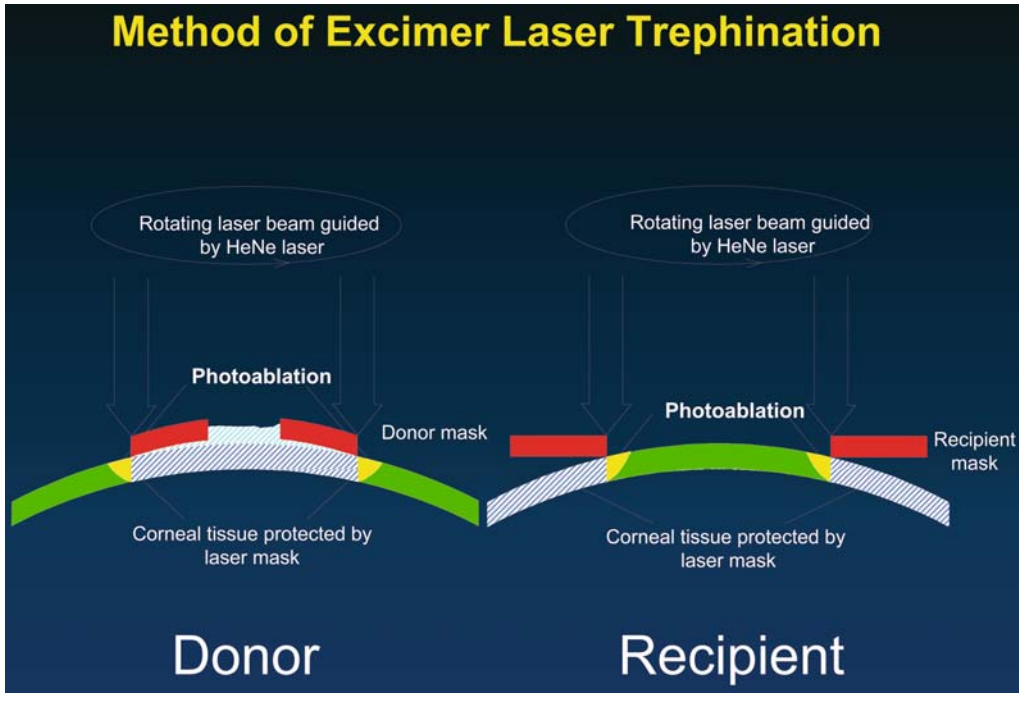

Fig. 10.15. Principle of excimer laser trephination in donor and recipient (schematic drawing, sagittal view)

10.3.3.1 The 193-nm Excimer Laser

Since 1989 more than 1650 human eyes have been treated successfully with the Meditec MEL60 excimer laser (Fig. 10.15). Keratoconus has been by far the leading indication (around 37%) for PKP with this non-contact technique (Table 10.7). For donor trephination from the epithelial side an artificial anterior chamber is used [41, 42, 58, 66].

Technique (Fig. 10.16). Before starting trephination, the limbus is centered on the perpendicular HeNe aiming beam in donor and patient to ensure a reproducible position of the eye relative to the laser and symmetric cut angles over the entire circumference without tilt. The horizontal positioning of the limbal plane can be controlled using the focusing device of the laser at 3, 6, 9, and 12 o'clock at the limbus before focusing the laser at the trephination edge ("triangulation"). "Horizontal torsion" of the graft may be reduced by employing eight orientation teeth at the donor trephination margin and eight corresponding notches in the recipient bed (a technique which allows the use of eight symmetric cardinal sutures) [2].

For *donor trephination* from the epithelial side using the 193-nm excimer laser MEL60

Table 10.7. Indications for 1656 consecutive non-mechanical excimer laser keratoplasties (06/1989 to 04/2005 in Erlangen)

Keratoconus	*607*	*(36.7%)*
Fuchs' dystrophy	323	(19.5%)
Bullous keratopathy	275	(16.6%)
Avascular scars	181	(10.9%)
Graft failure	77	(4.6%)
Corneal ulcer	64	(3.9%)
Stromal dystrophies	48	(2.9%)
Disabling astigmatism	40	(2.4%)
Others	41	(2.5%)

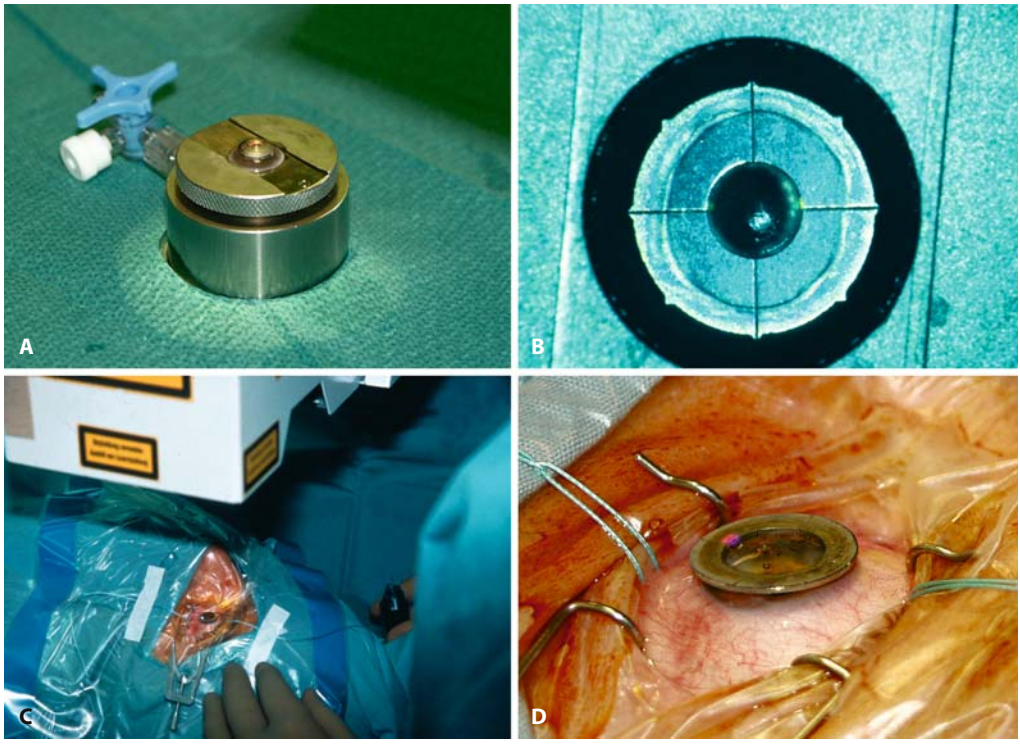

Fig. 10.16 A–D. Nonmechanical trephination using the 193-nm excimer laser in combination with metal masks with "orientation teeth/notches." **A** Curved donor mask on top of corneoscleral button fixed in a modified Krumeich artificial anterior chamber; **B** metal donor mask with eight "orientation teeth"; **C** laser arm and joystick for recipient trephination; **D** metal recipient mask with eight "orientation notches" on top of patient's cornea. A 1.5×1.5-mm laser spot is guided along the inner edge of the mask, half of the beam on the mask and half of it on the cornea

(Carl Zeiss Meditec, Jena, Germany), a circular round metal aperture mask (diameter 5.6–8.6 mm, central opening 3.0 mm for centration, thickness 0.5 mm, weight 0.2 g, eight orientation teeth 0.15×0.3 mm) is positioned on a corneoscleral button (16 mm diameter) fixed in an artificial anterior chamber (Polytech, Rossdorf, Germany) under microscopic control (Fig. 10.16 A, B). The pressure within the artificial anterior chamber is adjusted to 22 mmHg. An automated rotation device for the artificial anterior chamber is used.

For *recipient trephination* exclusively performed with the manually guided excimer laser, a corresponding metal mask is used (diameter 12.9 mm, central opening 5.5–8.5 mm), thickness 0.5 mm, weight 0.4 g, eight orientation notches 0.15×0.3 mm (Fig. 10.16 C, D). Before starting the trephination, centration relative to the limbus is achieved by lining up the eight notches with the eight lines of a radial keratotomy marker under microscopic control (Fig. 10.6).

Advantages (Table 10.8). The main advantage of this novel laser cutting technique performed from the epithelial side in donor and recipient is the avoidance of mechanical distortion during trephination, resulting in smooth cut edges (Fig. 10.17 A) which are congruent in donor and patient, potentially reducing "vertical tilt" [33]. Such cut edges in combination with "orientation teeth" (Fig. 10.17 B) at the graft margin [2] and corresponding notches at the recipient margin for symmetric positioning of the eight cardinal sutures minimize "horizontal torsion," thus potentially improving the optical performance

Table 10.8. Advantages of nonmechanical trephination with the 193-nm excimer laser along metal masks with "orientation teeth/notches" [41, 42, 58, 66]

1. No trauma to intraocular tissues
2. Avoid deformation and compression of tissue during trephination
3. Reduction of horizontal torsion ("Erlangen orientation teeth/notches")
4. Reduction of vertical tilt (congruent cut edges)
5. Reduction of host and donor decentration
6. Feasibility of "harmonization" of donor and host topography
7. Reduction of anterior chamber inflammation early after PKP
8. Reduction of astigmatism after suture removal
9. Higher regularity of corneal topography
10. Significantly better visual acuity with spectacle correction
11. Feasibility of trephination with unstable cornea (e.g., "open eye", descemetocele, after radial keratotomy, iatrogenic keratectasia after LASIK)
12. Arbitrary shape (e.g., elliptical) [28]

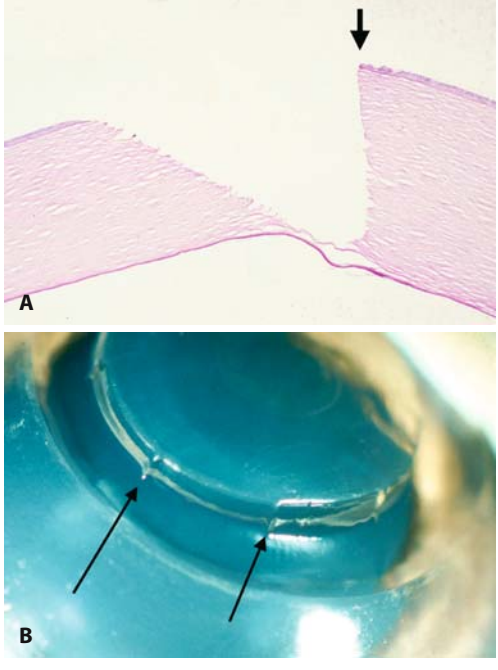

Fig. 10.17 A, B. Donor trephination immediately before perforation. **A** Histologic view with smooth almost perpendicular cut edge; **B** macroscopic view with smooth cut surfaces and "orientation teeth"

after transplantation [42]. Furthermore, recipient and donor decentration may be reduced [30, 61]. The use of metal masks allows for arbitrary shapes of the trephination [28].

These favorable impacts on major intraoperative determinants of post-keratoplasty astigmatism (cf. Table 10.2) result in lower keratometric astigmatism, higher topographic regularity and better visual acuity after suture removal. After sequential removal of a double running suture, keratometric astigmatism increased in 80% of eyes with conventional trephination, but further decreased in 52% of eyes with laser trephination [58]. In addition to less blood-aqueous barrier breakdown during the early postoperative time course after PK [26], laser trephination induces neither cataract formation nor higher endothelial cell loss of the graft. Likewise, the rates of immunologic graft rejection and secondary ocular hypertension are comparable using either technique. In addition, trephination of an unstable cornea, such as in (pre-)perforated corneal ulcers or after RK or LASIK, is facilitated [64].

Practical Considerations for the Microsurgeon [66]. The longer trephination time of around 6 min for the donor and around 4 min for the recipient are by far compensated for by practical advantages for the microsurgeon during the subsequent course of surgery: (1) injuries of in-

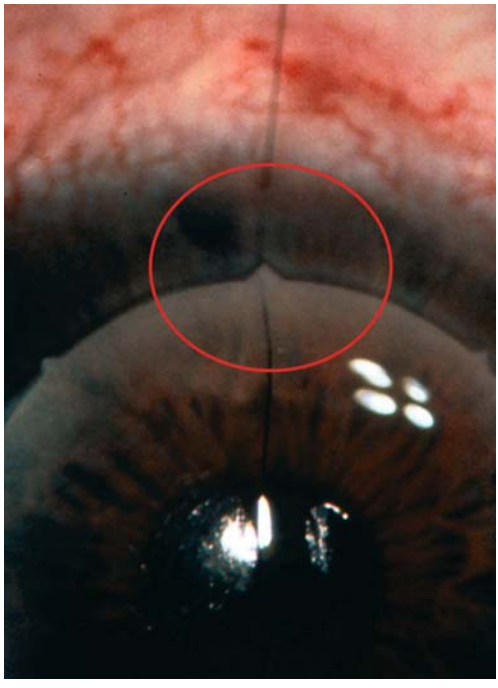

Fig. 10.18. Correct position of *second* cardinal suture (*arrow*) is facilitated by orientation tooth (donor) and corresponding notch (host)

traocular structures are impossible with the laser – even in beginner's hands – since the ablation stops as soon as aqueous humor fills the trephination groove after focal perforation. (2) The need for completion of the cut by scissors is reduced to a minimum. (3) The localization of the first eight cardinal sutures is unequivocally given by the "orientation teeth/notches" (Fig. 10.18). (4) Crescent-shaped tissue deficits at the graft-host junction (e.g., at other than round recipient openings in keratoconus) are avoided, thus achieving a latent watertight wound closure often as soon as after four cardinal sutures. (5) During further suturing the anterior chamber tends to remain stable. (6) The final double running suture needs very little tension to keep a watertight wound after removal of the eight cardinal sutures. (7) Therefore, only very rarely are additional single sutures with adverse effects on graft topography required at the end of surgery. (8) In addition, the so-called "barrel-top formation" at the proximal suture endings inducing a relative cornea plana and delaying optical rehabilitation can be avoided. (9) After removal of lid speculum and fixation sutures, the use of a Placido's disk often enables an almost round projection image to be achieved during intraoperative suture adjustment.

Summary for the Clinician

- **Nonmechanical trephination using the 193-nm excimer laser along metal masks has improved functional outcome after PKP with all-sutures-out**
- **The application of excimer lasers allows controlled trephination of unstable corneas such as perforated ulcers or iatrogenic keratectasia after LASIK**

10.3.3.2 The 2.94-µm Erbium:YAG Laser

The erbium:YAG laser was investigated to improve handling, reduce acquisition and maintenance costs, and provide solid state laser safety but keep the morphological advantages of the excimer laser trephination [1]. However, shrinkage effects due to thermal damage of the cut edges especially in the free-running but even with Q-switched laser pulses are major drawbacks of this infrared laser [69]. The induced thermal damage of the Q-switched mode erbium:YAG laser has been detected to be around 2–15 µm, in comparison to only 200 nm using the excimer laser [54, 68].

Summary for the Clinician

- **The erbium:YAG laser will probably not substitute the excimer laser for nonmechanical trephination in the near future without a loss of advantages**

10.3.3.3 The Femtosecond Laser

In contrast to the excimer laser, which allows only surface ablation, the femtosecond (= 10^{-15} s) laser allows the cornea to be cut within the stroma, enabling truly three-dimensional cuts without opening the eye and without thermal damage. No masks but an ultra-fast eye tracking system is required. There is no significant tissue loss to be compensated. For PKP especially in keratconus a non-contact approach of laser application is favored to avoid deformation.

Self-sealing keratoplasty wounds would be a major step towards rapid visual rehabilitation in PKP. Various kinds of nut-and-bolt configurations to fit in the donor including "orientation teeth" of the graft in the recipient bed are feasible using a femtosecond laser. We have introduced an *inverse mushroom shaped trephination* with the larger diameter of the graft at the level of Descemet's membrane (Fig. 10.19). Variation of the diameter of the "stipe" and the "cap" may help to produce the best individual compromise between the amount of transplanted endothelium and distance to limbal vessels and resistance to intraocular pressure [67].

In addition, *posterior lamellar keratoplasty* (PLKP) can be performed more easily with a femtosecond laser [63].

Fig. 10.19. Macrophotograph of *inverse mushroom shaped trephination* using a femtosecond laser [67]

Summary for the Clinician

- **Femtosecond laser application is the "*excitement of tomorrow*" in microsurgery of the cornea**
- **New nut-and-bolt type variants for potentially self-sealing donor/host appositions are on the horizon, offering a promising approach towards minimally invasive "*no-stitch keratoplasty*"**

10.4 Concluding Remarks

Today, expectations concerning the outcome after penetrating keratoplasty are not only restricted towards achieving a clear graft. The only criterion that counts for the patient is good vision preferably without the need for contact lenses but with an easily tolerable need for correction using spectacles. Therefore, transplant microsurgeons should not only consider all the means available to prevent high or irregular post-PKP astigmatism. Due to the lack of predictability of the refractive result in an individual patient after PKP, they should also familiarize themselves with the surgical techniques for correcting refractive errors after PKP in order to achieve the individually best outcome for a given patient.

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Infective Complications Following LASIK

11

Adam Watson, Sheraz Daya

Core Messages

- Infective complications following LASIK are a rare, potentially sight-devastating complication but often have good outcomes
- Early diagnosis helps prevent rapid steroid-related progression of infection
- Atypical organisms are common, especially non-tuberculous mycobacteria
- Early presenting cases (7–10 days) and late presenting cases (>10 days) have a different microbiological profile
- Intact epithelium inhibits antibiotic penetration. Flap lift, antibiotic soak and epithelial defect creation are useful strategies
- Reculture, biopsy and flap amputation may be necessary for worsening keratitis despite treatment
- Informed consent and attention to risk factors are crucial

11.1 Introduction

Since its development in 1989 by Pallikaris followed later by FDA approval in the United States in 1999, LASIK (laser-assisted in situ keratomileusis) has become an extremely commonly performed surgical procedure. Infective complications are rare [4] but present special challenges. Infective keratitis following LASIK often involves organisms unusual in other forms of infective keratitis. It usually occurs in the flap interface and may be relatively inaccessible to topical antibiotics. Bilateral infection, although not common, occurs at least partly due to the common practice of performing bilateral simultaneous LASIK procedures. Clusters of infection have also been reported [3, 7, 11]. Finally it should be noted that it is a vision-threatening complication occurring in people with generally high visual expectations, adding to its gravitas.

11.2 Frequency and Presentation

The reported frequency of infection following LASIK ranges from 0% to 1.5%, with the frequency in most large case series being less than 0.2% [4]. Gram-positive and non-tuberculous mycobacterial infections are commonest, with these organisms accounting for 26% and 47% of culture-positive infections respectively in a review of published cases [4]. Mycobacterial infections are probably overrepresented due to reporting bias but do represent a strikingly high proportion of cases of post-LASIK infection. Gram-negative organisms, by contrast, account for very few cases. Fungal and *Acanthamoeba* infections have also been described (Table 11.1).

There are almost certainly predisposing factors for post-LASIK infection. Uncontrolled meibomian gland dysfunction and blepharitis probably contribute to staphylococcal infection [12]. Performing LASIK on eyes that have previously undergone photorefractive keratectomy (PRK) seems to be a risk factor [4]. Post-LASIK trauma is undoubtedly associated with infection. However, the commonest association with reported infections is a breakdown in sterility during the procedure, with systematic contami-

Table 11.1. Organisms reported to have caused post-LASIK keratitis

Bacteria
Staphylococcus aureus
Streptococcus pneumoniae
Streptococcus viridans
Coagulase-negative staphylococci
Pseudomonas aeruginosa
Nocardia asteroides
Mycobacteria
Mycobacterium chelonae
Mycobacterium mucogenicum
Mycobacterium abscessus
Mycobacterium szulgai
Fungi
Candida albicans
Curvularia lunata
Scedosporium apiospermum
Fusarium solan
Fusarium oxysporum
Colleotrichum (*Fusarium*-like)
Other
Acanthamoeba

nation of the surgical field probably being responsible for three reported clusters of mycobacterial infection [3, 7, 11].

11.3 Characteristics

Patients with post-LASIK infective keratitis tend to present with varying combinations of pain, photophobia, discomfort, redness and discharge. Deterioration in postoperative visual acuity is commonly noted and may be the sole presenting symptom. Patients may also be asymptomatic with the infection identified at a routine postoperative examination.

The timing of the onset of symptoms varies – between zero days and several months [4, 12]. Post-LASIK infections may usefully be divided into early and late groups depending on the length of time from surgery to the onset of symptoms. Those presenting early occur in the first 7–10 days and are more likely to be caused by "typical" Gram-positive bacteria. Late infections, presenting beyond 10 days, are more likely to be atypical infections, especially non-tuberculous mycobacteria but also including fungal infection.

Flap interface infiltrate is the commonest sign evident on examination although infiltrate may be confined to the lamellar flap or the underlying corneal stroma [4]. Other features of infection that may be present are those found in other forms of infective keratitis, including anterior chamber reaction, keratic precipitates, corneal abscess and epithelial defects. Epithelial defects are found far less frequently in post-LASIK keratitis and tend to be associated with Gram-positive infection. The lack of an epithelial defect has important implications for treatment, as topical antimicrobial penetration is poorer in the absence of a defect. An intact epithelium presents a relatively impermeable barrier to topical antibiotic penetration.

Crystalline keratopathy has also been reported in several cases associated with *Mycobacterium chelonae* infection [2, 22]. This appearance is highly suggestive of *M. chelonae* infection.

11.4 Differential Diagnosis

11.4.1 Diffuse Lamellar Keratitis (DLK, "Sands of the Sahara")

DLK, a non-infectious inflammation occurring after LASIK in approximately 2–4% of cases [13], may present with mild pain, redness and photophobia in the 1st week after surgery. In the milder stage 1 and stage 2 forms of DLK the infiltrates are light and diffuse and unlikely to be confused with infection. More severe stages of DLK involve clumping of cellular infiltrates and, in stage 4 cases, stromal melting. The possibility of infection should always be considered in these cases and, since treatment of more severe DLK involves flap lift and irrigation, it is prudent to take a scrape sample for microbiology when lifting the flap [16]. Use of topical steroids for presumed DLK may lead to initial apparent improvement in infective keratitis with subsequent rapid progression of infection and destructive stromal necrosis.

11.4.2 Steroid-Induced Intraocular Pressure Elevation with Flap Oedema (Pseudo-DLK)

This uncommon phenomenon generally presents with decreased visual acuity, flap oedema and variable inflammation and may be mistaken for DLK. Increased frequency of steroid use then leads to worsening of the condition. The centrally measured intraocular pressure (IOP) is often normal and careful examination may reveal a fluid cleft in the flap interface. Peripheral IOP measured with a Tono-Pen (Medtronic-Solan) reveals an elevated IOP and the condition will resolve with control of IOP, usually with topical agents, and tapering or cessation of steroids [10, 15].

11.5 Management

The principles of management are similar to those in regular infective keratitis, namely:

- Suspect infection
- Obtain a microbiological sample prior to starting treatment
- Give broad spectrum empirical therapy initially
- Tailor therapy depending on clinical response and microbiological results (Gram and other stains, culture, sensitivities)
- If there is a worsening clinical situation and no microbiological information to guide, consider temporary withdrawal of treatment for rescrape or corneal biopsy

Post-LASIK infective keratitis differs from regular infective keratitis in that:

- Atypical infections (non-tuberculous mycobacteria) are relatively common
- Antibiotic penetration may be poor due to an intact epithelium
- Flap complications such as striae, epithelial ingrowth, flap melt and dehiscence may be problematic, related to infection or flap lift

We propose a management algorithm that takes some of these factors into account (Fig. 11.1).

11.5.1 Flap Lift

This should be carried out in most circumstances. An exception is if the focus of infection is very peripheral and associated with overlying flap necrosis allowing an adequate microbiological sample and debridement of infectious material (Fig. 11.2).

The flap may be lifted completely or partially, depending on the extent and location of infiltrate. Flap lift should be carried out beneath an operating microscope under sterile conditions with or without patient sedation. Some prefer to initiate the flap lift at the slit lamp where the flap border may be more easily identified. Initiation of flap lift is generally with a blunt spatula or Sinskey hook to break the epithelium and open the interface for one or two clock hours, then completed with non-toothed LASIK flap forceps.

11.5.2 Specimen Taking

Gentle scraping of material for microbiological examination and culture and to debride infective debris follows this. A hypodermic needle, number 15 Bard-Parker blade or Kimura spatula may be used. The authors prefer to plate the specimens themselves on culture media immediately. We suggest as a minimum, if the amount of material allows, an air-dried slide for immediate Gram stain, blood, chocolate and Sabouraud's agar plates and brain-heart infusion broth. If *Mycobacterium* is suspected, then culture on Lowenstein-Jensen medium should be considered. Useful additional stains for late-presenting cases include auramine-rhodamine for acid-fast bacilli [22] and periodic acid–Schiff (PAS) for fungi [21].

- **A microbiological specimen prior to treatment is essential**
- **Flap lift is usually necessary**

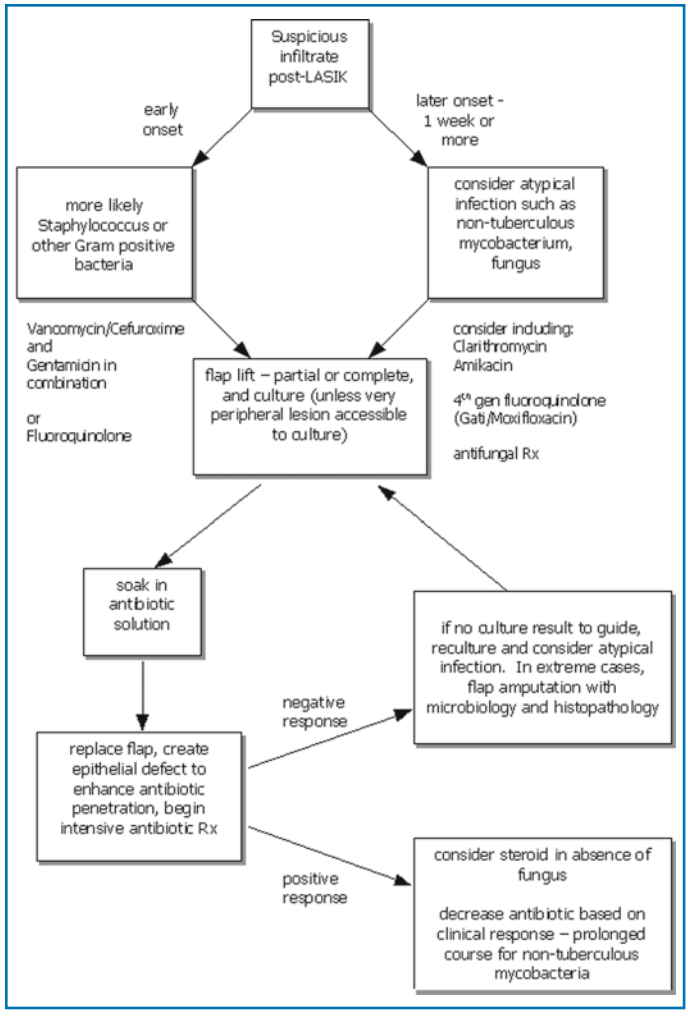

Fig. 11.1. Algorithm outlining an approach to post-LASIK infective keratitis

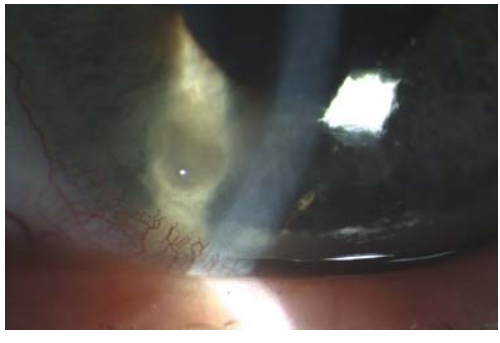

Fig. 11.2. Peripheral infiltrate 3 weeks after LASIK with focal flap melt

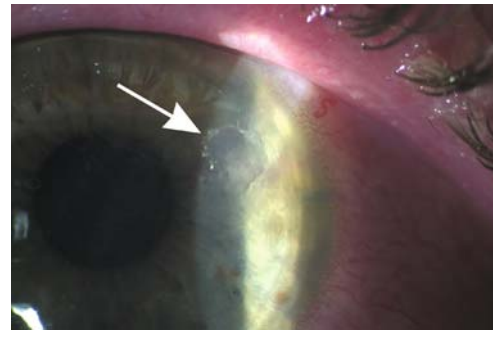

Fig. 11.3. *Arrow* indicates an epithelial defect created over a peripheral interface infiltrate after raising part of the flap for an interface scrape. The defect aids antibiotic penetration

11.5.3 Treatment

A moistened lint-free sponge may be used to remove residual debris, followed by "soaking" of the flap and stromal bed in antibiotic solution. The choice of antibiotics may depend on whether the keratitis falls into the early or late group (Fig. 11.1). Soaking should be for 2 min or more with each antibiotic solution in turn, followed by careful relaying of the flap. If there is little or no epithelial defect overlying the suspected infection, an epithelial defect should be created to aid antibiotic penetration (Fig. 11.3).

Intensive topical antibiotics should then be started (hourly alternating around the clock). The choice of antibiotics will be partly determined by the resistance characteristics of bacteria in the local region. Specialist microbiologist advice should be sought if there is doubt. Suggestions for treatment choice are given in Fig. 11.1.

