Histologic, Ultrastructural, and Immunofluorescent Evaluation of Human Laser-Assisted In Situ Keratomileusis Corneal Wounds

Daniel G. Dawson, MD; Theresa R. Kramer, MD; Hans E. Grossniklaus, MD; George O. Waring III, MD; Henry F. Edelhauser, PhD

Objective: To evaluate human corneas after laser-assisted in situ keratomileusis at different postoperative intervals.

Methods: Thirty-eight postmortem corneas from 20 patients with postoperative intervals from 2 months to 6.5 years after laser-assisted in situ keratomileusis surgery were collected from eye banks. The corneas were trisected and processed for conventional histologic analysis, transmission electron microscopy, and immunofluorescence.

Results: Light microscopy and transmission electron microscopy showed focal undulations in Bowman layer, focal epithelial hypertrophic modifications, and a variably thick (range, 0.4-16.4-µm) lamellar stromal interface scar in all specimens. The flap wound margin, which was adjacent to the epithelium, healed by producing an approximately 8-µm-thick hypercellular fibrotic scar, whereas the central and paracentral wound regions healed differently because a thinner (approximately 5-µm) hypocellular primitive stromal scar was present in all the corneas examined. Immunofluorescence identified increased type 3 collagen and myofibroblasts in the hypercellular fibrotic scar regions and decreased or absent levels of all corneal stromal components other than type 1 collagen in the hypocellular primitive scar regions.

Conclusions: After laser-assisted in situ keratomileusis surgery, the keratocyte-mediated production of a variably thick lamellar corneal stromal scar occurs, resulting in 2 regional types of scarring. The hypercellular fibrotic scar at the wound margin is usually visible clinically and functions to hold the flap in place, while the more central hypocellular primitive scar is not visible clinically and allows easy lifting of the flap postoperatively.

recent study evaluated 26 human donor corneas with previous uncomplicated LASIK using slitlamp, specular, and light microscopy.18

We examined a total of 92 postmortem eye-bank corneas from 48 patients who had LASIK and died for unrelated reasons. We previously described the histopathologic analysis and ultrastructure from 4 corneas of 2 patients19 and have submitted another publication that surveys the entire LASIK cornea to describe the prevalence and spectrum of pathologic findings found in 48 corneas from 25 patients.20 In the present study, 38 additional corneas of 20 patients were evaluated to describe in detail the changes that occur in the cellular and extracellular wound healing components of the lamellar LASIK wound interface. We correlate these findings with the ages of the wounds and suggest how they might affect the clinical appearance and function of the human cornea after surgery.

METHODS

After getting approval from the Emory University institutional review board, we obtained 38 postmortem corneoscleral buttons from 20 patients with a history of LASIK surgery from various eye banks in North America. The specimens were received in Optisol-GS solution (Chiron Ophthalmics, Irvine, Calif) within 6 days of death (mean ± SD time of preservation, 3.53±1.54 days). Review of preoperative, intraoperative, and postoperative clinical examinations was performed when available. Four postmortem normal corneas stored in Optisol-GS from 2 patients (mean time of preservation, 39 hours) and 2 postmortem normal corneas from 1 patient stored in moist chambers (mean time of preservation, 21 hours) were obtained from the Georgia Eye Bank (Atlanta) as controls.

TISSUE

Immediately after receiving the specimens, we evaluated the corneoscleral buttons for gross abnormalities with a dissecting microscope using direct transillumination and retroillumination techniques. The buttons were then oriented with the hinge superiorly and were trisected (Figure 1). The central portion was placed in 2.5% glutaraldehyde for transmission electron microscopic (TEM) processing. The left portion was placed in 10% neutral buffered formalin for conventional histologic processing. The right portion was frozen in optimal cutting temperature compound (Tissue-Tek-II; Miles Inc, Elkhart, Ind) for immunofluorescent histologic processing.

CONVENTIONAL HISTOLOGIC PROCESSING

The formalin-fixed specimens were processed by dehydration with a graded series of alcohol solutions, cleared with xylene solution, infiltrated with paraffin, and embedded into a paraffin block for sectioning. Four-micrometer-thick sections were then cut and stained with hematoxylin-eosin or periodic acid–Schiff according to routine protocols. Histopathologic findings from light microscopic examination were recorded, including endothelial cell density (determined by counting and averaging the number of endothelial cell nuclei in 5 high-power fields [HPFs; ×400] and converting to cell density using a previously derived nomogram17), epithelial thickness (taken from a representative area that had the least amount of artifacts and contained all 3 distinct types of epithelial cells: basal, wing, and superficial epithelial cells), flap thickness, and residual stromal bed thickness measurements using a calibrated ocular reticule and an Olympus BH-2 microscope (Olympus Ltd, Tokyo, Japan). A 30-µm-wide HPF column was used to quantitatively count the number of keratoocytes at the following 8 positions of the cornea (Figure 2A): (1) peripheral lamellar wound at the flap margin, (2) central lamellar wound, (3) posterior aspect of the flap centered at a depth of 100 µm from the anterior surface of the Bowman layer or 30 µm in front of this position if the 100-µm-deep column included the LASIK scar, (4) anterior aspect of the flap centered at 15 µm below the posterior surface of the Bowman layer, and (5-8) the corresponding 4 internal control positions at least 100 µm away from the lamellar scar.

