Studies on ophthalmic surface laser surgery and corneal regeneration following excimer laser treatment

Ph.D. Thesis

A cornea felszíni-lézersebészet körülményeinek és a cornea regenerációjának tanulmányozása excimer-lézer kezelés után

Egyetemi doktori értekezés

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Contents

Abbreviations

7-AAD	-7 amino-actinomycine D
ArF	-argon fluorid
Cx	-connexin
D	-dioptria
EGFR	-epithelial growth factor receptor
EM	-electron microscopy
FITC	-fluorescein-isothiocyanate
FLFC	-femtosecond laser flap creation
FLK	-femtosecond laser keratomileusis
FSLS	-femtosecond surgical laser system
HMW	-high molecular weight
ICR	-intracorneal ring
kD	-kiloDalton
LASIK	-laser in situ keratomileusis
LIOB	-laser induced optical breakdown
LMW	-low molecular weight
Nd-YAG	-neodynium-yttrium-aluminium-granit
Nd-YLF	-neodynium-yttrium-litium-fluorid
PBS	-phosphate buffered saline
PMMA	-polymethyl-metacrylate
PRK	-photorefractive keratectomy
PTK	-phototherapeutic keratectomy
RK	-radial keratotomy
TBS	-tris-buffered saline
UV	-ultraviolet

List of full papers related to the subject of the thesis:

I. Mohay J, Süveges I, **Ratkay I**, Füst Á, Bor Zs, Szabó G, Rácz B, Virág Sz: Szaruhártya scanning elektronmikroszkópiás vizsgálata excimer laser keratectomiát követõen. *Szemészet* 129:31-33,1992.

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X. **Ratkay I**, Förster W, Busse H, Kolozsvári L: Tapasztalataink a szaruhártya excimer lézeres fototerápiájában. I. Disztrófiás és hegesedéssel járó esetek. *Szemészet* 135:146-151,1998.

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XIII. Ferincz IE, **Ratkay-Traub I**, Bor Zs: Age and intended correction dependence of effective ablation rate during photorefractive keratectomy. *Laser Physics* 10:485-488,2000.

XIV. Spooner GJR, Juhasz T, **Ratkay-Traub I**, Djotyan G, Horvath C, Sacks Z, Marre G, Miller D, Williams AR, Kurtz R: New Development in Ophthalmic Applications of Ultrafast Lasers. *Proceedings of SPIE* 3934:62-72,2000.

XV. **Ratkay-Traub I**, Hopp B, Bor Zs, Dux L, Becker DL, Krenacs T: Regeneration of rabbit corneal epithelium following excimer laser photorefractiv keratectomy: the role of gap junctions, epithelial junctions, soluble factors and cell proliferation. (submitted to Exp Eye Res)

XVI. Juhasz T, Kurtz R, Horvath C, **Ratkay-Traub** I, Nordan L, Jotyan G, Mourou G: Initial demonstration of high precision, subsurface femtosecond laser surgery in humans. (submitted to Science)

XVII. Ferincz IE, **Ratkay I**, Bor Zs: Age dependence of the ablation rate during photorefractive keratectomy (submitted to J. Refr Surg)

XVIII. **Ratkay-Traub I**, Ferincz I, Kiss K, Juhasz T, Kurtz R: The first clinical results with femtosecond laser microkeratome. (submitted to J. Refr Surg)

Summary

Studies on the biophysical effects of excimer laser in the cornea

Excimer lasers have been used for corneal surface surgery since 1988, however, the biophysical mechanism of photoablation of the cornea is still incompletely understood. In a team with laser physicists we analyzed and visually dissected the biophysical interaction between individual laser pulses and the corneal surface. A high-speed laser-based photographic arrangement was constructed, with a temporal resolution of <1nsec, which could also be converted to a shadowgraph setup. By utilizing these facilities we could detect the ejection of the ablation plume and the formation of shock waves in the air above the eye caused by individual laser shots. In addition, we could demonstrate, for the first time, high-amplitude surface waves propagated in the targeted corneal surface.

Our further studies were focused on the surgical use of the excimer laser. Laser parameters were tested on *ex vivo* pig eyes and the wound healing process was studied *in vivo* in rabbit eyes. Scanning electron microscopy proved the high spatial accuracy of the 193nm UV laser in pig corneas. In preclinical studies, we found positive correlation between the etching depth and the number of laser shots and between the etching rate and the logarithm of the energy density. Based on these results we could calculate the value of absorption coefficient and the range of the fluence applicable for safe corneal surgery. Further studies, by treating groups of *ex vivo* sheep and lamb corneas or a polymethyl-methacylate (PMMA) test resin system, showed that: 1. with increased diameter of the ablation zone the ablation rate is significantly decreased, 2. there is no correlation between the repetition rate (up to 30Hz) and the ablation rate when energy density is constant, and 3. there is a positive correlation between the energy density and ablation rate at constant ablation diameter.

Molecular and morphological studies on the healing rabbit cornea after laser ablation

The visual performance of corneas following excimer laser photorefractive keratectomy is highly dependent on the rapid and co-ordinated rebuilding of its epithelium and stroma which is based on intimate interactions between the cells, matrix and soluble factors involved. There had been only few experimental reports studying corneal regeneration after excimer laser treatment and very limited attention focused on the role of direct cell-cell communication in wound healing. In a series of experiments we investigated the healing process in rabbits by devoting particular attention to gap junction expression correlated with the rearrangement of other cell junctions, cell proliferation and epidermal growth factor receptor (EGFR)

expression. We found two types of gap junctions, connexin43 (Cx43) and Cx26, the latter of which had not been described in the cornea before, appearing as early as in the migrating, highly proliferating epithelium which also expressed high level of EGFR. In untreated corneas Cx-s were localized to different regions of the same basal epithelial cells, Cx26 (β2) junctions were concentrated on the basolateral side while Cx43 (α1) types on the apical cell membranes. Since the two isotypes represent evolutionary different families of Cx-s (Cx43 is α1 and Cx26 is β2) they can not form heteromeric functional channels and they have selective permeabilities to molecules of ~<1kD. Therefore, they may represent alternative pathways for fine tuning direct cell-cell communication between basal epithelial cells.

During wound repair, the expression of both Cx-s were transiently upregulated by appearing in upper cell layers also, which suggested the involvement of direct cell-cell communication in corneal wound healing. Cell proliferation in corneal epithelium is facilitated by growth factor receptor activation including EGFR, particularly upon wound healing. Proliferating cell pool in both unwounded and wounded corneal epithelium had been thought to be restricted to the limbal basal cell region where EGFR expression is also elevated. In our experiments not only epithelial cells adjacent to the wound but those migrating towards and over the wounded area were found to show and excessive proliferation rate and high level of membrane-bound EGFR expression. Mitotic activity in the migrating and pharmacologically not influenced corneal epithelial cells is another important and novel finding in our experiments which is most probably the sign of the excessive demand for new epithelial cells not met by the proliferating limbal stock alone, due to the relatively large size of the wound. Our results showed that gap junctional communication is most probably involved in the regulation of wound repair, and the proliferative capacity of the basal corneal epithelium is highly flexible and it is not restricted to the peripherial areas when robust healing is required.

Clinical use of excimer laser for phototherapeutic and photorefractive

To improve the efficiency of photorefractive keratectomy (PRK) we monitored the long-term refractive outcome at several hundreds of our treated patients. We found that in patients older than ~40 years the effective ablation rate proportionally increased with age, therefore, the intended correction calculated on the basis of the corneal refraction before treatment may cause overcorrection. As opposed to them, in the younger group up to ~30 years of age, the calculated intended correction may result in undercorrection. These observations may be explained by the decreasing hydration rate of the cornea with age, since there is a negative

correlation between the ablation rate of the laser and the hydration of the corneal stroma. In cooperation with physicists colleagues, we elaborated nomogram based on a calculation equation which summarizes these correlations and assist in calculating the value of necessary compensation. By using this we can effectively reduce the risk of over- and undercorrection.
We also observed, that larger ablation diameter caused less side-effect including halos, and regressions, however, deeper ablation due to the increased diameter may have a risk of haze. We used the excimer laser also for therapeutic purposes to treat superficial corneal diseases in 252 eyes, suffering from recurrent erosion, band keratopathy, map-dot-fingerprint-, crystalline dystrophy, amyloidosis, scars or pterygium. In comparison to PRK, PTK is not a standardized procedure and in many cases only the symptoms are treated. To reduce the risk of hyperopic effect, we modified our earlier surgical strategy for combining the possible largest ablation zone with the most superficial PTK. We also found diclofenac sodium to effectively reduce postoperative pain and the need for systemic analgesics after PTK and PRK without any delay in wound healing and without using a contact lens.

Animal studies and clinical trials with an ultrafast femtosecond laser

By now the laser-assisted in situ keratomileusis (LASIK), where flap creation preceds excimer laser ablation of the corneal stroma, has become the most commonly performed refractive surgical technique.
Flap creation with a mechanical microkeratome is the source of most LASIK complications. To overcome this a high repetition-rate femtosecond laser with computer controlled scanning optical delivery system was developed. Femtosecond laser offers clear advantages for flap creation including the flexible adaptation of the size and depth of the flap, the lack of unwanted increase in intraocular pressure. All of these promise better reproducibility and postoperative flap stability, greater clinical safety and less pain and side effects. I have had the privilege to test this revolutinary new laser, for the firs time, in animal studies and later, in a clinical trial in non-sighted and partially sighted eyes. We could optimize the dissection and surface quality, the side cut and hinge architecture of the flaps produced by femtosecond laser, and succesfully used the Pulsion FS1 instrument for femtosecond laser keratomileusis, IntraLasik, intracorneal ring implantation and intrastromal corneal refractive surgery. The latter intervention is based on the speciality of the ultrafast femtosecond laser which allows to perform surgical cuts in the corneal stroma without any interference with the overlying cell layers.

Aims of the dissertation

1. To better understand the biophysical mechanisms of action of the 193nm excimer laser in order to more accurately set up laser parameters for clinical applications and therefore, to improve the efficiency, safety and reproducibility of the laser treatment.
2. To reveal how the changing physiology of the human cornea with age require corrections of the laser parameters in order to achieve the intended ablation rate and by doing so the necessary myopic correction.
3. To analyze the molecular and cellular mechanisms involved in the corneal wound repair process following excimer laser ablation. To reveal the possible role of direct cell-cell communication through gap junctions in normal corneal physiology and in the healing cornea in correlation with other epithelial cell junctions, epithelial cell proliferation and epidermal growth factor receptor expression.
4. To establish the potential in ophthalmologic surgery of a revolutionary, ultrafast laser type, the Nd-glass femtosecond laser through *in vitro* studies, animal experiments and early clinical trials.
5. To utilize research results of points 1-4 for clinical applications and validate laser treatment protocols by determining their medical efficiency on a large number of patients undergone phototherapeutic or photorefractive laser surgery.

1. Introduction

1.1. Background

The use of laser technology in the medical practice has developed rapidly. Excimer laser, for instance, has gained widespread applications in ophthalmologic surgery worldwide and found its way into the ophthalmologic practice in Hungary in the last decade. By now several thousands of corneal laser treatments are done in one year in Hungary alone for photorefractive (photorefractive keratectomy, PRK) and therapeutic (phototherapeutic keratectomy, PTK) purposes (Süveges et al. 1994, Német et al. 1994, Nagy et al. 1996, 1998, Ratkay et al. 1998a,b,c, Hassan et al. 1999).