Topical steroids should be avoided in the early stages of treatment and only instituted, if at all, when there is clear clinical evidence of improvement (e.g. less pain, diminishing and coalescing infiltrate, fewer keratic precipitates, healing epithelial defect), suggesting sterilisation of the offending organism. Introduction of any steroid should generally be in low dose (e.g. twice daily prednisolone sodium phosphate 0.5%) and the response closely monitored for signs of worsening infection, e.g. satellite infiltrates. Steroid use without concomitant antibiotic has been implicated in the recrudescence of infection after apparent sterilisation of *Pseudomonas* keratitis [8]. Steroid use should be avoided in cases of fungal keratitis.

Topical antibiotic choice may be altered when microbial sensitivities are available. If the infection is clinically improving, there may be no need to change the antibiotics other than tapering the frequency of use after 2–3 days. If the infection is improving and sensitivity data are available, it may be reasonable to discontinue one of the antibiotics (e.g. gentamicin in a vancomycin/gentamicin combination when treating a staphylococcal infection) to minimise epithelial toxicity and promote healing.

The use of preservative free lubricants to preserve epithelial health should be considered. A cycloplegic (preservative free cyclopentolate or homatropine) should be added if there is significant anterior chamber inflammation.

- **Choose antibiotics to cover atypical organisms in late-presenting cases**
- **Antibiotic penetration is aided by an epithelial defect**
- **Avoid steroid use unless there is unequivocal improvement suggesting sterilisation of infection**
- **Avoid steroids in fungal infection and without concomitant antibiotic use**

11.5.4 No Improvement

Failure of the infection to show signs of improvement after several days of treatment should prompt a re-evaluation. An attempt at reculturing the infective agent is mandatory, by further corneal scrape or corneal biopsy. If the infection is severe and judged to be threatening the eye, flap amputation may be necessary, with half the flap being sent for histological examination and staining for organisms, the other half being sent for microbial culture. A high suspicion for atypical infection exists at this point and mycobacteria, fungi and *Acanthamoeba* should be specifically looked for.

Failure to control the infection despite treatment, as with regular infective keratitis, may require further surgical intervention including therapeutic penetrating keratoplasty, and intraocular instillation of antimicrobial drugs in the case of perforation with suspected endophthalmitis, with or without lensectomy and vitrectomy depending on the involvement of intraocular structures.

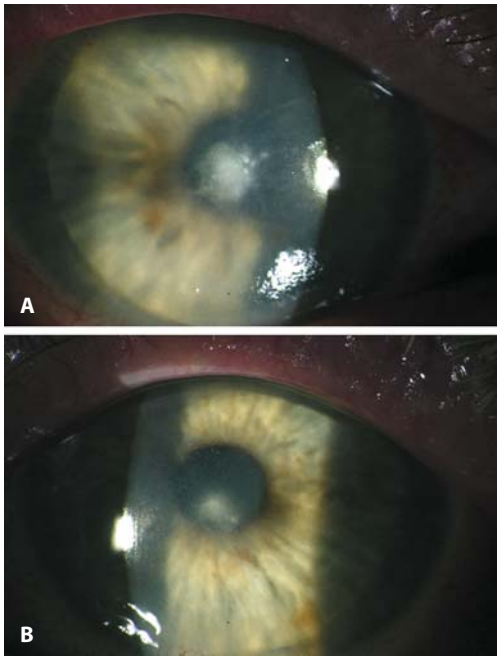

Fig. 11.4 A, B. Bilateral central *Mycobacterium chelonae* post-LASIK keratitis (right eye **A**, left eye **B**). Note central interface infiltrates

Fig. 11.5. Subsequent right central flap melt in the case of *M. chelonae* keratitis shown in Fig. 11.4

11.6 Special Considerations

11.6.1 Mycobacteria

Topical clarithromycin and amikacin have generally been the agents of choice for treatment of *M. chelonae* keratitis. Tobramycin and the fluoroquinolones are also often effective. There has been recent interest in the fourth generation fluoroquinolones, including moxifloxacin [1] and gatifloxacin, as having greater activity against non-tuberculous mycobacteria. The authors have experience of treating a case (unpublished) of bilateral moxifloxacin-resistant *M. chelonae* post-LASIK keratitis (Figs. 11.4, 11.5). This highlights the benefit of using multiple agents to treat infection empirically until the organism's sensitivities are known, with continued use of multiple antibiotics to which the organism is sensitive to prevent recrudescence. Treatment may need to be continued for 6 months or more with a gradual taper, monitoring closely for signs of recurrence. Viable mycobacteria have been cultured from an amputated LASIK flap despite 9 weeks of appropriate treatment for *M. chelonae* keratitis [19].

11.6.2 Fungal Keratitis

Fungal infections comprise about 14% of reported cases of post-LASIK keratitis [4]. Identification of hyphae, pseudohyphae or yeasts may be possible from direct microscopic examination of appropriately stained slide preparations of a scrape; or culture may yield fungal growth. An additional approach, maybe more applicable in the future, is PCR testing of specimens for fungal DNA, providing a quicker result than fungal culture. This method, while sensitive, does suffer from poor specificity [21].

Treatment of fungal infections should be determined in collaboration with a microbiologist and based on the organism's sensitivities when available. Common topical agents are natamycin 5% and amphotericin B 0.15%, both polyenes with a broad spectrum of activity against filamentous fungi and yeasts although natamycin may be slightly more effective and the preferred choice where available [21]. Topical econazole 1% is also being used where appropriate. Topical treatment should generally be combined with a systemic agent, e.g. one of the azoles such as ketoconazole or itraconazole. Voriconazole, a relatively new triazole agent, has

been reported to have superior activity against *Scedosporium* infections [18].

The use of topical steroids may cause fungal keratitis to progress rapidly to widespread corneal involvement and perforation. Steroids should be avoided when treating fungal infections, at least until effective antifungal treatment has been continued for several weeks. Antifungal therapy needs to be prolonged for at least 6 weeks – agents are generally fungistatic rather than fungicidal at the concentration achieved in the corneal stroma, and elimination of fungus depends ultimately on the host immune response.

11.6.3 Viral Keratitis

Case reports of apparent reactivation of *Herpes simplex* keratitis following LASIK have been published [5, 17]. It is not clear whether the LASIK procedure and/or the postoperative use of topical steroids were causative. However, ultraviolet radiation exposure has been associated with reactivation of latent *Herpes simplex* [20, 6]. In addition to a short-term topical antiviral, consideration should be given to longer-term systemic antiviral prophylaxis (e.g. oral acyclovir 400 mg twice daily).

11.7 Visual Outcome

The visual outcome following post-LASIK keratitis is highly variable. Approximately 50 % of reported cases have no clinically significant worsening of best-corrected Snellen visual acuity. Twenty-five per cent suffer a severe reduction [4]. Gram-positive infections are associated with better visual outcomes while fungal infections (excluding *Candida albicans*) are more likely to be associated with severe visual reduction. Reported cases of *C. albicans*, on the other hand, had a good visual outcome – with a best corrected visual acuity average of 20/25 [16]. Reported mycobacterial cases tend to be intermediate between Gram-positive and fungal infection in terms of visual outcome.

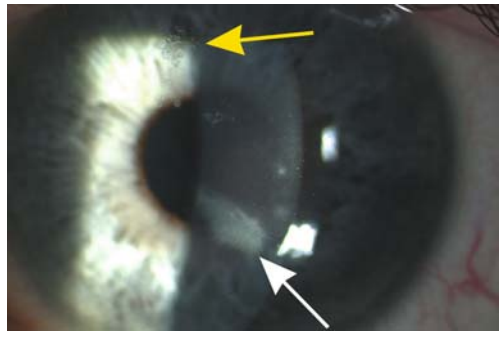

Fig. 11.6. Eye 9 months following treatment for *M. chelonae* post-LASIK keratitis. *Arrows* point to stromal scarring (*white*) and stable interface epithelial inclusions (*yellow*). The uncorrected visual acuity is 6/7.5

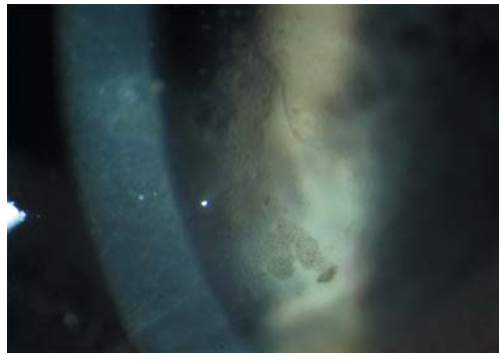

Fig. 11.7. Interface epithelial ingrowth arising from a flap defect in a case of *M. chelonae* post-LASIK keratitis. Tongues of epithelium are progressing peripherally

11.8 Management of Sequelae

Common sequelae of post-LASIK infection include scarring (Fig. 11.6), irregular astigmatism and varying degrees of epithelial ingrowth arising from flap lift or flap melt (Fig. 11.7).

Once the infection has settled, the goal of treatment is to optimise visual acuity in the affected eye. How this is achieved will vary markedly from case to case. Correction of refractive error should initially be explored using glasses, soft contact lens and rigid gas permeable lenses. Significant epithelial ingrowth in-

ducing astigmatism needs to be cleared from the flap interface prior to any further attempts at surgical correction. Irregular astigmatism resulting from scarring may be amenable to contact lens correction.

Consideration of further excimer laser refractive surgery should be approached with caution. In addition to likely patient concern about a repeat procedure, further LASIK will require recutting of a deeper flap to avoid the scarred and irregular interface inevitably present, and PRK or laser epithelial keratomileusis (LASEK) is associated with a high risk of development of haze in an environment with activated keratocytes.

Significant opacity affecting the visual axis, on the other hand, may need to be cleared. Options for this include homoplastic automated lamellar therapeutic keratoplasty (HALTK, a useful technique for opacities limited to the anterior one-third of the corneal stroma) [9], deep anterior lamellar keratoplasty [14] and penetrating keratoplasty.

11.9 Prevention

Rare cases of post-LASIK infective keratitis are inevitable. Attention to patient eyelid hygiene with control of blepharitis, careful patient instruction regarding pre- and postoperative care and avoidance of trauma, and meticulous attention to equipment sterility and operating environment hygiene are likely to lead to fewer cases. The authors strongly advise that separate blades and microkeratome heads be used if carrying out simultaneous bilateral LASIK to diminish the linked risk of bilateral infection. Above all, careful informed consent of the patient prior to surgery is mandatory.

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Treatment of Adenoviral Keratoconjunctivitis

12

Jost Hillenkamp, Rainer Sundmacher, Thomas Reinhard

Core Messages

- The clinical course of adenoviral keratoconjunctivitis (AKC) should be divided into an acute phase with conjunctival inflammation of varying intensity with or without corneal involvement and a chronic phase with corneal opacities
- AKC is caused by many different serotypes and is highly contagious during the acute phase
- The economic and social price of AKC as an community epidemic is high
- Corneal opacities, the hallmark of the chronic phase, are usually self-limited
- Topical steroids should be avoided because they prolong viral replication, frequently lead to long-lasting dry-eye symptoms, and corneal opacities almost always recur after discontinuation of topical steroids
- There is currently no effective and clinically applicable topical antiviral agent for the treatment of the acute phase of AKC
- Topical cidofovir is the first antiviral agent which has effectively reduced the incidence of corneal opacities, but local toxicity rules out its clinical application
- Recently, NMSO3, a sulfated sialyl lipid, has demonstrated a greater antiviral potency against adenovirus in vitro than cidofovir exhibiting minimal cytotoxicity
- Topical cyclosporin A (CsA) appears to be effective in the treatment of persistent corneal opacities
- Topical interferon might be effective as a prophylaxis of infection
- Topical interferon is currently not commercially available due to unsettled patent issues
- Adequate infection control measures should be followed as prevention and to reduce epidemic AKC outbreaks

12.1 Introduction

12.1.1 Etiology and Clinical Course of Ocular Adenoviral Infection

Adenoviral keratoconjunctivitis (AKC) was first described by Fuchs in 1889 [7]. In 1955, Jawetz identified adenovirus as the cause of the disease [20]. Other authors isolated adenovirus serotypes 8, 19 and 37 as the most frequent causative adenovirus subtypes [5]. Overall, many of the more than 40 adenovirus serotypes cause infections of the ocular surface with and without general symptoms. Pharyngoconjunctival fever is a conjunctivitis with upper respiratory tract involvement caused by serotypes 1, 3–7, and 14. The term "epidemic keratoconjunctivitis" describes an infection of the ocular surface without general symptoms caused by serotypes 2–4, 7–11, 14, 16, 19, 29, and 37. Unspecific follicular conjunctivitis is probably caused by all the above and serotype 34. In principle, neither is there a clear-cut distinction in this clinical

classification nor can the various serotypes be clearly associated with a distinct clinical presentation [5, 40].

The course of AKC must be divided into an acute phase and a chronic phase.

Summary for the Clinician

- **Many different serotypes of adenovirus cause AKC**

12.1.1.1 The Acute Phase

The acute phase has a wide spectrum of duration and intensity of local symptoms. It is principally self-limited. The intensity of symptoms varies between a picture of a mild unspecific conjunctivitis and intense conjunctival injection with marked chemosis and hemorrhagic involvement of the conjunctiva and the eyelids (Figs. 12.1–12.5). After an incubation period of 2–14 days symptoms usually begin in one eye and the other eye becomes symptomatic after 2–4 more days (Fig. 12.1). The mild forms of adenoviral conjunctivitis are clinically difficult to differentiate from any other unspecific conjunctivitis. The more pronounced cases can be readily diagnosed by the clinician. They present with a typical picture of conjunctival hyperemia and chemosis, swelling of the conjunctival plica, and intense serous or muco-serous tearing (Fig. 12.2). Conjunctival pseudomembranes occur in some cases. Ipsilateral preauricular lymphadenopathy is a fairly typical sign observed in many patients. The more severe cases of ocular adenoviral infections are characterized by a highly distressing morbidity [5] (Figs. 12.3–12.5). The cornea is not necessarily involved in the acute phase. If it is, a superficial punctate keratitis with small epithelial punctatae or larger stellata-type coarse punctatae and subepithelial infiltrates may develop [40]. Rarely, the cornea becomes involved with large epithelial erosions during the acute phase of the infection. Also rarely, the corneal endothelium becomes involved in the form of an endotheliitis with marked temporary corneal edema which usually subsides spontaneously [40].

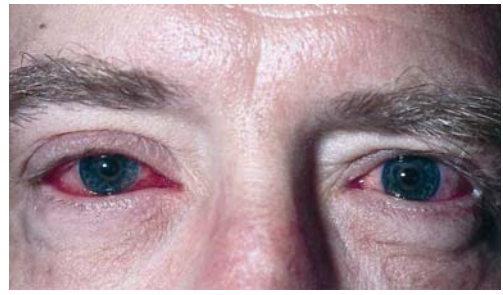

Fig. 12.1. Acute phase of AKC 1 week after onset of symptoms. Symptoms obviously first appeared in the right eye; the left eye became involved 2 days later

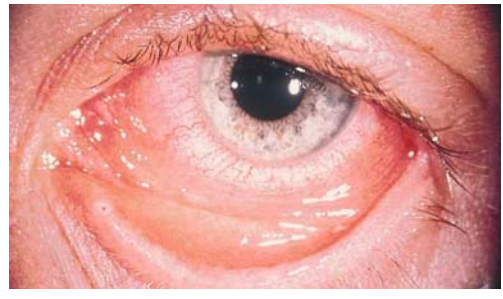

Fig. 12.2. Typical presentation of the acute phase of AKV with serous conjunctivitis

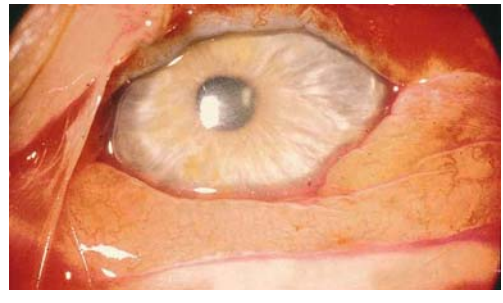

Fig. 12.3. This presentation with severe chemosis is fairly typical for the acute phase of AKC. An infection with herpesvirus cannot be clinically excluded whereas this picture clearly differs from allergic chemosis

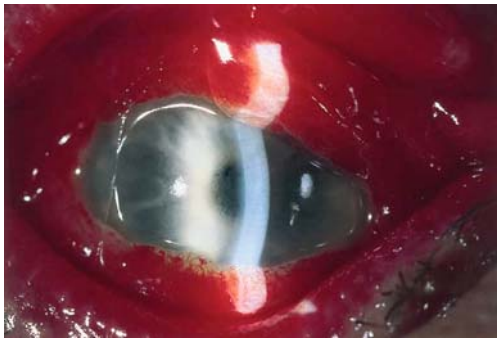

Fig. 12.4. This presentation with pronounced hemorrhagic chemosis is also fairly typical for the acute phase of AKC

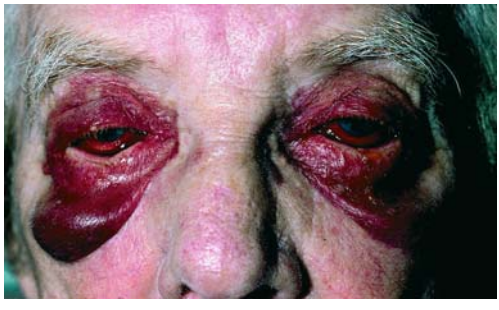

Fig. 12.5. This is an exceptionally severe hemorrhagic involvement of the eyelids in the acute phase of a case of proven AKC

Summary for the Clinician

- **The acute phase of AKC has a wide spectrum of intensity and duration of symptoms**
- **The acute phase of AKC is self-limited**
- **The cornea may or may not be involved in the acute phase of AKC**

12.1.1.2 The Chronic Phase

It is the corneal involvement during the chronic phase of the disease that sets AKC apart from other forms of virus conjunctivitis. Typically, during the course of the infection, approximately 10 days after onset of symptoms, corneal subepithelial opacities frequently appear (Fig. 12.6). These nummular opacities or infiltrates can impair visual function, and may persist for months to years [40, 28]. Histopathological investigation of focal biopsies revealed subepithelial infiltrates of lymphocytes, histiocytes and fibroblasts accompanied by a disruption of the collagen fibers of Bowman's layer [15, 25]. The pathogenesis of the nummular opacities most likely includes a persisting viral replication in subepithelial keratocytes triggering an immunological host reaction [40]. This hypothesis is supported by the clinical observation that opacities usually resolve with topical steroid treatment but recur when steroids are discontinued [39].

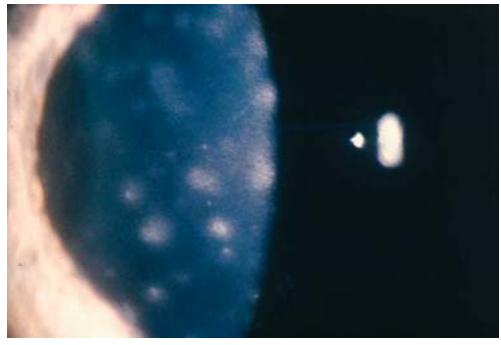

Fig. 12.6. Corneal opacities are the hallmark of the chronic phase of AKC. Histopathology revealed subepithelial infiltrates of lymphocytes, histiocytes and fibroblasts accompanied by a disruption of the collagen fibers of Bowman's layer [6, 7]. The pathogenesis of the nummular opacities most likely includes a persisting viral replication in subepithelial keratocytes triggering an immunological host reaction which causes the visible, steroid-sensitive opacities

Summary for the Clinician

- **Corneal opacities are the hallmark of the chronic phase of AKC**
- **Corneal opacities are probably caused by an immunological host reaction against persisting virus in keratocytes**
- **Corneal opacities almost invariably spontaneously resolve mostly within 1 year**

12.2 Socioeconomic Aspect

AKC is a highly contagious disease which occurs worldwide sporadically and epidemically. While not permanently blinding, adenoviral ocular infections remain the most common external ocular viral infection worldwide. The economic and social price of this community epidemic also remains high [6]. Public institutions such as schools or kindergartens must be closed following the outbreak of an epidemic. Many work hours are lost every year.

- **The economic and social price of AKC as a community epidemic is high**

12.3 Treatment

The clinical investigation of candidate treatments of AKC in patient studies has been hampered in the past by the very variable intensity and duration of the clinical symptoms and the self-limited nature of the acute phase of AKC. Furthermore, most studies have failed to apply laboratory tests for adenovirus as the cause of the disease and therefore the etiology of the treated diseases remains questionable. A possible treatment must therefore be evaluated by adequately designed large prospective controlled clinical trials with reliable tests of adenovirus as the underlying cause of the treated disease. Also, treatment of the acute phase of AKC must be distinguished from treatment and prophylaxis of corneal opacities, the hallmark of the chronic phase. Currently, no clinically applicable specific antiviral therapy is available to shorten the course of the infection, to improve the distressful clinical symptoms, to stop viral replication, and to prevent the development of corneal opacities. The effect of several topical agents has been investigated. Only steroids [42] and cidofovir [16, 17] have demonstrated a certain therapeutic effect and will therefore be further discussed. Other measures, such as topical povidone-iodine, topical interferon [26, 30, 31, 38, 41, 47], topical non-steroidal anti-inflammatories [13, 37], topical cyclosporin A or topical trifluridine, have been shown not to be more effective than topical lubrication [16, 17, 19, 46].

12.3.1 Treatment of the Acute Phase

12.3.1.1 Topical Steroids

Treatment of the acute phase of the infection with topical steroids has been widely recommended because of the clinical experience that the distressing local symptoms subside earlier with steroids. The effect of topical steroids has been investigated in a prospective randomized clinical study [42]. The results of this study confirm the clinical impression that the improvement of the local symptoms is accelerated with steroids. Furthermore, the number of corneal opacities per affected cornea in the chronic phase was reduced as compared to controls [42]. However, the number of patients affected by corneal opacities was not reduced and corneal opacities appeared later in the course of the disease. This finding suggests that steroids may prolong the persistence of adenovirus in the cornea. This undesired effect was confirmed by Romanowski et al., who found an increased viral replication under topical steroids [32]. Also, a significantly greater number of patients treated with topical steroids experienced long-lasting, distressing dry eye symptoms as compared to controls [42]. These findings lead to the conclusion that the negative effects of topical steroids outweigh the positive effect of an earlier relief of the distressing local symptom [32, 40, 42]. Therefore, topical steroids should be avoided in the treatment of the acute phase of AKC.

- **Local symptoms subside with topical steroids but viral replication is probably prolonged**
- **Topical steroids frequently lead to long-lasting dry-eye symptoms**
- **Topical steroids should be avoided**

12.3.1.2 Topical Cidofovir

Principally, an effective antiviral agent to shorten the course of the infection, to improve the distressful local symptoms of the acute phase, and to prevent the development of corneal opacities of the chronic phase would represent the ideal treatment of AKC.

Topical ganciclovir was only mildly effective in vitro and in the cotton rat model and was thus not further investigated as a potential treatment [43, 44].

Cidofovir or HPMPC, a broad-spectrum antiviral agent, demonstrated a significant inhibitory effect on adenovirus types 1, 5, 8 and 19 isolated from patients with AKC in vitro [8]. The efficacy of cidofovir was also documented in vivo. Topical cidofovir demonstrated significant antiviral activity in the AD 5 McEwen/NZ rabbit ocular model with 0.2% as the lowest effective concentration [11, 10]. Gordon et al. first reported clinical efficacy and safety of topical cidofovir 0.2% in the treatment of a single patient with proven AKC [12].

These results encouraged us to investigate the effect of topical cidofovir in a 0.2% concentration in the same preparation as described by Gordon et al. [16] Topical cidofovir 0.2% proved to be a well tolerated drug which did not cause any discomfort but did not have a statistically significant effect on the course of the acute or the chronic phase of adenoviral keratoconjunctivitis. In particular, the frequency of corneal infiltrates at the end of the 21-day treatment period was not altered by cidofovir 0.2% [16].

There are several possible explanations for the failure of cidofovir 0.2% to show the clinical efficacy that may have been expected from the antiviral activity demonstrated in vitro [8] and in the rabbit ocular model [11, 10].

Concentration of Cidofovir. Cidofovir 0.2% was administered 4 times daily. Gordon and Romanowski et al. showed that cidofovir 0.2% administered 4–5 times daily limited adenoviral replication in the adenovirus type 5/New Zealand rabbit ocular model. Cidofovir 0.5% and 1% administered only twice daily was not superior, but equally effective [11].

Serotype Dependency. Adenovirus demonstrated serotype-dependent differences in invitro infectivity titers and clinical course. AD8 was the most frequent serotype in a total number of 106 positive adenoviral cultures, causing significantly more often a severe clinical course with marked eyelid edema than other serotypes. AD3 and AD4 were associated with higher infectivity titers than other serotypes. Infectivity and the clinical course of AKC are serotype dependent [33].

Cidofovir proved to be effective against adenovirus types 1, 5 and 6 in the rabbit model [11, 29], but a relative resistance of serotype 19 to cidofovir was reported [8]. Variants of adenovirus serotype 5 with different sensitivity to topical treatment with cidofovir 0.5% in the rabbit ocular model have been described by Araullo-Cruz et al. [1]. Consequently, the efficacy of cidofovir in patients may also be serotype dependent. The patients enrolled in clinical studies were probably infected with various adenovirus types.

Pharmacokinetics. Eyedrops may be washed out by intense tearing; additionally regular conjunctival absorption of eyedrops may be impaired in patients with severe conjunctival chemosis and swelling of the conjunctival plica.

Viral Replication and Onset of Treatment. The New Zealand rabbit ocular model of adenovirus type 5 infection showed a duration of viral replication of 9 days with a peak on day 3. The symptoms of AKC after the phase of viral replication are thought to be caused by the host's immune response [9]. Treatment with cidofovir in the rabbit model began 24 h after inoculation [29]. The treatment of study patients began 1–7 days (mean: 3.5 days) after onset of symptoms when they first presented to the clinic [16]. Cidofovir may have been effective in the rabbit ocular model because treatment began early in the course of the infection while treatment of our patients may have failed because treatment did not start until the phase of viral replication had almost been completed.

The fivefold concentration of cidofovir tested in our second pilot study did not alter the course of the acute phase but appeared to be

Fig. 12.7. Local side effects of topical cidofovir 1%: conjunctivitis and erythematous inflammation of the eyelids

effective in the *prevention* of severe corneal opacities [17]. So far, only topical immunosuppressants such as steroids [39] or cyclosporin A [28] have been shown to be effective in the *treatment* of existing corneal opacities. These agents probably act by suppressing the immunological response directed against viral antigens which persist in the cornea. By contrast, as an antiviral agent, cidofovir treats the underlying cause of corneal opacities. As opposed to a treatment with steroids, or less so with topical cyclosporin A [28], it could therefore possibly prevent their occurrence, thereby avoiding the problem of recurrences after discontinuation of treatment [39]. Unfortunately, local toxicity forbids the clinical application of cidofovir in the 1% concentration. We observed an increased prevalence of conjunctival pseudomembranes as well as conjunctivitis and erythematous inflammation of the skin of the eyelids. These changes subsided completely after 7–28 days (mean 13.7 days) with topical lubrication and dexpanthenol ointment applied to the affected skin areas [17] (Fig. 12.7). Others have additionally described lacrimal blockade following the application of topical cidofovir [34]. These results are disappointing, however, the study demonstrated for the first time the principal efficacy of a topical antiviral agent as a treatment of AKC. This positive aspect of the study encourages further research for an effective yet tolerable antiviral agent.

Summary for the Clinician

- **Topical cidofovir is the first antiviral agent which effectively reduces the incidence of corneal opacities**
- **Local toxicity rules out the clinical application of topical cidofovir in an antivirally effective concentration**

12.3.2 Treatment of the Chronic Phase

12.3.2.1 Topical Steroids

The pathogenesis of the nummular corneal opacities of the chronic phase most likely includes a persisting viral replication in subepithelial keratocytes triggering an immunological host reaction. Topical steroids suppress the host reaction and thus lead to a quick disappearance of the opacities. Unfortunately, opacities almost invariably reappear when topical steroids are discontinued. The opacities frequently recur probably because steroids effectively suppress the immunological host reaction but lack a concomitant antiviral effect [40, 42]. Suppressing the immunological host reaction may therefore even enhance and prolong viral persistence within keratocytes. Even prolonged tapering of topical steroids failed to prevent recurrences of corneal opacities, even years after the initial acute phase of the disease [39]. For this reason and because of unwanted side effects of prolonged use of topical steroids such as cataract formation and secondary glaucoma, steroid treatment for the chronic phase of AKC cannot be recommended. There are no controlled clinical studies documenting the natural course of corneal opacities in AKC, but in the pre-steroid era Thygeson reported that corneal opacities in AKC invariably spontaneously disappear within 1 year. This is in accordance with our own clinical experience. Persistence of opacities without treatment for more than 1 year is a rarity. Also rarely, persistent, scarred corneal opacities cause irregular astigmatism which can be effectively corrected with hard contact lenses. Penetrating keratoplasty is usually not required [40].

Summary for the Clinician

- Corneal opacities almost always recur after discontinuation of topical steroids
- Even careful and prolonged tapering of topical steroids failed to prevent the recurrence of corneal opacities
- Corneal opacities mostly resolve spontaneously within 1 year

12.3.2.2 Topical Cyclosporin A

Cyclosporin A (CsA) is a well-established immunosuppressant which has been used in the prevention of transplant rejection for 25 years [3]. Topical CsA was used effectively in the treatment of Mooren's ulcer [48], vernal keratoconjunctivitis [2], ulcerative keratitis associated with rheumatoid arthritis [24], anterior uveitis [18], and Thygeson's punctate keratitis [27]. Side effects of topical CsA have not been described. CsA may also have some antiviral potency as CsA has been shown to inhibit herpes simplex virus in vitro [45]. In a non-controlled study topical CsA 2% led to a disappearance of corneal opacities in two-thirds of 56 treated patients with persisting opacity [28]. The opacities slowly responded to this treatment over several weeks, a response significantly slower than the rapid response to topical steroids [28]. Topical CsA was well tolerated, and only 7% of all treated patients discontinued the medication because of a local burning sensation. After slow tapering of topical CsA, one-third of the initial responders suffered a recurrence of the opacities. In all of these patients the opacities could be effectively abolished with another course of topical CsA on a low maintenance level of 1–2 drops/day [28].