ULTRASTRUCTURAL EVALUATION

The glutaraldehyde-fixed specimens were rinsed in cacodylate buffer and postfixed in 1% osmium tetroxide for 1 to 2 hours at room temperature. The tissue was then dehydrated and embedded in LX 112 epoxy resin according to standard techniques. Thick sections (1.5 µm) were then stained with toluidine blue and evaluated by light microscopy to find the lamellar LASIK wound. The blocks were trimmed around areas of interest and thin sections (70-80 nm) were cut, placed in a copper grid, double-stained with...
uranyl acetate–lead citrate, and examined with a JEM-100 CXII TEM (JEOL Ltd, Tokyo) at magnifications ranging from $4750$ to $90,000$. Additionally, 3 corneas were stained with a 2.5% glutaraldehyde solution containing 0.5% cupromeronic blue in 25mM sodium acetate buffer, pH 5.6, containing a critical electrolyte concentration of 0.1M magnesium chloride, according to published techniques, prior to routine TEM processing as described earlier to specifically identify and evaluate proteoglycan ultrastructure. After reviewing the TEM results, we recorded ultrastructural findings, including detailed descriptions and measurements (using an ultrastructure calculator) of the lamellar LASIK wound, with particular emphasis on the extracellular and cellular structures present in the wound.

**IMMUNOFLUORESCENCE**

The frozen specimens were cut into 5-µm sections using a cryostat at −20°C and mounted on coated glass slides that were dried overnight at 37°C. The sections were then fixed in acetone at 4°C for 20 minutes prior to immunofluorescence studies using a double-staining, indirect immunofluorescent method that evaluated 2 different antigens per section.

Briefly, the slides were immersed in 5% normal goat serum (for monoclonal antibody studies) or 5% normal horse serum (for polyclonal antibody studies) to block nonspecific background binding, incubated with a selected primary monoclonal or polyclonal antibody (Table 1), rinsed with phosphate-buffered saline solution, and incubated with the appropriate fluorescein isothiocyanate conjugated secondary antibody. Subsequently, the specimens were rinsed in phosphate-buffered saline solution, incubated with a second selected primary antibody for 2.5 hours at room temperature. Subsequently, the specimens were rinsed in phosphate-buffered saline solution, incubated with the appropriate fluorescein isothiocyanate conjugated secondary antibody and a cupric sulfate and ammonium acetate solution for 20 minutes at room temperature, and rinsed with distilled water prior to mounting for evaluation with the MRC-1024 laser confocal microscope (Bio-Rad Microscience Ltd).

**Table 1. Primary Antibodies Used for Immunofluorescence Evaluations**

<table>
<thead>
<tr>
<th>Antibody and Specificity</th>
<th>Source</th>
<th>Company</th>
<th>Titrated Concentration Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antihuman collagen type 1 polyclonal Ab</td>
<td>Rabbit</td>
<td>Chemicon, Inc, Temecula, Calif</td>
<td>1 in 40</td>
</tr>
<tr>
<td>Antihuman collagen type 3 polyclonal Ab</td>
<td>Goat</td>
<td>Chemicon, Inc</td>
<td>1 in 10</td>
</tr>
<tr>
<td>Antihuman collagen type 5 polyclonal Ab</td>
<td>Goat</td>
<td>Chemicon, Inc</td>
<td>1 in 20</td>
</tr>
<tr>
<td>Antihuman keratan sulfate monoclonal Ab</td>
<td>Mouse</td>
<td>Chemicon, Inc</td>
<td>1 in 100</td>
</tr>
<tr>
<td>Antihuman dermatan sulfate monoclonal Ab</td>
<td>Mouse</td>
<td>United States Biological, Swampset, Mass</td>
<td>1 in 2000</td>
</tr>
<tr>
<td>Antihuman smooth muscle actin monoclonal Ab</td>
<td>Mouse</td>
<td>DAKO, Carpinteria, Calif</td>
<td>1 in 50</td>
</tr>
</tbody>
</table>

Abbreviation: Ab, antibody.
Hemel Hempstead, UK). Normal autopsy skin specimens or internal normal corneal stroma served as positive controls while slides processed by omitting the primary antibody steps served as negative controls.

STATISTICAL ANALYSIS

All quantitative results were presented as mean±SD. When internal control measurements were obtained, we performed statistical analysis comparing the surgical areas measured with the internal controls using paired $t$ tests. The level of significance was $P≤.05$.

RESULTS

CLINICAL DATA

We could not get the complete histories of the patients who had undergone LASIK for the majority of the specimens because of limitations such as Health Insurance Portability and Accountability Act regulations and insufficient medical examiner histories. Clinical data that were available for every specimen included the patient's age, sex, cause of death, a history of having had uncomplicated myopic LASIK, and the postoperative interval since LASIK. The 20 patients (12 men, 8 women) ranged in age from 29 to 70 years (mean±SD, 46.9±10.2 years). The postoperative interval after LASIK ranged from 2 months to 6.5 years.

GROSS EXAMINATION

Gross examination of the corneoscleral buttons revealed a semicircular ring of haze along the wound margin of LASIK flaps in 34 of 38 corneas (Figure 1B and C). In all 4 corneas where a ring of haze was not present, the peripheral LASIK incision could be identified using retroillumination techniques, but it was very subtle to detect.