I had the privilege to be part of the first Hungarian study group, including laser physicists, Zsolt Bor, Gábor Szabó, Béla Rácz and Béla Hopp (Department of Optics and Quantum Electronics) and ophthalmologist colleagues, Ildikó Süveges, Judit Mohay and Ágnes Füst (Department of Ophthalmology), which formed in 1991 at the University of Szeged. Our group played a leading role in Hungary in studying the physics and biological effects of the 193nm excimer laser in experimental corneal surgery in order to properly set up laser parameters for clinical applications (Mohay et al. 1992, Bor et al. 1993a,b,c).

My research experience gained in animal models could later be extended to the clinical practice when I spent nearly two years starting in 1993 at the Department of Ophthalmology of the Westfalien Willhelms University in Münster. There, my scientific interest was focused on applied research on a large number of patients in connection with the use of the 193nm excimer laser for therapeutic and refractive purposes (Förster et al. 1995b, 1996a,b, 1997b, Ratkay et al. 1998a,b).

Recently, in a joint project with the Department of Pathology, University of Szeged we have investigated the molecular basis of corneal wound healing in a rabbit model after excimer laser treatment, with particular attention to the direct cell-cell communication through gap junctions (Ratkay et al. 1998d, 2000). In another cooperation with a research group from the USA we have been studying the circumstances for the clinical applications of a revolutionary new laser type, Nd-glass infrared femtosecond laser, which offers further improvements of the available ophthalmologic laser treatment protocols and chances for introducing new and more efficient treatment techniques (Juhasz et al. 1996, 2000, Spooner et al. 2000, Ratkay-Traub et al. 1999a,b, 2000a,b,c).

1.2. Principles of ophthalmic surface lasers

The 193nm ArF excimer laser is an ultraviolet (UV) laser of short repeated pulses. At this wavelength each photon has an energy of 6-4 electron volts, which exceeds the binding force of carbon-carbon bonds, therefore a single absorbed photon may lead to breakdown of these bonds (Marshall et al. 1985). Since UV radiation in this spectral domain does not propagate well in air and at any biological interfaces, the photons are virtually all absorbed within a few micron thick surface layer of the corneal tissue leading to its ablation (**Figure 1**). It has no harmful effect outside the targeted tissue layers of the eye (Trockel et al. 1983, Marschall et al. 1988, McDonald et al. 1990).

While other clinical lasers work by concentrating laser energy into a focused point, the excimer laser beam has a large cross-sectional area with the potential in a scanning mode to produce homogenous tissue ablation through a 5-7mm diameter treated area (Mohay et al. 1992, Ratkay et al. 1994, Füst et al. 1994). Based on this, the 193nm excimer laser has been utilized for the selective removal of superficial corneal tissue for photorefractive (Munnerlyn et al. 1988, Seiler et al. 1990, McDonald et al. 1991) and phototherapeutic (Campos et al. 1993, Dausch et al. 1994, Gartry et al. 1991, Förster et al. 1997b) utilizations. There have been several versions of the 193nm excimer laser instruments available.

Infrared Neodynium-Glass femtosecond laser (ultrafast) uses infrared light similar to a Neodynium-YAG laser, which latter is widely used in invasive surgery, except that in a femtosecond laser each laser pulse is approximately one hundred thousand times shorter in duration, lasting only about ten to the minus thirteenth seconds (Vogel et al. 1994, Juhasz et al. 1996, Spooner et al. 2000).

Unlike photothermal and photoablative lasers, the high peak intensities of the femtosecond laser allow it to create a plasma inside of transparent tissues, like the cornea, without interfering with the surface cell layers (**Figure 2**). Femtosecond laser pulses require significantly less energy to produce photodisruption when compared to longer pulsewidth lasers in the picosecond and nanosecond regimes (Krueger et al. 1998, Kurtz et al. 1998a). This lower energy threshold translates into smaller cavitation bubble size (termed microcavitation) (**Figure 3**), allowing nearly contiguous placement of laser pulses. Computer controlled, high precision delivery system optics, capable of scanning a focused beam over a 10 mm working diameter with micron-range accuracy have been developed recently, making the ultrafast lasers ready for biological applications. Our research group was one of the first to perform *ex vivo* and *in vivo* animal studies for potential corneal applications of this new laser

type (Kurtz et al. 1998b, Yen et al. 1999). Most recently, also for the first time, we have started up human clinical trials with a femtosecond laser instrument following the approval from the Food and Drug Administration (FDA) in the United States and permissions from the Hungarian health authorities (Ratkay-Traub et al. 1999a,b, 2000a,b,c). Additional trials with the femtosecond laser for surgical applications in the sclera and lens have also shown significant potential (Vogel et al. 1994, Spooner et al. 2000).

1.3. Biophysics of laser ablation of the cornea

High-power ultraviolet (193nm, ArF) excimer lasers are capable of producing precise optical etching of the cornea. The technique can be used for PRK and for smoothing the anterior corneal surface (Trockel et al. 1983; Marshall et al. 1985). Though excimer lasers were extensively used for corneal surgery in some countries by the early 90'-s, the biophysical mechanism of action during photoablation was still incompletely understood (Puliafito et al. 1985, Krauss et al. 1986).

Our laser physicists constructed a high speed laser-based photographic arrangement which had a temporal resolution better than 1ns. The setup could work as a Schlieren arrangement, which is sensitive to the refractive index change caused by the laser shock wave above the targeted corneal cells (Longhurst et al. 1973). With this setup we had the chance to detect different stages of the ejection of ablation plume and the formation of shock waves in the air above the eye within a microsecond and right after a high amplitude surface waves propagating on the corneal surface (Bor et al. 1993a,b).

We also studied the precision achievable with the excimer laser in comparison to traditional diamond knife surgery. A variety of incision and ablation wounds were produced in the cornea of enucleated pig eyes and the wound edges and surfaces were analyzed with scanning electron microscopy (Mohay et al. 1992). In line with the results of other groups (Seiler et al. 1990) there was a high degree of accuracy (1μm) and sharp wound-edges, little cell loss in the surrounding epithelium, and only small amount of cell debris in the stroma (Bor et al. 1993a, Ratkay et al. 1994).

For clinical applications, the correlation between the etching rate and the fluence (energy density) as well as between the etching depth and the number of laser shots, were also studied and established (Bor et al. 1993b,c). In further studies we determined the correlation's of the frequency of laser shots (repetition rate), the energy density of the laser (fluence), and ablation diameter to the ablation rate *ex vivo* and *in vitro* by using sheep and lamb eyes as well as a PMMA (polymethyl-metacrylate) model system (Förster et al. 1995a).

The long term refractive outcome of PRK may depend on several factors such as age, intended correction, corneal hydration, and the course of wound healing (Dougherty et al. 1994, Rasik et al. 1996). Basically, the refraction correction achieved depends on the amount of tissue removed, modified by wound healing. Unexpected variations in corneal wound healing and in the corneal ablation rate due to changing corneal physiology with age, however, affect the predictability of PRK. In a study we have defined the effective ablation rate as the etching rate calculated from the difference between intended correction and the achieved correction measured 6 months after PRK (Ferincz et al. 1999, 2000).

1.4. Morphological and molecular studies on corneal wound healing following excimer laser treatment

1.4.1. Gap junctional communication in stationary and wounding cornea

Re-epithelialization and stromal regeneration after corneal ablation seems to happen in a highly organized way which may reflect an intimate cooperation between the cells, matrix and soluble factors involved. After wounding a cohesive sheet of epithelium migrates rapidly over the rebuilding stroma to fully cover the wound (Gipson 1992, Shultz et al. 1992, Wilson et al. 1999). Proliferation of the epithelial cells followed by the rearrangement of their cytoskeleton and interactions between them and their microenvironment characterize this process until stratified epithelium highly resembling to that before treatment has been reformed in a few days (Hanna 1966).

Besides growth factors, direct interactions through cell-matrix and cell-cell adhesion molecules and adherent junctional complexes are most probably involved in the regulation of the coordinated repair process (Gipson et al. 1993, Shi et al. 2000). A most recent work calls attention to the possible importance in the wound repair of the syncytial arrangement of epithelial cells mediated by gap junctional coupling (Matic et al. 1997). Gap junctions formed by transmembrane connexins (Cx) represent the substrate of direct cell-cell communication by allowing the immediate passage of ions and small molecules (~ <1 kD) between coupled cells (Kumar and Gilula 1996). In this way cell meshworks may form with synchronized functions within tissues. Connexins are encoded by a multigene family consisting of at least 19 members in vertebrates (Simon and Goodenough, 1998). So far, there have been only two Cx isotypes, Cx43 and Cx50, implicated in the corneal functions including the repair process (Dong et al. 1994, Matic et al. 1997).

In a pilot study we found Cx26 in rabbit cornea, another Cx isotype typical of stratified epithelia, which could not be detected yet by others (Risek et al. 1994, Matic et al. 1997). In

addition, our sensitive reactions and the projected series of virtual sections in confocal laser scanning microscopy offered a much clearer resolution of the localization of particulate gap junctions (Krenács et al. 1997) that had been achieved in earlier works. The chance for revealing additional Cx isotypes and refining existing data has driven us to further study direct cell-cell communication in normal/stationary and regenerating rabbit corneas (Ratkay et al. 1998d, 2000). In our model we used laser keratectomy, which has nowadays much more relevance to clinical applications and has a slightly different mechanism of action than traditional surgery and has not yet been investigated in rabbit in this context.

1.4.2. Proliferation, growth factor receptor expression and cell interactions in wounding corneal epithelium

Studies on corneal wound repair usually focus on only one or limited aspects of the regeneration process, e.g. cell proliferation, growth factors and their receptor expression, epithelial adherence, gap junctional communication etc., respectively (Schultz et al. 1994, Matic et al. 1977, Wilson et al. 1999). It is, however, very difficult to fully correlate bunches of individual results without using identical experimental conditions for all the analyzed factors.

In our work we used comprehensive approach for studying the spatio-temporal distribution of epithelial cell junctions during the regeneration process in rabbit corneas following excimer laser keratectomy with particular attention to the communicating channels (Ratkay et al. 1998d, 2000). Early inflammatory and stromal responses, epithelial movements, desmosomes, hemidesmosomes and gap junctions were studied ultrastructurally, in semithin resin sections and in frozen sections with immunofluroescence labelling and confocal laser scanning microscopy. Since gap junctions have been implicated in the control of cell proliferation and the regulation of cell growth it seemed logical to correlate gap junction data with the proliferation activity and epidermal growth factor receptor (EGFR) expression of the wounding epithelium.

1.5. Clinical results with ophthalmic surface lasers

1.5.1. Excimer laser phototherapeutic keratectomy

In the treatment of superficial corneal diseases the surgeon has to face two major problems. Firstly, the normal cornea's optical, mechanical and biological properties should be maintained. Secondly, corneal surgery, like any surgery, should be safe, effective and reproducible.

PTK has been used to treat different corneal diseases such as recurrent erosions, band keratopathy, anterior stromal dystrophies and scarring from post-infectious and post-traumatic causes (Sher et al. 1991, Campos et al. 1993, Fagerholm et al. 1993, O'Brart et al. 1993, Dausch et al. 1994). The smoothing of a rough anterior corneal surface involves the use of a masking fluid to fill localized depressions, and subsequently only the elevated corneal tissue is ablated (Kornmehl et al. 1991). We report on therapeutic indications and strategies, postoperative regimens and the results of PTK treatment in 252 eyes (Förster et al. 1997b, Ratkay et al. 1998a,b).

To reduce the pain after PTK and PRK we have also applied topical diclofenac sodium and made a retrospective study about its analgesic and epithelial wound healing effects (Förster et al. 1997a).