Summary for the Clinician

- Topical CsA appears to be effective in the treatment of persistent corneal opacities
- So far, no side effects of topical CsA have been described

12.3.3 Prophylaxis

12.3.3.1 Topical Interferon

Interferons have multiple immunomodulative effects and therefore their effect on the course of viral infections is difficult to predict [40]. Treatment of the acute phase of AKC with topical interferon has been shown not to be effective [26, 30, 31, 38, 41, 47]. However, the consequent application of one drop per day of topical interferon (Berofor®) to all unaffected hospital staff and inpatients in a nosocomial viral epidemic keratoconjunctivitis which was recurrent in spite of hygienic prophylactic measures seemed to effectively prevent further spread of the infection [35]. In view of the epidemic character of AKC, prophylactic topical interferon seems to be an effective measure to protect yet unaffected individuals. However, topical interferon is currently not commercially available. Berofor® has been removed from the market due to unsettled patent issues.

- Interferons have multiple immunomodulative properties
- Topical interferon might be effective as a prophylaxis of infection

12.3.3.2 Infection Control and Hygienic Measures

It has been shown that infection control programs including specified methods of patient screening and isolation, handwashing, instrument disinfection, medication distribution, and furlough of infected employees are associated with decreased rates of nosocomial AKC outbreaks and outbreak morbidity in a large teaching eye institute [14]. A number of precautions should be observed to ensure safety in any ophthalmologist's office. Paramount among these is hand washing, both immediately after contact with patient's eyes and again between patients [4]. Highly concentrated alcohol-based hand rubs and hand gels have been demonstrated to

inactivate adenovirus within 2 min [21], povidone-iodine, peracetic acid, and formaldehyde have also demonstrated antiviral activity as disinfectant agents against adenovirus although the resistance of individual serotypes varies and the genomes of adenoviruses showed considerably more chemical resistance than the complete viral particle [36].

Careful disinfection of contact tonometry devices between patients is important. Furthermore, contact lens fitting is a typical procedure carrying the risk of transmitting adenovirus [4, 21]. A recent study investigated the effect of chemical, hydrogen peroxide, and heat sterilization systems on contaminated hard and soft contact lenses. Only heat sterilization was effective. As heat sterilization is not readily available, it may be prudent for patients with AKC to dispose of unclean contact lenses [21].

12.4 Conclusion and Outlook

Following topical cidofovir's failure in clinical development, the need for an antiviral to treat AKC persists.

It must be the aim of future work to investigate the therapeutic properties of an effective yet non-toxic topical antiviral agent for the treatment of all non-herpetic viral infections of the ocular surface. The acute phase of AKC calls for an antiviral monotherapy whereas the chronic phase with corneal opacities may require supplementation with an immunosuppressant such as topical cyclosporin A.

Because of the wide spectrum of duration and intensity of local symptoms of the natural course of the acute phase of AKC, a possible topical treatment will ultimately have to be investigated in an adequately designed prospective controlled clinical trial once the necessary in vitro studies and investigations in the already established rabbit ocular model have been completed.

Recently, $NMSO_3$, a sulfated sialyl lipid, has demonstrated a greater antiviral potency against adenovirus in vitro than cidofovir exhibiting minimal cytotoxicity [22]. The application of $NMSO_3$ 10 % also effectively inhibited viral replication in the established Ad5/NZW rabbit ocular model although to a lesser degree than cidofovir [34]. Further dosage and toxicity studies of $NMSO_3$ are required before this agent can be tested in humans.

12.5 Current Clinical Practice and Recommendations

Until treatment with an adequate antiviral agent has been established the following therapeutic strategies can be recommended:

- The acute phase of AKC may be treated with copious topical lubrication alone to alleviate distressful local symptoms.
- Topical cyclosporin A may be applied in patients with functionally relevant corneal opacities that fail to spontaneously disappear after months. This treatment must be tapered very slowly over several weeks to avoid recurrences.
- Prophylaxis of infection of exposed, yet unaffected individuals with topical interferon can be recommended.
- Topical steroids should be avoided in both the acute and the chronic phase of the disease.
- Adequate infection control measures should be followed as prevention and to reduce epidemic AKC outbreaks.

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In Vivo Micromorphology of the Cornea: Confocal Microscopy Principles and Clinical Applications

13

Rudolf F. Guthoff, Joachim Stave

Core Messages

- In vivo confocal microscopy permits cell density quantification for all corneal subpopulations
- Tear film dynamics can be studied using non-contact confocal biomicroscopy, and the generation of dry spots can be evaluated
- Corneal epithelial thickness can be measured with high precision
- Dendritic cells can be displayed and quantified
- The influence of contact lenses on epithelial cell densities can be evaluated
- Inflammatory cells associated with bacterial and viral infections of the cornea can be displayed and quantified
- Follow-up after refractive corneal surgery permits exact measurement of flap thickness and wound healing reactions in the interface
- Functional imaging at the cellular level using sodium fluorescein may be helpful for understanding metabolic activities in corneal epithelium
- Corneal nerves can be displayed three-dimensionally and perhaps quantified on the basis of nerve fiber density

13.1 Introduction

With good reason, biomicroscopy in ophthalmology is dominated by slit-lamp examinations. The foundations laid by Gullstrand were outlined in exemplary fashion in Vogt's *Lehrbuch und Atlas der Spaltlampenmikroskopie des lebenden Auges* [*Textbook and Atlas of Slit-Lamp Microscopy of the Living Eye*], first published in 1921 [19]. The second (1930) edition of that standard textbook placed particular emphasis on information yielded by focal illumination of scatters, described as *Hornhautkörperchen* (corpusculi corneae) [78]. However, the maximum magnification achievable with this technique – approx. ×40 – limited further subdifferentiation and did not reveal clinicomorphologic correlations at the cellular level.

Specular microscopy was also first described by Vogt [78], but did not gain popularity until photographic and video techniques allowed documentation and quantification of corneal endothelial cells [5, 7, 8, 31, 41]. Although the specular microscope is useful for the in vivo examination of the cornea, its applications are restricted to the endothelial cell layer.

In 1968, the same year that Maurice described the first high-powered specular microscope [41], the first scanning confocal microscope was proposed [54]. This device was characterized by high *z*-axis resolution and provided high-resolution microscopic images of cells within living tissues of patients without the need for fixation or staining.

Alongside this tandem scanning confocal microscope, a slit-scanning confocal design has also been produced [35, 58, 64, 73]. Currently, the tandem scanning design is manufactured by the Advanced Scanning Corporation (New Orleans, LA) and the most recently developed version of the slit-scanning design is produced by Nidek Technologies Srl (Vigonza, Italy). The authors have been accumulating experience with the slit-scanning design since 1994 [58, 64] and have attempted to modify the system in order to achieve reproducible and quantifiable results [4].

13.2 Principle of In Vivo Confocal Microscopy Based on the Laser-Scanning Technique

The development of in vivo confocal laser-scanning microscopy in the late 1980s permitted precise three-dimensional (3-D) visualization of microstructures of the ocular fundus in particular, with its optic nerve head and the peripapillary retina. Modern digital image processing technology enables quantitative data to be collected non-invasively, rapidly and with a low level of illumination. The precision of laser-scanning ophthalmoscopy in this context is based on the principle of the confocality of the examined object with the light source and the detector plane. A laser light source is focused through a pinhole diaphragm to one point on the object. The reflected laser light is separated by a beam splitter from the incident laser beam path and deflected through a second confocal diaphragm to reach a photosensitive detector. Because of the confocal design, light originating from outside of the focal plane is highly suppressed, and only the object layer located at the focal plane contributes to the image. In order to build up a two-dimensional image perpendicular to the optical axis of the device, the laser beam has to scan the sample point by point. This is achieved by introducing two oscillating mirrors into the beam path. Figure 13.1 provides a schematic illustration of this principle. By moving the focal plane optically, an image can be acquired from a deeper layer of the examined object, thus enabling a data cube to be built up in a successive series.

By contrast, in slit-lamp biomicroscopy examination of the cornea, an optical section that is essentially perpendicular to the corneal

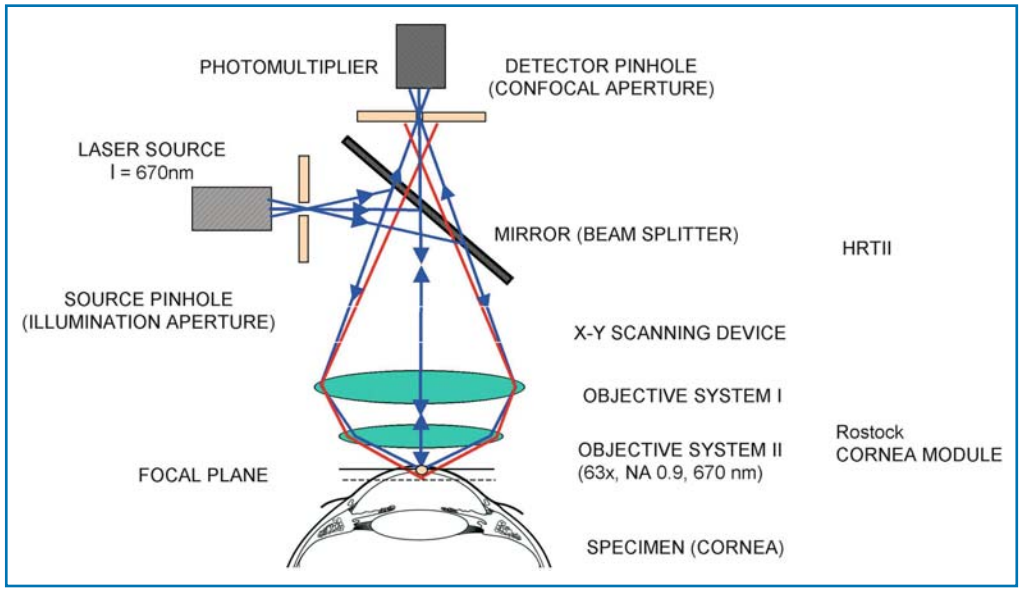

Fig. 13.1. Principle of confocal laser-scanning microscopy

surface is seen in up to ×50 magnification or, with an additional lens for endothelial viewing (specular microscopy), in up to ×200 magnification. Nowadays, documentation is generally performed using digital photography. All other cellular structures, e.g., the epithelium, cannot be imaged with this technique because of the high proportion of scattered light.

Optical tomography perpendicular to the incident light path has only become possible with the adaptation of confocal microscopy, as described above, for the examination of the living eye [6, 9, 10, 28, 43, 73, 74]. This yields images of the endothelium, for example, that are comparable to those obtained with specular microscopy. In this case the most pronounced source of scattered light is the cytosol of the endothelial cells, with the result that the cell borders appear dark. Only light reflected from the focal plane contributes to the image. In this way, cell structures in the stroma, nerves and corneal epithelium [81] can also be imaged in fine optical sections.

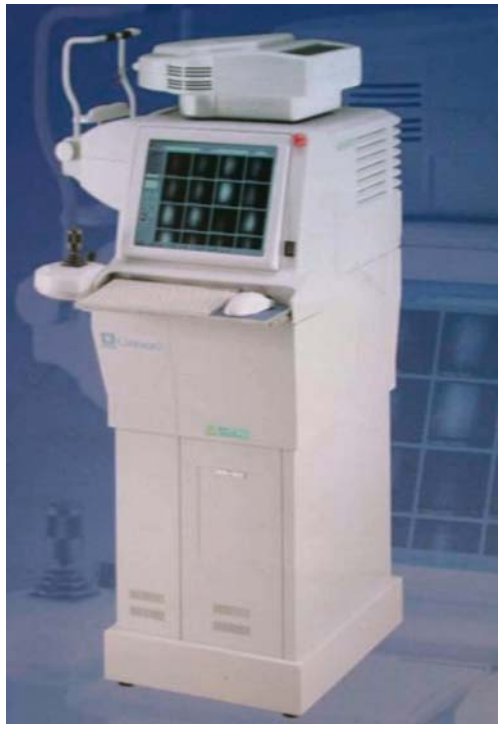

Fig. 13.2. Confocal slit-scanning microscope (ConfoScan 3/NIDEK)

13.2.1 Slit-Scanning Techniques

Techniques based on the principles of the rotating NIPKOW disk or tandem slit-scanning were used initially for confocal microscopy of the anterior segment of the eye. Figure 13.2 shows a slit-scanning microscope of this type incorporating the use of a halogen lamp. For the purposes of corneal assessment these microscopes can be used to image confocal sections with an optical layer thickness of approx. 5–10 µm. Synchronization of slit-scanning [35, 73, 80] with the video rate of a residual light camera yields sharp and motion-independent image sequences at 25 frames/s [34, 36] (Fig. 13.3).

Three-dimensional image distortion due to eye movements with this non-contact microscopy technique can only be minimized by faster image acquisition. However, more rapid movement of the confocal plane along the optical axis (z-scan) is accompanied by a loss of resolution. Loose optical coupling to the cornea using a gel (Fig. 13.4) limits the precision of depth information relating to the optical section in the cornea, and hence the 3-D reconstruction of cell structures [44, 53, 64]. Systematic errors such as inhomogeneous image illumination and image distortion also arise as a result of the electromechanical slit-scanning technique used. Linear movement along the z-axis during individual image acquisition also produces distortion along the z-axis. These fundamental sources of error can only be prevented by a rapid laser scanning system unhampered by mass inertia and incorporating a serial dot raster technique with stepwise advance of the confocal plane during the z-scan.

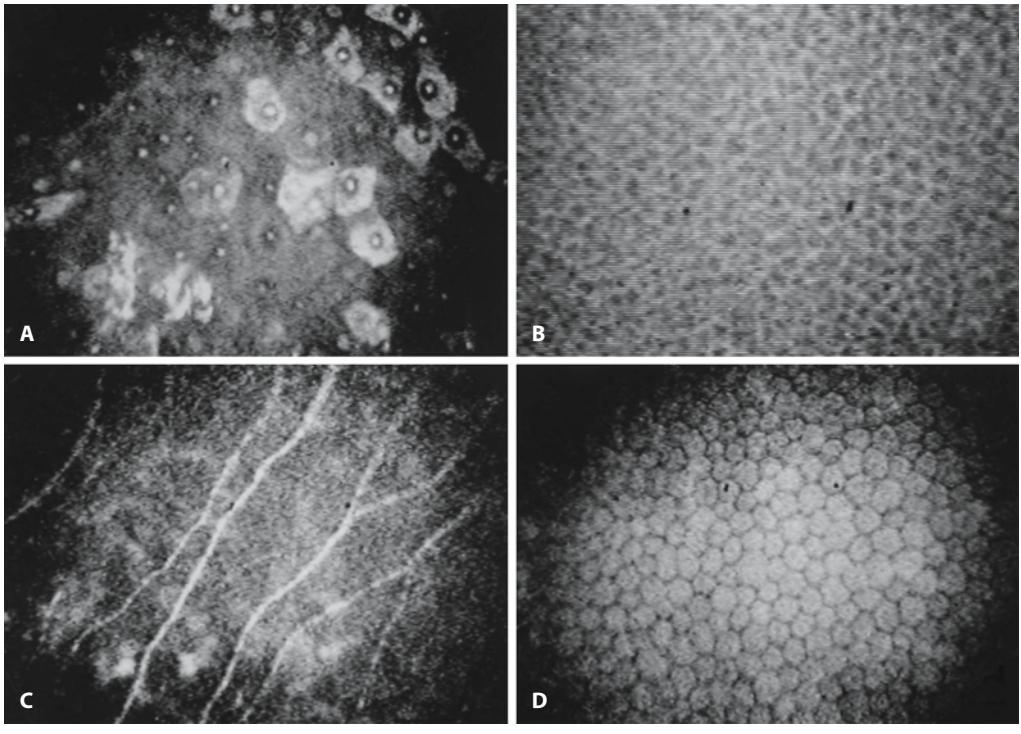

Fig. 13.3 A–D. Slit-scanning microscopy: **A** superficial and **B** basal cells; **C** nerve plexus with keratocytes and **D** endothelium

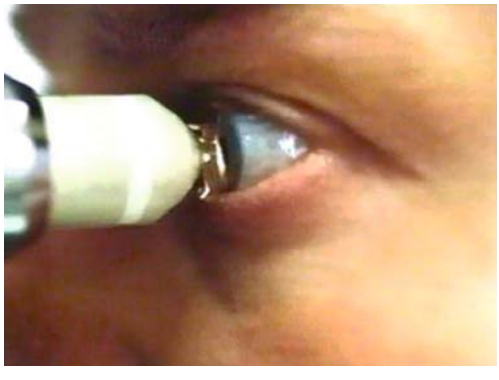

Fig. 13.4. Gel coupling between lens and eye (ConfoScan/NIDEK)

13.2.2 Laser-Scanning Microscopy and Pachymetry

As an alternative to confocal slit-scanning microscopes, a confocal laser-scanning microscope for the anterior segment of the eye was developed at the Rostock Eye Clinic on the basis of an already commercially available laser-scanning system. Not least because of its compact construction, the *Heidelberg Retina Tomograph II* (HRT II, Heidelberg Engineering GmbH, Germany) was selected as the basic device for a digital confocal corneal laser-scanning microscope.

In laser-scanning ophthalmoscopy of the posterior segment, the optically refractive media of the eye forms part of the optical imaging system. For anterior segment applications, a high-quality microscope lens is positioned between the eye and the device, providing a laser

Fig. 13.5. Rostock Cornea Module (RCM) (confocal laser-scanning microscope)

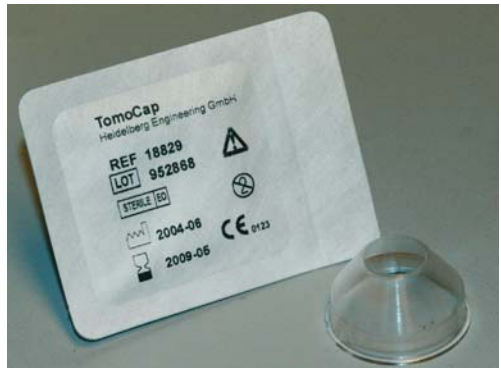

Fig. 13.6. TOMOCAP contact cap (Rostock Cornea Module)

focus less than 1 µm in diameter. The result is a high-resolution, high-speed, digital confocal laser-scanning microscope permitting in vivo investigation of the cornea (Fig. 13.5). Movement of the confocal image plane inside the cornea can be achieved manually at the microscope lens or by using the automatic internal *z*-scan function of the HRT II. Laser-scanning tomography is consequently possible in the anterior segment of the eye.

This technique permits rapid and reliable visualization and evaluation of all the microstructures in the cornea, including the epithelium, nerves, and keratocytes, as well as the endothelium and bulbar conjunctiva. For the first time, the dendritic (or Langerhans') cells can now also be visualized in vivo with an image quality that permits quantification [70, 83]. In principle, any body surface that can be reached by the lens system is a suitable candidate for examination, with the result that potential applications also exist outside ophthalmology (e.g., the skin, tongue surface and oral mucosae).

The original functions of the basic HRT II device for evaluating the optic nerve head in glaucoma are fully retained when the system is modified into the confocal laser microscope. Software adapted to the special requirements of scanning microscopy of the cornea has also been developed. This permits the acquisition of individual section images, image sequences of section images, and volume images with internal *z*-scan over a distance of approx. 80 µm. The digital properties of the device offer good patient and image data administration, also delivering rapid access to, and hence comparison with, data from previous examinations.

The Heidelberg Retina Tomograph HRT II has been modified with a lens system attachment known as the Rostock Cornea Module (RCM; J. Stave, utility model no. 296 19 361.5, licensed to Heidelberg Engineering GmbH). The module is combined with a manual *z*-axis drive to move the focal plane inside the cornea. This enables a cell layer at any depth to be imaged and, for example, selected as the starting plane for the automated internal *z*-scan. During the examination, pressure-free and centered contact with the cornea can be monitored visually using a color camera.

The distance from the cornea to the microscope is kept stable using a single-use contact element in sterile packaging (TomoCap). Optical coupling is achieved via the tear film or by applying protective gel to the eye (Fig. 13.6). The TomoCap is a thin cap with a planar contact surface made from PMMA and is coupled optically to the lens with the aid of a gel.

In the 3-D imaging mode, the distance between two subsequent image planes is approximately 2 µm in the cornea. A 3-D image consists of 40 image planes, thus covering a depth range of 80 µm. The acquisition time for a volume image is 6 s, and each individual section image is recorded in 0.024 s. In the image sequence acquisition mode up to 100 images can be stored with variable frame rates (1–30 frames/s). It thus becomes possible to document dynamic pro-

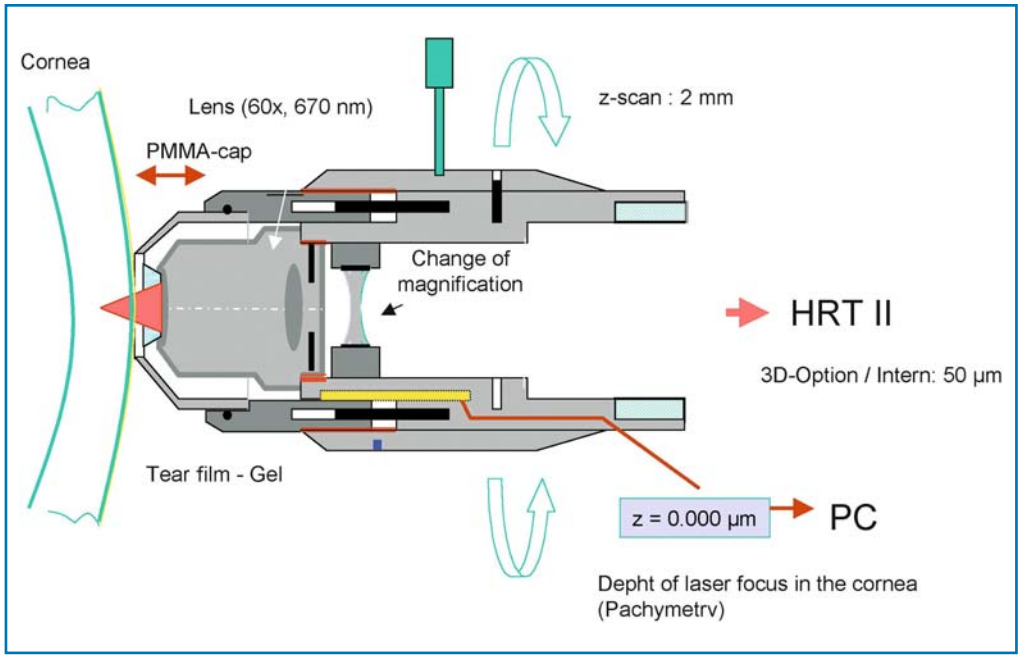

Fig. 13.7. Rostock Cornea Module (RCM)

cesses in the tissue (e.g., blood flow in the sclera). When the Rostock Cornea Module is used to set a plane manually at a desired depth, e.g., at the LASIK interface after laser surgery to correct refraction, image series from this depth can be acquired with almost 100 % image yield and precise depth allocation [32, 45, 46, 77].

With the HRT II, *z*-axis movement between images in the internal *z*-scan is performed – for the first time – in a stepwise manner, i.e., during acquisition of one section image the *z*-setting remains constant. This is a major advance and a prerequisite for generating distortion-free images of structure from one plane during the *z*-scan. A crucial prerequisite for undistorted 3-D reconstructions has therefore been achieved [26].

A short focal length water immersion microscope lens with a high numerical aperture was used to achieve high magnification (Achroplan 63× W/NA 0.95/AA 2.00 mm, 670 nm, Carl Zeiss; alternatively, LUMPLFL 60× W/NA 0.90/AA 2.00 mm, Olympus).

To optimize image quality, the Zeiss microscope lens was customized with special anti-reflection coating appropriate for the laser wavelength.

With the aid of an additional lens positioned between the HRT II and the microscope lens, the field of view of the scanning system (fixed at 15° with the HRT II) is reduced to approx. 7.5° to allow for the necessary magnification (Fig. 13.7). Depending on the microscope lens and additional lens used, the size of the field of view in the contact technique can be 250 µm × 250 µm, 400 µm × 400 µm, or 500 µm × 500 µm. Dry microscope lenses can be used in non-contact, for example to image the tear film [66].

In particular, the compact construction of the HRT II simplifies its use as a confocal in vivo microscope because the view is virtually unobscured when monitoring the patient and bringing the microscope up to the cornea.

The precise perpendicular positioning of the cornea in front of the microscope within the micrometer range is facilitated by color camera control. By observing the laser reflex on the cornea, even when bringing the microscope to the eye, it is possible to make a lateral or vertical correction so that contact with the cornea is

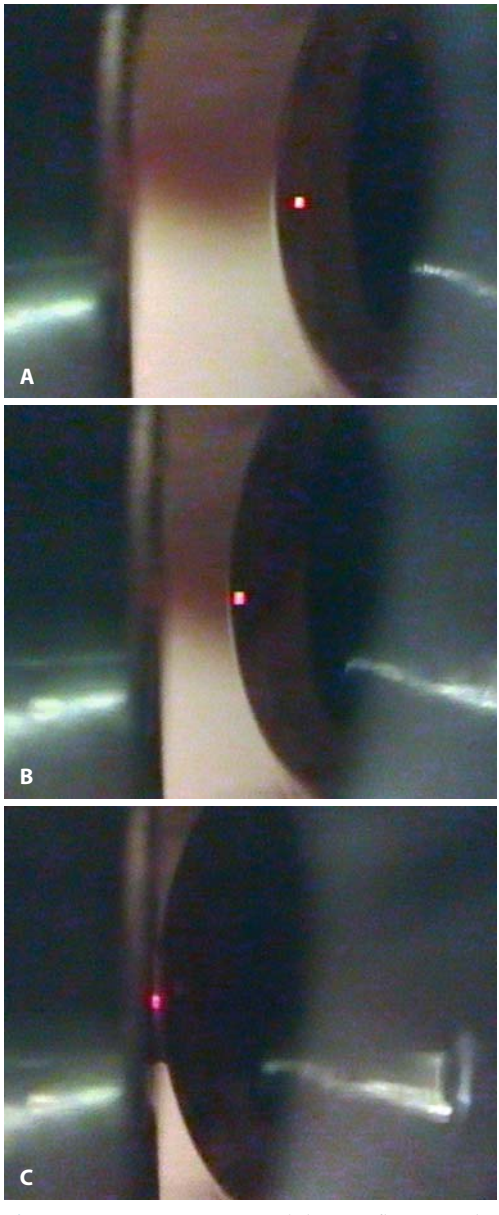

Fig. 13.8 A–C. Camera control: laser reflexes on the cornea – producing the immersion gel bridge between the objective lens/cap and the cornea

exactly in the optical axis (Fig. 13.8). As a result, images are captured only of cell structures that are in a plane parallel to the surface, i.e., transverse sectional images are acquired. The contact technique guarantees a fixed distance between microscope and cornea. The precise movement of the focal plane through the cornea with simultaneous digital recording of depth position relative to the superficial cells of the epithelium (at the corneal surface) thus makes exact pachymetry possible.

13.2.3 Fundamentals of Image Formation in In Vivo Confocal Microscopy

The laser light emitted constitutes an electromagnetic wave with a defined wavelength lambda (λ). This light wave is modified on passage through the cornea. Part of it migrates unchanged through all layers (transmission). At interfaces with changing refraction indices (n_D), the wave alters direction due to scatter and refraction. Light scattering is the basis of image formation in confocal microscopy where only backscattered light is used for the image. The amount of backscattered light is very small because scatter occurs predominantly in a forward direction [14]. Light-scattering interfaces are found in the cornea, for example, at the junction between cytoplasm or extracellular fluid with n_D=1.35–1.38 and lipid-rich membranes with n_D=1.47 in the form of cell borders, cell nucleus membranes and mitochondrial membranes [63] (Fig. 13.9). The amount of backscattered light depends on the structure of the interface surfaces. Rough surfaces scatter light in a broadly diffuse pattern, whereas directed waves with narrow scatter cones are formed on smooth structures. In addition, the confocal image is influenced both by the number as well as the size and orientation of scattering organelles or particles. Objects whose diameters are of the same order of magnitude as the wavelength of the laser light display Mie scatter, and very much smaller molecules display Rayleigh scatter, which is directed backwards to a greater extent than Mie scatter. Different orientations of elongated cell organelles can place different particle cross-sections in the path of the incident light beam, the result being that the light is backscattered to varying degrees. A high proportion of cell organelles also increases the amount of backscattered light [14].