LIGHT MICROSCOPY

Conventional histologic and toluidine blue–stained light microscopy evaluations revealed a 2- to 8-µm-thick lamellar interface scar in all the specimens (Figure 3). Qualitatively, the scar was easily identified in all specimens up to 3 years postoperatively; it became more dif-

Figure 3. Histopathology of a 6-month-old (A and B) and a 2-month-old (C) laser-assisted in situ keratomileusis corneal wound. The arrows indicate the lamellar wound. The stains and magnifications are hematoxylin-eosin, original magnification ×25; periodic acid–Schiff, original magnification ×25; and toluidine blue, original magnification ×25 for A, B, and C, respectively.
difficult to detect by light microscopy thereafter, especially on toluidine blue–stained sections. It was most easily identified in periodic acid-Schiff–stained sections because the scar stained more intensely than surrounding normal corneal stroma in all specimens. The scar also appeared more cellular at the wound margin and less cellular in the paracentral and central portions compared with that of normal adjacent corneal stroma (Figure 4A and B). At the wound margin, some corneas showed inward basal epithelial cell elongation filling gaps in the Bowman layer (Figure 5A), variable amounts of epithelial ingrowth into the lamellar wound (Figure 5), microscopic foci or islands of epithelial actually in the scar (Figure 5D), and variability in the alignment of the cut end of Bowman layer at the wound margin (eg, good alignment with a small gap at the break [Figure 6C], depressed or elevated ends [Figure 4A and Figure 5A], curled inward ends [Figure 5D], or good alignment with a wide

Figure 4. Histopathology of a 2-month-old laser-assisted in situ keratomileusis corneal wound (arrows) at the peripheral wound region (A) and at a more central, deeper region (B). A, Notice the thicker, hypercellular wound margin scar type (between the left arrowheads) compared with the thinner, hypocellular scar type present in the remaining aspects of the wound (between the right arrowheads and between all of the arrowheads in B). The epithelium in this cornea has severe artifactual changes (toluidine blue, original magnification ×100).
Figure 5. Histopathology of the peripheral wound region of 4 laser-assisted in situ keratomileusis corneas displaying varying degrees of epithelial ingrowth. A and B represent the more common appearances of the flap margin, while C and D are uncommon but informative appearances of the flap margin. A, Hypertrophy of basal epithelial cells producing an elongated cell type to fill the divot at the flap margin. B, Microscopic, presumably clinically inconspicuous, epithelial ingrowth. C, Large, presumably clinically apparent, epithelial ingrowth. D, An island of epithelial ingrowth. B and D have severe artifactual epithelial changes (toluidine blue, original magnification ×100).

Figure 6. Ultrastructure of a 4-month-old laser-assisted in situ keratomileusis (LASIK) corneal wound using low magnification (original magnification ×4750) (A) and moderate magnification (original magnification ×35,000) (B) of the typical LASIK wound margin. A, Notice that the initial 42-µm length of the 5.1-µm-thick (between arrowheads) scar (arrow) is hypercellular. B, The extracellular matrix of this scar is consistent with fibrosis because it predominantly is composed of a dense network of mildly disorganized collagen fibrils (arrows) with few patches of electron-dense granular material (arrowheads). Wound margin variants that had clinically transparent flap margins are shown in C and D. C, A well-aligned wound margin with minimal separation of the Bowman layer and minimal fibrotic scar tissue in the small 1.1-µm wound gap (original magnification ×4750). D, Epithelial ingrowth (arrowhead) of 80 µm in length is present in the wound margin with a small 5-µm surrounding zone of hypercellular fibrosis and an adjacent hypocellular primitive scar immediately beneath it (arrow) (original magnification ×4750). Bar indicates 1 µm.
gap at the break (Figure 6A). Mild epithelial hyperplasia was commonly present over the wound margin (Figure 7A–C). Although the epithelial hyperplasia was never found over the paracentral or central flap surface, there were focal areas of basal epithelial cell hypertrophic elongation causing mild thickening of the corneal epithelium. The basal epithelial cell elongation was most pronounced in valleys produced by undulations of the underlying Bowman layer as if the cells were stretching inward to fill in the depression, without altering the surface contour. These undulations in the Bowman layer were present in random focal areas over the flap in all corneas examined and are consistent with the clinical term of microstriae (Figure 7D). In the paracentral and central portions of the scar in 1 patient who had LASIK 2 months before dying, both corneas had small extracellular vacuoles in the lamellar scar that did not stain with periodic acid–Schiff, Alcian blue, or colloidal iron.

Quantitative light microscopy results are summarized in Table 2. Epithelial cell measurements were taken in 3 locations (Figure 7B): peripheral to the flap, over the flap margin (Figure 7C), and on the central flap surface (Figure 7C). The central thickness of the LASIK flap averaged 160±23 µm, the residual stromal bed thickness averaged 399±54 µm, and the endothelial cell density averaged 2307±974 cells/mm². Epithelial ingrowth into the flap margin was found in 20 (53%) of the 38 corneas examined using step sections. The length of epithelial ingrowth averaged 291±209 µm (range, 30-900 µm). Keratocyte cell counts per HPF obtained from 4 locations along the lamellar scar or in the flap were each statistically compared with 4 normal internal control values outside the surgical

<table>
<thead>
<tr>
<th>Pathologic Finding</th>
<th>Thickness, Mean ± SD</th>
<th>Range of Thickness</th>
<th>No. of Cell Layers, Mean ± SD</th>
<th>Range of Cell Layers, No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral epithelial thickness</td>
<td>33 ± 6</td>
<td>24-44</td>
<td>5.0 ± 0.9</td>
<td>4-7</td>
</tr>
<tr>
<td>Marginal epithelial thickness</td>
<td>43 ± 9</td>
<td>26-64</td>
<td>5.8 ± 1.4</td>
<td>4-9</td>
</tr>
<tr>
<td>Central epithelial thickness</td>
<td>29 ± 7</td>
<td>20-44</td>
<td>4.9 ± 0.9</td>
<td>4-6</td>
</tr>
<tr>
<td>Flap thickness</td>
<td>160 ± 23</td>
<td>110-216</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Residual stromal bed thickness</td>
<td>399 ± 54</td>
<td>290-460</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Endothelial cell density</td>
<td>2307±974 cells/mm²</td>
<td>1826-2843 cells/mm²</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Epithelial ingrowth</td>
<td>291±209 µm</td>
<td>30-900</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not applicable.

