The role of lumican and keratocan genes in persistent subepithelial corneal haze following excimer laser photorefractive keratectomy

Viktória Szabó,1 Katalin Balogh,2 Ildikó Süveges,1 Károly Rácz,2 László Hunyady,3 Zoltán Zsolt Nagy1

1 Department of Ophthalmology, 2nd Department of Medicine, and 3 Department of Physiology, Semmelweis University, Faculty of Medicine, Budapest, Hungary

Purpose: A retrospective clinical and a genetic study was carried out of severe subepithelial corneal haze occurring after photorefractive keratectomy (PRK). Since this clinical condition resembles the lumican-null mouse phenotype, mutation analysis of lumican and keratocan was carried out to investigate whether germline genetic alterations have an effect on development of severe corneal haze in humans. Corneal thickness, photoablation depth, and severity of persistent corneal haze were also analyzed. In vivo confocal microscopy examination was also performed to study corneal structure and endothelial cells.

Methods: Severity of corneal haze was evaluated by slit-lamp biomicroscopy according to Hanna’s scale. Corneal structure and endothelial cell shapes and density were viewed with a scanning confocal microscope. PCR-based mutational analysis was performed using temperature gradient gel electrophoresis (TGGE) and direct sequencing.

Results: Preoperative corneal thickness was normal (539±23.13 µm, mean±SD), and the photoablation depth was 88.94±18.64 µm (mean±SD). The most severe corneal haze was grade 2.0 on Hanna’s scale one year after PRK. In vivo confocal microscopy also showed normal endothelial cell density and morphology. Aside from an intronic polymorphism in a control, no genetic alterations were found in the lumican and keratocan genes.

Conclusions: There was no evidence that endothelial dysfunction and germline mutation of lumican and keratocan genes participate in the etiology of subepithelial corneal haze. Our findings suggest that the mechanisms of the development of severe corneal opacity are different in humans after PRK compared to the lumican deficient knockout mouse model.

Refractive surgery techniques that use the argon-fluoride (ArF) excimer laser to correct refractive errors of the eye have in recent years gained worldwide acceptance. The literature reports infrequent occurrences of complications after excimer laser treatment. However, in rare cases, despite careful preoperative examination and tailor-made intervention, some complications may occur. Subepithelial corneal haze development is one of the most prevalent complications following photorefractive keratectomy (PRK). This condition causes significant impairment of visual function, especially when the haze is to be found in the optical axis of the cornea (Figure 1). Experimental and clinical studies of refractive surgery have analyzed the wound healing response to PRK and concluded that the response shows variability among patients. The most intriguing question is why subepithelial corneal haze develops in some cases and not in others. These differences may partly be explained by genetic predisposition, environmental effects, or other changes in the avascular wound healing mechanisms [1-4].

Several steps of the avascular corneal wound healing response have been clarified, although it is still not exactly clear how the process of corneal wound healing is organized [1,5]. Three major corneal structures are involved differently during PRK. The epithelial barrier function appears to be affected at two weeks following PRK even though the corneal epithelium covers the ablated area [6]. Interactions of migrating epithelial cells and the extracellular matrix of the basement membrane also have a crucial role in the regeneration of injured tissues [7]. Examinations of the endothelial barrier function have shown no significant changes after excimer laser intervention [8,9]. In the excimer laser ablated stroma the keratocytes first undergo apoptosis, which is followed by inflammation, cell activation, and remodeling stages [10]. The major extracellular matrix components of the corneal stroma are collagens and proteoglycans. Collagen fibrils are organized into a lattice structure to minimize diffraction of incident light for optimal vision. Confocal microscopy studies have demonstrated the structural changes of photoablated areas in humans and in rabbits as well [11]. The hyperreflectivity of keratocytes and disorganized collagen structure were also observed by several authors [3,12]. Epithelial and stromal wound healing are influenced by extracellular matrix components, which are produced by activated stromal keratocytes. The small leucine-rich proteoglycan family has two major groups: (1) keratan sulfate proteoglycan which includes lumican, fibromodulin, keratocan, and mimecan; (2) chondroitin/dermatan sulfate proteoglycans, decorin and biglycan [13].