1.5.2. Excimer laser photorefractive keratectomy

Excimer lasers have gained their most widespread applications in ophthalmology for ablating corneal tissue in order to correct myopia (low, moderate and high) and myopic astigmatism (e.g. McDonald et al. 1991). We studied the influence of the diameter of ablation zone in myopic PRK by using two different instruments, a Schwind Keratome II and a VISX 20/20 excimer laser respectively (Förster et al. 1996b). I have also analyzed more than a hundred cases when rest myopia was corrected with excimer laser treatment following radial keratectomy (RK) (Ratkay et al. 1998c). Based on our research translated into the clinical practice I have so far performed more thousand laser PRK with a ~97% success rate. Refractive overcorrection, a possible complication of myopic PRK, may be corrected by using contact lenses or mechanical redebridement of the epithelium if it is persistent (Förster et al. 1996a).

1.5.3. Femtosecond laser for corneal surgery

The pulsed visible and near infrared lasers are not absorbed by the refractors of the eye, therefore laser light can access the bulbus without a surgical cut (Spooner et al. 2000, Juhasz et al. 2000). In co-operation with the University of Michigan (Ann Arbor, MI, USA) and the IntraLase Corporation (Irvine, CA, USA) we have performed animal experiments with a Pulsion^{TM} FS equipment and early clinical studies to prove, for first time, the efficiency of such kind of laser in ophthalmic surgery.

Laser assisted in situ keratomileusis (LASIK), based on creating a corneal flap (Pallikaris et al. 1991, 1997) by using the microkeratome, has been one of the most commonly performed

surgical procedures in the USA for correcting refractive errors. Though LASIK has a high success rate, complications have been reported (Seiler et al. 1998, Alio et al. 2000) in approximately 2-6% of cases, with visually significant consequences in about 10% of them. The majority of these are linked with the deficiencies of microkeratome performance.

A high precision laser keratome, such as the Pulsion FS Nd-glass laser using femtosecond pulses, might reduce the potential for the most common LASIK complications. I had the privilege to be part of a series of clinical trials to evaluate, for the first time, the efficiency of the Pulsion FS-1 laser as a flap-cutting microkeratome (IntraLasik^{TM}) (Ratkay-Traub et al. 1999a,b, 2000a,b) and as a resecting tool for the creation of intrastromal tunnels and entry cuts for placement of intracorneal ring (ICR) (Ratkay-Traub 2000c) segments. We also used the femtosecond laser for intrastromal ablation (femto-ISPRK) for treating low myopia and hyperopia without any damage of the upper layers including epithelium, nerves and the Bowman membrane, and for keratomileusis (FLK) to create a flap-cut and corneal lens removal to treat high myopia or hyperopia without excimer laser.

2. Materials and Methods

2.1. Biophysics of excimer laser ablation of the cornea

2.1.1. Plume emission, shock wave and surface wave formation

An ultra-fast stroboscopic system was set up for these experiments. The ArF laser beam (Lambda Physics EMG 102 MSC, wavelength: 193 nm; energy: 100 mJ; pulse duration: 15 ns; repetition rate: 1 pulse per second) was focused onto enucleated pig eyes by a fused silica lens of a focal length of 100 mm. The energy density was in the range between 0.3 - 1 J/cm^2.

The contour of the eyes was observed and recorded with a x24 magnification video camera (ITT Nokia VMC 3680 AF) set on a Brinnel microscope. The eye was illuminated by 1 ns pulse with a home-built N_2laser-pumped, short pulse dye laser operating at the orange spectral range (590 nm). The duration of the dye laser pulse was further reduced to 100 ps. The delay between the excimer and dye laser pulse ranged between 1-10μs. The timing was controlled by a photodiode and an oscilloscope.

The geometry of the illumination was arranged so that no direct light could enter the videorecorder, therefore the background of the photographs was dark. This optical system can be regarded as a stroboscopic Schlieren arrangement which can visualize the change of the refractive index of the air caused by the shock wave propagating above the eye. Due to the very short duration of the pulse (1 ns) there was no noticeable change in the illumination of

the eye, allowing frozen images of the shock waves to be recorded. The photographs were taken from the screen of the videomonitor.

2.1.2. The effect of laser parameters on the ablation rate

194 sheep eyes, 130 lamb eyes and 108 PMMA plates were ablated by using the Schwind Keratom I (Kleinostheim, Germany) 193 nm excimer laser. The eyes were kept on ice and immersed in a Dextran 250 solution for 5 min to avoid corneal swelling before surgery which was done within 2h of death. The preoperative intraocular pressure was standardised at about 18 Hgmm by injecting 0,9% NaCl solution into the corpus vitreum. The corneal thickness was calculated from the average of 3 measurements made just before ablation with an ultrasonic pachimeter (Storz Co., St. Louis, MO, USA). The laser beam was centered on the entrance pupil of the eyes.

Calculation of ablation rate was based on testing the effects of the gradual changes in one parameter at a time in three treatment groups where circular ablation was performed until perforation of the cornea was noted by the surgeon using the operating microscope. In each group 35 sheep eyes, 35 lamb eyes and 30 PMMA plates were used in five sets of seven-seven eyes and of six PMMA. In group 1 samples were ablated to 1.5, 3, 4.5, 6 and 7,5 mm diameters respectively in each set at a constant energy density (~200 mJ/cm^2) and pulse rate (10 Hz). In Group 2 ablations were performed by using repetition rates of 5, 10, 15, 20, 25 and 30 Hz respectively in each set at a constant energy density (~200 mJ/cm^2) and ablation area (6 mm). In Group 3 ablations were made by using different energy densities (88 - 126 - 167 - 201 - 224 mJ/cm^2) in each set at a constant (6 mm) ablation diameter and the repetition rate (10 Hz). No nitrogen gas was blown over the corneal surface. The total number of pulses necessary to perforate each cornea was recorded. The ablation rate was determined by dividing the preoperative corneal thickness by the total number of pulses.

2.1.3. Age dependence of the ablation rate during photorefractive keratectomy

Spherical myopic - and myopic-astigmatic PRK were performed by a Schwind Keratom 2F excimer laser on 613 eyes of 348 patients. Emitting radiation was at 193 nm, maximum pulse rate 10 Hz and radiant exposure 160 ± 4 mJ/cm^2. The software calculated ablation rate was 250 ± 5 nm/pulse which was calibrated, like the fluence, by perforating a known thickness of gelatin sheet with excimer laser shots. 311 cases were selected, where the follow-up was at least 6 months. Patients were assessed 1 week and 1, 3, and 6 months postoperatively and

their mean age was 30.2 ± 7.01 years. The intended spherical corrections were in the range of -1.0 D to -9.0 D. In 48% of cases, coexisting astigmatism was also corrected. In 70% of the cases (N=218) a preoperative spectacle-corrected visual acuity of 20/20 existed, in 19% of them (N=60) it was 20/25, and in 21% (N=33) it was at least 20/63.

Pre- and postoperative examinations included slit-lamp and tear film checking, cornea topography, ultrasonic biometry and pachymetry, direct and indirect ophthalmoscopy, uncorrected visual acuity, spectacle-corrected visual acuity using a Snellen chart, and manifest and cycloplegic refractions.

Surgery was performed under topical anesthesia. The lids were kept open with a lid speculum and the patients were asked to fix their eyes on a red, blinking light. Ablation area was marked, then epithelium was removed by mechanical scraping. The diameter of the ablation zone for PRK was at least 5.5 mm, depending on the required correction. In case of coexisting astigmatism the shorter axis was equal or larger than 5.5 mm (the maximum diameter was 8 mm). After the procedure the eyes received antibiotic drops and a pressure patch. For postoperative therapy patients had taken antibiotic drops for a week, and artificial tear film for 3 months. A week after PRK drops containing flouromethazon had been used for 2 months, 4 times daily. Follow-up were made1 week, and 1, 3, and 6 months after PRK.

The effective ablation rate can be calculated by the following equation: $\kappa_{eff} = 207 + 0.53 \cdot A - 3.16 \cdot D_i$, where **A** is the patient age in years, $\mathbf{D_i}$ is the intended correction in diopters, and $\boldsymbol{\kappa_{eff}}$ is the effective ablation rate in nm/pulse.

2.2. Morphological and molecular studies on corneal wound healing

2.2.1. Treatment protocol after excimer laser treatment on rabbit eyes

Three-month old New-Zealand albino rabbits were anesthetized intramuscularly with 1ml/ body weight Calypsol. The corneas of the right eyes of 22 rabbits were ablated with a 193 nm ArF excimer laser (Lambda Physik EMG 102 MSC). The constant ablation diameter was 6 mm, repetition rate 2 Hz, fluence 150+-10 mJ/cm^2 and the ablation depth 90 μm. Treated corneas collected 6, 16 hours; 1, 2, 3 and 7 days; and 1 and 3 month after surgery were removed in anesthesia. Based on earlier studies, special attention was focused on days 1,2 and 3 by checking 4 samples each. A 2 mm slice was cut throughout the central parts of each removed cornea, the cut pieces were halved in the middle to ensure that treated-untreated transitions could be properly subjected to electron microscopy and immunohistochemistry respectively. Pieces of removed corneas left behind were used for protein detection with

SDS polyacrylamide gel-electrophoresis. The left eyes of the animals were used as untreated controls some of which were cut including the corneo-scleral limbus. Animals were treated during the experiments in accordance with the Animal (Scientific) Act, 1986 and the regulations for animal experiments of the Ethical Committee at the University of Szeged.

2.2.2. Gel electrophoresis

Each of the treated corneas and the untreated controls were mechanically homogenised in 1ml phosphate buffered saline (PBS), their protein concentrations were measured at 280nm and they were further diluted in a sample buffer containing 50 mM Tris-HCl, pH 6.8; 2 % SDS, 10 % glycerol, 3 % 2-mercapto-ethanol and 0.001 % bromophenol blue. From each sample a final concentration of 8 μg/ml protein per well was subjected to polyacrylamide gel electrophoresis. Molecular weight markers of Pharmacia Biotech (USA, HMW: 212, 170, 116, 76, 53 and LMV: 94, 67, 43, 30, 20, 14) were also run in the gel. Ten cm gels containing 5 % (stacking gel), 12 % acrylamide (separating gel), 0.8% N,N'-bis-methylene acrylamide and 0.1 % SDS were polymerised with 0.025 % of tetramethyl-ethylenediamine (TEMED) and ammonium persulphate at room temperature (Laemmli, 1970, Nature). The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine and 0.1 % SDS. Electrophoresis was carried out with 8 mA current (50-60V) for 10-15 min in the stacking gel and with 12 mA (150-200V) in the separating gel until the bromophenol blue marker reached the bottom of the gel (about 75 min). The proteins were then fixed overnight in the gel with a solution containing 12% acetic acid, 50% methanol and 0.02% formaldehyde.

Silver-staining with a mixture of 0.2% silver nitrate and 0.03% formaldehyde for 25 min followed by development with a solution of 6% sodium carbonate, 0.02% formaldehyde and 0.0005% sodium thiosulfate for 3-5 min was used to reveal protein bands. Photographs on the stained gels were taken after fixation in a mixture of 50% methanol and 16% acetic acid.