*Values are in micrometers unless otherwise indicated.
field (Figure 2A). These results showed that a statistically significant ($P < 0.05$) increase of keratocytes (Table 2) occurred at the LASIK wound margin regardless of time from LASIK to death ($P < 0.02$) (Figure 2B), but no statistical difference was found along the central stromal wound ($P > 0.13$) compared with controls (Figure 2C). Keratocyte cell counts per HPF taken in the LASIK flap showed a statistically significant reduction (Table 2) at the anterior aspect of the flap compared with controls up to 6 months postoperatively ($P < 0.07$), and no statistical difference was found after 6 months in this region ($P > 0.19$). At all postoperative times, the posterior aspect of the flap had similar keratocyte densities ($P > 0.28$) as controls (Figure 2D).

ULTRASTRUCTURAL EVALUATION

Low-magnification ($\times 4750$) TEM results of the central (Figure 8A) and the paracentral lamellar wound (Figure 8B) showed a variably thick, hypocellular primi-
tive stromal scar present in all corneas with a mean cross-sectional thickness of 4.5 ± 3.1 µm (range, 0.4-11.4 µm). In LASIK wounds less than or equal to 6 months old, the keratocytes in or next to this scar predominantly had the morphological appearance of activated keratocytes (ie, prominent mitochondria, prominent Golgi apparatus, and extensive dilated endoplasmic reticulum). The proportion of keratocytes appearing activated and the extent of activation gradually diminished over time from 2 to 6 months after surgery. In all corneal wounds older than 6 months, only quiescent keratocytes with occasional small (0.25-2 µm in diameter), clear intracytoplasmic vacuoles were present. High-magnification ($\times 72,500$) TEM results showed that the extracellular matrix of the hypocellular scar was predominantly composed of an electron-dense granular material (Figure 8C) with only small amounts of loosely interspersed collagen fibrils slightly decreased in diameter (mean ± SD, 21.1 ± 0.8 nm) from normal (mean ± SD, 25.9 ± 1.4 nm). These interspersed collagen fibrils were randomly directed and severely and variably spatially disordered (Figure 8D) compared with normal corneal stroma. Transmission electron microscopy results from cupromeronic blue–stained tissue showed that the composition of this electron-dense granular material was a mixture primarily composed of abnormally large, non–fibril-bound proteoglycans (average size, 360 nm × 20 nm) with small, interspersed amounts of normal-size, fibril-bound proteoglycans (average size, 40-65 nm × 6 nm) and collagen microfibrils or molecules (Figure 9A and B).

At the wound margin, up to 75 µm of the initial length of the wound adjacent to the epithelium healed by pro-
ducing a hypercellular fibrotic stromal scar (Figure 6A and B). Interestingly, there were smaller areas of this scar type if the Bowman layer break was almost perfectly aligned (Figure 6C) or if epithelial ingrowth extended into the wound (Figure 6D). In low-magnification ($\times 4750$) TEM results, the typical LASIK flap wound margin showed a vari-

Figure 8. Ultrastructure of a 6-month-old laser-assisted in situ keratomileusis corneal wound (arrows) using low-magnification transmission electron microscopy (original magnification $\times 4750$) at the central scar region (A) and the paracentral scar region (B). A, Notice the variation in thickness of the central scar (5.8 µm thick between arrowheads at the left and 1.1 µm thick between arrowheads at the right). B, The paracentral scar is more uniform in thickness (5.5 µm thick between arrowheads at the left and 7.0 µm thick between arrowheads at the right). Higher-magnification views ($\times 72,500$) in the central scar showing that it is primarily composed of electron-dense granular material (arrows) are shown in C and D. C, The electron-dense granular material (arrow) appears to be produced by activated keratocytes (arrowhead) because it is most densely concentrated around these cell types and a few vacuoles inside these cells contain similar material. D, Notice the severe disorganization and the small amount of collagen fibrils (arrowheads) in this wound type. Bar indicates 1 µm.
ably thick hypercellular fibrotic scar (Figure 6A) with a mean cross-sectional thickness of 7.8±4.1 µm (range, 1.1-16.4 µm). In LASIK wounds less than or equal to 6 months old, the keratocytes in or next to this scar predominantly had the morphological appearance of activated keratocytes. Although the proportion of keratocytes appearing activated and the extent of activation gradually diminished over time from 2 to 6 months after surgery (similar to the

Figure 9. Ultrastructure using cupromeronic blue-stained tangential transmission electron microscopy (TEM) views of the interface scar (A) and 10 µm deeper into the residual stromal bed (B) of a 27-month-old laser-assisted in situ keratomileusis corneal wound. A, Notice the predominance of abnormally large, non-fibril-bound proteoglycans in the interface scar (arrows). B, The residual stromal bed shows a predominance of normal-size, fibril-bound proteoglycans (arrowheads) and fewer abnormally large proteoglycans (arrows). Bar indicates 1 µm (cupromeronic blue-stained TEM, original magnification x90 000).
central hypocellular scar type), the density and extent of keratocyte differentiation was greater in the hypercellular wound margin scar compared with the hypocellular primitive scar. In all corneal wounds older than 6 months, only quiescent keratocytes were apparent. High-magnification (×72 500) TEM results showed that the extracellular matrix of this scar type primarily was composed of a dense network of normal diameter collagen fibrils (mean±SD, 26.6±3.0 nm) with minimal interspersed patches of electron-dense granular material. The collagen fibrils of this scar type were mildly spatially disordered and randomly directed compared with normal corneal stroma (Figure 6B).

