Keratectasia After Laser In Situ Keratomileusis

A Histopathologic and Immunohistochemical Study

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Objective: To examine histopathologic and immunohistochemical features of human corneal buttons from patients who developed keratectasia after laser in situ keratomileusis (LASIK).

Methods: Five corneal buttons were obtained during penetrating keratoplasty from patients who developed keratectasia after LASIK. Histologic features were examined by hematoxylin-eosin staining using paraffin-embedded sections and by transmission electron microscopy. Immunostaining for α1-proteinase inhibitor, Sp1, and matrix metalloproteinases 1, 2, and 3 was performed with 2 healthy corneas and 2 corneas with keratoconus as controls.

Results: Central stromal thinning was observed after hematoxylin-eosin staining in all corneas with keratectasia. No histologic features specific to keratoconus, including Bowman layer disruption, were identified in the corneas with keratectasia. By transmission electron microscopy, collagen fibril thinning and decreased interfibril distance were observed in the stromal bed. Immunostaining intensity and/or pattern for α1-proteinase inhibitor and Sp1 in the corneas with keratectasia was comparable to that of healthy corneas and differed from that in the corneas with keratoconus. No significant staining with anti–matrix metalloproteinases 1, 2, and 3 antibodies was observed in either the corneas with keratectasia or the healthy corneas.

Conclusions: Histologic findings suggest that post-LASIK keratectasia results in collagen fibril thinning and decreased interfibril distance within the residual stromal bed. Discrepant results between keratectasia and keratoconus suggest that the pathogenesis of the 2 conditions differ.
LASIK keratectasia, the biochemical abnormalities found in keratoconus have not been studied in keratectasia. These biochemical alterations include decreased expression of the protease inhibitor α1-proteinase inhibitor (α1-PI) and upregulation of transcription factor Sp1. In addition, matrix metalloproteinases (MMPs), a group of enzymes that are involved in tissue remodeling, have recently been examined in keratectasia. Studies have detected MMP-3 in rare keratocones, and MMP-10 expression was elevated in the epithelium of ectatic corneas. The MMP expression patterns in keratoconus remain controversial, although overexpression of MMP-1 and MMP-2 has been recently reported in keratoconus corneas. In addition, MMPs have been implicated in various other corneal conditions, including wound repair and healing, ulcerations, and diabetic corneas.

In this study, we examined the histopathologic and biochemical characteristics of keratectasia after LASIK using light microscopy and transmission electron microscopy (TEM). Immunohistochemical analysis was also performed with antibodies against α1-PI and transcription factor Sp1. In addition, immunolocalization of major members of the MMP family, MMP-1, MMP-2, and MMP-3, was performed to determine if an MMP-mediated degradation process was involved in keratectasia after LASIK.

### Table 1. Patient Clinical Data After LASIK

<table>
<thead>
<tr>
<th>Patient No. (Eye)</th>
<th>No. of Enhancement Procedures</th>
<th>Onset Delay</th>
<th>Corneal Thickness, μm</th>
<th>Keratometry, D</th>
<th>Condition of Other Eye</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Right)</td>
<td>1</td>
<td>10 mo</td>
<td>260</td>
<td>59.5 × 90/greater 65 × 35</td>
<td>BCVA 20/50 after LASIK</td>
</tr>
<tr>
<td>2 (Right)</td>
<td>2</td>
<td>4 y</td>
<td>320</td>
<td>NA</td>
<td>–2.00 + 2.75 × 55 after LASIK</td>
</tr>
<tr>
<td>3 (Right)</td>
<td>0</td>
<td>18 mo</td>
<td>NA</td>
<td>45.50/49.00 × 118</td>
<td>Penetrating keratoplasty</td>
</tr>
<tr>
<td>3 (Left)</td>
<td>0</td>
<td>18 mo</td>
<td>NA</td>
<td>47.50/52.00 × 73</td>
<td>Penetrating keratoplasty</td>
</tr>
<tr>
<td>4 (Left)</td>
<td>1</td>
<td>3 y</td>
<td>318</td>
<td>63.9/56.5 × 147</td>
<td>BCVA 20/30, –3.75 + 0.75 × 050 refraction after LASIK</td>
</tr>
</tbody>
</table>