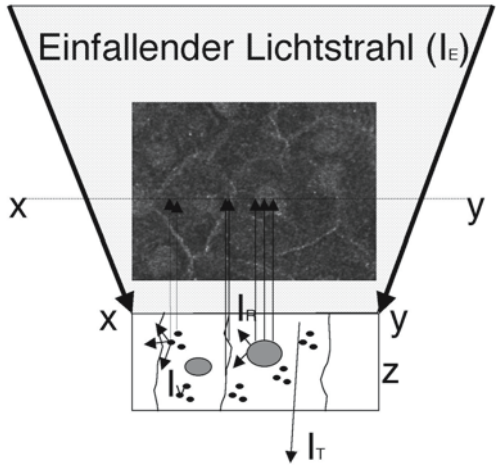

Fig. 13.9. The confocal image of one section (x, y) is produced by the sum of the backscattered light intensities (I_R) from the focal depth range (z) (I_V forward scatter, I_T transmission)

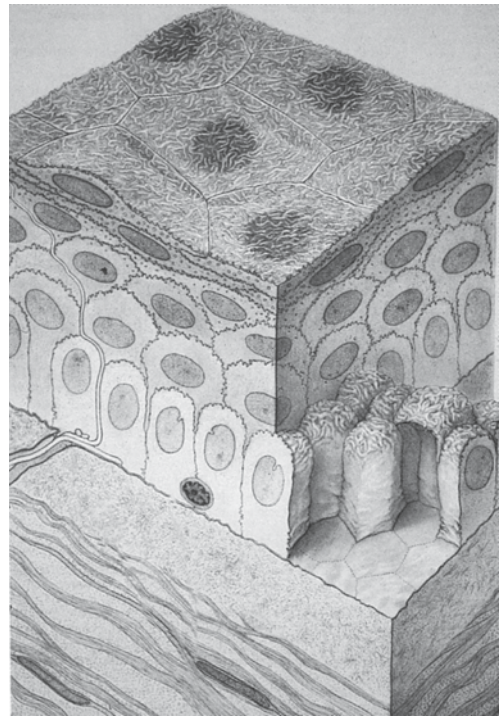

Fig. 13.10. Schematic illustration of the corneal epithelium and upper corneal stroma

13.3 General Anatomical Considerations

The *corneal epithelium* consists of five to six layers of nucleated cells that can be subdivided functionally and morphologically into three zones:

- *Superficial cells:* approx. 50 µm frontal diameter and approx. 5 µm thick. About 1/7 of these cells is lost by desquamation within 24 h. Before detachment the cytoplasm and nucleus undergo a change in their optical characteristics.
- *Intermediate cells:* 50 µm diameter and 10 µm thick. These cells form a contiguous polygonal, wing-shaped pattern (wing cells).
- Columnar *basal cells* have a flat basal surface, adjacent to Bowman's membrane, a frontal height of approx. 20 µm and a frontal diameter of 8–10 µm. Like endothelial cells, they can be quantified accurately because of their defined location in relation to the basement membrane (Fig. 13.10).

Bowman's membrane – which is clearly distinct histologically from the epithelial basement membrane – is 10–16 µm thick and remains amorphous on light microscopy. Its location on in vivo confocal microscopy is well defined by the subepithelial plexus (SEP).

The *stroma* accounts for some 90 % of total corneal volume. Ninety-five percent of the stroma consists of amorphous ground substance (glycoproteins, glycosaminoglycans: keratan sulfate and chondroitin sulfate) and collagen fibers. The remaining 5 % of stromal volume is accounted for by cellular structures known as keratocytes, which are specialized fibroblasts. Besides the nerves, their irregularly shaped nuclei are the only well-defined sources of scattered light in corneal stroma detected on confocal microscopy. Their widely branching cytoplasmic extensions are not visible in the healthy cornea (Fig. 13.11).

The cornea is the most densely innervated tissue in the human body. It is supplied by the terminal branches of the ophthalmic nerve in the form of 30–60 non-myelinated *ciliary nerves* (Fig. 13.12).

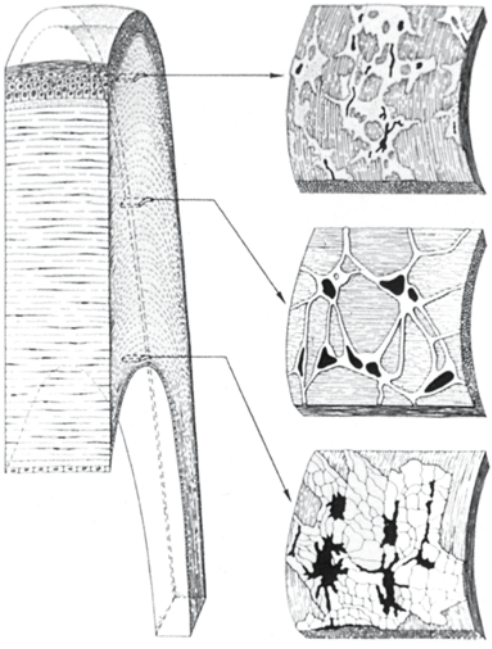

Fig. 13.11. Schematic illustration of the layered structure of the human cornea. The differently shaped keratocyte nuclei can be distinguished on in vivo confocal microscopy (adapted from Krstiè)

In the limbus region these are seen as whitish, filigree-like structures; their complex stromal and epithelial branchings are not visible by slit-lamp microscopy, but are relatively clear on confocal microscopy.

Like Bowman's membrane, *Descemet's membrane,* which should be regarded as the basement membrane of the endothelium, remains amorphous on light microscopy. It is 6–10 µm thick. On confocal microscopy it is defined optically by the easily identifiable endothelial cells.

The *endothelium* consists of about 500,000 hexagonal cells approx. 20 µm in diameter, 5 µm thick and with large, flattened, central nuclei. The high concentration of cell organelles is indicative of very intensive metabolic activity.

13.4 In Vivo Confocal Laser-Scanning Microscopy

The layered structure of the epithelium of the eye can be visualized with high contrast using the *Rostock Cornea Module* (RCM) attachment with the HRT II and, because of the good quality of depth resolution, can be imaged in optical sections a few micrometers thick. The same is true for the subepithelial plexus, the entire stroma including the keratocytes, and the endothelial fine structure (Fig. 13.13).

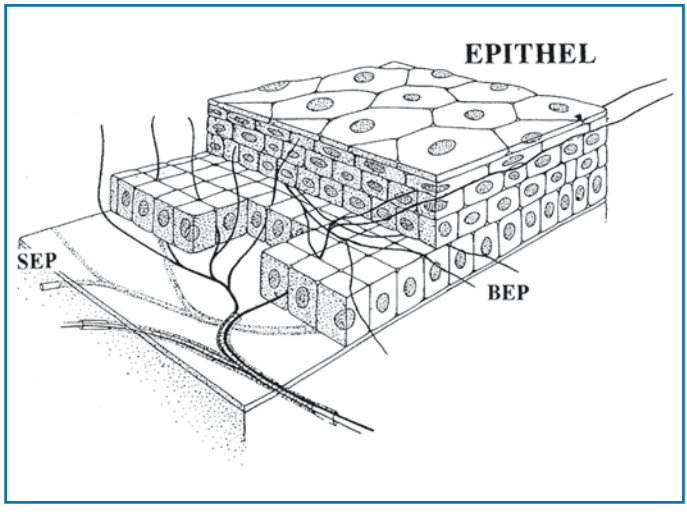

Fig. 13.12. Schematic illustration of the subepithelial plexus (*SEP*) and its branches in the corneal epithelial layers (*BEP* basal epithelial plexus)

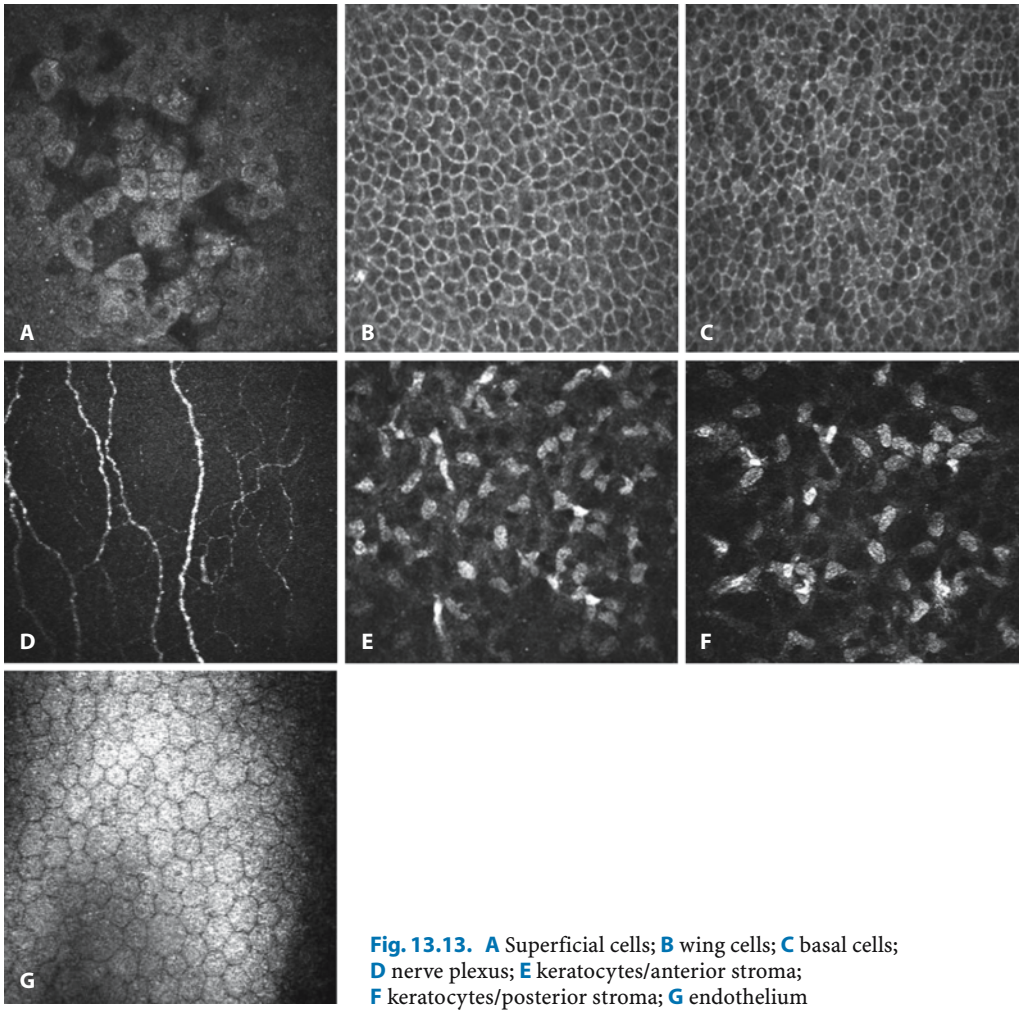

Fig. 13.13. **A** Superficial cells; **B** wing cells; **C** basal cells; **D** nerve plexus; **E** keratocytes/anterior stroma; **F** keratocytes/posterior stroma; **G** endothelium

13.4.1 Confocal Laser-Scanning Imaging of Normal Structures

13.4.1.1 Tear Film

The pre-ocular tear film with its complex fluid structure bathes the cornea and conjunctiva. Tear film structure and function are maintained by a highly differentiated system of secretory, distributive and excretory interactions [49, 60]. In particular, these function to smooth the corneal surface and maintain its optical clarity. The water content of the cornea is regulated by evaporation and the resultant osmotic gradient. The oxygen in the air is dissolved in the tear fluid and thus supports the aerobic metabolism of the epithelium.

The tear film is 7–10 µm thick and is characterized by a three-layered structure. The external lipid layer, which is produced chiefly by the meibomian glands close to the margin of the eyelids, prevents rapid evaporation of the aqueous layer and renders the surface hydrophobic. The inner mucin layer consists of glycoproteins. Its task is to make the epithelial surface hydrophobic and thus to guarantee wettability.

Replacing the contact system in the confocal laser-scanning microscope with a dry objective lens (Fig. 13.14) enables the fine structure of the tear film to be imaged (Fig. 13.15). The rapid imaging sequence in the device also permits dynamic processes to be recorded [39, 65, 75].

Fig. 13.14. Non-contact microscopy: laser reflex on the cornea

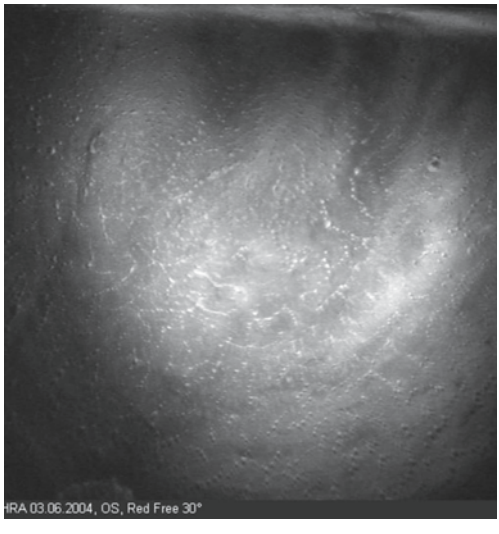

Fig. 13.15. Normal tear film

13.4.1.2 Epithelial Layer

13.4.1.2.1 Superficial Cells (up to approx. 50 µm in Diameter)

In the case of the most superficial epithelial cells, bright cell borders and a dark cell nucleus and cytoplasm are readily visualized on confocal laser-scanning microscopy. The cells characteristically display a polygonal – often hexagonal – shape. Cells undergoing desquamation are characterized by a highly reflective cytoplasm, in the center of which the brightly appearing (pyknic) cell nucleus with its dark perinuclear space is clearly visible (Fig. 13.16). The average density of superficial cells in the central and peripheral cornea is approx. 850 cells/mm².

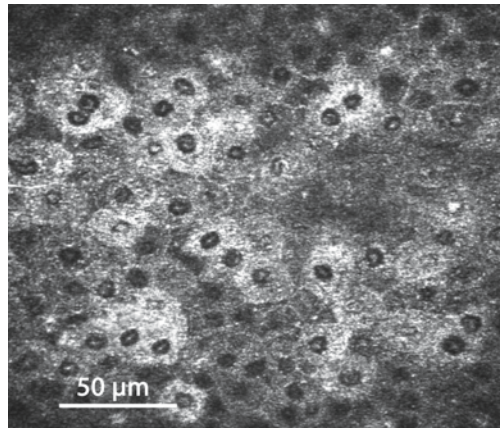

Fig. 13.16. Superficial cells: the cytoplasm and cell nuclei are visualized; cells in the process of desquamation possess a highly reflective cytoplasm, in the center of which the bright (pyknic) cell nucleus with its dark perinuclear space is clearly visible (z = 50 µm)

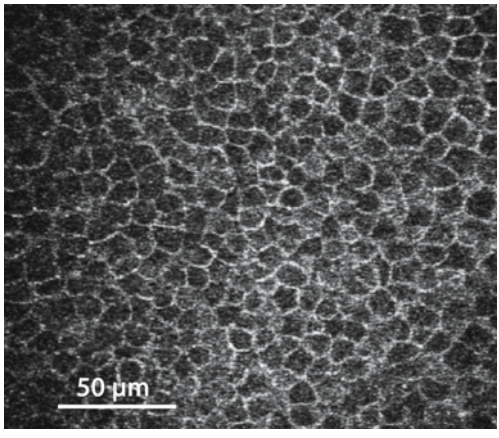

Fig. 13.17. Intermediate cells: the cells of the intermediate layers are characterized by bright cell borders and a dark cytoplasm. The cell nucleus can be identified only with difficulty. The wing cells display only minimal variation in terms of size and appearance

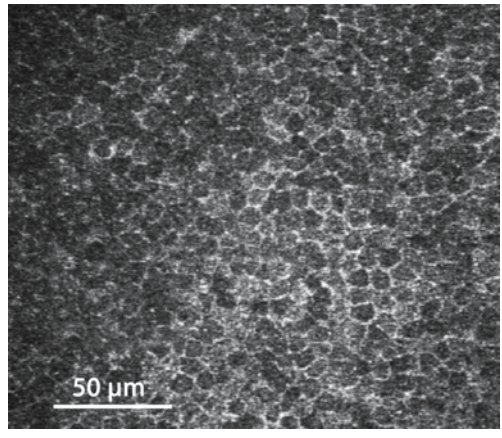

Fig. 13.18. Basal cells: these are regularly arranged cells with bright borders, but the cell nucleus is not visualized. Intercellular comparison reveals inhomogeneous cytoplasmic reflectivity

13.4.1.2.2 Intermediate Cells/Wing Cells (up to Approx. 20 µm in Diameter)

The cells of the intermediate layers are characterized by bright cell borders and a dark cytoplasm. The cell nucleus can be distinguished only with difficulty. In terms of size and appearance, wing cells in healthy subjects exhibit only minimal variation (Fig. 13.17). The average cell density is approx. 5,000 cells/mm² in the central cornea and approx. 5,500 cells/mm² in the periphery.

13.4.1.2.3 Basal Cells (up to Approx. 10 µm in Diameter)

The basal cells are located immediately above Bowman's membrane. They present as brightly bordered cells in which the cell nucleus is not visible. Between-cell comparison reveals inhomogeneous reflectivity of the cytoplasm. Like the wing cells above them, the basal cells display only minimal variation in shape and size (Fig. 13.18). The average cell density is approx. 9,000 cells/mm² in the center of the cornea and 10,000 cells/mm² in the periphery. In normal subjects, therefore, in terms of cell densities, the ratio between superficial cells, intermediate cells and basal cells is 1:5:10.

13.4.1.3 Langerhans' Cells

Confocal microscopy permits in vivo evaluation of Langerhans' cells (LCs) within the human cornea, with a particular emphasis on cell morphology and cell distribution.

LCs present as bright corpuscular particles with dendritic cell (DC) morphology and a diameter of up to 15 µm. LC distribution follows a gradient from low numbers in the center to higher cell densities in the periphery of the cornea. Moreover, in vivo confocal microscopy permits differentiation of LC bodies lacking dendrites, LCs with small dendritic processes forming a local network, and LCs forming a wire net via long interdigitating dendrites (Fig. 13.19). While almost all the cells located in the periphery of the cornea demonstrate long processes interdigitating with the corneal epithelium, those in the center of the cornea often lack dendrites, most probably underlining their immature phenotype [21]. Immature LCs are equipped to capture antigens, while mature forms are able to sensitize naive T-cells through MHC molecules and secretion of interleukin-12 as well as costim-

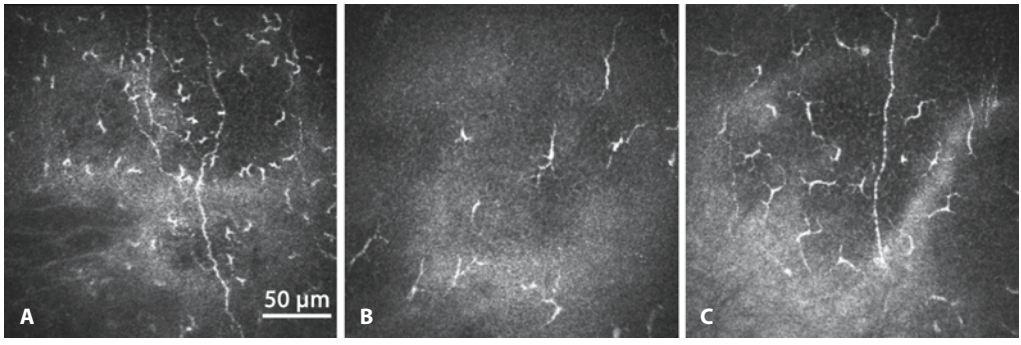

Fig. 13.19. In vivo confocal microscopic images, representing different forms of Langerhans' cells: **A** individual cell bodies without processes; **B** cells bearing dendrites; **C** cells arranged in a network via long interdigitating dendrites

ulatory molecules, and thus represent an integral part of the immune system [3].

The average density of LCs in normal subjects is 34±3 cells/mm² (range: 0–64 cells/mm²) in the central cornea and 98±8 cells/mm² (range: 0–208 cells/mm²) in the periphery [83]. In contact lens wearers, LC density varies from 60±16 cells/mm² (range: 0–600 cells/mm²) in the central cornea to 159±18 cells/mm² (range: 0–700 cells/mm²) in the periphery. LC densities differ significantly between healthy volunteers and contact lens wearers both in the central (p=0.03) and in the peripheral cornea (p=0.001), while the gradient of LC density from periphery to center was almost identical in both groups (unpublished data).

It has been suggested that LCs participate in immune and inflammatory responses, thereby determining cell-mediated immunity. In light of this theory, the present data on LCs in the human cornea provide a helpful basis for further investigations in ocular pathology.

13.4.1.4 Corneal Nerves

The cornea is one of the most sensitive structures in the human body, and even the most minimal contact provokes the lid reflex to protect the eye. This sensitivity is attributable to the large numbers of nerve fibers that pass through the cornea. Furthermore, the corneal nerves exert an influence on the regulation of epithelial integrity and on wound healing.

In vivo visualization of these nerve structures is possible by confocal corneal microscopy.

The cornea is innervated primarily by sensory fibers arising from the ophthalmic nerve, a side branch of the trigeminal nerve. Human corneal nerves are non-myelinated and vary in thickness between 0.2 and 10 µm.

The nerve fiber bundles, which enter the anterior and central stroma in the corneal periphery, run parallel to the corneal surface in a radial pattern before making an abrupt 90° turn in the direction of Bowman's membrane [47]. On confocal corneal microscopy these nerve fibers mostly present as thick, almost always stretched, highly reflective structures (Fig. 13.20). Frequently, the stromal nerves are found in close proximity to keratocytes. The deep stroma is devoid of nerves that can be visualized on confocal microscopy.

In the anterior stroma, immediately before Bowman's membrane, the nerve fiber bundles display three different patterns. Some of the nerve fibers ramify before reaching Bowman's membrane without penetrating it and form the subepithelial plexus [51] (see schematic illustration in Fig. 13.12). Other nerves penetrate Bowman's membrane either directly following a perpendicular or slightly oblique course, or just before penetration they ramify into several fine branchlets. After they have penetrated Bowman's membrane, they again make a 90° directional change and pass between the basal cell layer of the epithelium and Bowman's mem-

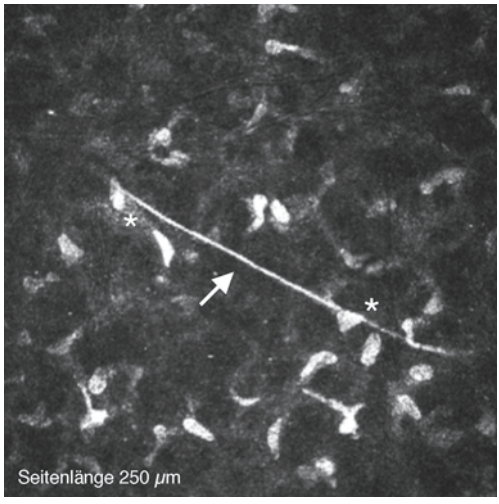

Fig. 13.20. Nerve fibers (*arrowed*) in the anterior corneal stroma. The stretched pattern of the stromal nerves is characteristic. The keratocyte nuclei are identifiable as hyperreflective oval structures, some of which are in close proximity to the nerve (*star*)

brane toward the corneal center and form the basal epithelial plexus (see schematic illustration in Fig. 13.12). In so doing, they give off many small side branchlets directed both toward the corneal surface, where they end freely, and toward the center [47, 48]. The nerve fibers of the basal epithelial plexus mostly run parallel to each other and often form Y- or T-shaped branches. Their predominantly granular, "string of pearl" structure is characteristic; more rarely they display a smooth surface. Unlike the stromal nerves, they are characterized by lesser reflectivity and frequently follow a meandering path (Fig. 13.21). Occasionally, a thicker nerve fiber bundle will divide into two finer nerve fibers, before these then reunite after a short distance into a single nerve fiber with the same thickness as before (Fig. 13.21 A). The finer branchlets also form connections between larger nerve fibers (Fig. 13.21 A, B).

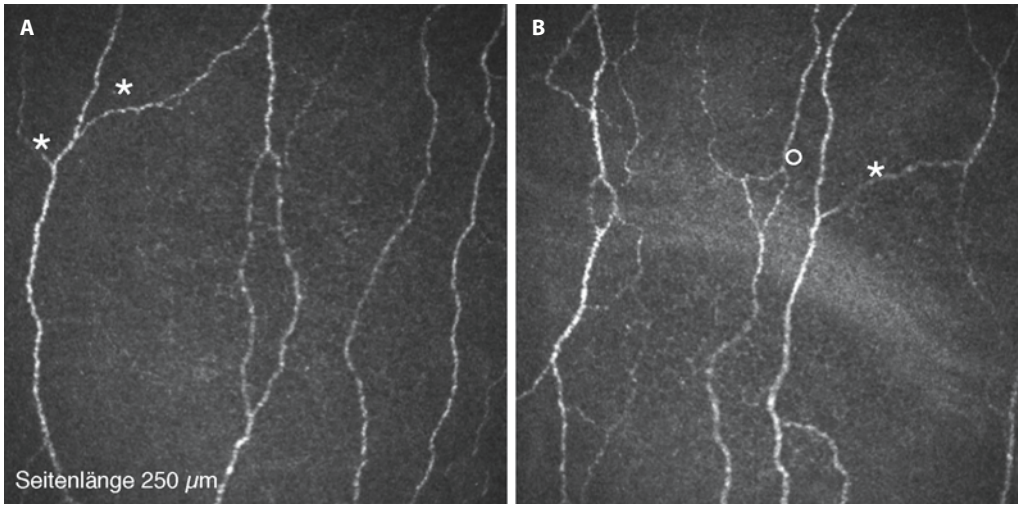

Fig. 13.21 A, B. Highly reflective nerves from the basal epithelial plexus located between Bowman's membrane and the basal cell layer of the corneal epithelium. The nerve fibers have their characteristic granular "string of pearl" appearance. In most cases they run parallel to each other and show T (*circle*)- and Y (*star*)-shaped nerve branchings that may produce connections between larger nerve fibers

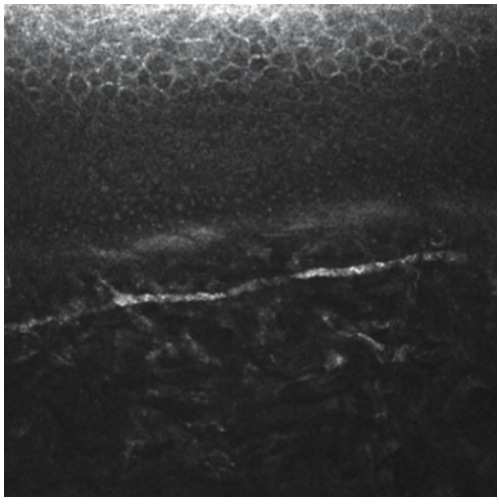

Fig. 13.22. Subepithelial nerve

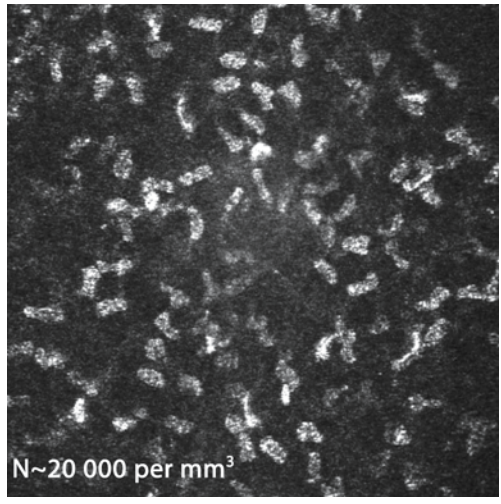

Fig. 13.23. Anterior stroma: in corneal stroma only the keratocyte nuclei are visualized; the density of the cell nuclei is highest of all in the anterior stroma (see Fig. 13.24); the size of the cell nuclei shown is approx. 15 µm

13.4.1.5 Bowman's Membrane

The anterior limiting membrane has an amorphous appearance. Its location can be established from the nerves of the basal epithelial plexus, which ramify there (Fig. 13.22).

13.4.1.6 Stroma

Apart from neural structures, only the highly reflective, sharply demarcated cell nuclei of the keratocytes are visualized on examination of the stroma. The cytoplasm of this fibroblast subpopulation and the collagen fibers produced by them are not visible. Keratocyte nucleus density is higher in the anterior stroma close to Bowman's membrane than in the central and deep stroma (Figs. 13.23, 13.24). Keratocyte density is highest in the anterior stroma, clearly declines toward the central stroma, and increases again slightly in the region immediately before Descemet's membrane.

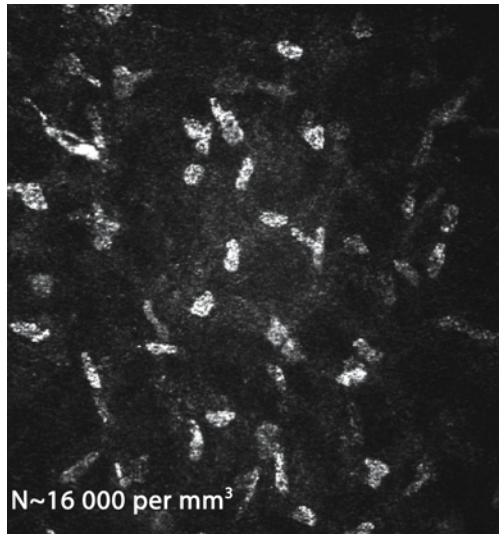

Fig. 13.24. Central stroma: clearly demarcated, highly reflective, oval-shaped nuclei of keratocytes in the central stroma; here cell nucleus density is the lowest in corneal stroma

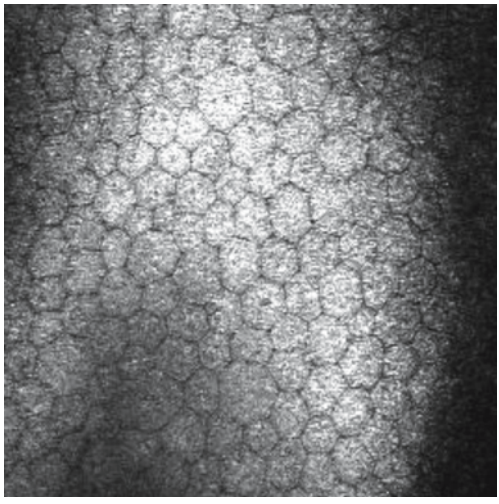

Fig. 13.25. Endothelium: a monolayer of regularly arranged hexagonal cells completely covering the posterior surface of the cornea. Unlike the basal cells (see Fig. 13.18), these cells have a brightly reflecting cytoplasm and dark cell borders. The cell nucleus is not visible

13.4.1.7 Descemet's Membrane

Like Bowman's membrane, Descemet's membrane has an amorphous appearance and is therefore not visualized in healthy subjects.