Ultrastructural evaluation also demonstrated in some cases empty spaces filled with foreign material suspicious for plastic particles23 in the peripheral scar (Figure 10A), occasional foci of microscopic (ie, 1-2 cells) epithelial cell implantation in the scar, extracellular spaces filled with lightly electron-dense fibrillar material in the scar (Figure 10B), occasional vacuolated cells containing fibrillar material next to the scar (Figure 10C), and foci of wide-spaced 110-nm banded collagen interspersed near the scar (Figure 10D). These last 3 findings were most prominent in the 2-month-old LASIK corneal wounds and were rarely found in wounds older than 6 months.

**IMMUNOFLUORESCENCE**

Immunofluorescent staining showed that collagen type 1 (Figure 11A) was present throughout the entire (ie, margin, paracentral, and central) lamellar scar at similar intensity levels compared with normal corneal stroma. Collagen type 6 and keratan sulfate were found in the wound margin at similar intensity levels to normal corneal stroma and slightly less than normal levels at the paracentral and central scar regions. Collagen type 5 was found in the wound margin at similar intensity levels to normal corneal stroma but was markedly reduced compared with normal levels at the paracentral and central scar regions. Collagen type 3 (Figure 11B) was present in the wound margin scar at higher-intensity levels than normal, and dermatan sulfate was present at the wound margin scar at similar intensity levels to normal corneal stroma, while both were absent in the paracentral and central scar regions. Used as a marker for myofibroblast differentiation, smooth muscle actin was found only in keratocytes at the wound margin scar.

Corneal wound healing using full-thickness,24 partial thickness,25 and superficial keratectomy wounds26 has been studied extensively in animal models and humans, especially...
in correlation to corneal transplantation, cataract extraction, radial keratotomy, and PRK. Those studies all showed that the cornea heals through a series of well-defined stages and steps: active wound healing (initial corneal injury; proliferation and migration of surviving corneal epithelial cells, stromal keratocytes, and endothelial cells; cellular differentiation with active removal, synthesis, and deposition of extracellular material) and tissue remodeling. Corneal function (ie, transparency and wound strength) usually returns to maximal levels, but not always normal levels, after completion of the remodeling stage.

Most of the cellular and extracellular details of LASIK wounds during the active wound-healing stage have been accurately addressed through animal histologic studies. Those studies have shown that LASIK-induced corneal stromal injury results in keratocyte apoptosis in a zone approximately 50 µm anterior and 50 µm posterior to the central and paracentral lamellar incision over the first 24 hours after injury. Animal studies have also shown that epithelial-stromal interactions significantly augment the initial wound response cascade only at the wound margin while the entire LASIK wound enters the phases of keratocyte proliferation (which starts by 4 hours after injury and ends by 1 week after injury), minor continued keratocyte apoptosis and minor keratocyte necrosis (24 hours to 1 week), inflammatory cell infiltration (4 hours to 1 week), keratocyte cell migration (3 days to 3 weeks), and keratocyte differentiation (1-6 weeks in the central flap and 1 week to 3 months or longer at the flap margin). Keratocyte differentiation is important for corneal transparency because the transient appearance or chronic persist-
uses up to 6 months postoperatively, with most corneas stabilizing between 3 and 6 months after surgery. Another study that evaluated full-thickness corneal wounds found that the remodeling stage occurs up to 4 years after surgery. Overall, these human studies are important because they imply that active corneal stromal wound healing should be completed by 6 months after LASIK, followed by a remodeling stage that may last up to 4 years.

Our study of postoperative (2 months to 6.5 years) human LASIK corneal wounds found the production of a stromal interface scar of variable thickness during the time of active corneal wound healing and an epithelium displaying focal outward compensatory epithelial hyperplasia only at the flap margin. Furthermore, our quantitative epithelial measurements did find evidence of a slight increase in the average thickness of central corneal epithelium of 1.3 to 3.2 µm compared with our normal control corneas. This epithelial thickening over the flap surface was identified as focal areas of inward basal epithelial cell hypertrophic elongation, most prominently seen filling in valleys produced by underlying microstriae (Figure 7D). This inward epithelial hypertrophy represents a milder compensatory mechanism of epithelial thickening than that of outward compensatory epithelial hyperplasia that usually is seen in cases with more abrupt contour changes or more excavated areas of stromal loss (eg, the LASIK wound margin). The cause for the epithelial thickening over the center of the flap after LASIK is important because it previously was suspected by several clinical LASIK studies to cause the regression of initial refractive effect. Those previous longitudinal studies showed a mean epithelial thickening of 6 to 8 µm over the center of the flap after LASIK, predominantly developing 1 week to 1 month after surgery. It was speculated from those studies that this epithelial thickening represented outward compensatory epithelial hyperplasia, which presumably resulted in a steepening of the curvature on the anterior corneal epithelial hyperplasia, which presumably resulted in a steepening of the curvature on the anterior corneal surface with resulting myopic regression. Although never addressed in those studies, it is possible that a large portion of the change in epithelial thickness in the previous clinical studies resulted from discontinuing chronic contact lens wear rather than the LASIK surgery itself. It has been previously reported that the epithelium thickens on average 6 µm over the first 33 days after extended-wear soft contact lens removal, likely caused by hypoxia relief. On the other hand, because our study is not longitudinal and tissue artifacts may have been introduced from storage and histologic processing (particularly the epithelial cell shrinkage induced by Optisol-GS storage), perhaps our post-LASIK central epithelial thickening results should be greater. Despite these possibilities, our study definitely found no evidence of outward epithelial hyperplasia over the surface of the flap except at the wound margin and only minor focal areas of epithelial thickening over the center of the flap from inward basal epithelial cell elongation.