2.2.3. Electron microscopy

Pieces of corneas were fixed in a mixture of freshly prepared 1% paraformaldehyde and 3% glutaraldehyde in neutral phosphate buffered saline (PBS) for 12-16 hours at 4^{o}C. Then the samples were washed in PBS, postfixed in 1% osmium tetroxide, contrasted with uranyl acetate-lead citrate, dehydrated in graded ethanol series completed with acetone and propylene-oxide, and infiltrated with an epoxy resin (TAAB 812, TAAB Laboratories, England). Finally, the tissue pieces were embedded in TAAB 812 perpendicular to the treated surface to ensure treated-untreated transitions to be localized in the middle of the cut

sections. One 1μm thick semithin sections stained with methylene blue-basic fuchsine were used to select areas for transmission electron microscopy which was performed in 70 nm thin sections with a Philips CM10 instrument.

2.2.4. Immunohistochemistry

For immunostaining, pieces of corneas oriented perpendicular to the treated surface were rapidly frozen in liquid nitrogen and 8 μm thick sections were cut in the cryostat. Frozen sections were let dry for 10 min and fixed in acetone at room temperature for 5 min. After further drying for 2-2 hours the sections were incubated with a blocking solution consisting of 1% BSA and 0.1 % sodium azide in neutral Tris-buffered saline (TBS). Immunohistochemical reactions included the following steps: 1) incubation with monoclonal mouse anti-Cx43 (clone: CX-1B1, 1:200, Zymed Laboratories Inc., South San Francisco, CA; or clone:Gap1, 1:200; DL Becker, UCL, London, UK), anti-Cx32 (clone:CX-2C2, 1:200, Zymed), anti-Cx26 (clone:Cx-12H10,1:200, Zymed), anti-epidermal growth factor receptor (NCL-EGFR, clone:EGFR.113, 1:30, NovoCastra, Newcastle, UK) or anti-Ki67 protein (clone:Mib1, 1:50, Immunotech, Marseille, France); or with rabbit anti-Cx43 (Gap15, 1:200), anti-Cx26 (Des3, 1:300; both from WH Evans, University of Cardiff, UK), anti-Cx40 (1:200, from RG Gourdie, MUSC, Charlestone, SC, USA) or anti-Cx37 (Rb442, 1:200, DL Becker) for 2 h; 2) incubation with biotinylated goat anti-mouse Igs (1:150, Dako), or biotinylated goat anti-rabbit Igs (1:150, Dako) for 40 min; 3) and finally with FITC-labeled streptavidin (1:100, Dako) for 30 min. For further details on Cx antibodies see Krenacs et al, 1995 and Becker et al, 1998. All incubation steps were at room temperature and between them the sections were washed for 3x5 min in TBS. All immunoreagents were diluted in the blocking buffer (*see above*). Finally, in some cases cell nuclei were stained with 7-amino-actinomycine-D (7-AAD, Molecular Probes Europe BV, Leiden, The Netherlands) to assist in the more accurate localization of gap junction plaques. Immunostained sections were mounted with a gelatine-based mountant Faramount (Dako) and analyzed in single and dual channel scanning modes with a Leica TCS SP (Leica Lasertechnique, Heidelberg, Germany) spectral confocal laser scanning microscope.

To calculate the size range of gap junctions digital images were magnified until individual pixels were clearly visible. The average pixel number through the diameter of autofluorescencing conjunctival red blood cells divided by 5, the accepted size of red blood cells in μm, resulted in the pixel number through 1μm at a given magnification. Thirty

particulate junctions were measured in 4 separate images for both Cx-s. The mitotic rate of epithelial cells was calculated in % by dividing the number of counted all basal cells with that of Ki67 positive cells through the same confocal images and multiplied by 100.

2.2.5. In vivo animal studies with Nd-Glass Femtosecond Laser

Intrastromal corneal photodisruption was performed in the eyes of Rhesus Macacus and owl monkeys and New-Zealand albino rabbits by tightly focusing infrared femtosecond laser pulses below the tissue surface *(for technical details see 2.3.4 later).* A computer-controlled delivery system allowed precise laser pulse placements to create corneal flaps and layered intrastromal disruption. The results were evaluated 1, 3, 14, 28, 56 and 84 days after surgery with routine ophthalmological methods *(for details see at 2.1.3)* and after enucleation cornea were also studied with light and scanning electron microscopy.

2.3. Clinical results with ophthalmic surface lasers

2.3.1. Excimer laser phototherapeutic keratectomy

For these clinical studies a Schwind Keratom1 was used. The fluence of the excimer laser was precisely set up to $160\underline{+}5$ mJ/cm^2. The internal repetition rate was 10 Hz, the diameter of the ablation zone was between 3-8 mm depending on the corneal disease. One hour before treatment, the patients received oral analgesia (paracetamol) and topical anesthesia (promethacaine hydrochloride 0.55%), which latter was repeated three times before surgery. Patients with pterygia received additional local anesthesia. Under laser treatment the patient were asked to fix their eyes on a diode laser with their eyelid fixed open by a speculum. The laser was focused on the anterior surface of the cornea and its irregularities of were smoothed under stit-lamp control. Postoperatively, all patients were given antibiotic ointment (ofloxacin), a semipressure binocular patch and oral analgesics until epithelial closure. Postoperative corneal scarring and haze formation was scored according to Hanna et al. (1992) by four ophthalmologists.

Group I: Recurrent erosions (92 patients, 103 eyes)

Most patients came with known trauma, several recurrences within 18 months, and had several unsuccessful attempts of traditional treatments. The affected epithelium was marked and mechanically removed with a hockey-knife. The ablation diameter of the following excimer laser was 1 mm larger than the debried area with a maximum of 8 mm. The whole area was

treated with 15-20 pulses. After epithelial closure, patients received 2% dexpanthenol ointment four times and arteficial tear-film ad libitum.

Group II: Pterygia

Two subgroups: a) pterygia removed using the ’bare-sclera’ technique (42 patients, 50 eyes) and b) pterygia removed by conjunctival transplant (32 patients, 36 eyes).

Scoring, 0: no pterygium, 1: pterygium at the limbus, 2: pterygium up to 2 mm, 3: pterygium up to 4 mm, 4: pterygium of larger than 4 mm. In group IIa, patients’ scores were 1 in 4, 2 in 15, 3 in 17 and 4 in 14. Twenty-four patients were from Central Europe, and 26 from Southern Europe, the Middle East or Africa. In group IIb, there were 12 patients in each group scoring 2, 3 and 4 respectively. Twenty patients were from Central Europe and 16 from Southern Europe, the Middle East or from Africa.

First, all patients underwent surgery appropriate to their grouping, under local anaestesia. Then excimer laser smoothing of the cornea was performed by using methylhydroxypropyl-cellulose masking fluid, with systematically reducing its concentration from 2%, 1%, 0.5%, down to 0.25%. The ablation zone was between 4-8 mm. Postoperatively the patients received prednisolone eye drops (Inflanefran forte) five times a day for 4 weeks. When automatic refraction could not be measured we used the subjective refraction to allow analysis of the induction of astigmatism.

Group III: Bandlike keratopathy (25 patients, 29 eyes)

Only non-sighted or poor-sighted eyes were treated. In all patients the preoperative refraction could not be measured exactly and they suffered from pain due to epithelial breakdown.

If the irregularities of the anterior surface of the cornea were very rough a preliminary smoothening with a hockey-knife was done. PTK and the following postoperative treatment were performed in the same way as in Group II. The ablation zone varied with a maximum diameter of 8 mm.

Group IV: Special indications including scarring (25 patients, 34 eyes)

Epithelial dystrophies were treated like recurrent erosions (*see Group I*).

For amyloidosis, scleroperikeratitis, scars and crystalline dystrophy we followed a treatment protocol similar to that used for bandlike keratopathy (*see Group III*). The ablation zone was between 7.5-8 mm.

2.3.2. Topical Diclofenac Sodium treatment after PTK

One hundred thirty-four patients receiving PTK (*see above*) were enrolled into the study. In 65 eyes topical diclofenac sodium (Voltaren Ophtha Sine, Ciba Vision, Wessling, Germany) was

given before and after surgery three times a day for 3-4 days. The 69 eyes had PTK before topical diclofenac sodium was approved for ophthalmic use with laser surgery in Germany. Patients remained medicated and hospitalized with binocular eye patching until they were pain-free or completely reepithelialized. All patients received paracetamol and topical proxymetacain 3 times in every 10 minutes before surgery. Ofloxacin eye ointment was administered immediately after ablation. Patients received 500mg paracetamol for the first 1-2 days after surgery. If pain persisted, tramadol, metamizol, pethidin, or tilidin were additionally used. The operative reports and patients' medical records were analyzed to assess epithelial wound healing and the use of systemic analgesics.

2.3.3. Excimer laser photorefractive keratectomy

Myopic PRK was performed on 150 eyes (follow-up 6-9 months) up to –9D with a Schwind Keratom I by using single zone ablations fixed either to be 5, 6 or 7 mm. The surgical treatment and medications were according to our standard protocol (*see at 2.1.3*). The results of these three groups were compared.

Another group of 98 patients (105 eyes; 57 male, 41 female), where rest myopia occurred 1-3 years following radial keratotomy (RK) with diamond knifes, were treated with PRK by using a Schwind Keratom 2F. The patients could not wear contact lens and they did not want to wear glasses. Before laser treatment standard eye examinations were done (*see at 2.1.3*), supplemented with slit-lamp photography. The intended corrections were between -1,75 D and -8,0 D. Group1: <-3.0 D (61 patients); Group2: < - 6.0 D (31 patients); and Group3: $\geq$ - 6.0 D (6 patients). The ablation zone was 5.0-6.0 mm. In astigmatism, it was between 6.6 x 5.5 mm and 8.1 x 5.7 mm. The ablations depth was between 23-106 μm. In most patients we tended to apply the larger ablation zones to minimize regression and to reduce halos. Patients were controlled 1 day, 1 week, and 1, 3, 6, 12 and 18 months after the treatment respectively. They received antibiotics eyedropps for 6 days, steroid eyedrops (fluoromethalon or dexamethason) for 1-4 months and artificial tear-film up to 3 months.

2.3.4. Femtosecond laser for human corneal surgery

A clinical trial using a Pulsion^{TM} FS1 equipment has been performed recently in partially sighted eyes. So far, IntraLasik treatment was performed on 46 eyes (46 patients) up to –14D; 5 patients each with one highly myopic and amblyopic eye, got femtosecond laser keratomileusis (FLK); 13 patients each with one myopic or hyperopic and amblyopic eye had intrastromal ablation; and 17 patients received intracorneal rings (Femto-ICR); under an

investigative protocol approved by both local (Hungarian) and U.S. institutional review boards (FDA). In four control patients of the IntraLasik group and one of the Femto-ICR group, no secondary refractive procedure (excimer ablation or ring insertion) was performed in order to evaluate potential refractive effects of the femtosecond laser procedure itself. Pre and post-operative examinations were performed as detailed above (see at 2.1.3) for at least one year.

The laser consists of a solid-state laser source and computer-controlled optical delivery system. The femtosecond (fs) laser operates at 1.05μm wavelength, it emits 500fs long pulses at 3-5 kHz repetition rate. Typical surgical energy was between 4-6μJ per pulse. The alignment of the system was first verified on a test surface. After placements of topical anaesthetics and antibiotic drops a suction ring was applied on the limbus and a contact lens assembly was used to applanate the cornea. The intraocular pressure was ~35 Hgmm. Laser pulses were delivered according to pre-programmed patterns to either create a flap, intrastromal ablation, lens or a channel. For corneal flaps, a spiral pattern was scanned at 160, 180, or 200μm depth, followed by a hinge cut to the surface with partial blocking of the beam. After completion of the laser procedure, suction was released and the contact lens elevated, allowing access to the resected corneal flap, flap and lenticule, or channel. In IntraLasik procedure a traditional excimer ablation (VISX 20/20, Santa Clara, CA, USA) was done under the elevated flap. In femto-ICR, ring segments (Intacs, KeraVision, Fremont, CA, USA) were introduced into the laser-created channels. In FLK, a lens-shaped block of corneal stroma, called lenticule, was removed manually from below the flap. Finally, the flap was easily repositioned due to the cone-shaped edge-cut made at 45°.