Abbreviations: BCVA, best-corrected visual acuity; LASIK, laser in situ keratomileusis; NA, clinical data were not available; UCVA, uncorrected visual acuity.
The protocol was approved by the University of Illinois at Chicago institutional review board. Five keratectasia corneal buttons were obtained from patients during penetrating keratoplasty from the University of Illinois Medical Center, Chicago, Evanston Northwestern Healthcare, Evanston, Illinois, and Armed Forces Institute of Pathology, Washington, DC. Two healthy human corneal buttons from donors (aged 29 and 69 years) were obtained from the Illinois Eye Bank, Chicago, within 24 hours of death. None of the donors had known ocular diseases, and their corneas were clear, with unremarkable gross and microscopic appearance. Two corneal buttons from patients with typical keratoconus features (aged 40 and 41 years) were obtained after transplantation from the cornea service at the University of Illinois at Chicago as another set of controls. The corneas excised from healthy human eyes and the keratectasia and keratoconus buttons were fixed in 10% buffered formalin, processed, and embedded in paraffin.

Paraffin sections 5 µm in size were deparaffinized, rehydrated, and stained with hematoxylin-eosin (H&E). Prussian blue staining was used to detect the presence of iron deposits. The thickness of the total stroma, flap, and posterior stromal bed for LASIK was measured in H&E-stained sections (AxioVision, version 4.4.1.0 software; Carl Zeiss MicroImaging Inc, Gottingen, Germany) after photographing under a Zeiss Axioskop 2 plus microscope using a Zeiss AxioCam HR camera. Immunohistochemical studies were also conducted. The sections were incubated at 4°C with primary antibodies for 16 hours.

The primary antibodies used in the study included (1) a polyclonal goat antibody to α1-PI (1:200; MP Biomedicals, Aurora, Ohio), (2) a polyclonal rabbit antibody to Sp1 (PEP 2, 1:100; Santa Cruz Biotechnology, Santa Cruz, California), (3) a polyclonal rabbit antibody to MMP-1 antibody (1:100; Lab Vision, Fremont, California), (4) a monoclonal mouse antibody specific to MMP-2 (A-Gel VC2, 1:30; Lab Vision), and (5) a monoclonal mouse antibody to MMP-3 (SL-1 IID4, 1:20; Lab Vision). Before primary antibody incubation for MMPs, an antigen retrieval method was applied on each section with 8-mol/L buffered urea (pH 3.5) at room temperature for 2 hours. Biotinylated donkey anti–goat IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania), donkey anti–rabbit IgG (1:250; Jackson ImmunoResearch Laboratories, Inc, West Grove, Pennsylvania), donkey anti–rabbit IgG (1:250; Jackson ImmunoResearch Laboratories, Inc), and donkey anti–mouse IgG (1:250; Jackson ImmunoResearch Laboratories, Inc) were used as secondary antibodies at room temperature for 45 minutes. The color reaction for the anti-Sp1 was performed with fast red TR/naphthol AS-MX phosphate (Sigma-Aldrich, St Louis, Missouri) after sections were incubated with alkaline phosphatase conjugates.
gated Extravidin (1:50; Sigma-Aldrich). For α1-PI, MMP-1, MMP-2, and MMP-3, sections were incubated with avidin-biotin complex (Vectastain; Vector Laboratories, Burlingame, California) for 45 minutes followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). After mounting in Permount (α1-PI, MMP-1, MMP-2, and MMP-3) or aqueous mounting fluid (Sp1), the staining intensity in each experiment was scored by 3 masked observers (D.P.E., B.Y., H.N.) on a scale from 0 to 3, with 0 indicating no staining and 3 the most intense staining. Experiments were repeated twice.