In the stromal wound, after the LASIK flap wound is created by the microkeratome (mechanical or laser), we found that there are 2 distinct types and regions of wound healing: a fibrotic reparative stromal wound healing occurring at the wound margin and a primitive reparative stromal wound healing in the remainder of the wound. The edge of the LASIK flap healed according to classic corneal stroma wound healing because a variably thick (mean±SD thickness, 7.8±4.1 µm) hypercellular fibrotic scar was produced. Our quantitative keratocyte measurements confirmed that a permanent but gradually diminishing statistically significant increase in keratocytes occurred in the wound margin scar compared with normal (Figure 2B). Additionally, the transient (ie, ≤6 months after surgery) and persistent alterations found in the keratocytes populating this scar type, the transient activation of large numbers of keratocytes, and the occasional persistent myofibroblasts were only present in this region of the wound. This presumably occurs because epithelial-stromal interactions (ie, chemotactic cytokines and growth factors from epithelial cells, keratocytes, and tears) only take place in this region of the wound, where the 2 tissues are in apposition. The extracellular matrix of this scar type was described as fibrotic because it was predominantly composed of a dense network of normal diameter (approximately 26 nm) collagen fibrils. Based on our immunofluorescence results, this scar contained more collagen type 3 compared with that normally present in the corneal stroma while possessing similar levels of collagen types 1, 5, and 6; dermatan sulfate; and keratan sulfate.

By comparison, the paracentral and central portions of the wound that have no epithelial cell influences healed similarly to early nonspecific corneal stroma wound healing steps that partially recapitulate events of a developing fetal cornea. Our quantitative keratocyte measurements confirmed that this scar type was hypocellular, but using a 30-µm-wide HPF column did not show a statistically significant decrease in keratocytes compared with normal (Figure 2C), probably because this scar type was much thinner than the measuring width of the HPF column. Only transient wound response keratocytes (ie, migratory and activated keratocytes) were found in this sparsely keratocyte-populated scar type. The extracellular matrix of this scar type is described as primitive because it primarily is composed of electron-dense granular material with only loosely scattered, smaller-than-normal-diameter collagen fibrils (approximately 21 nm). When our ultrastructural evaluations were arranged chronologically by the age of the wound, an increase in the amount and cross-sectional thickness of the electron-dense granular material was apparent in the hypocellular scar regions from 2 to 6 months old; otherwise, no other qualitative trends were apparent. This electron-dense granular material was found to be a mixture predominantly composed of abnormally large, highly sulfated, non–fibril-bound proteoglycans with small, interspersed amounts of both normal-size, fibril-bound proteoglycans and collagen microfibrils and molecules. Based on our immunofluorescence results, this scar contained similar amounts of collagen type 1, slightly decreased amounts of collagen type 6 and keratan sulfate, markedly decreased amounts of collagen type 5, and no detectable amounts of collagen type 3 and dermatan sulfate compared with those of normal corneal stroma.

Previous studies using cuprolinic blue staining in rabbit corneas with full-thickness wounds, superficial me-
clinical keratectomy wounds, superficial laser keratectomy wounds, and mechanical lamellar incisions have also shown the production of nonspecific, abnormally large sulfated proteoglycans during early stromal wound healing; however, in those studies the abnormal proteoglycans decreased in size and density as rabbit corneal wounds aged before disappearing altogether by 1 year after the injury. We did not detect a similar decrease in the size, density, or disappearance of these abnormal proteoglycans in adult human corneal wounds that developed a hypocellular primitive stromal scar, but it should be emphasized that our study was limited to only 3 curomeric blue evaluations (6-month-old, 27-month-old, and 5-year-old corneal wounds). Two rabbit studies also have demonstrated that the majority of the abnormally large proteoglycans were susceptible to digestion with chondroitinase ABC indicating that they most likely are a type of dermatan sulfate proteoglycan. Although this was confirmed in a rabbit study using chromatography techniques, 2 other chromatography studies in rabbits also discovered that abnormally large keratan sulfate proteoglycans are present in corneal stromal wounds as well but in reduced amounts compared with the normal corneal stroma. 

Interestingly, these abnormally large proteoglycans were found to be altered in molecular form from normal as the deramatan sulfate contained a 2-fold increase in iduronic acid content and was more highly sulfated than normal while the keratan sulfate was undersulfated compared with normal. This alteration of form may explain why our primary antibody for normal corneal deramatan sulfate failed to stain the hypocellular primitive scar. Overall, all of the proteoglycan alterations previously discussed and the reduced collagen fibril formation in the hypocellular primitive stromal scar may increase the water-absorbing and swelling capabilities of the cornea wound. This would then explain the preferential localization of corneal edema to the LASIK interface region in patients with endothelial cell dysfunction or high intraocular pressure.
Table 3. Pathologic-Clinical Correlation