3. Results

3.1. Biophysics of excimer laser ablation of the cornea

3.1.1. Mechanism and accuracy of tissue elimination

In a team involving laser physicists and ophthalmologists we have studied how the laser pulse interferes with the affected tissue. Shock wave and surface wave formation in the corneal tissue was dissected, and the spatial accuracy of the incisions was assessed.

With our high speed laser-based photographic arrangement we could visualize the refractive index change caused by the shock wave propagated in the air above the eye (**Figure 4**). Different stages of the shock wave formation could be monitored within microseconds. With minor changes the setup was converted into a shadowgraph providing negative images, the

ablation plume right after the shock wave propagating on the surface of the eye became also visible (**Figure 5**). The ejection velocity of the plume was found to be over 600 m/s. We could additionally show, for the first time, that the recoil forces of the plume are generating a wave moving out-center and resembling to a „shaken-carpet" (**Figure 6**).

In *ex vivo* pig and human corneas the etching rate was proportional to the logarithm of the energy density of the laser and etching depth in the cornea was also proportional with the number of laser shots. These data served to calculate the absorption coefficient of the cornea.

In further studies we determined the correlations of the frequency of laser shots (repetition rate), energy density of the laser (fluence) and the ablation diameter to the ablation rate *in vitro* by using sheep and lamb corneas as well as a PMMA (polymethyl-metacrylate) model system. There was only one factor changed at a time. In group I: With increased diameter of the ablation zone the ablation rate was significantly decreased. In group II: There was no correlation between the repetition rate (up to 30Hz) and the ablation rate when energy density was constant. In group III: There was a positive correlation between the energy density and ablation rate when the diameter of ablation was constant. Ablation rates showed significant differences between PMMA (0.17μm) and sheep and lamb eyes (0.37μm) with no statistical differences between animal eyes.

The analysis of the ultrastructure and the surface properties of the wound edges revealed that the excimer laser photoablation allows for a very high degree of accuracy (1μm), sharp wound edges and only a small amount of cell loss in the epithelium. There was no obvious damage in the surrounding epithelium, stroma and endothelium. Parallel, regular lamellae and pseudomembrane formation with relatively smooth surface were produced by the laser in experiments using a bronze grid on pig eyes (**Figure 7**).

3.1.2. Femtosecond laser

In preliminary studies with intrastromal laser surgery, a photodisruption through 10 parallel spiral layers of 10μm steps were performed in the corneal stroma of Rhesus monkeys. This was designed to demonstrate the volume of tissue removed with each spiral layers. The corneal slit-lamp photographs showed no significant haze or loss of corneal transparency three weeks after this procedure (Figure 8). Corneal pachymetry revealed an 80μm loss in stromal thickness compared to the untreated eye, which was remarkably stabile over a two-month follow-up period. Histologically, though stromal edema was present at day 1-2, little or no inflammatory response and only some active keratocytes and a thin layer of scar around

the treated area were seen at any point during the 3 months study. Bowman's layer, adjacent stroma, Descemet's membrane, and the endothelium remained intact throughout.

Highly precise and smoother cut surface at the corneal bed was achieved when femtosecond laser was used to create a corneal flap when compared to that produced with a mechanical microkeratome (Figure 9-10). Femtosecond laser proved also to be superior than a laser using picosecond pulses for producing and removing an intrastromal corneal lenticule through a flap. It produced a much higher degree of accuracy when compared with scanning electron microscopy (Figure 11).

3.1.3. Age and intended correction dependence of excimer laser ablation

The uncorrected visual acuity 6 months after PRK showed at least similar or slightly better values than the spectacle-corrected acuity before treatment (**Figure 12)**. **Figure 13** summarizes the induced post-operative refraction changes at 1 week and 1, 3, and 6 months after surgery. Before treatment the mean spherical refraction was -4.52 ± 2.14 D. One week after surgery the mean value reflected slight overcorrection (0.84 ± 1.11 D) which gradually decreased through emmetropia until stabilized slightly below zero (-0.40 ± 0.73 D) 6 months after PRK.

According to the equation set up for calculating the effective ablation rate *(see at 2.1.3)* the effective ablation rate showed a positive correlation both with the degree of myopia and the age of the patients, as it is summarized on **Figure 14**. For example, above 40 years of age and in case of the -6 D or higher intended correction, the increased effective ablation rate is very likely to lead to hyperopic results.

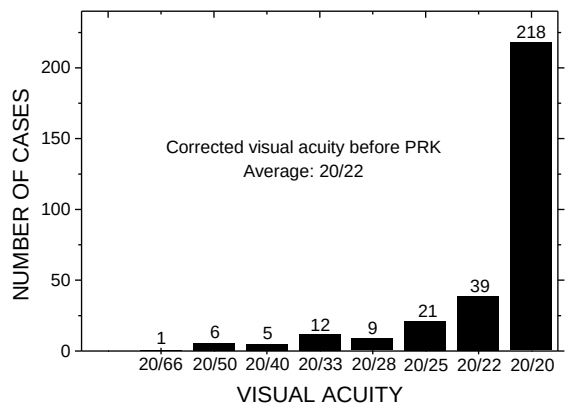

Figure 12a. Distribution of the best spectacle-corrected visual acuity before PRK.

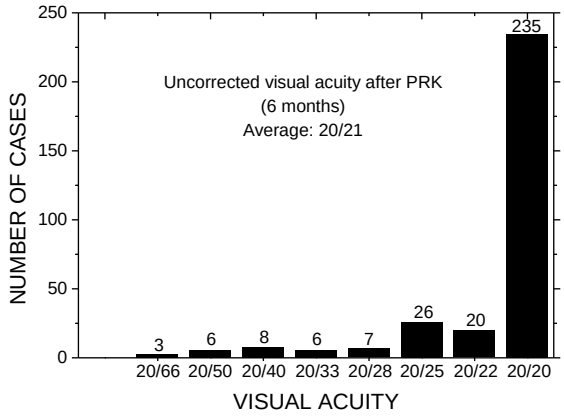

Figure 12b: Distribution of the uncorrected visual acuity 6 months after PRK.

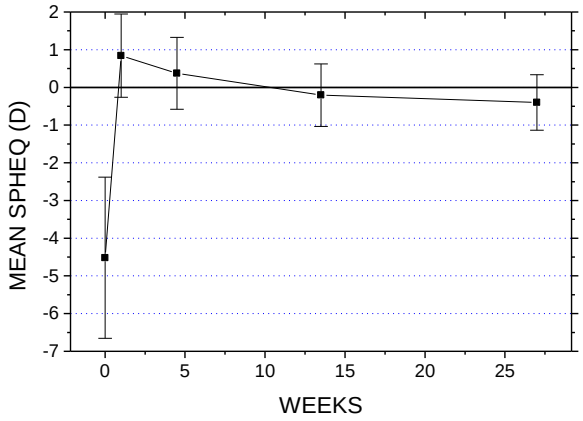

Figure 13: Changes in mean spherical refraction after treatment. The intended corrections were in the range of -1.0 D to -9.0 D.

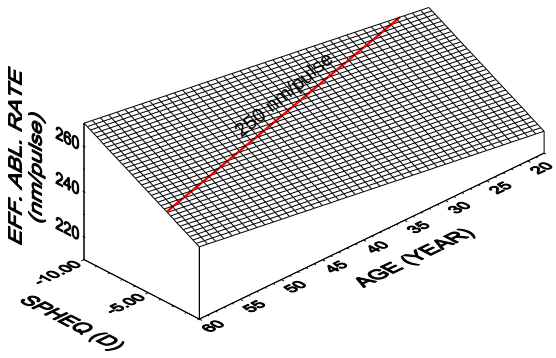

Figure 14. The correlation between effective ablation rate, age and intended correction.

3.2. Morphological and molecular studies on corneal wound healing after laser treatment

3.2.1. Gap junctions in normal rabbit cornea

The corneal epithelium in untreated rabbits consisted of 3-5 cell layers, a cuboidal basal layer covered with gradually flattened stratified cells *(see Figure 20b).*

Of the five connexin isotypes, Cx26, -32, -37, -40 and Cx43 tested, Cx26 and Cx43 were detected in rabbit corneas. Monoclonal antibodies gave similar immunostaining pattern to polyclonals but with much clearer background, therefore, they were used further. The size of immunostained particulate junctions, each representing hundreds of transmembrane channels, ranged between 0.6-1.2 μm for the Cx26 and 0.4-1.0 μm for the Cx43 isotypes.

Cx26 was localized on the basolatheral cell membranes between basal epithelial cells and a few randomly dispersed around the suprabasal/wing cells, but it was missing in the rest of corneal tissue (**Figure 15a**). In tangentially cut sections Cx26 positive junctions appeared as rings around the basis of basal epithelial cells (**Figure 15b**).

Cx43 was evenly distributed in the opposing cell membranes of basal epithelial cells and they were especially at a high density along the apex of basal cells, facing suprabasal/wing cells (**Figure 16**). Cx43 reaction was also seen along the epithelial basal lamina and a few were randomly dispersed throughout the rest of the epithelium. Outside epithelium Cx43 gap junctions were found in stromal fibroblasts and in the endothelial cells lining the inner edge of the cornea *(see Figure 27a later).*

3.2.2. Corneal regeneration and connexin expression

Early events, between 6-48h

Six hours after treatment the ablated stromal surface was covered with a „pseudomembrane" of fine molecular debris (**Figure 17**).

In 6-16h wounds the collagen stroma beyond the surface was infiltrated with acute inflammatory cells and a few histiocytes intermingled with activated fibroblasts (**Figure 18**). Elongated basal and suprabasal epithelial cells started stretching over the denuded stroma by forming a cone of diminishing cells toward its front (**Figure 19**). Few cells in the front were practically Cx negative. Behind them uneven intracytoplasmic staining and a few randomly arranged dots of both Cx reactions were seen (*see on Figure 22*). Ultrastructural hallmarks of hemidesmosomal junctions seen in resting basal epithelial cells almost completely disappeared near the wound front (**Figure 20**). On the other hand, interdigitations between stretching cells including the basal ones became more pronounced towards the front of the wound (**Figure 21**). Interdigitations between suprabasal cells were decorated with desmosomal plaques.

Between 16-48 hours of wounding, an elongated epithelial cell monolayer expressing both Cx isotypes protruded further over the ablated area (**Figure 22**) to completely cover the wound surface by 48h. Both Cx-s seemed upregulated adjacent to the wound edges but their cytoplasmic and particulate reactions were unevenly distributed and were not restricted to the basal epithelium only. The number of particulate signals and their level of organization showed positive correlation with the distance from the wounding front. Under the healing epithelium, keratocytes with the ultrastructural signs of increased protein synthesis were noted (*see e.g. on Figure 21a)* and they were irregularly arranged as it was also reflected by their Cx43 staining pattern (*see Figure 22h*). Some Cx43 signals, probably of stromal origin, were also seen near and on the surface of the wound not yet covered by epithelial cells (**Figure 23**). Basal and some suprabasal epithelial cells adjacent to the wound and all over it showed increased proliferating activity, including the migrating monolayer, as detected with a Ki67 protein specific antibody Mib1 (**Figure 24**). The mitotic rate reached a peak on day 1 after wounding, then it was gradually reduced, but was still elevated by the end of the first week (**Figure 25**). While EGFR expression in resting corneal epithelium was concentrated on the membranes of basal and the first suprabasal epithelial cells, a strong EGFR reaction was noted around all epithelial cells over the wound including the multilayered areas (**Figure 26**).