13.4.1.8 Endothelial Cells

The endothelium consists of a regular pattern of hexagonal reflective cells. The cell nuclei cannot usually be visualized. The cell borders reflect less light than the cytoplasm, with the result that a network of dark cell borders appears between areas of bright cytoplasm. Endothelial cell density can be determined by counting (Fig. 13.25).

13.4.1.9 Limbal Region

Because it forms a junctional zone with the conjunctiva, the limbal region is especially important. Inflammatory cells migrate across it into the cornea in immunological disease, it is the source of new inbudding corneal vessels and, not least, it also plays an important role in corneal regeneration as the site of origin of corneal stem cells.

The limbal region is where the corneal epithelium forms a junction with the conjunctival epithelium which comprises approx. 10–12 cell layers. This region also contains a radial arrangement of trabecular conjunctival processes (the limbal palisades of Vogt) that are considered to be the site of origin of corneal stem cells [12, 61]. Overall, the organization of the conjunctival epithelium is less uniform because different epithelial cell types (e.g., goblet cells) occur here and the arrangement of the individual cell layers is also not so strictly parallel with the surface [13].

On confocal microscopy, the epithelial cells of the conjunctiva, unlike those of the cornea, are more reflective, smaller and less well demarcated. Their nucleus is relatively large and bright. The junctional zone is characterized by inhomogeneous reflectivity and marked variation in cell shape and size (Fig. 13.26). The limbal palisades of Vogt can often be visualized as parallel trabecular extensions of the conjunctival epithelium (Fig. 13.27). In the immediate junctional zone the conjunctival epithelium also commonly exhibits tongue-like extensions which are mostly well demarcated, especially in the deeper layers, and at the end of which are located isolated cells or cell groups with very bright cell borders and a bright cytoplasm (Fig. 13.28). These may be secretory cells. Subepithelially, in the region of the conjunctiva close to the limbus, are the blood vessels of the limbal vascular plexus, in the lumen of which flowing blood cells can be seen (Fig. 13.29).

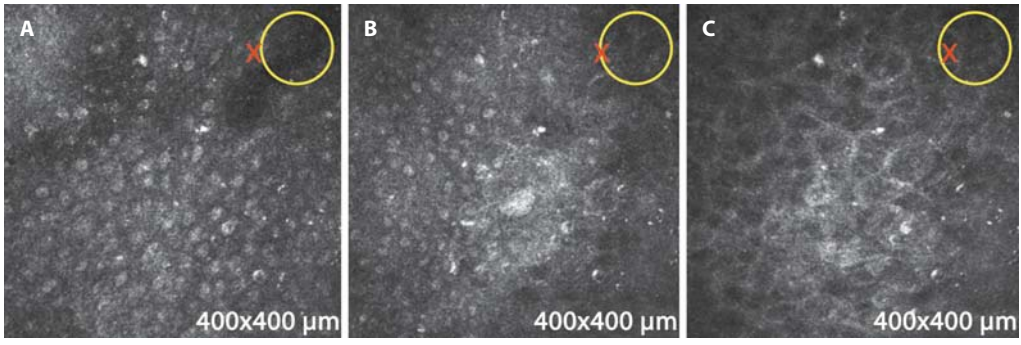

Fig. 13.26. Superficial epithelium of: **A** conjunctiva (cells up to approx. 30 µm in diameter, bright large cell nuclei; **B** transitional zone (variable morphology; **C** cornea (cells up to approx. 50 µm in diameter, bright cell borders) (x = Position on the cornea)

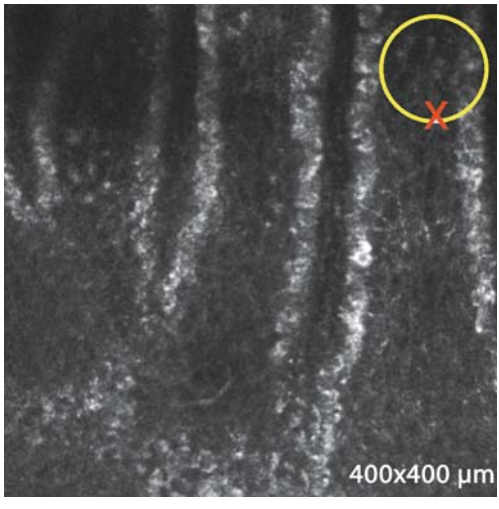

Fig. 13.27. Limbal palisades of Vogt. Trabecular extensions of the conjunctiva growing from outside (in this case from below) in a radial pattern toward the cornea

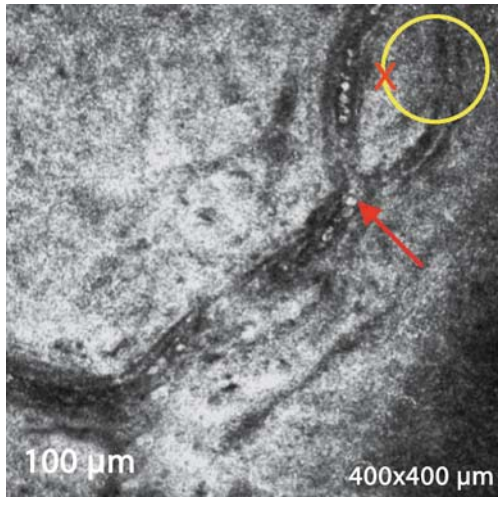

Fig. 13.29. Branched conjunctival vessel close to the limbus with erythrocytes visible (z = 100 µm, depth of laser focus in the cornea)

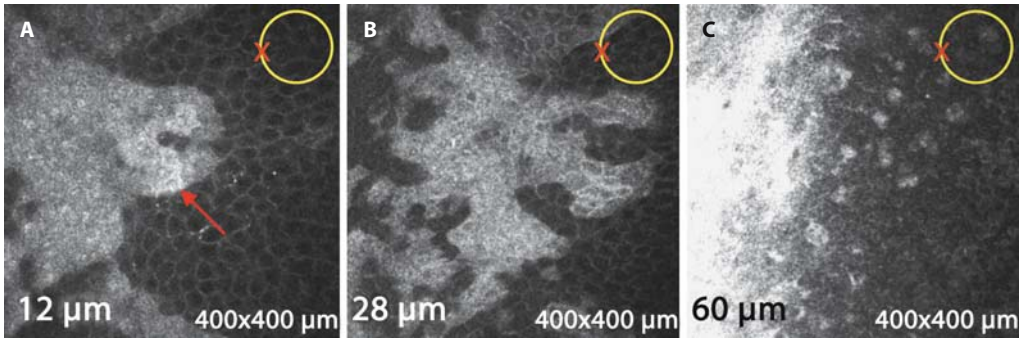

Fig. 13.28 A–C. Extensions of conjunctival epithelium at different depths. In the center of the image in **A** a cell group with strikingly bright cell borders is shown (*arrowed*). The basal conjunctival epithelium in **C** is much brighter with the result that structures are no longer identifiable

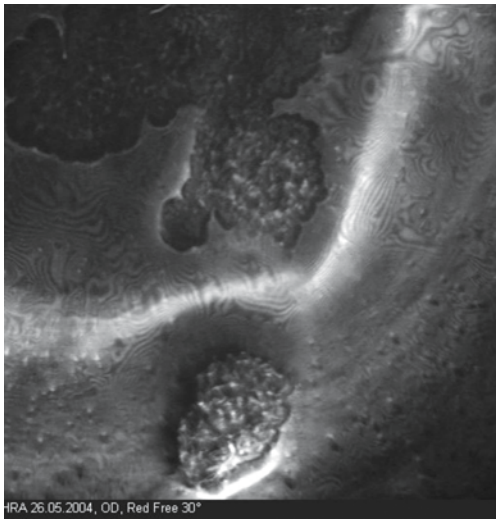

Fig. 13.30. Tear film/dry spots

13.5 Clinical Findings

13.5.1 Dry Eye

Disturbances of tear film secretion or tear film structure give rise to a condition known as *dry eye*. On microscopy such disturbances are evident as altered reflection or dry spots on the epithelium (Fig. 13.30).

As the most important component of the corneal diffusion barrier, the corneal epithelium displays differing permeability for aqueous ionic substances such as sodium fluorescein (NaF). Patients with diabetes, for example, have significantly increased permeability for NaF. NaF also penetrates areas of micro-erosions and pathologically altered cells. The literature reveals discrepant views concerning the nature of the penetration process. Most authors subscribe to the view that the fluorescein fills the "footprint" spaces vacated by cells that have been lost. However, others assume that fluorescein fills the intercellular space. At present only confocal slit-scanning microscopes and fluorophotometers are used to analyze this phenomenon.

The *Heidelberg Retina Angiograph* (HRA) in combination with the *Rostock Cornea Module* can be used for confocal *laser-scanning fluorescence microscopy* of the microstructure of the corneal epithelium and tear film using contact and non-contact techniques (Fig. 13.31) with a lateral resolution of 1 µm and up to ×1,000 magnification. The red-free reflection and fluorescence images display the intercellular microstructure with stained cell nuclei and altered cell surfaces and borders. The same area examined on the cornea can be visualized simultaneously in reflection and fluorescence mode. The penetration profile of NaF can be measured with precise depth resolution over a prolonged time using the contact technique. Autofluorescence measurements are also possible. Figure 13.32 shows the tear film (a) and epithelium (b) in fluorescence mode.

13.5.2 Meesmann's Dystrophy

Meesmann's dystrophy is a rare, bilaterally symmetrical epithelial condition inherited as an autosomal dominant trait and attributable to mutations in the keratin 3 (K3) gene [11] or keratin 12 (K12) gene [82] on chromosome 12 [27] or chromosome 17 [71]. Due to the rupture of mainly interpalpebral epithelial cysts, the condition causes episodic pain, photophobia, epiphora, blepharospasm and fluctuating visual acuity or a moderate decline in visual acuity, even in young children. No causal therapy exists.

13.5.2.1 Summary Evaluation

Clinically, Meesmann's dystrophy is characterized bilaterally by small cystic changes in the corneal epithelium, particularly in the interpalpebral zone, and individual superficial punctate opacities. Histological assessment and electron microscopy reveal exclusively intraepithelial cysts with cell debris (clumped keratin) which migrate to the corneal surface during normal epithelial regeneration and rupture there (Fig. 13.33). In addition, there are

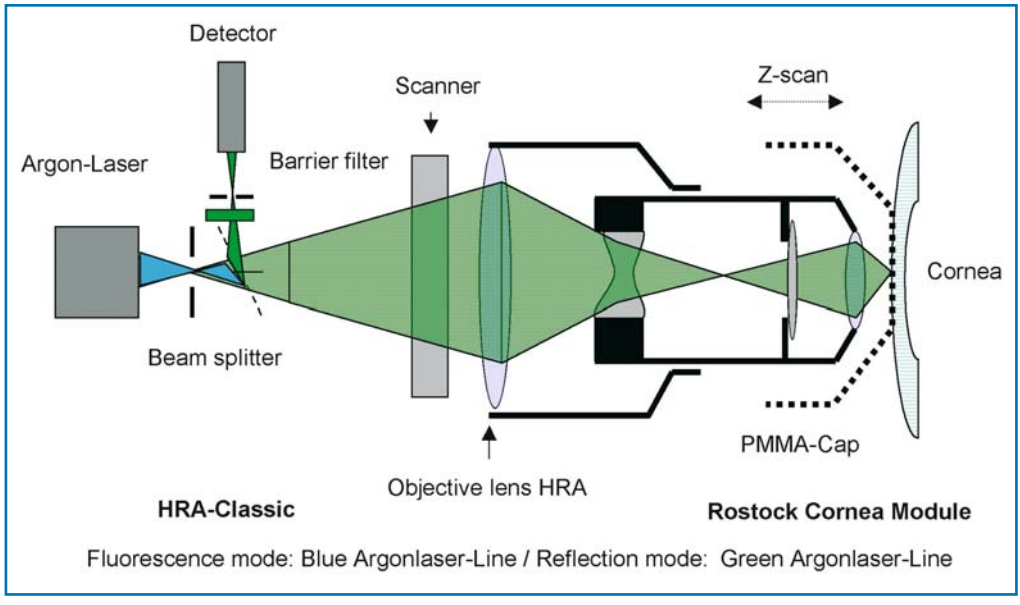

Fig. 13.31. Heidelberg Retina Angiograph HRA/RCM. Fluorescence mode: blue argon laser line; reflection mode: green argon laser line

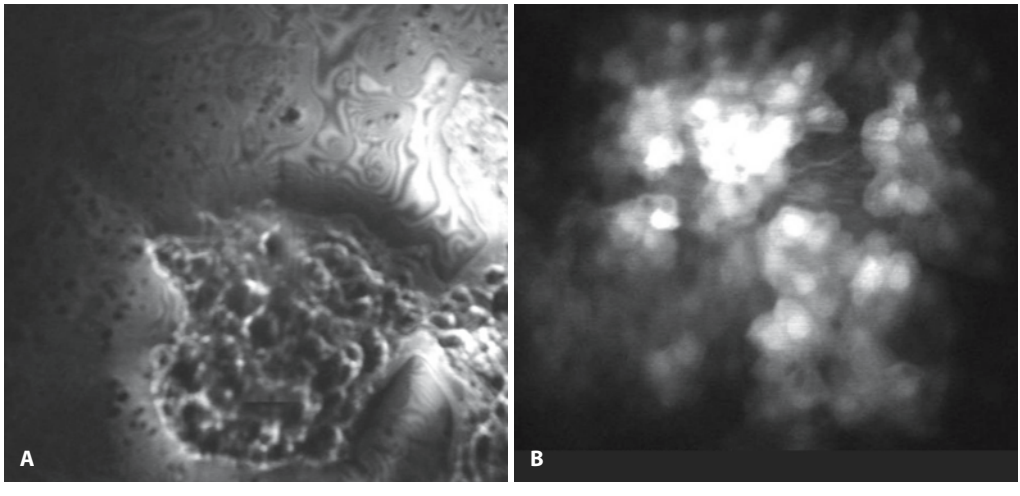

Fig. 13.32. **A** tear film (HRA Classic); reflection mode; **B** epithelium/NaF-stained (HRA Classic); fluorescence mode

irregular cell arrangements and granular deposits in the basal cells and a thickened basement membrane. The other corneal layers do not show any changes.

Cystic epithelial changes consistent with the histological findings can be visualized in vivo using the Rostock confocal laser-scanning microscope (Figs. 13.34, 13.35), and thus this non-invasive method contributes to confirming the diagnosis.

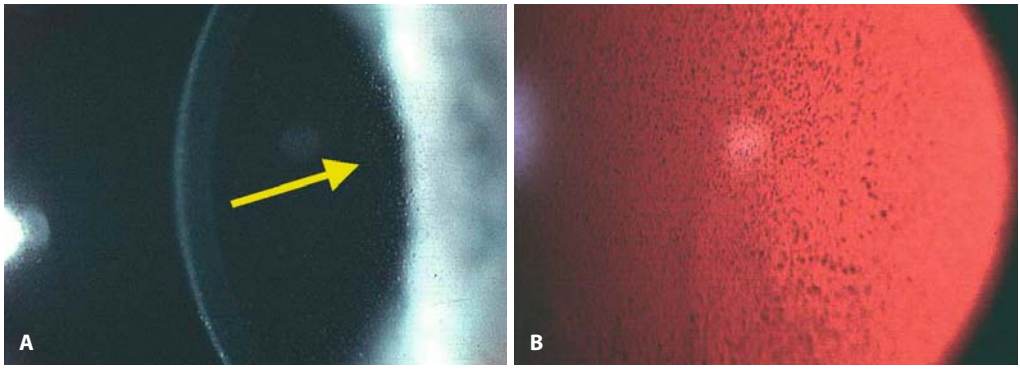

Fig. 13.33. **A** B.N., 12 years old: microcystic epithelial changes in the interpalpebral zone, isolated superficial punctate opacities, otherwise normal corneal structures. **B** Retroillumination (cf. **A**)

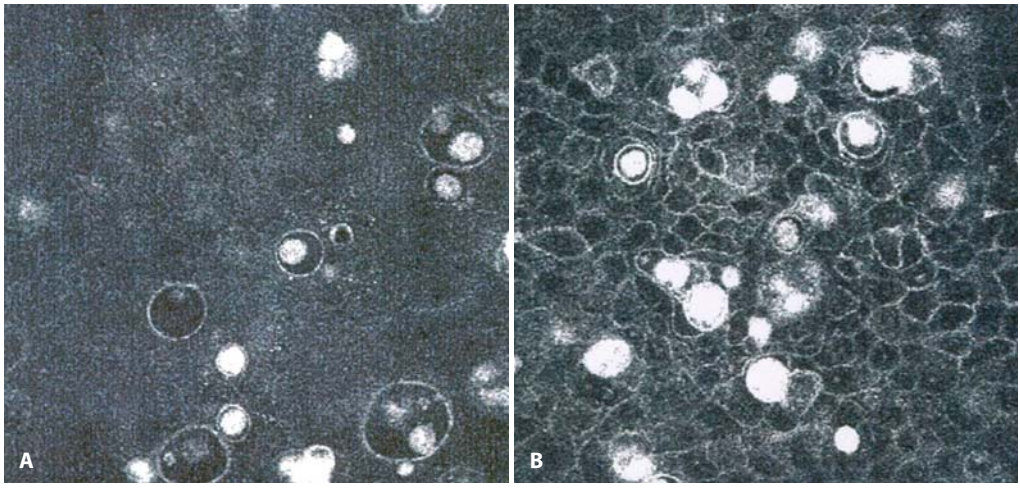

Fig. 13.34. **A** B.N., 12 years old: confocal microscopy of the epithelium in vicinity of the superficial cells (depth: 5 µm) showing cystic structures with spherical, highly reflective contents similar to the histologic sections (see Fig. 13.35). **B** B.N., 12 years old: confocal microscopy of the epithelium at a depth of 30 µm with increased visualization of spherical highly reflective and cystic structures

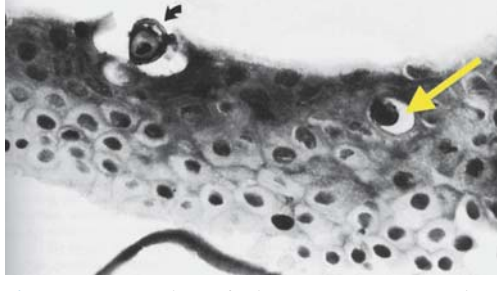

Fig. 13.35. Histologic findings in Meesmann's dystrophy (after Naumann: *Pathologie des Auges* [49]) with intraepithelial cysts containing cellular debris

13.5.3 Epithelium in Contact Lens Wearers

Distinct changes in corneal morphology, pachymetry and structure in contact lens wearers can be demonstrated by in vivo confocal laser scanning microscopy. These findings are best interpreted as resulting from mechanical or metabolic disturbances of the cornea.

All cell layers (superficial, intermediate and basal cells) are present and characterized by bright cell borders and uniformly dark cyto-

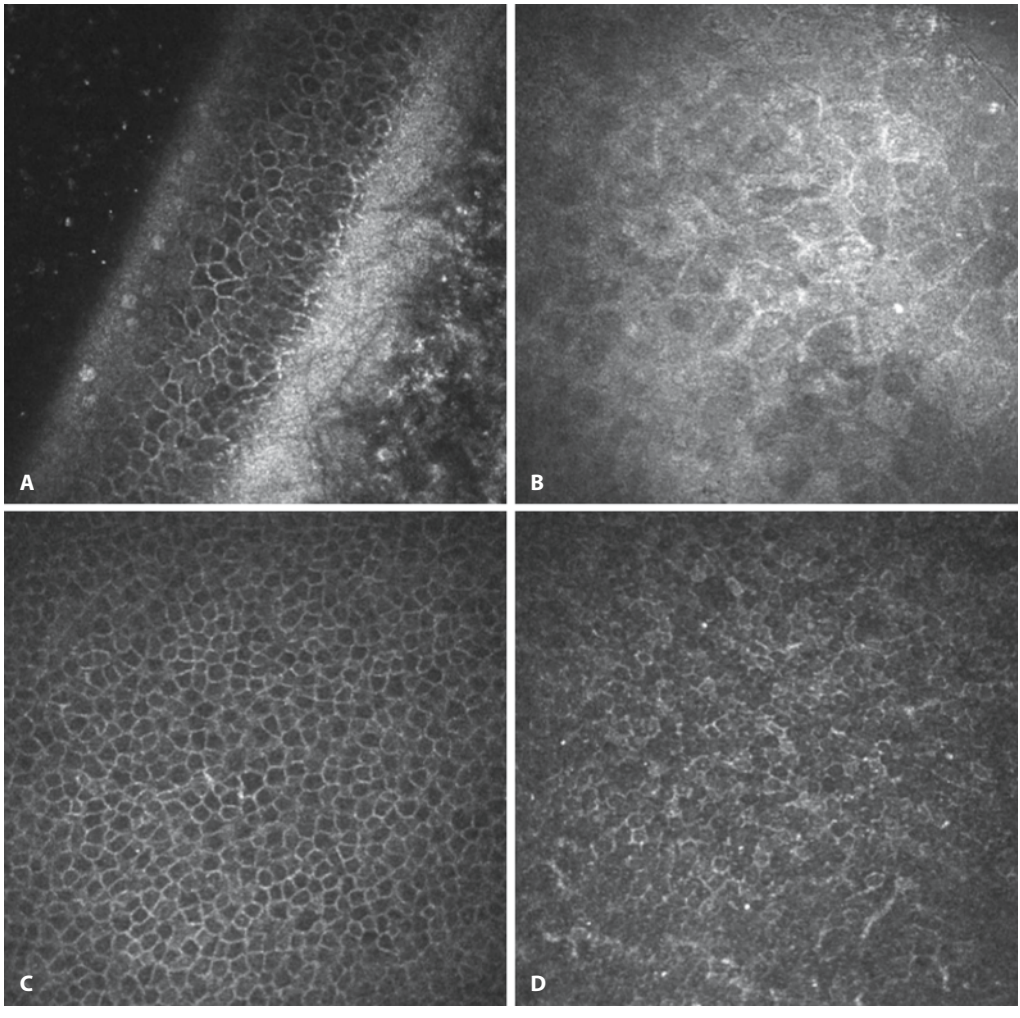

Fig. 13.36. Epithelium in contact lens wearers: **A** oblique corneal section: superficial, intermediate and basal cells, Bowman's membrane and anterior stroma; **B** superficial cells; **C** intermediate cells; **D** basal cells

plasm. The cell count increases with layer depth due to a decrease in cell diameter. Bowman's membrane and the subepithelial plexus display border structures between epithelium and stroma (Fig. 13.36 A).

Superficial cells are characterized by a dark nucleus, and the cytoplasm is generally darker than in the normal cornea. The polygonal structure is retained, but cell bodies are generally smaller (30 µm in contact lens wearers and up to 50 µm in the normal cornea) (Fig. 13.36 B).

Our data (unpublished results) show a significant increase in superficial cell density ($p<0.05$) both centrally and peripherally.

The intermediate cells do not show any morphological changes by comparison with findings in normal subjects: pale cell borders, invisible nucleus and dark cytoplasm were detected in both the lower and upper wing cells (Fig. 13.36 C). A significant reduction in the cell count was noted only in the periphery ($p<0.05$).

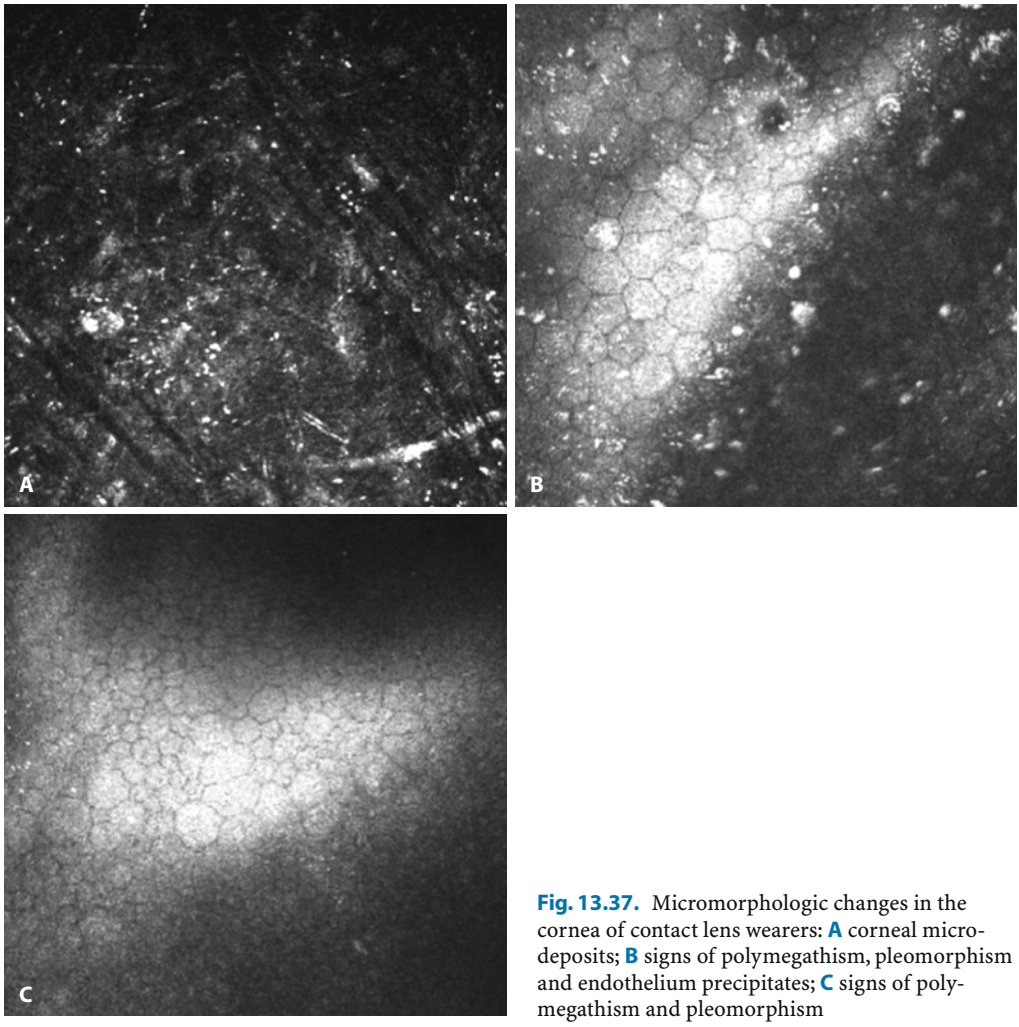

Fig. 13.37. Micromorphologic changes in the cornea of contact lens wearers: **A** corneal microdeposits; **B** signs of polymegathism, pleomorphism and endothelium precipitates; **C** signs of polymegathism and pleomorphism

Basal cell structure is characterized by an inhomogeneous cytoplasm and invisible nucleus; cell diameters are approx. 8–10 µm (Fig. 13.36 D). A significant reduction in the cell count was also detected in the peripheral cornea ($p<0.05$).

Analysis of the pachymetry data revealed reduced corneal thickness in the periphery compared to that in normal volunteers, especially in patients who had worn contact lenses for longer than 10 years. There were no age-related changes in cell count or epithelial thickness, but stromal thickness was reduced.

The type of contact lens (hard vs. soft) has no influence on corneal morphology; duration of contact lens wear was the factor with the greatest impact. Corneal microdeposits in stroma (Fig. 13.37 A) and signs of polymegathism, pleomorphism (Fig. 13.37 B, C) and endothelium precipitates (Fig. 13.37 B) are the most common findings.

As demonstrated in Fig. 13.38, alterations in Langerhans' cells also occur due to contact lens wearing.

In light of this, in investigations of the cornea in contact lens wearers, attention must focus on the cell density of each layer and on the thickness of the corneal epithelium, and results must always be compared between the center and periphery.