<table>
<thead>
<tr>
<th>Pathologic Finding</th>
<th>Clinical Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compensatory outward epithelial hyperplasia at the wound margin</td>
<td>Smooths contour of anterior corneal surface</td>
</tr>
<tr>
<td>Focal inward basal epithelial cell hypertrophy over flap surface</td>
<td>Fills in low spots produced by the underlying Bowman layer undulations and Bowman layer breaks</td>
</tr>
<tr>
<td>Focal undulations of the Bowman layer in the flap</td>
<td>Minor or secondary role in regression of refractive effect (early component 1 wk to 1 mo)</td>
</tr>
<tr>
<td>Hypercellular fibrotic wound margin scar</td>
<td>Postoperatively may increase flap thickness measurements</td>
</tr>
<tr>
<td></td>
<td>Represents how the corneal stroma of the flap adjusts to fill in the gap produced by the missing ablated tissue</td>
</tr>
<tr>
<td>Hypocellular primitive central and paracentral scar</td>
<td>Hazy gray semicircular ring may be clinically apparent (primarily due to myofibroblasts)</td>
</tr>
<tr>
<td></td>
<td>Strongest portion of scar (holds flap in place)</td>
</tr>
<tr>
<td></td>
<td>Major role in regression of refractive effect</td>
</tr>
<tr>
<td>Hypocellular primitive central and paracentral scar</td>
<td>Clinically invisible scar</td>
</tr>
<tr>
<td></td>
<td>Weakest portion of scar (allows easy lifting of flap for an indefinite time postoperatively)</td>
</tr>
<tr>
<td></td>
<td>Moderate role in regression of refractive effect</td>
</tr>
<tr>
<td></td>
<td>Increased capacity to absorb water and swell</td>
</tr>
<tr>
<td></td>
<td>Serves as a potential space for cells (eg, white blood cells, epithelial cells, red blood cells) or microbes to collect in</td>
</tr>
<tr>
<td></td>
<td>Major role in regression of refractive effect</td>
</tr>
<tr>
<td></td>
<td>Minor or secondary role in regression of refractive effect (early component 1 wk to 1 mo)</td>
</tr>
<tr>
<td></td>
<td>Increased capacity to absorb water and swell</td>
</tr>
<tr>
<td></td>
<td>Serves as a potential space for cells (eg, white blood cells, epithelial cells, red blood cells) or microbes to collect in</td>
</tr>
<tr>
<td>Transparent derivation</td>
<td>Transient decrease in corneal sensation, decrease in tear production, and neurotrophic epitheliopathy</td>
</tr>
<tr>
<td></td>
<td>Transient decrease in anterior keratocyte density</td>
</tr>
</tbody>
</table>

Wound tissue strength after LASIK can be addressed qualitatively from 2 vantage points. First, because our ultrastructural evaluations showed that the architecture of the hypacellular primitive stromal scar regions lacks collagen fibrils (ie, the insoluble connective tissue component important for resisting tensile stress) but contains more than a sufficient amount of altered proteoglycans (ie, soluble connective tissue component that is important in resisting compressive forces), we suspect that the LASIK scar is biomechanically weak in tensile cohesive strength compared with normal corneal stroma, except at the wound margin where the hypercellular fibrotic scar resides. A previous quantitative human corneal wound strength study using healed, sutured, full-thickness corneal or corneoscleral wounds of different ages found that the tensile wound strength of the scars increased gradually up to 4 years after injury before stabilizing at approximately 64% to that of normal stromal tissue. At the wound margin where the hypercellular fibrotic scar resides, the tensile wound strength of the scars increased gradually up to 4 years after injury before stabilizing at approximately 64% to that of normal stromal tissue. Although LASIK corneal wounds are unsutured, partial thickness, and oriented obliquely or parallel to the corneal surface, we presume that LASIK wounds probably reach similar wound strengths over the first 4 years after injury. Unfortunately, no quantitative human lamellar corneal wound studies have been published to address this issue, but qualitative observations from our study directly show that LASIK wounds are weaker than normal corneal stroma. We found that LASIK wounds never healed sufficiently to prevent artificial flap detachments, induced by routine histologic sectioning of tissue, from occurring (34% of our corneas developed partial or complete flap detachments, including many LASIK corneal wounds 4 years old or greater, whereas normal, uninjured corneal tissue rarely artifactually fragmented). We also found evidence for remodeling in LASIK scars at the wound margin (ie, gain in wound strength) because LASIK wounds 3 years old or greater never artifactually detached at the wound margin unless an overlying epithelial defect was present. Furthermore, our qualitative assessment of wound strength parallels the clinical literature, which cites numerous case reports or case series of late traumatic flap dislocations occurring up to 38 months after LASIK and from the success of relifting flaps up to 8 years postoperatively for enhancement procedures after manually debriding the overlying marginal epithelial surface and breaking the fibrotic flap wound margin stromal scar. Because 1 of us (G.O.W.) found that LASIK flaps can be relifted with little difficulty up to 11 years postoperatively, we suspect that it probably will be demonstrated in the future that LASIK flaps can be lifted indefinitely. In fact, when excimer lasers create a specific pattern in the surface of the residual corneal stromal bed, such as the concentric circles that are left behind by a large area dilating diaphragm laser, it is possible to see the pattern many years after initial surgery when the flap is relifted, attesting to the lack of fibrosis in the region of the hypacellular primitive scar.

Although the LASIK flap in our studies had no obvious extracellular matrix alterations or changes in the cellularity in the posterior aspect of the flap (Figure 2D), we did find that the anterior aspect of the LASIK flap showed a transient, statistically significant decrease in keratocytes in wounds less than or equal to 6 months old compared with normal; thereafter, the anterior flap remained slightly less cellular than normal but not statistically different (Figure 2D). As this correlates with the time of transient denervation of the flap, we suspect that this reduction in keratocyte density may be due to loss...
of neuropeptides produced by corneal nerves, such as sub-
stance P. Substance P has been found to have synergis-
tic effects with insulin-like growth factor-1 or epider-
mal growth factor.64 both of which are produced by
epithelial cells and keratocytes and are powerful ker-
atoctye chemotactic factors.65 Although a decrease in ker-
atocyte density in the anterior aspect of the flap has been
previously described,66,67 those studies both used in vivo
confocal microscopy to measure keratocyte density and
did not find statistically significant decreases until 6
months or longer after LASIK. We think that the differ-
ences between our histopathologic study (30-µm-thick
tissue section) and those confocal microscopy studies (9-
µm-thick optical section) probably result from sam-
plicity errors that occur when trying to measure such small
portions of the tissue that are so highly variable in cel-
ularity.