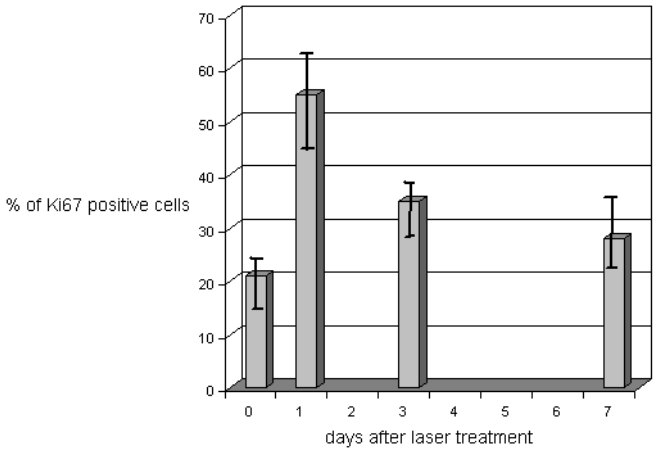

Figure 25. Mitotic rate in corneal epithelium during wound healing after excimer laser treatment based on the immunodetection of Ki67 protein. The number of basal cells were divided by the number of Ki67 positive cells on the same confocal images including those of the migrating monolayer up to the wound front. The number of Ki67 positive cells at least doubled 1 day after treatment.

Later events, between 48 h and 1 week

After 72 hours the wound was covered with 2-3 new layers of wavy-running epithelium in which Cx-s were distributed in a much less organized way than in normal (**Figure 27**). Clumps of immunostained Cx43 and Cx26 were concentrated in parts of the healing epithelium reflecting the uneven upregulation of gap junction proteins. Proliferating activity in newly formed epithelium was still elevated in the regenerated area (*see Figure 24e*).

From early regeneration, 6h on with a peak at 72h, a protein band of ~12 kD was isolated and identified in electrophoretic gels (**Figure 28**). The detected protein is most probably involved in the regulation of wound healing since it was not present in untreated corneas.

One week after treatment the regenerated epithelium consisted again of 3-5 layers with slight irregularities in the basal lamina and connexin distribution (**Figure 29**). There were still activated fibroblasts seen in the stroma under the wound.

Except a narrow scar, the corneal structure seemed completely normal 3 months after laser treatment.

3.3. Clinical results with ophthalmic surface lasers

3.3.1. Excimer laser phototherapeutic keratectomy

Group I: Recurrences were noted in 9 of 103 eyes (9%) operated with recurrent erosion during a 12-36 months follow-up period. Four patients did not follow the postoperative regimen and their recurrences occurred within the first 3 months. Three of them were retreated and there was no further recurrences within 6 months. The remaining five patients had recurrences outside the treated area. From six months on after surgery, we have not found any refractive change or decrease in the best-corrected visual acuity and there was no induced superficial haze at any patients.

In group IIa) all pterygia were nasal. Recurrences were noted in 26 eyes (52%). Nine of them, all from Southern Europe, the Middle East or Africa, had undergone previous surgery. Recurrences were more frequent in score 3 (47%) or score 4 (71 %) cases than in those with more moderate disease. The new pterygium appeared within 12 months. No patients had a decrease in the best-corrected visual acuity. Some eyes were initially inaccessible for refractive measurements. In patients with score 2 or more an irregular corneal surface was usually seen by topographic analysis, under the excised pterygium. One patient had massive scarring in the operated region. No induced central haze of more than stage 1 was visible in any of the patients.

In group IIb) 33 nasal and 3 temporal pterygia were treated with the free conjunctival transplant technique (**Figure 30**). Recurrences were detected in 12 of 36 eyes (33%). Ten of them (27%) had previous surgery and all were from Southern Europe, the Middle East or Africa. We found no decrease in best-corrected visual acuity in any of them.

Group III: All patients with bandlike keratopathy had a pain-free epithelial closure completed within 6 days after surgery. Recurrences were noted in 7 of 29 (24%) patients within 9 months, all in the peripheral region of the ablated zone. All of them had a rough type disease where not all calcifications could be removed. Refraction changes were difficult to assess since initial refractions could not be measured exactly. The maximum measured postoperative hyperopia was +4D and the maximum irregular astigmatism -4.5D. Reduction of pain was achieved in all cases as a result of the treatment. There was no recurrences in the 4 of 29 patients with smooth keratopathies.

Group IV included patients with special indications. Two (brother and sister) had corneal amyloid deposits which could be completely eliminated with PTK, which resulted in improved visual acuity from 10/200 to 20/30 in both. A patient with acanthamoebic keratitis was pain-free 4 months after epithelial closure, the visual acuity improved, and the central cornea was almost clear (**Figure 31**). All patients (10 eyes) with map-dot-fingerprint dystrophy were pain-free with no recurrent erosions within at least 12 months after PTK. The corneas were clear in the ablation area, and visual acuity improved. In the 2 patients with anterior crystalline dystrophy corneas were transparent but not entirely clear in the center after PTK (**Figure 32**). Visual acuity improved by 3-5 lines without drastic hyperopic shift. In patients with central scar (7 eyes) the corneas became clear after PTK and best spectacle--corrected visual acuity improved from 20/200 to between 20/100 and 20/20 (**Figure 33**).

3.3.2. Topical diclofenac sodium treatment after PTK

After PTK and PRK, postoperatively all patients suffered from pain and needed systemic analgesia. To reduce its dose we tested topical Diclofenac Sodium administration. Seventy-two hours after PTK, there was no statistical difference in epithelial closure between the diclofenac user and the control groups. Also, there were no systemic or local complications or allergic reactions. Besides paracetamol, in the control group the majority of patients (95%) required additional analgesics while in the diclofenac group only 28 of 65 (43%) needed further analgesic support.

3.3.3. Excimer laser photorefractive keratectomy

Myopic PRK was performed in 150 patients up to –9D by using different ablation diameters. Application of larger optical zone ablations resulted in better accuracy and personal satisfaction of the patients and reduced halos in all groups when compared to using smaller ablation diameters. In patients with myopia between –6D to –9D, larger optical zones were also responsible for less regressions, although in this group the accuracy of the corrections decreased at any ablation zones as compared to the results in patients up to –6D.

In a case with overcorrection after myopic PRK (+2.5D), mechanical debriment of the epithelium resulted in improvement in refraction to +1D, though on the expense of a transient central corneal haze.

Excimer laser PRK was used as a second treatment to correct rest myopia in 98 patients who received radial keratectomy earlier, but could not wear contact lenses after this. After PRK visual acuity substantially improved in all patients up to –6D, although the regeneration process was accompanied by a considerable transient haze for several months. Larger optical zones reduced unwanted side effects.

3.3.4. Femtosecond Laser for Corneal Surgery

In the 42 patients where femtosecond laser was used for flap creation followed by excimer laser treatment the refractive results achieved were comparable to those got after using a traditional LASIK procedure, by the same surgeon (**Figure 34**). A few early patients developed epithelial inclusion cysts between the cut edges made at 90° to the corneal surface, due to mild retraction of the edge of the corneal flap. The vertical side cut angle was then changed to 45°, with no retraction visible any longer. No free or buttonholed flaps, which might happen sometimes with traditional LASIK flaps, were observed and no epithelial ingrowth was seen. The flaps created with the femtosecond laser had more even thinkness

than traditional flaps, and they also showed excellent reproducibility and stability immediately after repositioning, without loss of corneal clarity.

In 16 patients intracorneal rings were implanted into corneal tunnels made with femtosecond laser (**Figure 35**). The refractive results were the same as by using traditional ICR procedure. However, unlike with ICR there were practically no operative complications and much less deposits found in the tunnels after femto-ICR with at least 1 year follow-up. The patients visual acuity improved immediately after surgery.

In the 5 patients receiving FLK surgery mild corneal edema occurred on the first 2 days. The centration of the procedures was excellent and high myopia was corrected without using excimer laser ablation (**Figure 36**). The corneal transparency and stability were high. The visual acuity of all patients was better, than the spectacular corrected values before treatment.

Intrastromal ablation of the cornea was performed in 13 highly amblyopic patients. The bubbles created during the surgery in the stroma disappeared within 1-2 hours after surgery and then the corneas were highly transparent. The refractive results were stabile, therefore the procedure seems promising for treating low myopic and hyperopic eyes. There was no haze detected throughout a 18 months follow-up period when using fluoromethalon eye drops for the first month postoperatively.

Figure 36. Corneal elevation cornea-topographic map (Orbscan) 3 months after femtosecond laser keratomileusis (FLK) revealing an improvement of about 10 diopters.

4. Discussion

4.1. Biophysics of laser ablation of the cornea

During the history of excimer lasers in ophthalmology, three different UV laser sources have been tested. One shot of xenon-fluorid (emission ~310nm), or krypton-fluorid (emission ~248nm) lasers were found to penetrate and be absorbed in a relatively deep layer of tissue, between 3-33μm, and consequently caused uncontrolled tissue damage (Marschall et al. 1985). However, the advantages of argon-fluorid lasers emitting at a wavelength of 193 nm have been claimed by numerous investigators, since this radiation is absorbed within 1-3μm of surface tissue which is less than the diameter of the smallest cell type, offering a precision means for surgical ablation (Trockel et al. 1983, Marschall et al. 1988, Munnerlyn et al. 1988). The limited penetration depth of 193 nm radiation and high energy of each of the incident photons cannot be overemphasized, as this is the single most important factor in the prevention of damage to the deeper corneal stroma, Descemet's membrane, and the corneal endothelium. It has been postulated by some investigators that the high corneal absorption at 193 nm wavelength could be explained only by assuming a significant absorption by peptide bonds of cell proteins, collagens (i.e. Jester et al. 1984). The high efficiency of 193nm excimer laser in corneal surgery is probably due to the high energy of the incident photons which is very effectively absorbed in the corneal tissue (Seiler et al. 1990).

By dissecting the biophysics of laser ablation, our group, for the first time, could demonstrate and measure the details the shock wave formation, plume emission and surface wave propagation by the laser hitting the corneal surface in timelapse studies (Bor et al, 1993a,b). It was found that the initial speed of the shock wave can be as high as 4000 m/s, and the ejection starts 70 ns after the excimer laser pulse and stops about 25 μs later. After the plume ejection, the rise of the surface waves of 0.15-0.4 mm amplitude was observed (Bor et al. 1993a,b).

Our scanning electron microscopic analysis demonstrated the high spatial accuracy of the 193nm UV laser in pig corneal tissue, which was in line with the results of other groups (Marschall et al. 1985, Mohay et al. 1992). In preclinical validation studies, we found that the etching depth was proportional to the number of laser shots and the etching rate was proportional to logarithm of the energy density (Bor et al. 1993b,c). Based on these results we could calculate the value of absorption coefficient and the range of energy densities applicable for safe corneal surgery.

First in animal studies and later in clinical trials we found a positive correlation between the ablation diameter used and the efficiency of the laser treatment (Förster et al. 1995a). Wider

ablation diameters caused less side-effects, however, caution had to be exercised to avoid haze due to the deeper ablation (Förster et al. 1996b).