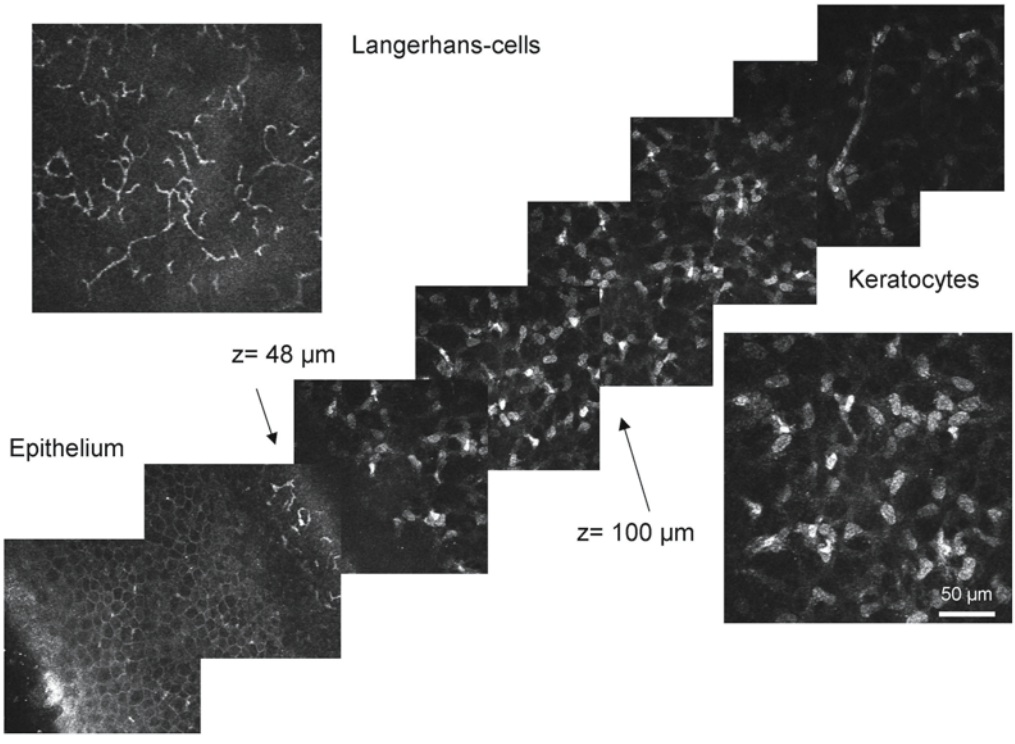

Fig. 13.38. Langerhans' cells and reflective keratocytes after contact lens wearing (3 years)

13.5.4 Epidemic Keratoconjunctivitis

Epidemic keratoconjunctivitis (EKC) is a highly contagious infection caused by type 8, 19, 37 adenoviruses; one of its chief complications is the development of nummular areas of subepithelial corneal opacity which, in exceptional cases, may lead to years of reduced visual acuity and to increased glare sensitivity [30, 69]. Histopathologic examination reveals that the nummular lesions consist of an accumulation of cells from the monocyte-macrophage system, such as lymphocytes, histiocytes and fibroblasts [18].

Viral persistence in the keratocytes is suspected as the cause for the continuing presence of the nummular lesions. The immune response induces focal infiltration of immune cells around the infected keratocytes. The complexes form the slit-lamp microscopic substrate of the nummular lesions [57] (Fig. 13.39 A, B).

Hyperreflective punctate structures can be visualized in the intermediate layer of the epithelium on confocal Rostock laser-scanning microscopy (Fig. 13.40 A). These may be lymphocytes, histiocytes and/or fibroblasts [33].

By contrast with physiologic findings, the basal cell layer is hardly distinguishable as such. In addition to a network of hyperreflective dendritic structures (Fig. 13.40 B), which becomes clearly less dense with increasing depth (Fig. 13.40 C, D), corpuscular changes with dendritic extensions are visualized (Fig. 13.40 C, D), some of which appear to be spread out between the nerve fibers (Fig. 13.40 E). Considering their location, size and shape, these are most probably the antigen-presenting Langerhans' cells [59], which are responsible for the induction of cell-mediated delayed-type immune responses. They assume an important role in triggering

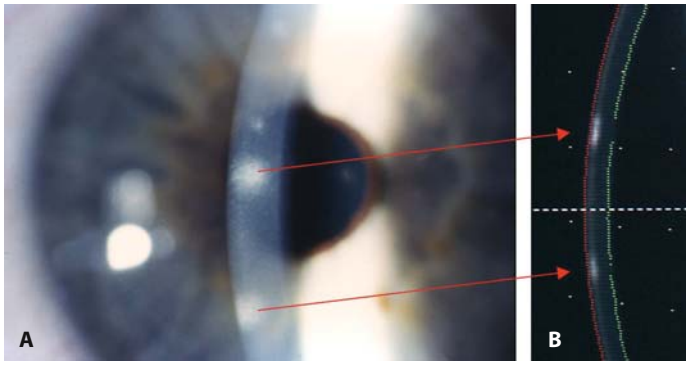

Fig. 13.39 A, B. Slit-lamp microscopy photograph: right eye of a 28-year-old female patient on day 14 after the onset of symptoms of epidemic keratoconjunctivitis, showing the subepithelial nummular lesions as fleecy-fused areas of opacity with unclear margins: **A** slit-lamp microscopy and **B** with the "Pentacam" Scheimpflug camera (Oculus)

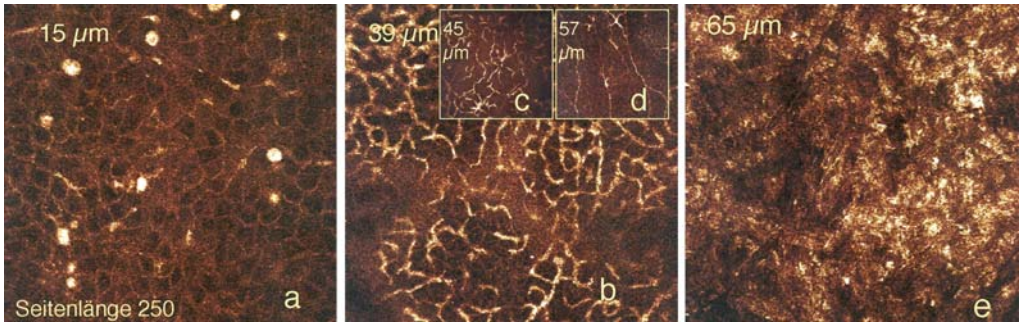

Fig. 13.40 a–e. Confocal image of the central cornea in epidemic keratoconjunctivitis; edge length of the image in vivo, 250 µm; focal planes moved axially from epithelium to endothelium. **a** Intermediate epithelial layer with isolated hyperreflective round structures located between the cells; **b** basal cell layer with hyperreflective dendritic network; **c, d** transition from basal cell layer to nerve plexus layer with dendritic cell structures, some of which appear to be spread out between the nerve fibers; **e** Bowman's membrane

contact allergies, rejection reactions and viral defense, and in the healthy cornea they are located in the epithelial layers of the conjunctiva, the limbus and peripheral cornea, but not in the central cornea. Migration of the Langerhans' cells into the central cornea may occur in response to traumatic, chemical or inflammatory stimuli [1, 18].

The changes visible beneath the nerve fiber layer are possibly scatter artifacts in the vicinity of the ruptured Bowman's membrane [33].

The superficial epithelial layer, stroma and endothelium do not display any abnormalities.

13.5.5 *Acanthamoeba* Keratitis

Numerous free-living phagotrophic amoebae cause opportunistic infection in humans. *Acanthamoeba* keratitis has been recognized as a potentially blinding disease, which is often only diagnosed at a late stage. The condition is sometimes confused with other types of infectious keratitis, particularly those of fungal and herpetic origin.

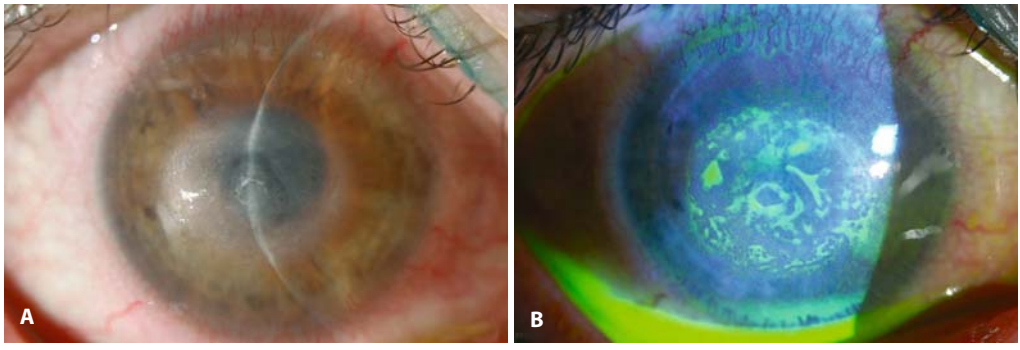

Fig. 13.41. Slit-lamp photograph from a 42-year-old female patient with a unilateral red, painful eye: **A** with epithelial defects, stromal ring infiltrate; **B** fluorescein staining positive; sensibility decreased. PCR (herpes zoster) and corneal scrapings (pathological agents including *Acanthamoeba*) negative

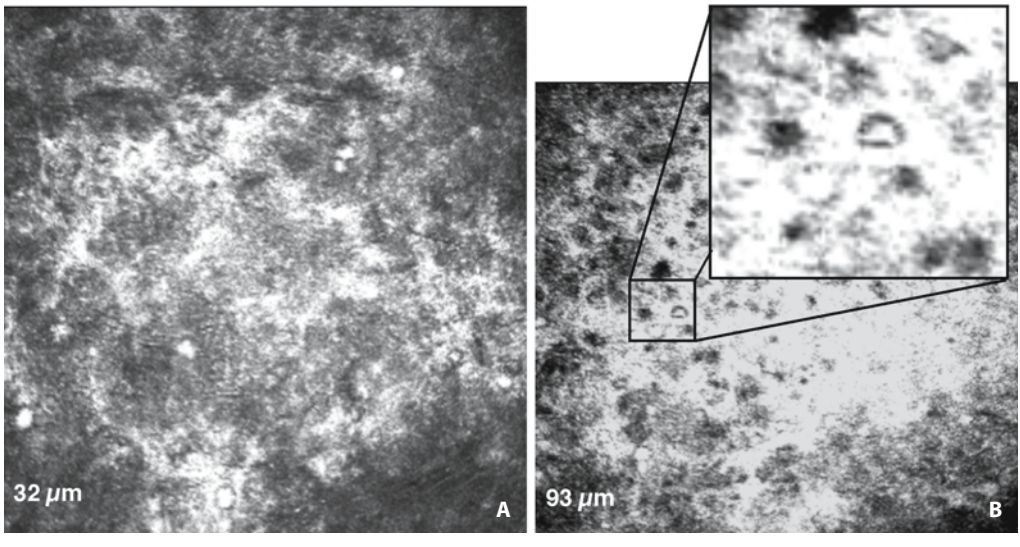

Fig. 13.42 A, B. Corneal microcysts (cystic stage of life cycle, round in shape, up to 10 µm, double wall) are visible at the level of the deep intermediate and basal cells (z = 32 µm) (**A**) and in the anterior stroma (z = 93 µm) (**B**)

Although not widely available, the confocal microscope can be helpful in establishing the diagnosis of *Acanthamoeba* keratitis, based on the visualization of pear-shaped cysts approx. 10 µm in length and irregular trophozoites [38, 55] (Figs. 13.41–13.43).

In vivo confocal microscopy permits identification of *Acanthamoeba* cysts in the cornea [40, 2]. The identity of findings with those from conventional ex vivo microscopy and PCR provides a basis for simple and reliable in vivo diagnosis (Fig. 13.44).

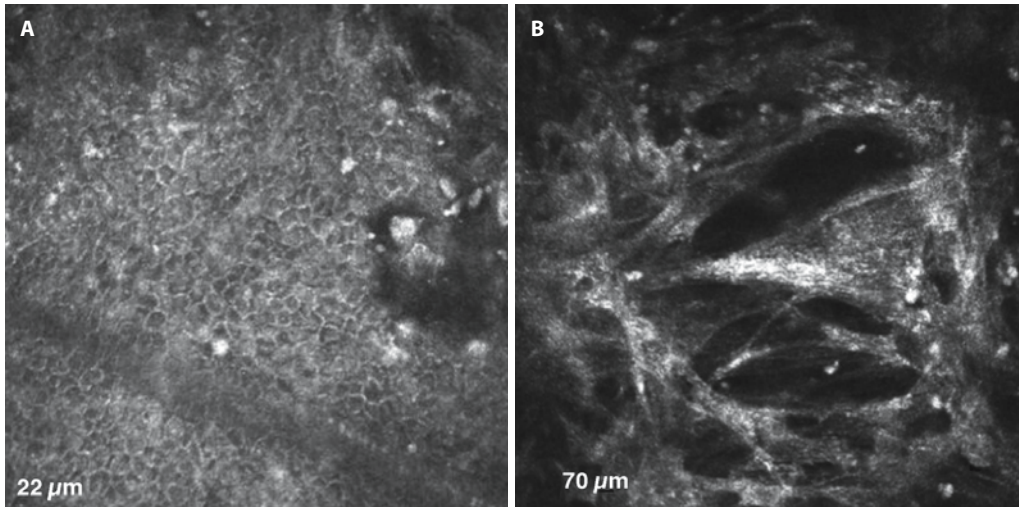

Fig. 13.43 A, B. The same areas of the cornea 3 months after specific therapy (propamidine isethionate/Brolene): no signs of cysts either in the epithelium (z = 22 µm) or in the stroma (z = 70 µm) . The stromal architecture is highly irregular (**A, B**)

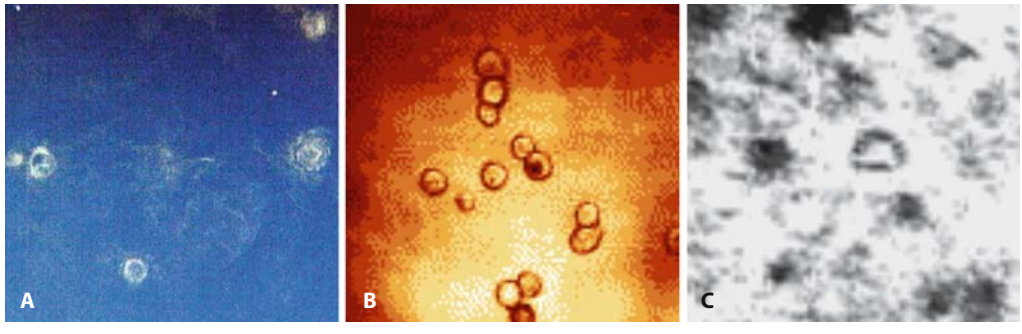

Fig. 13.44 A–C. Confocal microscopy as a non-invasive diagnostic method for in vivo identification of *Acanthamoeba* cysts in the cornea. The identity of findings with conventional ex vivo microscopy provides a basis for easy and reliable in vivo diagnosis of *Acanthamoeba* cysts. **A** Light microscopy in vitro; **B** confocal microscopy ex vivo; **C** confocal microscopy in vivo

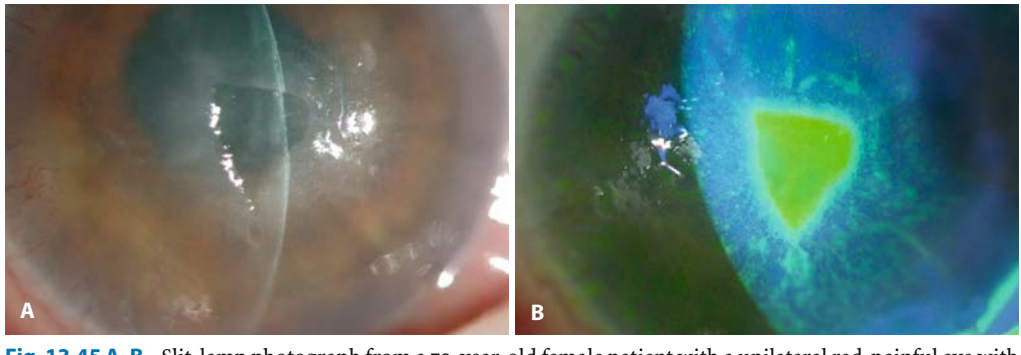

Fig. 13.45 A, B. Slit-lamp photograph from a 70-year-old female patient with a unilateral red, painful eye with epithelial and stromal defects: **A** infiltrated and blurred cornea in the ulcer area; **B** fluorescein staining positive

13.5.6 Corneal Ulcer

Little experience has been gained with confocal microscopy in unspecific corneal ulcers. Leukocyte infiltration may be demonstrated in the ulcer margins in both the epithelial and the superficial stromal region. Figure 13.45 is a slit-lamp photograph and Fig. 13.46 shows confocal microscopy. In vivo confocal microscopy of corneal ulcers provides additional information about corneal healing processes, and permits evaluation of epithelialization and reinnervation at the cellular level.

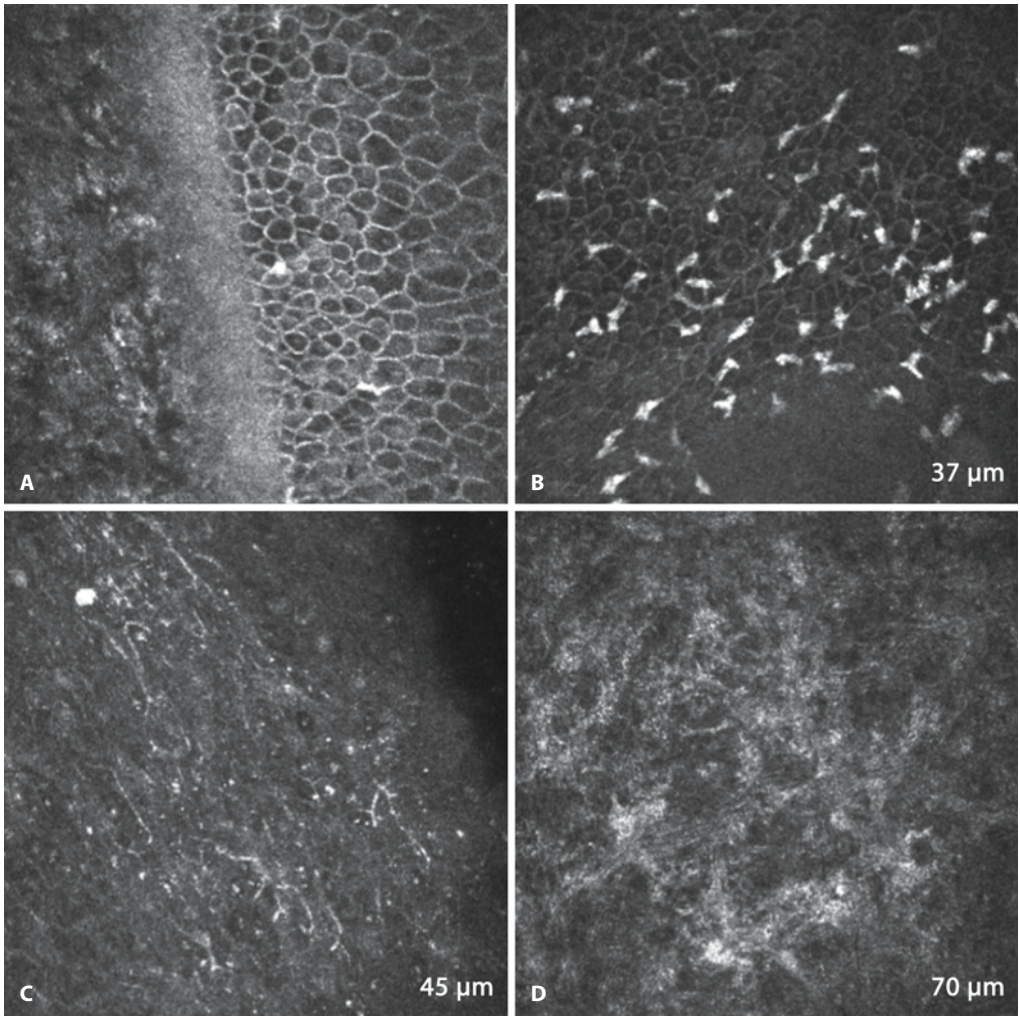

Fig. 13.46. **A** Oblique section of central cornea near the ulcer: regular epithelial structure, absence of subepithelial plexus, and distortion of anterior stroma; **B** at the level of deeper intermediate and basal cells: bright cellular structures, most probably leukocytes; **C** distortion of basal cell layer and other structures of the subepithelial plexus; **D** anterior stroma in ulcer area: keratocytes or cell nuclei are not visible, severe destruction of stromal structure

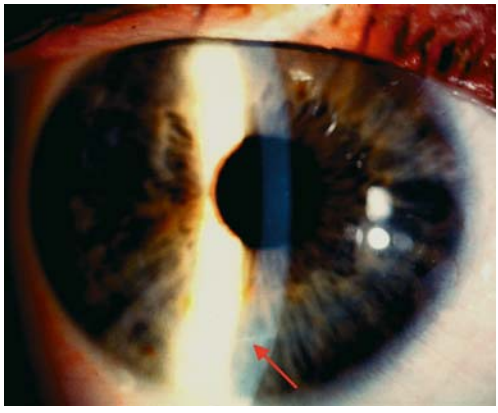

Fig. 13.47. Slit-lamp photograph from a 25-year-old male patient 1 year after laser in-situ keratomileusis (LASIK): uncorrected visual acuity 20/20, normal corneal morphology apart from a small circular stromal scar, representing the border of the former flap zone (*arrow*)

13.5.7 Refractive Corneal Surgery

The different methods of refractive corneal surgery are designed to reduce ametropia, where present. Depending on the technique used, refractive corneal surgery may result in morphologic changes and sometimes also in irritation and complications in the vicinity of the corneal epithelium or stroma that may lead to subjective disorders [56, 50]. The morphology and mechanism of wound healing processes following refractive corneal surgery are therefore of particular interest in this context [25, 15] (Figs. 13.47–13.49).

In vivo confocal microscopy of the cornea after refractive surgery yields information about the functional status of the keratocytes and the reinnervation of stroma and epithelium [37]. It is possible to define the precise depth location for corneal opacities and to measure changes in corneal thickness [45]. Even years later, the depth of the interface zone following laser in-situ keratomileusis (LASIK) can be identified on the basis of the morphologic changes visible there.

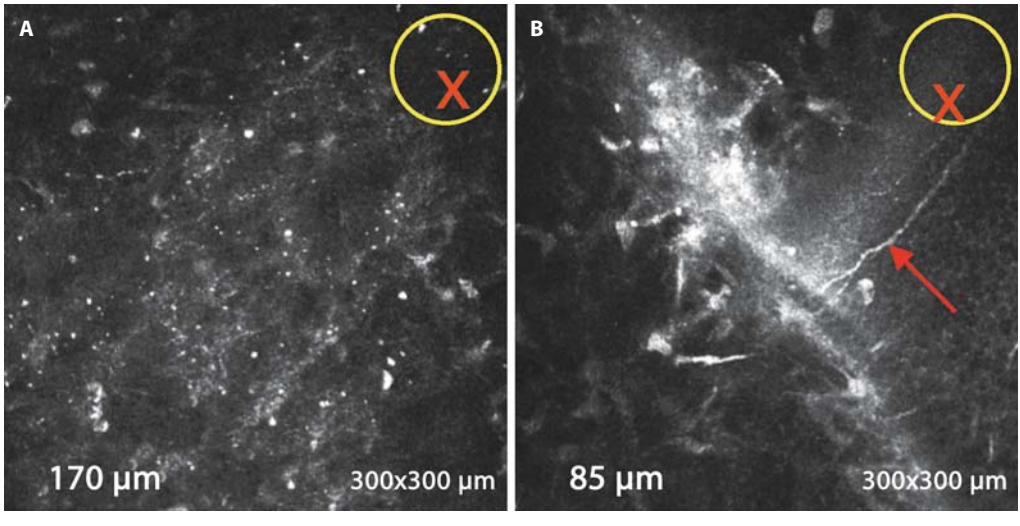

Fig. 13.48 A, B. Confocal images from the same patient: **A** level of the interface zone with diffuse hyperreflection and "microdot" structures; **B** region of the circular stromal scar with evidence of reinnervation of the flap (*arrow*) (z = 170 µm (**A**); z = 85 µm (**B**))

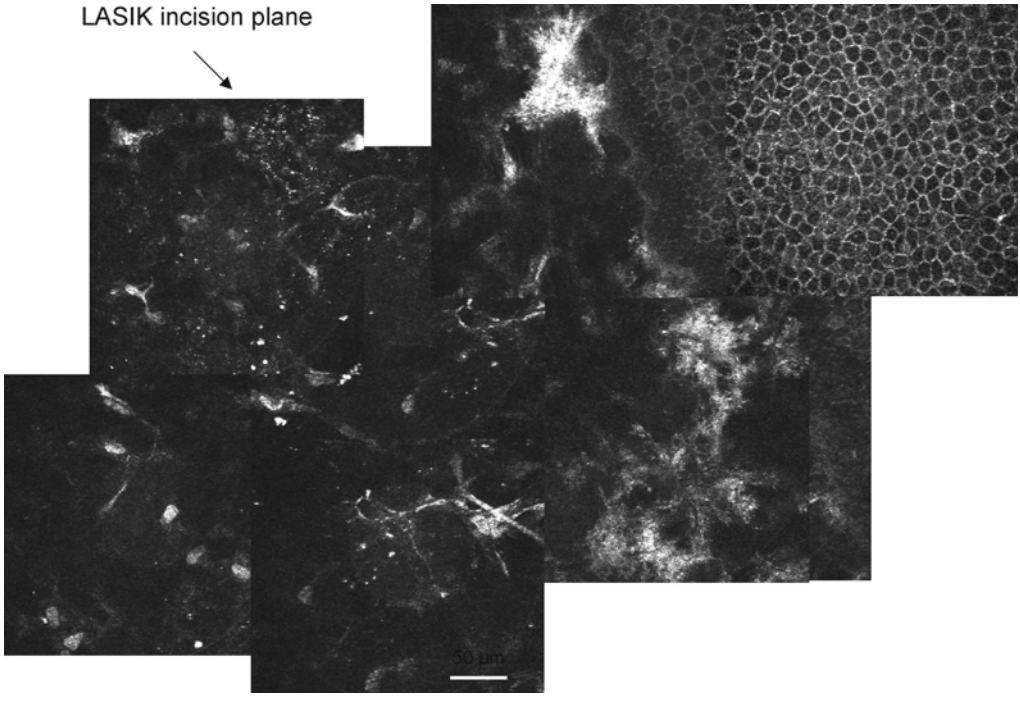

Fig. 13.49. Epithelium and keratocytes after LASIK

13.6 Future Developments

13.6.1 Three-Dimensional Confocal Laser-Scanning Microscopy

Many researchers have investigated the cornea with in vivo confocal microscopy [4, 22, 23, 24, 37, 72]. This sophisticated tool has been useful in augmenting our understanding of anatomy in the healthy and diseased human cornea. The limitations in magnification due to slight, unavoidable eye movements are obvious and therefore 3-D reconstruction is restricted on practical grounds. The step size is too coarse and magnification is too small. However, 3-D visualization and modeling would improve our understanding of the morphology of corneal architecture, e.g., of epithelial nerve structure.

This was our motivation for developing a fast, non-invasive, high-resolution method for the detailed, 3-D investigation of the human cornea. The further development of the confocal microscope [79] took the form of a modified confocal laser-scanning ophthalmoscope [66] based on a commercially available instrument (Heidelberg Retina Tomograph II, Heidelberg Engineering GmbH, Germany) [62]. A water-immersion microscope lens (Achroplan 63×/0.95W/AA 2.00 mm, Carl Zeiss, Germany) with a long working distance and high numerical aperture was used and coupled to the cornea via a PMMA cap by interposing a transparent gel (Vidisic, Mann Pharma, Germany) for in vivo imaging [66]. For 3-D imaging, an internal scanning device moves the focal plane perpendicularly to the *x-y* plane, in the same way as in optic disk tomography performed with the original HRT II configuration. During image capture the *z*-movement is stopped and the image plane is exactly perpendicularly to the *z*-axis. For the investigations presented, an acquisition time of 1 s with a scanning depth of 30 µm was used for all subjects; this is

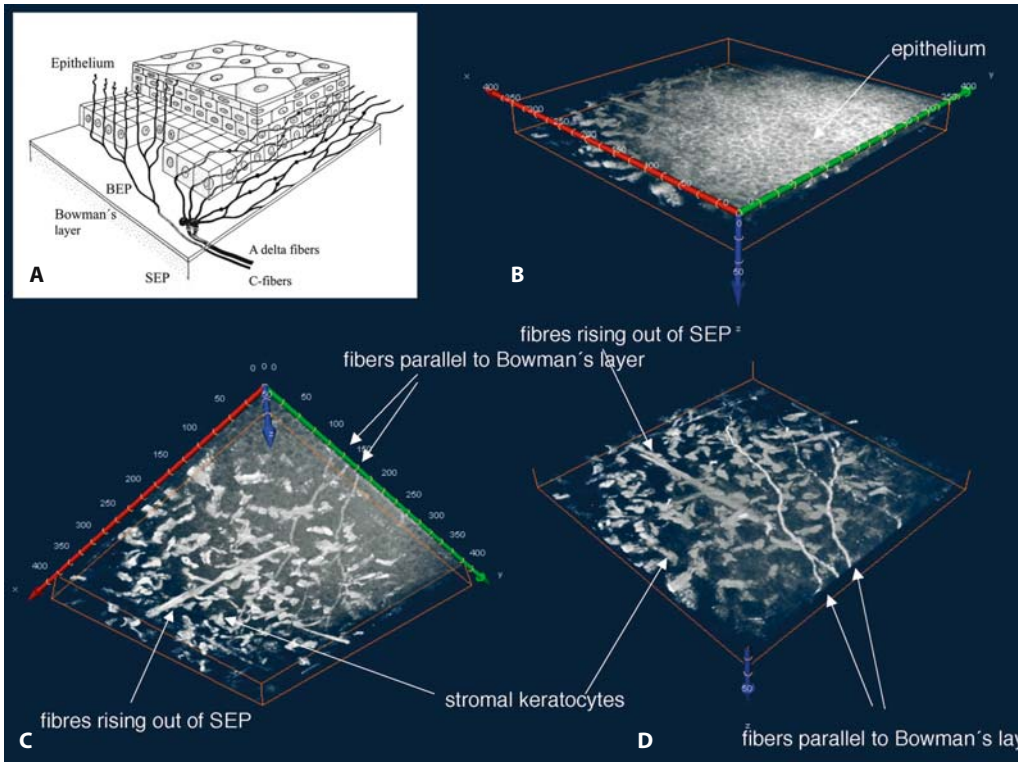

Fig. 13.50 A–D. Schematic illustration (**A**) and 3-D reconstruction (**B–D**) of the corneal epithelium with anterior stroma and nerves (healthy human subject): **B** anterior view, **C** posterior view, **D** anterior view with virtual removal of the epithelium. Thin nerves running parallel to Bowman's membrane in the basal epithelial plexus. Thicker fibers originating from the subepithelial plexus

currently thought to be the maximum when patient and examiner movements are taken into account.