In summary, our human LASIK study demonstrated
that active wound healing occurs in the corneal stroma
up to 6 months postoperatively, producing either a
hypocellular primitive stromal scar or a hypercellular
fibrotic stromal scar. The latter scar type presumably
occurs when epithelial-stromal interactions augment the
normal stromal wound healing steps. Additionally, our
ultrastructural evaluations and qualitative wound
strength observations suggest that remodeling of LASIK
wounds occurs up to 3 years postoperatively only in
hypercellular fibrotic stromal scar regions (ie, the flap
wound margin). Finally, because permanent histologic
and ultrastructural changes were universally found in all
our LASIK corneas, we have tried to explain how each
common microscopic finding potentially alters corneal
functionality (Table 3). The reason why human adult
corneal stromal wounds heal incompletely is currently
unknown.22 Based on the frequency and variable degree
of DNA damage found in normal adult keratocyte popu-
lations,68 it is possible that adult keratocytes may be too
altered at the genetic level to successfully repair the
corneal stroma completely using keratocyte injury path-
ways alone. This reason could additionally help explain
why wound-healing responses can occur to such unpre-
dictable degrees, even in the same cornea. It is fascinat-
ing that when corneal stromal injury is accompanied by
overlying epithelial injury, the superficial corneal stroma
near the epithelial cell surface heals by producing a
more cellular scar with a more completely assembled
extracellular matrix than the rest of the stromal wound.
Maybe the ectodermally derived epithelial cells make the
growth factors, enzymes, or structural proteins neces-
sary for completing extracellular matrix repair, which is
lacking from normal or damaged neural crest–derived
keratocytes. Two major deficiencies in this epithelial-
stromal pathway appear to be the potential to perma-
nently alter the transparency of wound response kerato-
cytes and the fact that this pathway only occurs over
short distances.

Submitted for Publication: May 10, 2004; final revision
received July 28, 2004; accepted September 28, 2004.
Correspondence: Henry F. Edelhauser, PhD, Emory Eye
Center, 1365-B Clifton Rd, NE, Atlanta, GA 30322
(ophthfe@emory.edu).

Funding/Support: This work was supported in part by
grants EY-00933, P30-EY06360, and T32-EY07092 from
the National Eye Institute, Bethesda, Md, and an unre-
stricted grant from Research to Prevent Blindness, New
York, NY.

Acknowledgment: We thank Nancy L’Hernault and
Xiaoyan Nie, Emory ophthalmologic pathology labora-
tory technicians, for the beautiful electron microscopy
and immunofluorescence evaluations. We also thank
Patrick de Leon, Emory University undergraduate stu-
dent, for his tremendous help with the illustrations
and photoediting and Linda Muller, PhD, Netherlands
Ophthalmic Research Institute (Amsterdam, the Neth-
erlands), for her assistance on the cupromeronic blue
staining technique.

REFERENCES

2. Pallikaris IG, Papatzanaki ME, Stathi EZ, Frerschocck O, Georgiadis A. Laser in
Presented at: Meeting of the International Society of Refractive Surgery of the
American Academy of Ophthalmology; November 14, 2003; Anaheim, Calif.
7. Ahm M, Wetzl W, Winter M, Uthoff D, Dunker GI. Histopathological compar-
ison of photorefractive keratometry and laser in situ keratomileusis in rabbits.
8. Park CK, Kim JH. Comparison of wound healing after photorefractive kerate-
25:842-850.
9. Nakamura K, Kurosaka D, Bissen-Miyajima H, Tsubota K. Intact corneal epith-
eum is essential for the prevention of stromal haze after laser assisted in situ
ogy of corneal wound healing after photorefractive keratectomy and laser in situ
11. Latvala T, Linna T, Tervo T. Corneal nerve recovery after photorefractive kera-
12. Wright JD, Neubar CC, Stevens G. Epithelial ingrowth in a corneal graft treated
13. Geggel HS, Tailey AR. Delayed onset keratactasia following laser in situ
14. Saichow DJ, Zirm ME, Pfaller K, Stiedorff C. Histology and keratometric studies
15. Wu WC, Stark WJ, Green WR. Corneal wound healing after 193-nm excimer la-
16. Philipp WE, Speicher L, Gottinger W. Histological and immunohistochemical find-
2003;29:808-820.
18. Mootha VV, Dawson D, Kumar A, Gleiser J, Qualls C, Albert DM. Stillamp, specu-
lar, and light microscopic findings of human donor corneas after laser-assisted
ings in human corneas after successful laser in situ keratomileusis. Arch Ophthal-
mol. 2002;120:288-293.
20. Kramer TR, Chuckpaiwong V, Dawson SG, L’Hernault NL, Grossniklaus HE, Edel-
hauser HF. Pathologic findings in postmortem corneas after successful laser in
21. Williams KK, Noe RL, Grossniklaus HE, Drews-Botsch C, Edelhauser HF. Corre-
lation of histologic corneal endothelial cells counts with specular microscopic
22. Muller LJ, Pels E, Schurmans LRHM, Vrensen GFJ. A new three-dimensional


Error in Figure. In the Clinical Sciences article by Dawson et al titled “Histologic, Ultrastructural, and Immunofluorescent Evaluation of Human Laser-Assisted In Situ Keratomileusis Corneal Wounds,” published in the June issue of the ARCHIVES (2005;123:741-756), an incorrect figure appeared as Figure 11 on page 751. The corrected Figure 11 is reprinted here. The ARCHIVES regrets the error.

Figure 11. Immunofluorescence of a 5-year-old laser-assisted in situ keratomileusis (LASIK) corneal wound (arrows) showing type 1 collagen throughout the entire interface scar (A) and increased type 3 collagen at the wound margin only (B). Using the primary antibody for /H9251 smooth muscle actin, myofibroblasts were found in a 75-µm zone in the wound margin scar in a 4-month-old LASIK wound (C) and in first cell layer below the epithelial surface in a 3-year-old LASIK wound (D) (original magnification ×100).