The effective laser ablation rate depend mainly on the corneal ablation rate and the wound healing process, both of which are affected by the patient age (Dougherty 1994). The age dependence of the effective ablation rate may be related to the corneal dehydration, changes is the tissue structure of cornea, and most probably the differences in the wound healing process of young and old people.

Above 40 years of age and in case of the -6 D or higher intended correction the increased effective ablation rate very likely leads to hyperopic result, since the effective ablation rate is equal or higher than the nominal etching rate. On the other hand, PRK of young patients often yields undercorrection. Best correction can be achieved by considering the age related factors we summarized on a nomogram when designing PRK treatment (Ferincz et al. 2000)

Femtosecond Laser

Only three years after development of the first infrared Nd-YAG lasers in 1960, ophthalmic laser procedures were already performed with these devices (see Puliafito et al. 1985). Since then, the ability to transmit light energy to almost any ocular structure, as well as the functional importance of vision, has continued to make the eye a favored target organ for laser surgery. However, through ophthalmology accounts for approximately 50% of all medical laser procedures, most ocular surgical procedures either do not use lasers at all, or combine them with more traditional mechanical devices. For example, LASIK treatment, the most commonly performed ophthalmic laser procedure nowadays, use 193 nm (UV) excimer laser (Pallikaris et al. 1991, 1997, Buratto et al. 1992, Knorz et al. 1996, Binder et al. 1997). However, since the 193 nm light is absorbed at the corneal surface, an automated mechanical blade (microkeratome) is first needed to cut and expose deeper corneal tissue, thereby avoiding damage to superficial corneal layers, which are responsible for most pain and healing responses.

Attempts to develop lasers that have precise surgical effects inside transparent or translucent tissues have been focused on so-called photodisruptive lasers. The principle of photodisruption is based on laser induced optical breakdown (LIOB), where a strongly focused and short duration laser pulse generates a high intensity electric field, leading to the formation of a mixture of free electrons and ions that constitutes the plasma state (Blombergen et al 1974, Vogel et al. 1986). The optically generated hot plasma expands with supersonic velocity displacing surrounding material. The rapid adiabatic expansion of the

generated plasma quickly decreases its temperature and the vaporized tissue forms cavitation gas bubbles within the focal volume of the laser beam. Using a high repetition rate femtosecond laser and computer controlled scanning optical delivery system, such localized micro-cavitations can be placed in a contiguous fashion to produce high-precision tissue separations inside transparent and translucent tissues (Juhasz et al. 1996, Kurtz et al. 1997).
Compared to nanosecond and picosecond laser pulses, femtosecond laser pulses require approximately one fourth to one tenth of the energy to produce photodisruption and display greater reproducibility (Krueger et al. 1998, Kurtz et al 1998a). The high energy required for photodisruption with nanosecond and picosecond pulses results in significant collateral tissue damage. In addition, the relatively large cavitation gas bubbles formed by these laser pulses does not permit contiguous photodisruption. Femtosecond laser, however, permits higher precision and inproved incision quality required for refractive and other novel ophthalmic procedures.

4.2. Morphological and molecular studies on corneal wound healing after laser treatment

4.2.1. Gap junctions, hemidesmosomes and desmosomes in normal and healing epithelium

The visual performance of cornea following excimer laser photorefractive keratectomy is highly dependent on the rapid and co-ordinated rebuilding of its epithelium and stroma. In this work we investigated the healing process in rabbits by focusing particular attention to gap junction expression correlated with the rearrangement of other cell junctions, cell proliferation and EGFR expression. We found two types of gap junctions, Cx43 and Cx26 of which the latter had not been detected in the cornea before, appearing as early as in the migrating, highly proliferating epithelium which expressed high level of EGFR. Both Cx-s were normally segregated within the basal epithelial cells, but they were transiently expressed by upper cell layers also during regeneration reflecting the involvement of direct cell-cell communication in corneal wound healing.
Gap junctional communication in the regenerating cornea has so far attracted only limited attention (Matic et al, 1997, Suzuki et al., 2000). By utilising our experience in gap junction research and using sensitive antigen detection combined with confocal laser scanning microscopy (Krenács and Rosendaal 1995, Krenács et al. 1997) we could refine earlier results and reveal new features of Cx expression in the cornea. Some of our findings suggest that our technique may be more appropriate for studying gap junctions in the cornea than those used earlier by others (Matic et al, 1997, Suzuki et al, 2000):

1.We detected Cx26 in the cornea with the same monoclonal antibody, which yielded negative result in the hands of others 2. The density of detected connexins we found was higher, with a primarily particulate pattern in the untreated cornea, than in that of earlier studies. These features provided better assistance in differentiating membrane versus intracytoplasmic Cx expression during wound healing. 3. Serving as an internal positive control, Cx43 was always detected in corneal keratocytes and endothelium throughout our study while stromal reaction was usually missing in the studies by others. 4. We found both particulate and intracytoplasmic Cx-s at a gradually increasing concentration from the wound front to the uninjured epithelium, while others could not detect noticeable amount of Cx-s in the migrating epithelium.

Gap junction channels have been show to serve several important roles in life including the control of cell proliferation and differentiation, nutrition in avascular tissues and the propagation of local signals through cell meshworks for synchronising their functions (Kumar and Gilula 1996, Simon and Goodenough 1998). Before our study, there were only two isotypes, Cx43 and Cx50 identified in the resting cornea and they were shown to be differentially expressed in the epithelium (Dong et al. 1994, Matic et al. 1997). In line with these reports we also detected Cx43 and another isotype, Cx26, which had not been reported in the cornea before. Connexin26 is a regular subtype found in other stratified squamous epithelia being expressed by the more differentiated upper layers in the skin (Risek et al. 1998).

In rabbit cornea, both Cx26 and Cx43 were confined to basal cells, but they were largely segregated to different parts of the cell membrane, Cx26 was concentrated basolaterally while Cx43 apically. Their size range, which can be assessed reliably on immunostained samples, were similar to those found in the skin or most other organs except the heart (Green et al, 1991, Krenács and Rosendaal 1995). Since the two isotypes represent evolutionary different families of Cx-s (Cx43 is α1 and Cx26 is β2) they can not form heteromeric functional channels (Kumar and Gilula 1996) and they have selective permeabilities to molecules of ~<1kD (Elfgang et al. 1995). Therefore, they may represent alternative pathways for fine tuning direct cell-cell communication between basal cells. Though Matic and her co-workers (1997) could not detect dye transfer between basal cells and between basal and wing cells by using scrape-loading of rabbit corneal epithelium with Lucifer Yellow, Williams and Watksy (1997) have established functioning gap junctions in these relations with the more accurate carboxyfluorescein microinjection technique. Cx43 gap junctions concentrated in the apical membrane of basal cells and probably shared by the membranes of basal and wing cells may

also be involved in basal-wing cells communication besides Cx50 which latter was detected earlier throughout the epithelium (Matic et al, 1997). Heteromeric channels have little chance to function between Cx43 and Cx50 either (White et al. 1994). In agreement with others we also found consistent expression of Cx43, but no Cx26, in the stromal and endothelial cells (Mohay and McLaughlin 1995, Petridou and Masur 1996).

Corneal healing of surgical wounds involves a series of coordinated events between cells, matrix and soluble factors. Soon after wounding basal epithelial cells adjacent to the damage relocates their hemidesmosomal junctions so that their ultrastructural hallmarks disappear. Detachment and redistribution of hemidesmosomal integrings ($\alpha 6\beta 4$) and BMP antigens allow basal cells to migrate towards and then over the denuded area (Gipson et al. 1993). During migration they exhibit only transient adhesion (focal contacts) to basement membrane collagen/laminin and upregulate fibronectin/fibrinogen receptors to drag themselves over the bare wound (Stepp et al. 1993). In the leading cells, prominent interdigitations, analogous to lammellipodia of migrating basal cells in adult skin wound, are seen as a result of continuous rearrangement of actin/stress filaments (Gipson and Anderson 1977, Takeuchi 1987, Nodden and Martin 1997). For the migration of a continuous sheet of epithelia cell adhesion must be maintained, which has been proven through immunohistologically detecting membrane-associated desmoplakin (Shi et al. 2000), E-cadherin and a tight junction protein (Suzuki et al. 2000). Consistent with this, we observed the ultrastructural equivalents of desmosomal plaques in the cells migrating over the wound. Cell adhesion through Ca-dependent adherins, E-Cadherin and the desmosomal desmoglein, is probably also a prerequisite for functional gap junction formation (Jongen et al. 1991, Krenács and Rosendaal 1995).

Unlike other research groups we detected both Cx43 and Cx26 throughout the healing corneal epithelium including the protruding monolayer (Matic et al. 1997, Suzuki et al. 2000) suggesting that migrating cells partly preserved both their cell-cell adhesion and to some extent their chance for gap junctional communication. This chance was probably low near the wound front, since most of the Cx-s there were intracytoplasmic, but might increase towards the original wound edge as reflected by the elevated number of particulate Cx reactions there. Communication between migrating cells is possible as it was demonstrated in carboxyfluorescein microinjected keratocytes in healing corneal stroma (Watsky 1995). Furthermore, wounding caused upregulation and rearrangement of both Cx-s around the original wound edges including the limbal areas. This was manifested by the loss of the original segregation and spreading to upper cell layers of the particulate reactions after 24h

and uneven concentration of Cx-s in the basal layer after 3 days. Similar upregulation and overlapping relocation of Cx26 and Cx43 expression has been demonstrated in rodent skin during rapid epidermal growth and differentiation forced by mitogen activation (Risek et al. 1998). These and other results showing dynamic modulation of gap junction expression accompanying cell proliferation and differentiation may reflect the regulatory role of direct cell-cell communication in the coordinated rebuilding of stratified epithelia (Goliger and Paul 1995, Gibson et al. 1997). Whether the transient upregulation of Cx-s happens to control proliferating cells and force them out of cycle needs further investigations. The short half-life of connexins, e.g. ranging between 1-3h for Cx43, assists to the rapid adaptation of gap junctions to changing needs (Saffitz et al. 2000). The reason why other groups neither detected Cx-s in the migrating epithelial layer nor observed Cx upregulation around the original wound edges (Matic et al. 1997, Suzuki et al. 2000) is most probably technical (see above). Due to the limited sensitivity of their techniques they might have only the chance to study the top of the iceberg concerning the Cx43 and Cx26 immunofluorescence. Missing desmoplakin reaction at the same location by Suzuki's group (2000) which, however, was detected by Shi and co-workers (2000) though by using a cocktail of antibody clones, may further support our view.

4.2.2. Corneal epithelial cell proliferation and epidermal growth factor receptor expression

Elevated cell proliferation during epithelial healing is a prerequisite for the rapid substitution of the eliminated cells and isolation of the wound from the outside environment (Haaskjold et al. 1989, Sandvig and Haaskjold 1993, Chung et al. 1999). We observed massive increase in cell proliferation 24h after wounding, which involved the migrating epithelial monolayer covering the wounded area. For the first sight, this finding was in controversy with the results of some other groups who found proliferating cells only adjacent to the wound, particularly at the limbus (Chung et al. 1999, Zieske 2000). However, comparison of our models suggested that the method of wounding and the size and spatial arrangement of the wound may affect the scenario of healing. At smaller and more superficial wounds (∅ 3mm, epithelial debriment) elevated proliferation in the basal epithelium outside the wound seems to cope alone with the demand for new cells (Sandvig and Haaskjold 1993, Chung et al. 1999). At larger diameter and deeper ablation wounds, such as we used (∅ 6mm), the urgent need for wound isolation, reflected also by the extended monolayer of elongated epithelial cells, resulted in the proliferation of the migrating cells also. Similar massive replication was demonstrated in migrating epithelial cells right behind the leading cells 12-24h after making

a 10mm diameter epithelial defect where healing was supported by biosynthetic hEGF (Kitazawa et al. 1990). Healing corneal endothelial cells are also able to migrate and replicate at the same time (Petroll et al. 1999). Though Ki67 protein specific clones are prone to suggest an overestimate of the proliferating pool, their reactions follow the same trend as thymidine incorporation and thus they are the most used and reliable immunohistological markers of cell proliferation (Scott et al. 1991, McCormick et al. 1993).