Lumican is present in large quantities in the corneal stroma (OMIM 600616) [14-17]. In the cornea, lumican interacts with collagen, especially with type I collagen, and limits the diameter-growth of fibrils. Furthermore, because of its keratan sulfate-containing glycosaminoglycan side chains, lumican also
plays an important role in the regular spacing of collagen fibrils, primarily in limiting lateral growth [18-20]. Lumican may regulate cell proliferation, apoptosis, and corneal inflammatory response [21,22], and it also may modulate cell migration and adhesion, thus contributing to corneal epithelial wound healing [23]. It is essential, along with other proteoglycans and collagens, for the maintenance of corneal transparency [14,19,20,23,24]. Lumican also affects corneal transparency by influencing corneal hydration. Gene-targeted lumican-null mice developed corneal opacity and skin fragility, and furthermore abnormally thick collagen fibrils and irregular interfibrillar spacing were described in the corneal stroma. The corneal haze found in lumican-null mice was coincident with the presence of a disorganized collagen fibril structure in the stromal extracellular matrix [20,23,25,26].

In adults, keratocan is a cornea-specific keratan sulfate proteoglycan. It is one of the major components of the extracellular matrix in vertebrate corneal stroma (OMIM 603288) [27]. Keratocan, similar to lumican, is also important to corneal transparency due to playing a pivotal role in matrix assembly; however, it binds to different sites of collagen fibrils [28]. Lumican has been demonstrated to have a regulatory role in keratocan expression at the transcriptional level [29]. Generally, keratocan ensures the appropriate corneal shape and it is expressed by stromal keratocytes. Like lumican, keratocan is downregulated during wound healing in the injured cornea. Keratocan knockout mice have been shown to exhibit a less severe corneal phenotype. Corneal transparency was normal, but the collagen fibrils were disorganized and stromal fibril diameters were larger in comparison with wild-type littermates examined by transmission electron microscopy [28]. In humans, mutations of the keratocan gene are associated with autosomal recessive cornea plana (CNA2) that manifest in flattened convex curvature of cornea [30-32].

There is currently no method available for predicting those patients who might develop severe haze due to a pronounced wound-healing response. Identification of genetic changes may provide additional insights into corneal haze formation. The genetic background of corneal wound healing has not been

![Persistent subepithelial corneal haze (Hanna’s scale 2.0) found in the optical axis of a male patient following excimer laser photorefractive keratectomy.](image)

**Table 1.**

<table>
<thead>
<tr>
<th>Number</th>
<th>Age</th>
<th>Gender</th>
<th>Preoperative refraction</th>
<th>Ablation depth (mm)</th>
<th>Haze 3 m</th>
<th>Haze 6 m</th>
<th>Haze 12 m</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45</td>
<td>M</td>
<td>-6.5</td>
<td>78</td>
<td>2.0</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>M</td>
<td>-9.0</td>
<td>82</td>
<td>3.0</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>44</td>
<td>M</td>
<td>-8.875</td>
<td>104</td>
<td>2.5</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>M</td>
<td>-8.0</td>
<td>72</td>
<td>1.0</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>M</td>
<td>-9.0</td>
<td>88</td>
<td>1.0</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>37</td>
<td>F</td>
<td>-6.0</td>
<td>79</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>39</td>
<td>M</td>
<td>-14.0</td>
<td>128</td>
<td>1.5</td>
<td>1.25</td>
<td>2.0</td>
</tr>
<tr>
<td>8</td>
<td>28</td>
<td>M</td>
<td>-10.0</td>
<td>89</td>
<td>1.5</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>21</td>
<td>F</td>
<td>-12.125</td>
<td>130</td>
<td>1.0</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td>10</td>
<td>41</td>
<td>F</td>
<td>-5.5</td>
<td>60</td>
<td>0.75</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**Table shows clinical data of patients participating in the study: age, gender, preoperative refractive error (spherical equivalent; D sph + D cyl/2), and progression of subepithelial corneal haze during the one-year follow-up. Patients 2, 3, and 7 had unilateral excimer laser photorefractive keratectomy.**
examined in clinical mutation analysis studies. In this study, we carried out a retrospective clinical examination among excimer laser-treated myopic patients with persistent subepithelial corneal haze grade 1.0 or higher according to Hanna’s scale [33]. Lumican and keratanan genes were candidates for mutation analysis because they play a critical role in the maintenance of corneal transparency. To the best of our knowledge, our present study is the first to evaluate the role of lumican and keratan genes in subepithelial corneal haze development following excimer laser PRK. We analyzed central corneal thickness, photoablation depth and severity of persistent corneal haze, and also performed in vivo confocal microscopy examination to study corneal structure and endothelial cell morphology and density.