Ultrastructural examination of 2 corneal specimens with keratectasia (right eye in patient 2 and left eye patient 4) and 2 healthy corneal specimens was performed by TEM. The areas of study were obtained from the midperipheral cornea in the healthy specimen and the specimen with keratectasia. The central area was not included because most of the ectatic area was included in the paraffin block. The specimens were fixed in 2% glutaraldehyde at 4°C, processed, and embedded in epoxy resin. Photographs were taken using a Gatan Digital CCD camera and Gatan Digital Micrograph 2.5 version acquisition software (Gatan Inc, Pleasanton, California). Corneal collagen fibril diameter and interfibril distance were measured using ImageJ 1.36b software (National Institutes of Health, Bethesda, Maryland). The fibrils were measured from the central cornea in the area of thinning and in areas where the fibrils were well oriented, which accounts for the disparity in the number of fibrils measured. The number of fibrils measured for diameter in each specimen was 21 in patient 2, 40 in patient 4, and 80 in the healthy controls. The number of interfibril distances measured were 39 in patient 2, 52 in patient 4, and 104 in the healthy controls. Statistical analysis was per-
formed using the Mann-Whitney U test to compare the fibril diameter and interfibril distance of each of the keratectasia corneas with healthy controls. $P < .05$ was considered statistically significant.

**RESULTS**

**CLINICAL HISTORY**

All patients in this study were women, ranged in age from 44 to 57 years, and had varying degrees of myopia. Preoperative pachymetry was available only in patient 3 and revealed corneal thicknesses of 532 µm in the right eye and 515 µm in the left eye. The salient findings at the time of penetrating keratoplasty for post-LASIK keratectasia are summarized in Table 1 and Figure 1.

**HISTOLOGIC ANALYSIS**

Measurements of corneal stromal thickness were performed on histologic sections of all 5 keratectasia corneas. Full stromal, residual stromal bed, and flap thicknesses were measured centrally and peripherally. Peripheral measurements were taken just central to the microkeratome incision point on all specimens. Mean (SD) central and peripheral full stromal corneal thickness measurements in keratectasia were 293 (69.4) µm and 440 (36.7) µm, respectively. In comparison, healthy corneal thickness was 399 (5.7) µm centrally and 431 (7.8) µm peripherally. Additional results of thickness measurements (Table 2) showed stromal thinning in the flap and the residual bed.

The 5 H&E-stained specimens had similar histopathologic features, which are summarized in Table 3. These features included the presence of an epithelial iron ring, a lack of Bowman layer disruption that is typically seen in keratoconus, a lack of inflammation or scarring, a faint demarcation line between the flap and residual stromal bed, and an unremarkable Descemet membrane and endothelium. In addition, areas of peripheral epithelial ingrowth at the site of the microkeratome incision were observed in patients 2 (right eye) and 4 (left eye). The central corneal epithelium showed thinning on patient 3 (left eye) and patient 4 (left eye); macrostriae were seen in the residual stromal bed of patients 3 (right eye) and 4 (left eye). Patients 2 and 3 are depicted in Figure 2 and Figure 3, these images are representative of the spectrum of histologic changes observed in our series of keratectasia cases.

**TRANSMISSION ELECTRON MICROSCOPY**

Examination of patients 2 (right eye) and 4 (left eye) by TEM revealed ultrastructural changes in the midperipheral corneal stroma (Figure 4). The mean (SD) diameter of the collagen fibrils in the residual stromal bed was $27.6 (2.7)$ nm in patient 2 and $27.5 (2.3)$ nm in patient 4, and these collagen fibrils were significantly thinner than collagen fibrils of healthy controls ($32.9 [2.8]$ nm, $P < .001$). Interfibril distances observed in keratectasia patients 2 and 4 were $23.4 (9.6)$ nm and $23.5 (7.0)$ nm, respectively, and were also significantly less than interfibril distances seen in healthy controls ($25.9 [6.1]$ nm, $P = .031$).