Upon corneal wounding proliferation and migration of epithelial cells are facilitated by a range of endogenous growth factors including EGF (Schultz et al. 1992, Wilson et al. 1999, Zieske et al. 2000). EGF produced by all three major cell lines in the cornea finds its receptor restricted to the epithelial cells (Zieske et al. 2000). In agreement with others we detected abundant membrane-bound expression of the EGFR protein in the basal cell layer and a gradually decreasing concentration in the upper layers. During regeneration we also noted a high level of EGFR expression in the migrating cells over the wound (Beuerman and Thompson 1992), where the staining was equally strong in the upper layers also. Finding the majority of the EGFR positive cells in a replicative state is in line with the notion that EGFR activation promotes DNA synthesis and cell proliferation (Kitazawa et al. 1990). In experimental models using smaller and more superficial wounds migrating cells were neither proliferating nor expressed membrane associated EGFR which supports the same conclusion (Zieske et al. 2000).

In an effort to find any differentially expressed protein in our wounding model, we detected an exclusive band of ~12kD in electrophoretic gels 6-72h post-wounding which disappeared by 1 week. Since this band was not seen in untreated corneas we suggest that the protein(s) it represents might have a role in the healing process. Fibroblast growth factor and hepatocyte growth factor gene products, thought to be involved in the regulation of corneal wound healing, may fall into this molecular range (Winkles et al. 1993, Wilson et al. 1994, 1999, Chen et al. 2000). The exact nature of the upregulated protein needs to be further clarified and correlated with differential gene expression (Yu et al. 1995).

Besides specificity, the sensitivity of the immunohistological reaction, can not be overemphasized since low sensitivity results may lead to inaccurate conclusions. Acetone, as the least harmful precipitating fixative, helps retaining antigen at its native location while preserving antigenicity for immunodetection. In our experience, immediate acetone fixation after cutting and prompt starting of immunoreactions within 60min improves the chance for reliable detection of Cx-s. Other antigens also benefit of this care. Though some epitopes survive short formaldehyde fixation and better resist against tissue damage (Krenács and

Rosendaal, 1995). The biotin-streptavidin-FITC system bear also a clear sensitivity advantage compared to fluorencein labelled anti-mouse (rabbit) Ig-s. Finally, confocal laser scanning microscopy offers a much better resolution for the fine localization of the immunostained junctions and all other immunolocalized antigens (Krenács et al. 1997).

In this work, particulate Cx26 gap junctions were detected, for the first time, in corneal epithelium. They were concentrated at different membrane regions in the same basal cells than the Cx43 types. During regeneration following laser ablation both Cx-s were expressed throughout the epithelium including the migrating cells. They were slightly upregulated and relocated to the upper epithelial layers. These findings suggested their involvement in the regulation of epithelial wound healing. Migrating epithelial cells over the wounded area also expressed membrane bound EGFR and they were highly proliferating. Mitotic activity in the migrating and pharmacologically not influenced corneal epithelial cells is a novel finding which is probably the sign of the excessive demand for new epithelial cells not met by the proliferating limbal stock alone, due to the relatively large size of the wound.

4.3. Clinical results with ophthalmic surface lasers

4.3.1. Excimer laser phototherapeutic keratectomy

In comparison to PRK, PTK is not a standardized procedure. Existing reports describe variety of different surgical strategies. It is necessary to note that in many cases the symptoms of the corneal disease are treated rather than the cause, with the exception of superficial scars after injury (Sher et al. 1991, Campos et al. 1993).

We have achieved similar efficiency to other groups in treating recidive erosions with a low rate (9%) of recurrences (O'Brart et al. 1994). Postoperatively, it is recommended to use ointments and artificial tear film as lubricants for 6 weeks, to compensate for the increased rigidity of the patient's tear film (Süveges et al. 1994).

Using supplementary excimer laser PTK after pterygium surgery with the "bare-sclera" technique, we found much smoother corneal surfaces than others with traditional surgery but recurrence rates were not very different, around 50% (Schrage et al. 1993, Förster et al. 1995b). Additional treatment with Mitomycin C, a known suppressor of mitosis, might reduce recurrences to zero, as reported by Seiler and co-workers (1992), however, side effects may be expected.

The results with free conjunctival transplants in severe pterygia are more promising: we saw a relatively low recurrence rate of 30% within at least 9 months. The efficiency of this kind of

surgery might be further improved if excimer laser PTK and limbal autograft transplantation are combined, as proposed by Kenyon and Tseng (1989).

Our results achieved in the treatment of bandlike keratopathy correlated well with those reported by Gartry and co-workers (1991) and O'Brart and co-workers (1993). All patients became pain-free after epithelial closure. To avoid extreme hyperopic shifts, caused complete elimination of the stromal defects as reported by Campos et al. (1993), we did not intend drastic ablation, meanwhile achieved similar results with rare haze formation (Lohmann et al. 1991). Recently, Salah et al. (1996) reported significant improvement in visual acuity of six eyes when they used PTK for eliminating calcifications in band keratopathy before cataract surgery. Despite these successes, a prospective study comparing PTK with traditional treatment protocols is still lacking.

An important problem after PTK is a consequent hyperopic shift. This risk may be reduced by modifying the surgical strategy and making some allowances for the sake of visual acuity (Förster et al, 1996a). In the group treated for special indications as well as in band keratopathy we demonstrated that avoiding deep ablation helps avoiding the risk of hyperopic shift. This and the risk of severe irregular astigmatism can be overcome by using the possible largest optical zone (8 mm) combined with the possible minimum of ablation depth (Ratkay, 1998a,b).

Postoperative pain until epithelial closures a major problem after PTK. Due to reasons of possible infectious and non-infectious (sterile) keratitis as well as effects on epithelial healing, contact lenses are not generally used (Faschinger et al. 1995). Diclofenac sodium reduces the need for systemic analgesics after superficial corneal surgery without coincident use of bandage contact lenses (Sher, Lindstrom et al. 1993). We found no difference in epithelial wound healing after 3 days or incidence of sterile infiltrates when using diclofenac sodium in association with PTK (Förster et al. 1997a). However, topical diclofenac sodium, 3 times a day for 3-4 days, significantly reduced pain which result was in line with that found by Sher et al. (1993) after PRK.

The use of excimer laser is not recommended for the treatment of scars caused by recurrent herpes simplex infections since the latent virus is probably reactivated by the ultraviolet laser light (Shimomura et al. 1993). Though the laser ablation of malignant tumors is possible, as reported by Dausch and co-workers (1994), from a pathological point of view, it is contra-indicated due to the loss of diagnostic tissue for proper tumor subtyping.

Based on our experiences we recommend excimer laser PTK as a alternative surgical technique in treating superficial corneal diseases (Förster et al. 1995b, 1997b, Ratkay et al. 1998a,b).

4.3.2. Excimer laser photorefractive keratectomy

Photorefractive keratectomy was first used to treat myopia in sighted human eyes by McDonald et al. in 1988. Initially, the maximum correction attained was -13.0D, but later the results indicated that regression is usually substantial when the correction exceeds -6.0D. As with any new procedure, safety, efficacy, stability and predictability had to be investigated (Trockel et al. 1983, Seiler et al 1990, McDonald et al. 1991, Lohmann et al. 1996). Since then several studies have been done and still under way world-wide to investigate newly modified excimer laser instruments aimed at further improving treatment standards.

Our first results on myopic PRK by using different optical zones in 150 eyes suggested that larger optical zones reduce the risk of unwanted side-effects including halos and regressions (Förster et al, 1996b). For corrections above –6.0D, other techniques than PRK, such as Lasik treatment is preferred (Pallikaris et al. 1991).

In a few cases mechanical debriment after PRK was able to reduce overcorrection by inducing a secondary healing, but haze formation could not been excluded (Förster et al. 1996a).

We have also utilized excimer laser PRK for correcting rest myopia following RK with a similar efficiency achieved by other groups (Campos et al. 1992, Ratkay et al. 1998c).

4.3.3. Femtosecond laser for corneal surgery

I had the privilege to be the first refractive surgeon ophthalmologist in a research team to test femtosecond laser for corneal surgery in human clinical trials (Spooner et al. 2000, Juhasz et al. 2000).

Initial experience with the femtosecond laser surgical system reveals a number of clinical advantages over traditional techniques. For example, corneal flap creation with a mechanical microkeratome is the source of most LASIK complications, including thin, thick, irregular, buttonholed, partial flaps or metal fragment deposit (Seiler et al. 1998, Alio et al. 2000). The reproducibility and ability to vary laser-created parameters (such as thickness, diameter, hinge location, hinge angle and side-cut architecture), maintain normal intraocular pressure, and increase the reproducibility and postoperative stability of the flap can provide greater clinical safety and flexibility (Ratkay et al. 1999a,b, Spooner et al. 2000). The flaps we created in animal experiments and in the human clinical trial displayed excellent stability immediately

after repositioning, without loss of corneal clarity. We could optimize the dissection and surface quality, the side cut and hinge architecture and the centration techniques (Ratkay et al. 2000a,b). With these results we applied for and got approval from the FDA for this procedure.

In the second refractive procedure, FLK, the anterior curvature of the cornea flattens due to the removal of an intrastromal tissue lens, resulting in a refractive change analogous to current LASIK techniques. Compared to LASIK, FLK is a single technology performed in the reverse order, under a permanent low vacuum fixation of the eye, which makes centration highly reproducible. For clinical use of FLK, a surgical nomogram describing the shape, size and position of the lenticule to be eliminated has been designed as the basis for accurate calculations by using an analytical model of the human cornea (Jotyan et al. 2000). The accuracy, reproducibility, stability and safety of this procedure must be determined in larger series of patients.

We have also demonstrated that a femtosecond laser can be used for implanting intrastromal corneal rings with similar results to those of traditional ICR (Schanzlin et al. 1997), but with much less operative complications (Ratkay et al. 2000c). This procedure is the only reversible refractive surgery technique. FDA approval for this procedure is under way.

Femtosecond laser intrastromal corneal refractive keratectomy is a revolutionary new method for correcting low myopia and hyperopia without any cuts at the corneal surface. The epithelium and Bowman membrane stay intact, so the patients do not have any pain or discomfort feeling after the procedure. Since the refractive results are stabile, the patients satisfaction is excellent, this treatment has a very promising future after clinical validation in a large number of patients.

Concerning further potentials of femtosecond laser surgery there are several exciting areas to explore. It might have a significant potential in improving corneal transplantation (anterior, posterior lamellar- and full thickness transplantation), in surgically manipulating other ocular tissues, such as the sclera (glaucoma surgery) and the lens (cataract surgery). Continuous improvements of ultrafast lasers, including the recently developed solid-state based higher energy femtosecond lasers, and the increasing demands from ophthalmologists to improve available surgical technologies and develop new means predict ultrafast laser technology a very bright future.

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