**METHODS**

*Patient selection:* Ten patients who had a severe corneal haze graded 1.0-2.0 according to Hanna’s scale one year after PRK were selected from 5,316 myopic patients treated with the excimer laser (Mel 70, Zeiss Meditec, Jena, Germany) between January 1, 1994 and December 31, 2003. Seven males and three females with persistent subepithelial corneal haze participated in the study (two of the females were sisters). Clinical and biological features of the cases are shown in Table 1. The purpose of each operation was a correction to attain emmetropia. Postoperatively, reepithelization of the cornea was normal in every case. All patients received ciprofloxacin (0.3%) eyedrops up to the fifth postoperative day, thereafter fluorometholone (0.1%) was used five times daily for three to five months in a gradually tapered dose. Peripheral blood was collected from the patients and from two controls one year after PRK. The controls were myopic patients who had normal corneal wound healing responses after PRK. All subjects completed a questionnaire that included questions on family and personal history of abnormal dermal wound healing, connective tissue disorders, autoimmune, and other diseases. Oral comments of patients were also collected. The study was approved by the Research Ethics Committee of the Hungarian Ministry of Health. Written informed consent in accordance with the tenets of the Declaration of Helsinki was obtained from all patients who participated in the study.

*Clinical examinations:* The preoperative assessment included medical history, uncorrected and best corrected visual acuity, automated refractometry, slit lamp examination, ultrasound pachymetry, topography, and Goldmann applanation tonometry. The postoperative assessment included uncorrected and best corrected visual acuity as recorded during the follow-up period. The severity of subepithelial corneal haze was graded by the same observer according to Hanna’s scale using slit-lamp biomicroscopy.

Central corneal thickness measurement was carried out using a Humphrey Model 855 (Zeiss, Oberkothen, Germany) after giving topical anesthetic eyedrops.

*In vivo confocal microscopy:* Eyes (13) were examined from affected patients with persistent corneal haze. Six eyes from patients treated with PRK but who developed no haze, and five nontreated control eyes without history of ocular inflammation and contact lens wear were examined using an in vivo scanning slit corneal confocal microscope (Confoscan 3, NIDEK Technologies, Gamagori, Japan) with a 40x/NA=0.75 immersion objective (Achromplan, Zeiss, Oberkothen, Germany). Each image represented a coronal section approximately 300x200 µm, and according to manufacturer’s specifications, the effective depth resolution of the optical sectioning (z-axis) was 10 µm with the objective used. Selected images of the corneal layers were evaluated for the cell size and shape, light scattering, and reflection of deposits. Counts of the endothelium were performed manually. Each counting area was analyzed three times and the average value was taken for the analysis. By convention, objects overlapping the edges of the boundary were only counted on two sides of the section area. Endothelial cell density was expressed as cells per unit volume for each image (cells/mm²). Data of endothelial cells of post-PRK corneas and healthy control corneas were compared by the paired Student t-test. A p value less than 0.05 was considered statistically significant.

*Polymerase chain reaction:* Genomic DNA was extracted from peripheral blood leukocytes by the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Polymerase chain reaction (PCR) amplification of the lumican gene was carried out using self-designed oligonucleotide primers (DNAsstar, Madison, WI; GenBank NM_002345) Primers for amplification of the keratan gene (GenBank NM_007035) were previously described [34], but for prescreening we modified one of the primers with a 40-mer GC-clamp depending on melting behavior calculated by POLAND [35] software. The GC-rich