Images are presented in the form of a series of 2-D grayscale images (384×384 pixels, 8 bit) representing optical sections through the cornea. The original raw image stacks were converted using ImageJ (NIH, USA) for 3-D reconstruction using Amira 3.1 (TGS Inc., USA). The voxel size is around 0.8×0.8×0.9 µm using the above-mentioned acquisition parameters. The Amira volume-rendering software package provides an interactive environment allowing features such as volume orientation for viewing planes and 3-D perspectives, segmentation and determination of distances and surfaces. The image stacks were carefully aligned and modified to eliminate unspecific information by adapting the gray values in the depicted spectrum. Shadows and illumination were manipulated after assigning density values to gray values to more clearly visualize the spatial arrangement without loss of information.

As a first in vivo application of the new device in combination with 3-D reconstruction techniques, nerve fiber distribution was characterized in healthy human corneal epithelium. The spatial arrangement of epithelium, nerves and keratocytes was visualized by in vivo 3-D confocal laser-scanning microscopy (CLSM) (Fig. 13.50). The 3-D reconstruction of the cornea in healthy volunteers yielded a picture of the nerves in the central part of the human cornea. Thick fibers arise from the subepithelial plexus, and the nerves further subdivide di- and trichotomously, resulting in five to six thinner fibers arranged parallel to Bowman's membrane and with partial interconnections

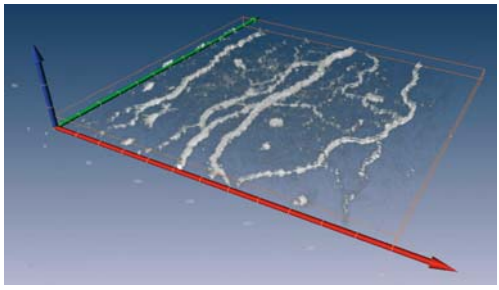

Fig. 13.51. Nerve fibers of the basal epithelial plexus, in strict alignment parallel to Bowman's membrane

(Fig. 13.51). Branches penetrating the anterior epithelial cell layer cannot be visualized.

In conclusion, 3-D CLSM is the first technique to permit visualization and analysis of the spatial arrangement of the epithelium, nerves and keratocytes in the living human cornea. The method developed provides a basis for further device refinements and for studies of changes in cellular arrangement and epithelial innervation in corneal disease. For example, CLSM may help to clarify gross variations of nerve fiber patterns under various clinical and experimental conditions.

13.6.2 Functional Imaging

In conventional microscopy the possibility of using dyes to visualize specific anatomic structures yields major information gains. This is especially true when techniques of fluorescence microscopy or immunohistochemistry are used in combination with confocal techniques [67]. Because these methods are well suited for investigating the functional status of tissues, they are also interesting for in vivo microscopy in humans, for example, for studies of wound healing or inflammation processes. However, problems arise due to the necessity for "real time" investigation because of involuntary movements on the part of the subjects and due to the selection of suitable non-toxic vital stains. Nevertheless, successful initial steps have already been taken toward confocal in vivo fluorescence microscopy of the anterior eye segments [20, 29], and

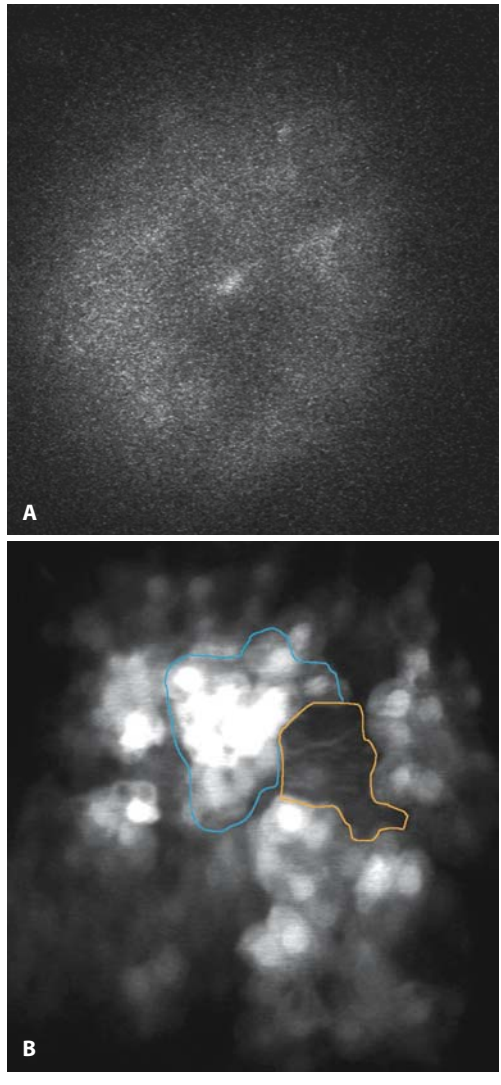

Fig. 13.52 A, B. Fluorescence micrographs of the superficial corneal epithelial cells: **A** intact corneal epithelium without appreciable fluorescence; **B** stippled epithelium after contact glass examination, *blue marked area* with fluorescein-stained cells, *orange marked area* with unstained intact cells

these may be regarded as a further enhancement of corneal assessments by slit-lamp microscopy following fluorescein or rose bengal staining [68, 17, 16, 42, 52].

To achieve this, a Heidelberg Retina Angiograph (HRA/C, Heidelberg Engineering GmbH, Germany) has been modified with a lens attach-

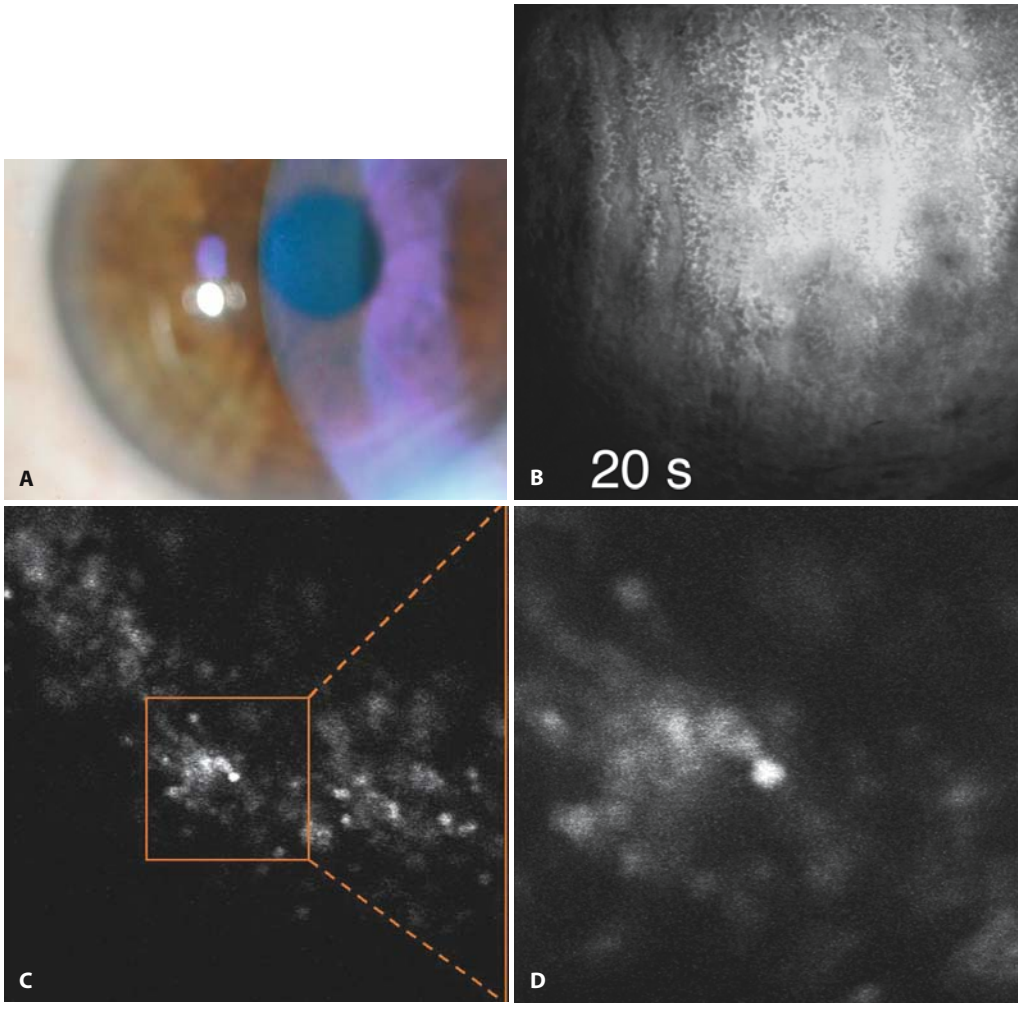

Fig. 13.53 A–D. Patient with intact corneal epithelium: **A** intact corneal epithelium, slit-lamp microscopy photograph; **B** reflection mode, tear film still intact 20 s after eyelid opening; **C** fluorescence mode, superficial corneal epithelium, only minimal fluorescence of individual cells; **D** fluorescence mode, higher magnification

ment (Rostock Cornea Module) so that the laser focus is shifted to the anterior eye segments. This enables fluorescence microscopy images to be obtained after staining with the non-specific stain sodium fluorescein and excitation with blue argon laser light (wavelength 488 nm) and addition of a barrier filter (500 nm). Using a green argon laser (514 nm) in reflection mode with the same device, it is also possible to visualize break-up phenomena of the tear film [29, 75, 76] (Figs. 13.52–13.54).

The result is a technique that enables further-reaching investigations of damaged corneal epithelium and of the associated wound healing processes. In future, confocal fluorescence microscopes specially designed for in vivo investigations in humans will perhaps permit high-quality functional imaging that is even more comprehensive and specific.

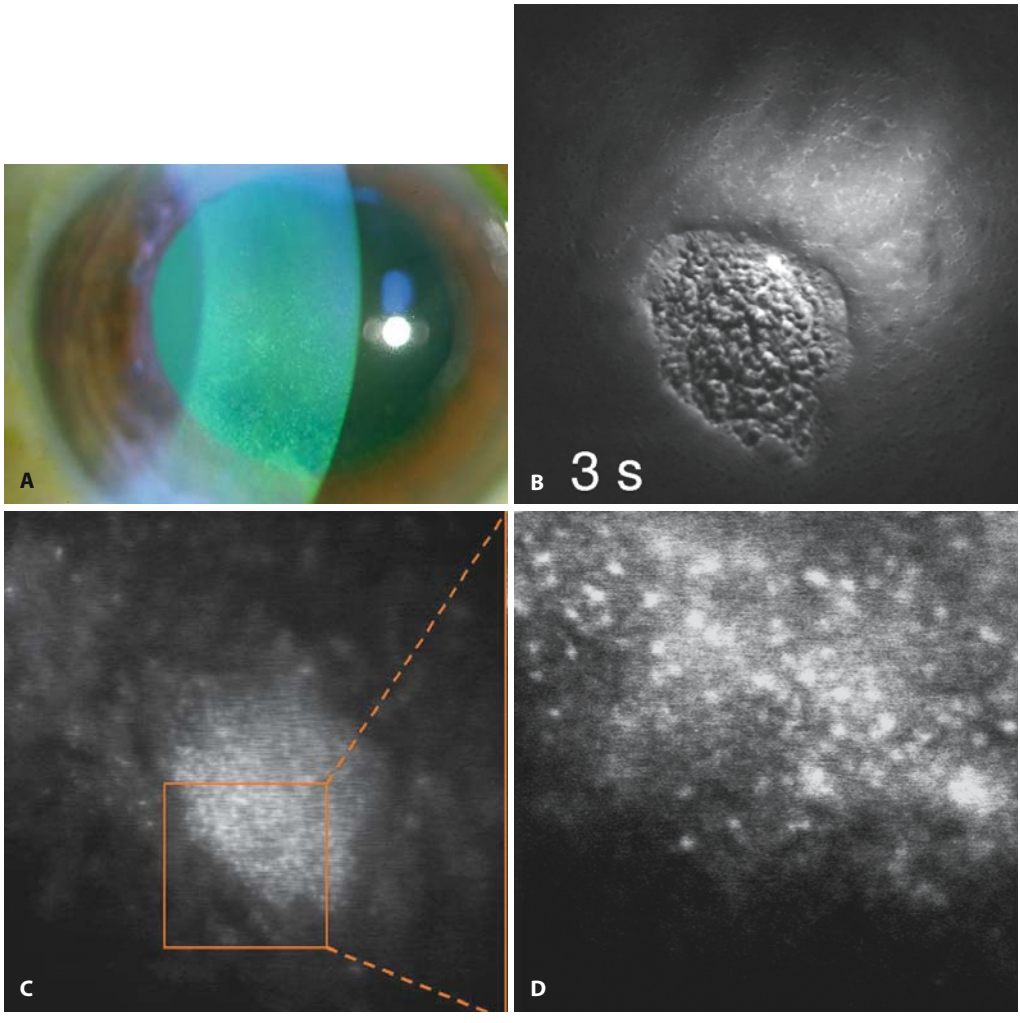

Fig. 13.54 A–D. Same patient as in Fig. 13.52 after application of a local anesthetic and following applanation tonometry: **A** slit-lamp microscopy photograph with corneal stippling; **B** reflection mode, tear film defect (dry spot) just 3 s after eyelid opening; **C** fluorescence mode, same area as in **B**, superficial corneal epithelium, area with marked fluorescence; **D** fluorescence mode, higher magnification

Summary for the Clinician

- Confocal high-resolution biomicroscopy will be used for the in vivo description of corneal pathology at the cellular level
- It will enable degeneration and repair mechanisms under various conditions to be examined so that the findings can be correlated with those from conventional slit-lamp biomicroscopy
- This will generate enhanced quality in clinical evaluation
- The use of vital staining substances, e.g., sodium fluorescein or etidium homodimer or calcein, may give insights into the metabolic activities of a variety of cells under different wound healing or degenerative conditions

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Allergic Eye Disease: Pathophysiology, Clinical Manifestations and Treatment

14

Bita Manzouri, Thomas Flynn, Santa Jeremy Ono

Core Messages

- Allergic eye disease affects a reported 20% of the population worldwide and may be increasing in line with other atopic diseases, such as asthma, as a result of environmental factors
- Other pathological mechanisms, in addition to the standard type I hypersensitivity reaction, have been recently implicated in the pathogenesis of allergic eye disease
- Established treatments have targeted mast cells, but as a result of our greater understanding of the mechanisms involved in eye allergy, researchers are now concentrating on other cell types, such as eosinophils and dendritic cells, as potential targets for immunomodulation
- Other areas of investigation to elucidate novel treatment strategies include the study of the genetics of ocular allergy, the role of environmental factors in the pathogenesis of ocular allergy, and the use of immunostimulatory DNA sequences that can inhibit the allergic response

14.1 Introduction

Owing to the fact that the eye is one of the first organs to encounter environmental allergens, allergic eye disease has become a common ocular problem, estimated to affect about 20% of the population worldwide [51]. Allergic eye disease is one of a spectrum of diseases that share a common initiating mechanism and pattern of inflammation and is a problem that is widespread among individuals who suffer with allergies. Although the incidence of allergic eye disease varies by geographical location, its prevalence is difficult to gauge as allergies tend to be underreported. A recent survey conducted by the American College of Allergy, Asthma and Immunology found that 35% of families interviewed in the United States experienced allergies, 50% of whom reported associated eye symptoms [48]. However, this prevalence is set to increase probably as a result of environmental factors. For example, the morbidity and mortality of asthma have increased with this, coinciding with the increase in house dust mite levels, and are greatest in communities exposed to high allergen levels [32].

Geographical variations, the lack of any clear-cut objective diagnostic criteria and the difficulty over the diagnosis – especially when it is the sole manifestation of atopy – have made it difficult to report the incidence rates for different forms of allergic eye disease. In the past, clinical features were used to classify allergic eye disease, but recent work that has defined the underlying pathogenic mechanisms has provided an understanding of the cellular and mediator mechanisms involved, thereby enabling a better understanding of the disease process and the development of more effective treatments.

Allergic conjunctivitis is typically divided into five types: seasonal allergic conjunctivitis (SAC), perennial allergic conjunctivitis (PAC), vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC) and giant papillary conjunctivitis (GPC). The latter is an iatrogenic disease associated with foreign bodies on the eye, such as contact lenses and ocular prostheses.

Although not always included in this grouping, it is thought to have a possible allergic mechanism because of the predominance of mast cells. GPC invariably resolves when the cause is removed and keratopathy is rare.

The aim of this review will be to focus on the underlying mechanisms of allergic eye disease and the current classification of the various disease manifestations. Treatment modalities, both well established and new innovations, will also be discussed.

14.2 Pathophysiology

Ocular allergic disease is typically associated with immunoglobulin E mediated mast cell activation (type I immediate hypersensitivity reaction) in the conjunctival tissue. However, recent data from several groups indicate that other additional mechanisms can also be involved in causing a red, allergic eye.

14.2.1 Type I Hypersensitivity

The allergic response begins when allergen is encountered by an antigen presenting cell (APC), either directly or as part of an immune complex with immunoglobulin. The APCs then process and present the allergen to CD4+ T cells as a peptide fragment in association with the major histocompatibility (MHC) class II molecule. These T cells are then polarized into T helper type 1 (Th1) cells and T helper type 2 (Th2) cells. The Th2 cells produce a variety of interleukins, two of which – IL-4 and IL-13 – stimulate immunoglobulin class switching of B cells from producing IgM to producing IgE. This immunoglobulin binds to high affinity receptors (FceRI) on the surface of mast cells and basophils. Subsequent encounter with this allergen results in the cross linkage of IgE bound to FceRI on the surface of mast cells and a cascade of signal transduction with a resultant release of preformed and newly synthesized mediators. Tissue fibroblasts and epithelial cells are also triggered by Th2 cells to produce chemokines such as monocyte chemoattractant protein-1 (MCP-1), eotaxin-1, or the protein regulated on activation normal T-cell expressed and secreted (RANTES), resulting in the migration of inflammatory cells into the site of allergen exposure [5].

This sensitized mast cell mediated response is responsible for many of the symptoms seen in SAC and PAC – such as itching, redness and eyelid swelling – with most of these patients having a positive family history of atopy and raised levels of allergen specific IgE in the serum and tears [32]. Immunohistochemical studies have shown that in SAC there is a significant increase in the numbers of conjunctival mast cells, which correlates with the patient's severity of symptoms [32]. A number of proinflammatory cytokines are released by mast cells and these include histamine, leukotriene C_4, prostaglandin D_2, platelet-activating factor (PAF), tryptase, chymase, cathepsin G and other eosinophil and neutrophil chemoattractants in what is termed the early phase response [32]. This response lasts for a maximum of 20 min after allergen activation and includes enhanced tear levels of histamine, protease tryptase, and leukotrienes, and an increase in the number of eosinophils [46]. At about 6 h a late phase response occurs which includes a second peak of tear histamine (without an increase in tryptase) and an increase in tissue adhesion molecules E-selectin and interstitial cell adhesion molecule 1 (ICAM-1), which is followed by an influx of inflammatory cells such as neutrophils, T cells, basophils and eosinophils [46]. The presence of tear histamine and the absence of tear tryptase in the late phase response may indicate that basophils, as opposed to mast cells, are involved.

Mast cells are also known to synthesize, store and release a number of cytokines such as IL-4, IL-5, IL-8, IL-13 and TNFα [46]. Cytokine involvement, particularly the Th2 cytokines, has been the focus of many studies recently looking into the mechanisms of ocular allergy. It is known, for example, that IL-4 plays a key role in allergic inflammation by promoting T-cell growth, by inducing the production of IgE from B cells, by upregulating the adhesion molecule vascular cell adhesion molecule 1 (VCAM 1), and by regulating the differentiation of the Th2

subset, which is essential for the allergic reaction [19, 31].

Physiologically, mast cells represent a heterogeneous population. They are subdivided on the basis of their ultrastructural characteristics, protease content, and T-lymphocyte dependency [49]. In humans, mast cells that contain tryptases, chymases, carboxypeptidase A, and cathepsin G are designated MC_{TC} and those that contain tryptase only are designated MC_T. Although both subtypes develop from the same CD34+ mononuclear precursor, the MC_T subtype is dependent on the presence of T lymphocytes, present at mucosal surfaces, and increases in number in aeroallergen driven allergic disease, whilst the MC_{TC} subtype appears to be independent of T cells but its development requires fibroblastic derived growth factors, which are predominant in connective and perivascular tissues, and is characteristic of fibrotic processes [32]. Normally, approximately 80 % of conjunctival mast cells are of the MC_{TC} phenotype and are mainly subepithelial in distribution, with the rest being MC_T, but during allergic inflammation such as that seen in SAC, VKC or AKC, the numbers of the latter type increase in the epithelial and subepithelial layers [37]. In the chronic and fibrosing condition AKC, however, the MC_{TC} subtype predominates, perhaps indicating an important transition from a simple mediator driven disorder to that of chronic inflammation leading to conjunctival fibrosis [37].

14.2.2 Ocular Inflammatory Reaction: Late Phase

A late phase reaction sustained by a complex network of inflammatory cells and mediators can also occur in the eye. This has been demonstrated in humans using allergen for conjunctival provocation of allergic subjects [10]. Allergen challenge caused the typical early-phase reaction within 20 min, with the initial reaction being dose dependent. With smaller doses of allergen the reaction was not so pronounced and spontaneous recovery occurred within a brief period. With larger doses, the reaction was more persistent and progressed to a late-phase reaction. Typically, high doses of allergen induced a continuous reaction manifested by burning, redness, itching, tearing and a foreign body sensation that began 4–8 h after challenge and persisted for up to 24 h. This clinical reaction was accompanied by a significant recruitment of inflammatory cells in tears. Neutrophils first appeared about 20 min after challenge, with eosinophils and lymphocytes increasing in prominence 6–24 h after challenge.

The eosinophil predominates in the late phase reaction. It is a powerful effector cell, releasing arginine rich toxic proteins capable of causing corneal epithelial damage [32]. Normally, eosinophils are not found in the conjunctival epithelium of non-atopic subjects but the numbers are increased in the conjunctival epithelium, subepithelium and tears of patients with AKC and, to a greater extent, VKC patients. Furthermore, this increase in eosinophils and eosinophil products [e.g. eosinophil peroxidase, eosinophil cationic protein (ECP)] is also present in both skin test positive and skin test negative VKC and is not confined to ocular tissues. This suggests that, in at least some forms of allergic conjunctivitis such as VKC, eosinophilic infiltration – and not IgE sensitization – is the more relevant feature of the disease and is associated with signs of systemic activation of eosinophils [10].

14.2.3 Non-specific Conjunctival Hyperreactivity

Non-specific stimuli can also cause target organ hyperreactivity and this is thought to play a role in allergic diseases of the eye. It is postulated that "non-specific conjunctival hyperreactivity" may represent a distinct pathophysiological abnormality in allergic eye disease [10]. The variability of symptoms experienced in allergic conjunctivitis which do not correlate with environmental changes such as the levels of sensitizing allergens, as well as the ocular reaction induced by non-sensitizing stimuli, may well be explained by this non-specific hyperreactivity. Natural non-specific stimulation with agents such as wind, dust, and sunlight may act only as

triggers of an abnormal non-specific reactivity of the conjunctiva in allergic patients [10].

Furthermore, multiple physical, chemical, infectious, or antigenic factors may stimulate the biological responses of mast cells, leading to the release of several mediators. Rubbing of the eyes, exposure to UV light, and increase of ocular surface temperature may lead to acute degranulation of the mast cells and release of their mediators. The local generation of stimuli that induce different patterns of mast cell cytokine release may represent another method of biological, non-specific activation of mast cells [42]. It has been observed that whenever a patient with VKC is exposed to the sun, signs and symptoms recur. Furthermore, the symptoms of allergy become most severe in children with VKC who develop bacterial conjunctivitis. Certain types of lipopolysaccharides of bacteria may cause degranulation of mast cells, leading to the release of their mediators that cause exacerbation of the allergic process.

14.2.4 T-Cell-Mediated Hypersensitivity in Allergic Eye Disease

Both CD4+ and CD8+ T cells populate the subepithelial tissue of the normal human conjunctiva. In the active forms of SAC and PAC, the T cell profile remains virtually unchanged compared to the normal milieu, but in chronic allergic disorders such as VKC, AKC and GPC, CD4+ T cells but not CD8+ T cell numbers are increased, with a mixed cellular infiltrate containing many mast cells, eosinophils, neutrophils, and macrophages [32]. In chronic allergic diseases there is no clear-cut difference between the allergen specific IgE responses and the nature and severity of the allergic responses; hence it is likely that non-IgE mechanisms are contributory, with the involvement of cell mediated responses [32].

Most of the T cells in normal conjunctiva are naïve, but in chronic allergic conditions 90 % of the T cells are memory T cells [35]. Corresponding with this rise in activated T cells, there is also upregulation of markers present on antigen presenting cells.

CD4+ T cells can be further subdivided into two distinct subsets based on their pattern of cytokine production. The first subset, Th1 cells, produce IL-2, IL-3, TNFβ and interferon γ (IFNγ) and are more associated with classic delayed type hypersensitivity. The second subset, Th2 cells, produce a range of cytokines encoded on chromosome 5, such as IL-4 and IL-5, which promote immediate hypersensitivity responses through their ability to stimulate proliferation, B cell IgE production and eosinophil production, activation and survival [32]. It has been shown that in AKC there is increased numbers of both Th1 and Th2 lymphocytes as opposed to in VKC where lymphocytes secreting cytokines typical of the Th2 subset are found. This observation suggests that VKC results from a maturation shift of CD4+ T cells towards a pattern of secretion of cytokines which drives a mast cell and eosinophil mediated inflammatory response [34].

14.3 Clinical Syndromes of Allergic Eye Disease

Allergic diseases of the eye comprise a number of different inflammatory conditions that share common features such as seasonal variation, association with atopic disease and presumed involvement, to a greater or lesser extent, of the type I hypersensitivity mechanism in their pathophysiology. They are traditionally classified, as outlined above, into five distinct entities: SAC, PAC, VKC, AKC, and GPC (Fig. 14.1).

As previously mentioned, recent evidence suggests that the traditional type I hypersensitivity reaction may be less important in some of these diseases than others. However, these diseases share many symptoms in common and it is therefore reasonable to group them in the same broad category of “allergic eye disease”. The cardinal feature of all allergic eye disease is itching – in the absence of this symptom one should be wary of making this diagnosis. Other symptoms such as tearing, burning and foreign body sensation may be present in variable degrees in all of these conditions. Despite similarities in the symptoms, it is important to distinguish, where possible, between the different

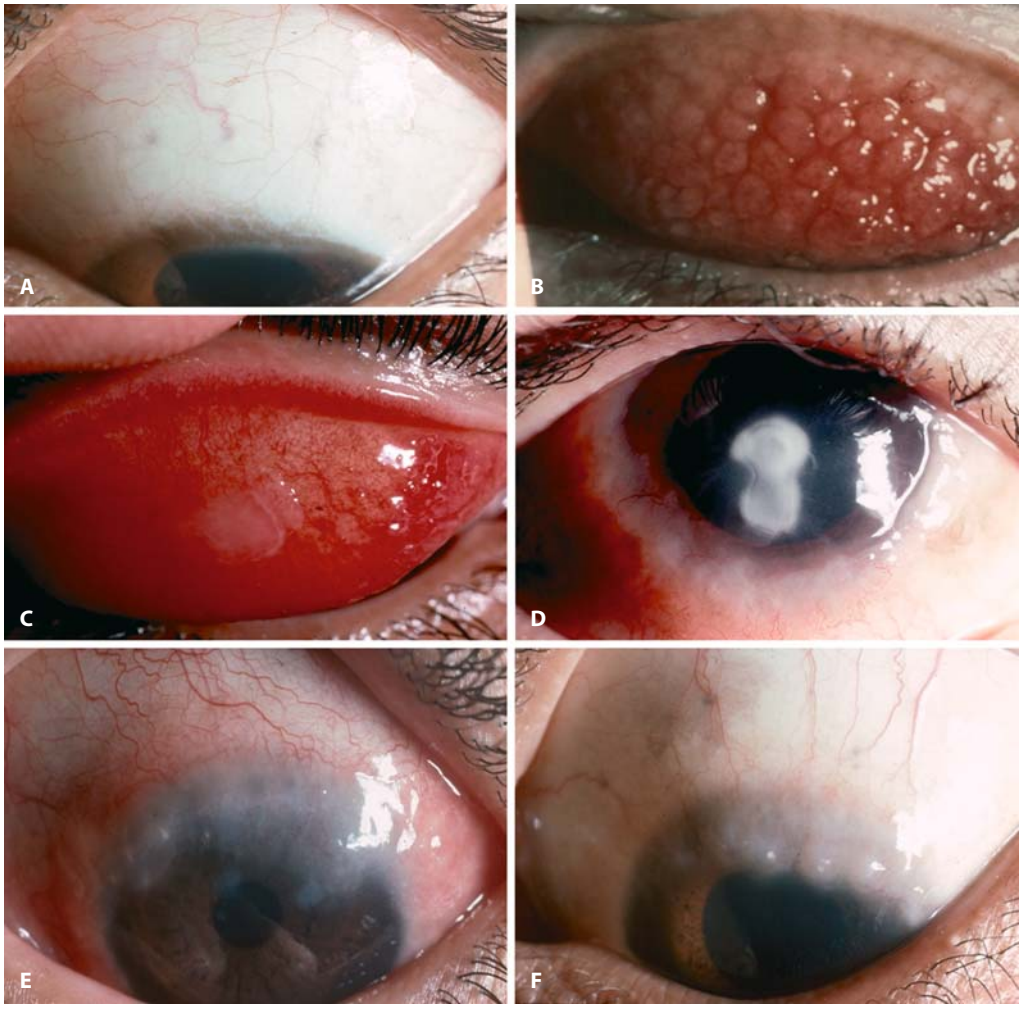

Fig. 14.1. **A** Normal bulbar conjunctiva; **B** giant papillae in GPC; **C** typical appearance of superior tarsal conjunctiva in a severe case of SAC; **D** corneal ulcer in VKC; **E** early stages of corneal pannus in AKC; **F** Horner-Trantas dots seen in AKC. (Pictures courtesy of Dr. Mohammed Siddique, Institute of Ophthalmology, London)

types of allergic eye disease as each of them has a different visual prognosis. Accurate diagnosis will allow appropriate counselling of patients.