**IMMUNOHISTOCHEMICAL ANALYSIS**

Immunohistochemical experiments demonstrated positive staining of α1-PI in the epithelium of keratectasia corneas. The intensity of the staining was similar to that found in healthy human corneas. The epithelial staining of α1-PI in corneas with keratoconus, in the meantime, was markedly decreased as expected (Figure 5). Immunohistochemical analysis for Sp1 in corneas with keratectasia and healthy human corneas revealed much weaker staining than that in corneas with keratoconus (Figure 6). The epithelium of healthy human corneas and corneas with keratectasia displayed nearly absent staining for MMP-1, whereas strong staining was observed in the corneal epithelium of keratoconus cases. Both MMP-2 and MMP-3 immunolabeling were at the background level or absent in all corneal specimens. However, the positive controls (placenta) showed strong positive staining (data not shown). In general, the MMP immunostaining pattern in the keratectasia corneas resembled that seen in healthy controls (Figure 7). Semiquantitative immunostaining results for all antibodies are displayed in Figure 8.
The continuously growing popularity of refractive surgery procedures, namely LASIK, has caused increased concern regarding the serious complication of keratectasia. Little is known about the mechanism of this rare complication. In this study, we analyzed 5 corneal buttons from patients who developed this complication using histologic analysis, immunohistochemical analysis, and TEM to better understand the disease process of keratectasia.

**Figure 6.** Immunostaining for Sp1. A, Healthy control. B, Cornea with keratectasia. C, Cornea with keratoconus. Note that the intensity of the Sp1 nuclear staining is much lower in the epithelium and kerocytes of the healthy cornea and the cornea with keratectasia compared with the cornea with keratoconus (chromagen fast red TR/naphthol AS-MX phosphate, original magnification ×126).

**Figure 7.** Immunostaining results for the matrix metalloproteinases (MMPs). A-C, MMP-1 in healthy corneas, corneas with keratectasia, and corneas with keratoconus, respectively. D-F, MMP-2 in healthy corneas, corneas with keratectasia, and corneas with keratoconus, respectively. G-I, MMP-3 in healthy corneas, corneas with keratectasia, and corneas with keratoconus, respectively. Note that the intensity of the MMP-1 staining is very low in the epithelium of the healthy corneas and the corneas with keratoconus. By contrast, strong epithelial staining of MMP-1 is present in the keratoconus case. The MMP-2 and MMP-3 staining was at the background level or absent in healthy corneas, the corneas with keratoconus, and the corneas with keratectasia (chromagen 3,3-diaminobenzidine tetrahydrochloride, original magnification ×126).
In addition, we sought to compare keratectasia with keratoconus given the similarities, both clinically and topographically, between the 2 conditions.

The histologic findings from H&E staining of all 5 of our specimens of post-LASIK keratectasia were consistent with those reported in the literature, including stromal thinning of both the residual bed and flap and the lack of signs of inflammation or corneal scarring in the posterior stroma. Furthermore, Seitz et al reported macrostriae in the residual stroma of a case of keratectasia after LASIK; similar macrostriae were observed in patient 3 (right eye). Also in patient 3 (right eye) epithelial thinning was observed in the central cornea; a similar finding was reported by Kim et al. The presence of an iron ring after LASIK-related keratectasia observed in our cases was likely due to tear pooling at the edge of the ectatic cornea; this finding has also been previously reported. The Bowman layer in all 5 of our corneas did not show disruptions that are typically seen in keratoconus. Most previous histologic studies on post-LASIK keratectasia also reported a lack of Bowman layer disruption.

Immunohistochemical analysis performed on corneas with keratectasia, corneas with keratoconus, and healthy corneas disclosed that the expression pattern of α1-PI and Sp1 in keratectasia was similar to that of healthy corneas. The previously reported decrease in α1-PI expression and upregulation of Sp1 that occur in corneas with keratoconus were not observed in our cases of post-LASIK keratectasia. Therefore, LASIK-related keratectasia and keratoconus, despite similar clinical presentations, appear to be biochemically different, suggesting that the mechanisms by which progressive post-LASIK keratectasia develops differ from those in keratoconus.