<table>
<thead>
<tr>
<th>Fragments</th>
<th>Primer 5’-3’</th>
<th>Annealing temperature (°C)</th>
<th>Fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LUM exon 1 F</td>
<td>TGGTTGCAAATTCAAACATATAC</td>
<td>60</td>
<td>499</td>
</tr>
<tr>
<td>LUM exon 1 R</td>
<td>ATTGGCAAAATTGCTCTGGA</td>
<td>58</td>
<td>353</td>
</tr>
<tr>
<td>LUM exon 2.3 R</td>
<td>CCACTTCGGCCTCCCAAAATG</td>
<td>54</td>
<td>409</td>
</tr>
<tr>
<td>LUM exon 2.2 R</td>
<td>TGTTGCTGATCTTATTGTTGTCTA</td>
<td>56</td>
<td>428</td>
</tr>
<tr>
<td>LUM exon 1 F</td>
<td>ATGTTGCAAATTGAATGTCTT</td>
<td>56</td>
<td>424</td>
</tr>
<tr>
<td>LUM exon 2.1 F</td>
<td>CCCTTTTATCTTGGAGTTTTCTA</td>
<td>56</td>
<td>494</td>
</tr>
<tr>
<td>LUM exon 2.2 F</td>
<td>AAGGCCTTGGAGAATACCTG</td>
<td>56</td>
<td>409</td>
</tr>
<tr>
<td>LUM exon 2.1 R</td>
<td>CCCTTTTATCTTGGAGTTTTCTA</td>
<td>56</td>
<td>468</td>
</tr>
<tr>
<td>LUM exon 1 F</td>
<td>TATCACATTTACATTTTTTC</td>
<td>50</td>
<td>354</td>
</tr>
<tr>
<td>KERA exon 1.1 R-GC</td>
<td>GC clamp-AACATTCTCTCTTTAATTCC</td>
<td>50</td>
<td>354</td>
</tr>
<tr>
<td>KERA exon 1.1 R-GC</td>
<td>GC clamp-AGGCTTTCATCTCTTATTGG</td>
<td>56</td>
<td>468</td>
</tr>
<tr>
<td>KERA exon 1.2 R-GC</td>
<td>GC clamp-CGATGTTTCATCTCTTATTGG</td>
<td>54</td>
<td>494</td>
</tr>
<tr>
<td>KERA exon 1.2 R-GC</td>
<td>GC clamp-CGATGTTTCATCTCTTATTGG</td>
<td>54</td>
<td>494</td>
</tr>
<tr>
<td>KERA exon 1.1 F</td>
<td>ACAATTTTTCTCCTCTC</td>
<td>50</td>
<td>468</td>
</tr>
<tr>
<td>KERA exon 1.1 R-GC</td>
<td>GC clamp-ACTGCCTCTCTCATTGG</td>
<td>56</td>
<td>468</td>
</tr>
<tr>
<td>KERA exon 2.2 F-GC</td>
<td>GC clamp-CTGCAGTATGAAGCTAGAAGAG</td>
<td>58</td>
<td>617</td>
</tr>
<tr>
<td>KERA exon 2.2 R</td>
<td>TAAAGACATACGGG</td>
<td>58</td>
<td>617</td>
</tr>
<tr>
<td>KERA exon 3 F-GC</td>
<td>GC clamp-CTGCAGTATGAAGCTAGAAGAG</td>
<td>58</td>
<td>447</td>
</tr>
<tr>
<td>KERA exon 3 R</td>
<td>CTGCAAAGACATACGGG</td>
<td>58</td>
<td>447</td>
</tr>
</tbody>
</table>

sequence increased the number of melting domains, therefore all melting domains were analyzed during the prescreening method (temperature gradient gel electrophoresis; TGGE) except for the domain with the highest melting temperature. During electrophoresis with temperature gradient, the GC-clamp held the two strands together at one side. On the opposite site the DNA strand opened and the double strand slowed down. At high temperature it separated irreversibly into single strands. Details of the primers, size of the amplified fragments and annealing temperatures are given in Table 2. Primer pairs yielded PCR amplicons which included the 5'-UTR, the coding regions, flanking intronic sequences, and the initial part of the 3'-UTR of the lumican and keratocan genes (197 bp and 161 bp, respectively). Specificity of the primers was confirmed by a BLAST search.

PCR was performed using genomic DNA in a Ter0 Hybaid PxE thermal cycler (Thermo Hybaid, Franklin, MA) in a reaction volume of 50 µl, containing 5X GoTaq Reaction Buffer (pH 8.5, 1.5 mM MgCl2; Promega Corporation, Madison, WI), 25 pmol of each primer (InVitrogen Life Technologies, Glasgow, United Kingdom), 0.2 mmol/l of each dNTP (Promega), and 2.5 U GoTaq polymerase (Promega). For exon 1, for the first and second fragments of exon 2, and for exon 3 of the lumican gene, there was a pre-denaturation step at 95 °C for 5 min. PCR cycles for the lumican gene are as follows: 35 cycles of 1 min denaturation at 95 °C, 1.5 min annealing at the appropriate temperature and 1.5 min elongation at 72 °C. The final extension was performed at 72 °C for 10 min. Amplification of the third fragment of exon 2 proved to be problematic, so it was performed by the afore described protocol, but using three different annealing temperatures during the first 10, and the following 15 and 20 cycles, respectively. PCR conditions for the keratocan gene were as follows: pre-denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at the appropriate temperature for 1.5 min and 1.5 min elongation at 72 °C. The final extension was performed at 72 °C for 10 min. A denaturation at 94 °C for 5 min, then incubation at 25 °C for 1 h is required for heteroduplex formation.