The most common type of allergic eye disease, seasonal allergic conjunctivitis (hay fever conjunctivitis), is also the least serious in terms of visual outcome. SAC and PAC together account for 98 % of allergic eye disease [41]. VKC and AKC, although much rarer, are more likely to lead to visual impairment, with AKC being the most destructive disease and having the worst visual prognosis. The emergence of newer treatments based on an increasing understanding of the individual pathogenic mechanisms of each disease also underlines the importance of accurate diagnosis. The different types of allergic eye disease can usually be distinguished by history and examination alone.

14.3.1 Seasonal Allergic Conjunctivitis

Of the allergic eye diseases, SAC represents the most "pure" form of type I hypersensitivity. As the name suggests, the symptoms and signs are intermittent and occur rapidly following exposure to a specific allergen, with patients often having a personal or family history of atopy. In the absence of prolonged exposure to allergen, attacks are short lived. The commonest seasonal allergen is pollen, with tree pollen predominating in spring, grass pollen in summer and ragweed pollen in autumn. Symptoms are typically absent during winter. The severity of signs and symptoms varies from patient to patient depending on the specific allergen and the exposure.

14.3.1.1 Symptoms

Patients usually complain of intense itching of the eyes associated with a watery discharge.

14.3.1.2 Signs

There may be eyelid oedema. Conjunctival vessels may be injected and conjunctival chemosis may give the conjunctiva a "milky" appearance. Symptoms and signs are usually bilateral although they may be asymmetrical. Young children can present with dramatic unilateral lid oedema and chemosis.

14.3.2 Perennial Allergic Conjunctivitis

PAC is less common than SAC. Although the symptoms and signs of these diseases are the same, the distinction between them lies in the timing of the symptoms. Whereas SAC sufferers have symptoms for a defined period of time, PAC sufferers are sensitive to allergens that are present year-round and so are perennially symptomatic. "Household" allergens such as the dust mite or pet dander are the usual offenders in PAC. These patients may also be sensitive to seasonal allergens and so there may be a superimposed seasonal element to their symptoms.

14.3.3 Vernal Keratoconjunctivitis

A disease of childhood, VKC accounts for 0.5% of allergic eye disease [32]. Like AKC it has a male preponderance but onset is much earlier, typically late in the first decade. It is seen most commonly in temperate climates such as those of the Mediterranean, South Africa and North America. However, genetic as well as environmental factors are important. Even in cooler northern climates the disease is more commonly seen in people of African or Asian descent [39]. There is frequently a personal or family history of atopy but this association is not as strong as in other types of allergic eye disease, with a large proportion of VKC patients having no such history.

In the majority of cases the disease shows seasonal variation with symptoms typically appearing in spring and lasting about 6 months. Additional recurrences in winter are common. In some cases the disease evolves over time into a more chronic, perennial form of inflammation with up to one-quarter of VKC patients having a perennial form of the disease from the outset [11]. Although serious visual complications may occur, VKC is a less destructive disease than AKC and usually burns itself out by the early twenties [30].

14.3.3.1 Symptoms

Symptoms are usually bilateral but may be asymmetrical and, like all allergic eye diseases, itching is a cardinal feature. Photophobia is also prominent and patients may complain of tearing and a mucoid discharge. Depending on the severity of corneal involvement, they may also complain of a foreign body sensation or pain.

14.3.3.2 Signs

In contrast to AKC, the periorbital skin is usually unaffected. The disease is further classified into tarsal, limbal or mixed VKC depending on the location of the conjunctival inflammatory signs.

Tarsal. The inflammation is predominantly in the superior tarsal conjunctiva although the bulbar conjunctiva may show non-specific signs such as injection or chemosis. The superior tarsal conjunctiva develops a papillary reaction. Papillae are typically large (>1 mm) and diffuse, giving a "cobblestone" appearance. These tarsal papillae tend to persist even when the disease is quiescent but become hyperaemic and oedematous during periods of disease activity. The presence of a thick, mucoid, white secretion associated with these papillae is another indicator of disease activity. Papillae may enlarge to several millimetres in diameter and may give rise to ptosis. In severe forms of the disease, linear subepithelial scars (Arlt's lines) may appear parallel to the lid margin.

Limbal. Limbal VKC is characterized by single or multiple gelatinous, pale infiltrates in the limbal conjunctiva. The extent of limbal involvement is variable. Infrequently, there may be 360° limbal inflammation. There is usually injection of the surrounding bulbar conjunctival vessels. Aggregates of degenerating eosinophils at the apex of the infiltrates are seen as small white spots (Horner-Trantas dots) – both the limbal infiltrate and the Horner-Trantas dots are transient.

In mixed VKC both limbal and tarsal signs may be observed. Although limbal and tarsal VKC are believed to be variants of the same disease, certain differences have been observed in their demographics and natural history. Limbal VKC is particularly common in people of African or Asian descent. There is mixed evidence as to which, if either, of the variants is more responsive to treatment [11, 52]. Patients with tarsal disease are certainly more likely to develop sight-threatening corneal ulceration [52].

Cornea. Sight-threatening complications occur less frequently in the cornea than in AKC. However, both non-specific and pathognomonic corneal signs are seen. In a follow-up series of 195 patients with VKC, 9.7% developed corneal ulcers and 6% developed a permanent decrease in visual acuity [11]. Abnormalities of the central and superior cornea are most commonly seen in tarsal disease. In its earliest form there may be only punctuate epithelial erosions. These may, with time, coalesce to form larger erosions that may in turn evolve into the characteristic "shield" ulcer of VKC. Shield ulcers are non-infectious and occur in the central/superior cornea. At first they are shallow with a transparent base. Over time the ulcer becomes filled with inflammatory debris and the base opacifies. Further accumulation of inflammatory debris leads to plaque formation. The pathogenesis of these ulcers is incompletely understood. Mechanical abrasion of the epithelium by large papillae on the superior tarsal conjunctiva is thought to play a role, as is epithelial corrosion by toxic granule proteins released from eosinophils in the tarsal conjunctiva and tear film. In persistent or recurrent limbal disease, peripheral corneal signs such as pannus or opacification (pseudogerontoxon) may develop. Limbal lesions may also cause significant astigmatism.

14.3.4 Atopic Keratoconjunctivitis

First described in 1952 [22], AKC constitutes a more relentless form of conjunctival inflammation than either SAC or VKC. Atopic dermatitis (eczema), a pruritic skin condition that affects 3% of the population, is present in 95% of patients with AKC [7]. Conversely, 25–40% of atopic dermatitis patients have AKC [18]. Typically patients have had atopic dermatitis since childhood with ocular symptoms developing at a later stage. Symptoms may begin in the late teens or early twenties but the peak incidence is between the ages of 30 and 50. Males are more commonly affected than females and there is often a personal or family history of other atopic diseases. Unlike SAC, and most cases of VKC, the symptoms are perennial. It differs from PAC in

that the symptoms are less intermittent. Although there may be periods of relative quiescence, signs of disease activity are usually present to some degree.

14.3.4.1 Symptoms

Bilateral itching of the eyelids and periorbital skin is the most frequent symptom. Patients also complain of tearing, photophobia, burning and blurred vision. Increased mucus and inflammatory debris may thicken the tear film and contribute to a stringy discharge. Depending on the severity of corneal involvement, patients may complain of a foreign body sensation and pain.

14.3.4.2 Signs

Invariably there are signs of disease on the eyelids and periorbital skin. Ocular surface inflammation in AKC may, as the name suggests, affect the conjunctiva and cornea. In many cases the disease is mild and corneal signs may actually be absent or minimal. Such cases have been termed atopic blepharoconjunctivitis (ABC) [53].

Eyelids. The periorbital skin typically has the dry, indurated and scaly appearance of eczema. Eyelid swelling may contribute to the generalized wrinkling of the skin and the development of a fold in the lower lid skin (Dennie-Morgan fold). In severe cases there may be fissures at the lateral canthus and/or absence of the lateral part of the eyebrow (Herthoge's sign). The latter signs may be induced or aggravated by vigorous eyelid rubbing. Lid margins may be thickened (tylosis) and may develop meibomian gland dysfunction. Colonization of the lid margin with staphylococcus with resultant staphylococcal blepharitis is common [54].

Conjunctiva. There is typically a papillary reaction on the tarsal conjunctiva, which, in contrast to VKC, is usually more prominent on the inferior, rather than the superior, tarsal conjunctiva. The bulbar conjunctiva may show non-specific signs of inflammation such as hyperaemia or chemosis. Rarely, papillary hyperplasia of the limbal conjunctiva occurs, resulting in a gelatinous limbal nodule similar to those seen in limbal VKC. Associated Horner-Trantas dots have been seen. Prolonged or severe inflammation may result in conjunctival cicatrization. This is most commonly seen in the lower fornix and may result in shallowing of the fornix and symblepharon. Activation of fibroblasts by mast cells has been proposed as a mechanism for conjunctival scarring in allergic disease [47]. Several cases of squamous cell carcinoma/CIN have been reported in patients with atopic dermatitis or AKC [20, 24] although the mechanism of tumourigenesis remains unclear.

Cornea. Visual deterioration in AKC is most commonly caused by corneal complications. Corneal scarring in AKC may result from vascularization, infection or keratoconus. A broad spectrum of corneal disease may be seen depending on the severity and chronicity of inflammation. Punctate epithelial erosions are seen early in the course of the disease. The severity of the corneal erosions correlates with the number of inflammatory cells (especially eosinophils) in brush cytology samples from the superior tarsal conjunctiva [50]. Peripheral corneal vascularization, which may be associated with opacification, is common. These changes may occur as a result of limbal stem cell deficiency. Rarely, corneal vascularization may encroach on the visual axis and cause visual impairment. Epithelial erosion may coalesce to form non-infectious corneal ulcers. Toxic granule proteins derived from conjunctival eosinophils have been implicated in the pathogenesis of these ulcers [33]. Staphylococcal colonization of the lid margins coupled with a decrease in barrier function [56] also puts AKC patients at increased risk of developing bacterial infectious corneal ulcers. They are particularly vulnerable to herpes simplex keratitis [16]. Chronic eye rubbing may be an important factor in the association between AKC and keratoconus [6].

Other Causes of Visual Deterioration in AKC. AKC is associated with the development of premature bilateral cataracts. Typically the lens

opacity develops in the anterior subcapsular region and has well defined margins. It is often referred to as a "shield" cataract. A rarer cause of visual impairment in AKC is that of retinal detachment [57]. The reasons for this association are not well understood. Finally, chronic use of topical steroids in the treatment of AKC may result in posterior subcapsular cataracts and glaucoma (see below).

14.3.5 Giant Papillary Conjunctivitis

The term giant papillary conjunctivitis describes the advanced stages of the conjunctival response to the prolonged presence of a foreign body on the ocular surface. It was first observed and characterized in contact lens wearers [3] and was later reported in patients with ocular prostheses and exposed suture ends. Nowadays, it is seen commonly in contact lens wearers, and most of the knowledge of this condition arises from experience with these patients. Wearers of soft contact lenses are most likely to develop giant papillary conjunctivitis, but it has been estimated that 1–5 % of rigid gas-permeable lens wearers may also be affected [26, 27]. The condition shows no age or gender preference and there does not appear to be a strong association with allergy [28].

14.3.5.1 Symptoms

Earliest symptoms are of mucus discharge in the morning and itching on removal of the lenses. As the disease progresses these symptoms become more marked and may be associated with a foreign body sensation. Patients complain of blurred vision as a result of coating of the lens with mucus and increasing lens mobility and instability. As the disease advances patients become increasingly intolerant of their contact lenses.

14.3.5.2 Signs

Giant papillary conjunctivitis is characterized, in the late stages, by the presence of abnormally large (>0.3 mm) papillae on the superior tarsal conjunctiva. In the earliest stage, however, when the patient first becomes symptomatic, the conjunctiva may appear normal. As the disease progresses the superior tarsal conjunctiva becomes thickened and hyperaemic. Small papillae develop first which increase in size and number over time. The distribution of giant papillae varies according to the type of lens worn. In wearers of soft lenses papillae emerge first at the superior edge of the tarsal plate. Wearers of hard lenses, which are smaller, develop papillae closer to the superior lid margin [23]. The bulbar conjunctiva and inferior fornix are usually normal.

The symptoms and signs of this disease may resemble those of VKC. Important factors in the history, which could help to distinguish these conditions, include contact lens history and patient age since VKC is seldom seen after the early twenties.

14.4 Treatment of Allergic Eye Disease

The mainstays of treatment for the majority of allergic eye disease symptoms are topical eye drops, and for this purpose a wide range of topically administered agents have been developed to treat the milder disease varieties. These include antihistamines, mast cell stabilizing agents and anti-inflammatory agents. Additionally, topical nasal decongestants are also available. Of the topical eye drops, it is antihistamines and mast cell stabilizers that have been extensively studied to assess their therapeutic value in a large number of comparative clinical trials over the years. Furthermore, as the chemical and cellular infiltrates in both acute and chronic allergic eye disease become better characterized, there are significant implications for treatment of these conditions. Efficacy of all of these agents varies from patient to patient and the choice of agent used depends on a number

of variables, such as the underlying state of health of the eye being treated, drug costs and availability, contact lens wear, and the potential for compliance [8].

The preferred treatment modality in mild diseases such as SAC and PAC is topical therapy, since neither is sight threatening, and their pathogenesis involves mast cell degranulation and the release of histamine. Topical treatment offers several advantages: the ease of application directly to the site affected by the disease process, the general lack of systemic side effects, and the washout effect of the drops themselves aiding the removal of the inflammatory mediators.

14.4.1 Antihistamines

The first line of treatment of ocular allergy includes the avoidance of allergens, the use of cold compresses for symptom relief (especially itching), and regular lubrication of the eye to wash out tear histamine and other inflammatory mediators, thus diluting their effects and aiding the patient's comfort. Topical therapy may start with the use of antihistamines or mast cell stabilizers. Considering the former, the stimulation of H1 receptors in the conjunctiva mediates the symptom of itching whereas H2 receptor activation results in vasodilation. Second generation H1 receptor antagonists are used for the topical treatment of the benign forms of allergic conjunctivitis, and these include levocabastine, azelastine and emedastine. They all bind selectively to H1 receptors in the conjunctiva and have little or no effect on dopaminergic, adrenergic or sertotoninergic receptors [46]. Of this new generation H1 receptor antagonists, topical azelastine has been shown to be a powerful topical antihistamine, decreasing eosinophil and T lymphocyte activation, having an inhibitory effect on a broad array of other mediators, and being a potent suppressor of itching and conjunctival hyperaemia after conjunctival provocation with an allergen, with an onset of action seen within 3 min and a duration of effect of at least 8–10 h [32, 46]. Although topical antihistamines can be used alone to treat allergic conjunctivitis, combining an antihistamine with a vasoconstrictor is more effective than either agent alone. The vasoconstrictors commonly used in combination with topical antihistamines are phenylephrine or naphazoline [8].

14.4.2 Mast Cell Stabilizing Agents

The most common topical drugs invariably used by ophthalmologists for all forms of allergic conjunctivitis are the mast cell stabilizing agents. These include sodium cromoglygate, lodoxamide, ketotifen, nedocromil sodium and the newly introduced olopatadine. Mast cell stabilizers are effective in the milder forms of allergic eye disease and have very few side effects, either locally or systemically, but for patients to receive long-term benefit from them such that expected exposure to allergen reduces the tryptase and inflammatory cells after allergen challenge, treatment is needed for many years [46].

Sodium cromoglygate is the prototypic mast cell secretion inhibitor. It is the oldest and most widely used agent of this family of drugs. However, despite its extensive use, the mechanisms of its action are still unclear. The efficacy of the medication appears to be dependent on the concentration of the solution used [9]. Nedocromil sodium has been shown to be able to inhibit chloride ion flux in mast cells, epithelial cells and neurons. This feature may explain how it can prevent responses such as mast cell degranulation. Others have suggested the inhibition of IgE production by B cells as an alternative mechanism [46]. Newer agents such as lodoxamide have become available, which are faster acting and approximately 2,500 times more potent than sodium cromoglycate in the prevention of histamine release, that also act to reduce tear tryptase and inflammatory cells after allergen challenge [8]. In a comparative trial with sodium cromoglygate and lodoxamide in subjects with the more severe forms of allergic eye disease (VKC, AKC and GPC), lodaxamide was found to be superior for symptom relief. It was also found to be effective in the long-term

treatment of VKC especially in cases with an epitheliopathy [17, 43].

14.4.3 Dual-Acting Agents

Dual-acting agents are named for their antihistamine effects and their inhibition of mediator release. They are the newest generation of antiallergic agents. The advantages of these drugs lie in the rapidity of symptomatic relief given by immediate histamine receptor antagonism coupled with the long-term disease modifying benefit of mast cell stabilization. Not all of these agents are equivalent and in selecting a dual-action agent, one should look for a potent and long-lasting agent that relieves the signs and symptoms of allergy, including itching, redness, lid swelling and chemosis [41].

Clinical studies have demonstrated the efficacy and tolerance of olopatadine for the management of allergic conjunctivitis or in a conjunctival allergen model [1, 4, 13]. This agent both acts as a mast cell stabilizer and has antihistamine activity. This dual mode of action has been shown to be advantageous for the management of allergic conjunctivitis, and as a topical preparation has been subjectively preferred by patients [4, 44]. Furthermore, a direct anti-inflammatory property for this drug has been suggested by a study which showed that olopatadine inhibited the anti-IgE antibody-mediated release of TNFα from human conjunctival mast cells [14].

14.4.4 Non-steroidal Anti-inflammatory Drugs (NSAIDs)

Prostaglandins, especially PGE_2 and PGI2, lower the threshold of the human skin and conjunctiva to histamine-induced itching. NSAIDs, by inhibiting the production of prostaglandins, help to alleviate this itching but also reduce pain and inflammation of the eye associated with allergic reactions [9, 46]. NSAIDs used in the topical treatment of allergic ocular conditions include ketorolac, diclofenac, fluribrofen and indomethacin. These agents, unlike corticosteroids, do not mask ocular infections, affect wound healing, increase intraocular pressure, or contribute to cataract formation [8]. However, of these agents, only ketorolac tromethamine (Acular) has been approved by the Food and Drug Administration for the management of acute SAC [15]. It acts to significantly reduce tear tryptase levels and the number of eosinophils and lymphocytes in tear specimens after conjunctival provocation [29].

Ocular NSAIDs have been associated with a low-to-moderate incidence of burning and stinging [9]. The concern of NSAID-induced asthma does not appear to be a problem except in patients who have the triad of asthma, nasal polyposis and aspirin sensitivity [45].

14.4.5 Topical Corticosteroids

Topical steroid preparations are the most effective therapy for moderate to severe forms of VKC, but their use should be strictly limited for severe cases and carefully monitored since their long-term use is associated with an increased risk for the development of cataracts and glaucoma and can potentiate ocular herpetic infections. In fact, topical steroids are responsible for the 2% incidence of glaucoma in VKC patients [12]. In T cell dependent AKC and VKC, sodium cromoglycate has been used either prophylactically or as maintenance therapy to control mild symptoms only, but is ineffective in acute exacerbations. In acute exacerbations, even the newer class of mast cell stabilizers may not be enough, and under these circumstances steroids (fluoromethalone or dexamethasone) tend to be used in doses of up to one drop hourly to reverse corneal epitheliopathy caused by the release of epithelial toxic mediators from eosinophils and neutrophils [32]. Once control of the acute phase of the disease has been achieved, steroids should be discontinued and alternative topical treatment, as outlined previously, should be started [12].

Two modified corticosteroids have recently been investigated for their efficacy in allergic conjunctivitis: rimexoline (a derivative of pred-

nisolone) that is quickly inactivated in the anterior chamber of the eye, thus improving efficacy and decreasing the safety concerns, e.g. raised intraocular pressure; and both low-dose and high-dose loteprednol etabonate are highly effective as prophylaxis against, and in the acute phase of, allergic conjunctivitis [8].

14.4.6 Calcineurin Inhibitors

Two calcineurin inhibitors are currently in clinical use:

1. Cyclosporin A (CsA) is a fungal antimetabolite and anti-CD4+ agent that decreases the clinical signs and symptoms of the chronic forms of VKC and AKC. It acts to control ocular inflammation by blocking Th2 lymphocyte proliferation and IL-2 production, by inhibiting histamine release from mast cells and basophils, and by reducing the production of IL-5, thereby reducing the recruitment and effects of eosinophils on the conjunctiva [12]. Although systemic CsA has been used for the treatment of severe AKC and keratoconjunctivitis sicca, topical cyclosporin causes ocular irritation with burning, tearing, erythema and itching. This is due to the fact that since the drug is lipophilic, it has to be dissolved in an alcohol base which causes the ocular irritation [8]. However, the topical form of this drug is not yet generally available.

   CsA has been evaluated in patients with steroid dependent AKC. In one study, 12 patients were randomized to treatment with CsA and 9 patients to a vehicle treatment group. The results showed that in the CsA group, 9 out of 12 patients were able to cease steroid therapy as compared to 1 out of 9 in the vehicle group [21]. Furthermore, the final steroid use was significantly lower in the CsA group versus the vehicle group. This study concluded that CsA is an effective and safe steroid sparing agent in AKC and is also capable of improving the symptoms and signs of AKC. In another randomized trial the short-term efficacy and safety of topical CsA 0.05% was evaluated in the treatment of patients with severe, steroid resistant AKC [2]. Patients were randomly assigned to treatment with topical CsA 0.05% or placebo for a period of 28 days with the symptoms and signs of AKC recorded on the day of enrollment and at the end of the treatment period. The results, recorded by a composite score computed by summing the severity grade of all five symptoms and six signs of AKC, showed a greater improvement in the CsA group relative to the placebo group at the end of the treatment period. It was hence concluded that topical CsA 0.05% is safe, and may actually have some effect in alleviating the signs and symptoms, in severe AKC that is resistant to topical steroid treatment.
2. Tacrolimus (FK-506) is a macrolide antibiotic with potent immunomodulatory properties which has already been used to treat the immune mediated problems encountered with corneal graft rejection, ocular pemphigoid and uveitis. It acts on T lymphocytes to block the production of lymphokines, such as IL-2, IL-2, IL-5, TNFα and interferon-ɣ a. It also blocks the degranulation of mast cells and several mast cell cytokines, such as IL-3 and IL-5 [8].

14.4.7 Future Drug Developments

The aims of future drug development will focus on steroid-sparing agents that control the immune response. These may be administered alone, or in combination with newer drugs that have already demonstrated their efficacy in the management of these conditions, such as antihistamines and mast cell stabilizers.

Our understanding of the pathophysiology of allergic conjunctivitis has increased greatly over the last 3 years. New areas of investigation to elucidate novel treatment strategies include the study of the genetics of ocular allergy, since it has been known for some time that different mouse strains are more or less responsive to specific allergen challenge in the eye, and linkage analysis of these mice is being pursued to define disease susceptibility genes for ocular allergy [41]. A few studies have addressed the

role of environmental factors in the pathogenesis of ocular allergy. For example, it has been shown that there is a positive association between the dietary intake of n-6 polyunsaturated fatty acids and seasonal allergic rhinoconjunctivitis [55]. Other studies have focused on the genetics of allergic conjunctivitis. One of the earliest published studied approximately 117 families with probands with allergic conjunctivitis [40]. Evidence was found, by analysis of the genomic DNA, for genetic linkage of allergic conjunctivitis for chromosomes 5, 16 and 17. This genetic linkage for allergic conjunctivitis was shown to differ from that reported for atopic asthma, and hence it was concluded that there were likely to be organ specific disease susceptibility genes, which, together with general atopy genes, target the allergic response to specific mucosal tissues.

Resident dendritic cells in the conjunctiva have also been the focus of recent research since it has been shown that dendritic cell activation by an allergen is a very early step in disease pathogenesis, with dermal allergy being used as the prototype [41]. Other areas of interest lie in the activation and mediator release from human conjunctival mast cells on FceRI cross-linking. A recombinant humanized monoclonal anti-IgE antibody, omalizumab, was recently developed which binds specifically to the IgE binding site on human FceRI and thereby blocks the binding of IgE to mast cells and basophils [5]. Studies have shown that this agent benefits patients with moderate to severe allergic asthma who remain symptomatic despite treatment with systemic or inhaled corticosteroids [5]. Additionally, omalizumab has been shown to be safe and well tolerated.

One of the most innovative treatment advances has been in the use of immunostimulatory DNA sequences that can inhibit the allergic response. Both bacterial DNA and synthetic oligodeoxynucleotides containing specific motifs centered on a CpG dinucleotide have been shown to be potent immunostimulatory agents [46]. It is likely that these sequences represent a signal to the immune system, resulting in a powerful Th1 response and this can be used to switch an allergic response from a Th2 dominated immune profile towards a Th1 profile [46]. Miyazaki et al. evaluated the therapeutic potential of immunostimulatory sequence oligodeoxynucleotide (ISS-ODN) administration in ocular allergy using a mouse model of ragweed-specific conjunctivitis [36]. They concluded that ISS-ODN was an effective treatment for ocular allergy when administered systemically or conjunctivally. Systemic treatment markedly inhibited clinical parameters of SAC and blocked conjunctival eosinophilia in the late phase reaction. Additionally, it also effectively blocked neutrophilia, which is a hallmark of the late phase reaction.

Other areas of potential therapeutic value which require further research include the use of antagonists of the action of macrophage inflammatory protein-1α (MIP-1α) and the use of IL-1 receptor antagonists. Data have shown that MIP-1α constitutes an important second signal for mast cell degranulation in the conjunctiva in vivo and consequently for acute phase disease [38]. Therefore, antagonizing the interaction of MIP-1α with its receptor (CCR1) or signal transduction from this receptor may hold promise for future treatment of both acute and late phase reactions. Similarly, in a mouse model of allergic eye disease, IL-1 inhibition using an IL-1 receptor antagonist was found to downregulate the recruitment of eosinophils and inflammatory cells by decreasing the concentration of attractant chemokines [25]. This research also offers a potential novel treatment for the prevention and treatment of allergic eye disease.

14.5 Conclusion

Allergic eye disease represents a heterogeneous group of diseases that share a common symptomology but different pathogenesis. They are further distinguished by their long-term visual prognosis, with diseases such as SAC and PAC having no long-term effects on sight whereas VKC and AKC, through corneal involvement and subsequent scarring reactions, can adversely affect visual prognosis. Future work needs to increase our understanding of the genetics and mechanisms of mast cell cytokine expression and mediator release, the regulation of the

cellular inflammatory response and the B cell regulation of IgE secretion. Armed with this knowledge, more ways of treating allergic eye disease will be developed which will target more specific components of the allergic response. Most novel therapies so far have been directed at controlling the allergic response in the bronchial airways and the nasal mucosa, but it is hoped that new strategies will begin to focus treatment on ocular disease to downregulate the allergic response rather than to control its effects.

Summary for the Clinician

- **Allergic eye disease is a common problem. It is reported to affect about 20% of the population worldwide but this may be an underestimate of the true prevalence of the condition due to geographical variations and the lack of any clear cut objective diagnostic criteria**
- **There are five main syndromes of allergic eye disease, two of which (vernal and atopic keratoconjunctivitis) have sight-threatening complications; hence it is important to strive to make an accurate diagnosis due to the prognostic implications**
- **The majority of patients have an atopic tendency or a family history of atopy. There is a particularly strong association between atopic dermatitis and atopic keratoconjunctivitis**
- **The mainstays of treatment for the majority of allergic eye disease symptoms are topical eye drops, including antihistamines, mast cell stabilizers and anti-inflammatory agents**
- **Topical steroid preparations are the most effective therapy for moderate to severe forms of allergic eye disease but their use should be limited to these cases and the eye monitored carefully for steroid related side effects such as cataracts and glaucoma**
- **Topical calcineurin inhibitors may be of benefit as steroid sparing agents or in the treatment of allergic eye disease where the disease is failing to respond to steroid treatment**

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