The delayed onset and progressive nature of the development of keratectasia after LASIK led us to postulate that the condition may be related to alteration in protein degradation by the various MMPs. Our results showed that the MMP-1, MMP-2, and MMP-3 immunostaining in corneas with keratectasia was weak and similar to that observed in healthy corneas. A recent study, however, showed an increased expression of MMP-3 in the kerocytes of 2 corneas with post-LASIK keratectasia using an alternative antibody on cryofrozen sections and the immunofluorescence procedure. Despite a lack of immunolabeling for MMP-3 in our keratectasia specimens, immunostaining for MMP-3 in antigen-retrieved paraffin-embedded human placenta sections (positive control) consistently showed obvious positive staining. Interestingly, immunostaining for MMP-1 in keratoconus corneas showed a higher intensity in our study. This finding differs from that reported in a previous article. The disparity may be related to the adoption of antigen retrieval techniques specific to MMPs in the present study. Further research with a
larger sample size and possibly zymography would be required to investigate the expression of MMP-1 and MMP-3 in corneas with post-LASIK keratectasia.

Two of the 5 corneas with keratectasia (patient 2 [right eye] and patient 4 [left eye]) were examined by TEM in the midperipheral cornea (close to the edge of the area of central thinning) and compared with the healthy controls. A statistically significant decrease in the diameter of collagen fibrils within the residual stromal bed was demonstrated in comparison to collagen fibril diameter measurements conducted on healthy controls. In addition, interfibril distances in the cases of keratectasia were significantly decreased when compared with interfibril distances in healthy corneal specimens. These new findings imply that the development of keratectasia after LASIK may be due to stretching of the collagen fibrils in addition to compression of the cornea, which is evident even in corneal segments away from the area of ectasia. The thinning of the actual collagen fibrils along with depletion of interfibril space may suggest that the fibril and surrounding extracellular matrix in keratectasia cannot resist ectatic changes due to intraocular pressure. Currently, it is recommended that at least 250 µm of residual stroma should be left after laser ablation to provide the cornea with sufficient mechanical strength to resist ectatic changes.6,10 This value is not an absolute cutoff point because several reports have described corneas with a residual stromal bed thickness of more than 300 µm that developed ectasia, and conversely other reports have described corneas with a residual stromal bed thickness of less than 250 µm after laser ablation that did not develop keratectasia.1 This leads us to believe that each cornea has its own threshold of residual stromal thickness needed to resist deformation by healthy intraocular pressure. A biomechanical instability, however, does not explain the delayed onset and progressive nature of the disorder in some cases. Although we did not find an increase in degradative enzymes in our cases of keratectasia, Maguen and colleagues22 reported an increased expression of MMP-3 in keratocytes and MMP-10 in the epithelium of 2 ectatic corneas after LASIK. This finding suggests that biochemical changes may occur in the corneal stromal matrix before biomechanical failure in some cases. Further studies with a larger number of LASIK-related ectatic corneas are needed to further strengthen this idea.

In summary, the 5 corneas with keratectasia showed histologic changes consistent with those reported previously, including stromal thinning in the residual bed and flap, lack of inflammation or corneal scarring, macrostriae in the residual bed, presence of an iron ring, and a lack of Bowman layer disruption typically seen in keratoconus.16,18-23 Immunohistochemical studies revealed that corneas with keratectasia and healthy corneas showed similar staining patterns for α-1-PI and Sp1, distinctly different from those seen in corneas with keratoconus. The expression of MMP-1, MMP-2, and MMP-3 in keratectasia was also demonstrated to be similar to that in healthy corneas. In addition, we demonstrated the thinning of the collagen fibrils with depletion of the interfibril space in keratectasia by TEM. These results may imply that after LASIK the collagen fibrils in the residual stromal bed of corneas with keratectasia may be mechanically stretched while the surrounding extracellular matrix is concurrently compressed because of loss of structural resistance to intraocular pressure.

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