The amplicons were analyzed on 1% agarose gel, stained with ethidium bromide and purified using Roche High Pure PCR Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s protocol. PCR-amplified DNA of the lumican gene was sequenced by direct nucleotide sequencing using the Big Dye Terminator Cycle- Sequencing v3.1 Kit (Applied Biosystems, Foster City, CA) and run on an automated sequencer (ABI PRISM® 310 Genetic Analyzer; Applied Biosystems).

**Prescreening method:** TGGE (TGGE Maxi System; Biometra, Goettingen, Germany) is an effective method for screening double-stranded DNA fragment through a polyacrylamide gel with a gradient of increasing temperature with constant concentration of denaturant (urea). Homoduplex and heteroduplex DNA strands are separated depending on their different melting profile caused by mutations or polymor-
phisms. The molecules with mismatches start to denature at different times compared to wild-types and electrophoretic mobility decreases as DNA denaturation begins. The keratocan gene was examined in five fragments. We performed a perpendicular running for all fragments on polyacrylamide gel containing urea, which provided an optimal melting range (Figure 2A). Melting ranges for all fragments are shown in Table 3. Parallel running at optimal melting temperature range was carried out (Figure 2B), and polyacrylamide gels were stained using silver staining. Detected genetic alterations were sequenced by direct nucleotide sequencing using the Big Dye Terminator Cycle-Sequencing v3.1 Kit and run on an automated sequencer (ABI PRISM® 310 Genetic Analyzer).

RESULTS
The questionnaires were evaluated. None of the patients had any previous signs of delayed dermal wound healing, connective tissue disorder, autoimmune disease, or any other type of genetic disorder. Postoperatively, two patients were dissatisfied with their regression of myopia and all patients with hazy corneas complained about glare and blurred vision.

Slit-lamp biomicroscopy: Slit-lamp biomicroscopy showed normal epithelium in all 10 patients one year following treatment with excimer laser. There was no surface irregularity, but stromal involvement was found in all PRK-treated corneas. The severity of the subepithelial haze was evaluated according Hanna’s scale (Table 1). The most severe haze was 3.0, three months after PRK in a young male patient who complained about glare and impairment of quality of vision. The most severe haze one year after PRK was 2.0 on Hanna’s scale, found in two cases, both with high grade myopia. It was difficult to evaluate the extent of the anterior stromal involvement by slit-lamp examination. No corneal alterations were found in nontreated control eyes. In PRK-treated control eyes a central iron line indicated only the previous surface ablation treatment. The average photoablation depth was 88.94±18.64 µm (mean±SD, range: 56-130 µm). Attempted corrections ranged from 4.75 D to 14.0 D of myopia (mean±SD: -8.5±2.3 D).
Preoperative corneal thickness was 539±23.13 µm, which decreased to 463.18±16.44 µm, on average.

Confocal microscopy: Confocal microscopy identified no epithelial change in any of the eyes. In the photoablated area, Bowman’s membrane was absent and there were hyperreflective cells and nonhomogeneous, reflective deposits in the subepithelial extracellular matrix of the PRK-treated eyes (Figure 3B,C) compared to nontreated healthy eyes (Figure 3A). A bright reflection from the photoablated surface exhibited below the epithelial-stromal surface. Increased fibrosis-like extracellular matrix reflectivity (Figure 3D), bright keratocyte nuclei, and uneven cell distribution were observed. Deposits appeared as small punctate deposits or granular bodies, and their density was almost constant from the subepithelial area to the posterior region of the anterior stroma. Anterior stroma was affected in its entire thickness in six eyes, in those patients who underwent more than 100 µm deep photoablation. The lesions were mainly in the central cornea, leaving the periphery transparent, corresponding with the 6.0 mm treatment zone. Subbasal nerves were also clearly visible. The most aggressive wound healing response was observed in two corneas (patients seven and nine in Table 1) that developed clinical haze grade 2.0 on Hanna’s scale. Keratocytes remained quiescent in the mid and posterior stroma. Descemet’s membrane and the endothelium were apparently normal. Endothelial cell density was measured by manual analysis, the average cell density was 2,214.96±223.7 cells/mm² (mean±SD, range: 1,620-2,765 cells/mm²) and the mean coefficient of variation was 0.34±0.057. There was no significant difference (p >0.05) in endothelial cell density of PRK-treated hazy corneas compared to healthy control corneas (Figure 4). Confocal microscopic images of PRK-treated control eyes showed mild stromal alterations in the cornea (Figure 3B). Endothelial cell density and morphology appeared identical with the healthy control corneas.

Genetic examinations: Exons and the flanking intronic regions of the lumican gene of the patients were analyzed by DNA sequencing, and the obtained genomic DNA sequences were compared to the corresponding reference sequences in the Genbank. None of these patients and the control individuals had any germine genetic alterations in the 5’-UTR sequences in exons 1 and 2, in the coding region of the lumican gene in exons 2 and 3, and in the initial part of the 3’-UTR sequence in exon 3.

Three exons of the keratocan gene, including the 5’-UTR, the coding regions, the initial part of the 3’-UTR, and flanking intronic sequences, were evaluated with TGGE and DNA sequencing. An unusual TGGE pattern was detected in one control individual, which was identified after DNA sequencing as an intronic polymorphism (IVS1). No other genetic alterations were detected in the keratocan gene.

DISCUSSION
In humans, subepithelial corneal haze may normally occur one month after PRK, peaking at about three to six months, and progressively disappearing during the postoperative nine to 12 months. Following PRK, the grade of haze is usually less than 1.0 on the Hanna’s scale. But in rare cases, subepithelial corneal haze may remain for years [3,36,37]. Visually, significant haze may persist in 3-5% of all PRK-treated patients [38]. The exact pathogenesis of subepithelial corneal haze after PRK still needs to be elucidated. Corneal light-scattering after PRK may be caused by disruption of collagen fibril structure [18,19,24,39], by activated keratocytes [3,38], or the result of inappropriate deposition of extracellular matrix components [1,40]. The probability and severity of haze may be related to the photoablation depth, although multiple factors, such as UV-B exposition during the early postoperative period might be associated [4,38,41].

In our study, patients avoided UV-B exposure (sunbathing or solarium treatment) and were protected by sunglasses with UV-filters, therefore environmental UV-B played no significant role in the augmented wound healing response of the
cornea after excimer laser treatment. Despite UV protection we found that in 10 patients pathological subepithelial corneal haze (1.0-2.0 on Hanna’s scale) remained one year after the PRK and that the patients complained about significant impairment of quality of vision. Preoperative corneal thickness was normal and also the endothelium was intact in all patients, therefore it could be presumed that these factors did not have a major influence on development of corneal haze in our patients.

We assumed that in these 10 myopic patients with persistent subepithelial corneal haze, genetic predisposition may have played a role in the abnormal corneal stromal wound healing response. Two signs supported the hypothesis. Two sisters out of the ten patients had persistent subepithelial corneal haze, and all patients who underwent bilateral PRK developed bilateral subepithelial corneal haze at the similar clinical severity in both eyes.

To exclude the role of endothelial dysfunction in loss of corneal transparency, we performed in vivo confocal microscopy examination. Confocal microscopy allows a noninvasive optical sectioning of living corneal tissue in real time. In the current study, the epithelium was fully restored without hyperplasia. All photoablated corneas showed enhanced hyperreflectivity within the PRK-treated anterior stroma, suggesting that corneal haze occurred due to disorganized collagen structure and cellular hyperreflectivity of activated keratocytes producing extracellular matrix. Metabolic activity of keratocytes indicated that wound healing processes were active several years after PRK surgery, and the reconstruction of the cellular and fibrillar structure of corneal stroma was incomplete. The endothelium was normal with no evidence of an impact on corneal clarity. Taken together, in vivo confocal microscopy showed that pathologic changes in the cornea were limited to the stroma. Our findings were concordant with previously described findings [3,12]. Also, in lumican-deficient mouse corneas diffuse light-scattering of the stroma was observed using confocal microscopy through focusing [42]. Altered interaction between epithelial and stromal cells and also neural dysfunction are presumed to be involved in subepithelial haze formation [43].

Previous examinations on the cytotoxic and mutagenic effect of 193 nm excimer laser have shown no significant changes in mouse corneal fibroblast, Chinese hamster ovarian (CHO), and human fibroblast cell lines and skin model [44-46]. This led to the conclusion there is no potential mutagen causing somatic mutations in the cornea during the 193 nm excimer laser treatment. However, the existence of germline mutations in different corneal genes may not be excluded.

In the corneal stroma, lumican interacts with collagen fibrils and helps maintain their crucial size and regular structure, as well as corneal transparency. Lumican and keratocan are uniquely abundant in the cornea, and might be the target genes of TGF-β2 signaling in vivo [47]. In lumican-null mice, the absence of lumican caused a delay of corneal epithelial wound healing and skin fragility [7]. Site-specific mutagenesis was performed in a cysteine-rich domain of the lumican gene transfected to a mouse corneal stromal cell culture. Ultrastructural analysis showed a different multilayered stromal matrix formed by the mutant cell line ex vivo, compared to stroma built by the wild-type cell line. The mutant cell line exhibited altered collagen fiber structure and packing [48]. Further knockout mouse models were also elaborated to investigate the role of other proteoglycans in the cornea. In keratocan knockout animals, corneal stromal thinning and a narrower cornea-iris angle of the anterior segment were observed. In mice, targeted disruption of the biglycan gene led to an osteoporosis-like phenotype. Decorin-null animals showed a skin fragility phenotype. Fibromodulin-null animals showed an abnormal tendon phenotype, but the cornea remained clear [26]. The ocular and scleral alterations in lumican- and fibromodulin-deficient double-null animals showed several phenotypic similarities with high grade myopia. Scleral thinning resulted in increased axial length, possibly an outcome of reduced scleral strength [25,26]. No evidence of polymorphism or pathologic mutation in lumican and fibromodulin was found in two high grade myopia pedigrees [49]. In summary, animals suffering from lack of lumican, keratocan, decorin, biglycan, or fibromodulin showed alterations in connective tissue organization, although only the lumican-deficient mice developed bilateral corneal opacity [23].

In this study, patients with severe persistent corneal haze and two controls were screened for genetic alterations in the human lumican and keratocan genes. We investigated whether genetic alterations of major leucine-rich proteoglycan genes may have a role in the development of subepithelial corneal haze in the group of our patients, because lumican has been previously reported as a candidate gene for causing corneal opacity and also keratocan plays a crucial role in collagen fibril assembly [14,18-20,23,25,26]. In vivo examination and corneal mRNA collection in PRK-treated patients can not be carried out. Therefore, we examined genomic DNA samples and performed a mutation analysis of the lumican and keratocan genes in ten myopic patients who suffered from severe subepithelial corneal haze 1.0 or higher grade according to Hanna’s scale one year following refractive surgery. In the lumican and keratocan genes no genetic alteration was found among patients with pronounced subepithelial corneal haze compared to the controls and Genbank database. Knockout mice studies have helped to identify unique and overlapping functions of genes, but several times show difference compared to examinations in humans. Although a previous study demonstrated that genetic deletion of lumican caused a phenotype similar to the one observed in our study, our data suggest that after keratocytic other factors are responsible for the development of corneal dysfunction.

There is a high patient to patient variability, but no methods are currently available to predict who might have an abnormal wound healing response and persistent subepithelial haze. A complicating factor in the search for disease-associated genes in the human population is that most diseases are clinically heterogeneous and that certain genetic factors may not alone cause susceptibility to a disease, but in association with other genetic and environmental factors. However, the
influence of changes of lumican or keratocan expression may not be excluded as a feasible factor in the corneal stroma after photorefractive keratectomy. The gene expression pattern of keratocytes varies with growth factors, cytokines, and environmental factors. Although in the present paper, no evidence was found that germline mutations of the two major leucine-rich proteoglycan are responsible for subepithelial corneal haze development in our set of patients, further genetic investigations on the corneal wound healing process may help in understanding the exact pathogenesis of corneal haze development.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Hungarian Ministry of Health (ETT 611/2003) and from the Ministry for Education (OTKA T 037452). The authors thank Adam Vannay, MD, PhD for helping in the primer design and Miklos Resch, MD for critical reading of the manuscript. The excellent technical assistance of Kata Morcz and Judit Fekete is greatly acknowledged.

REFERENCES


