Correlation Between Epithelial Ingrowth and Basement Membrane Remodeling in Human Corneas After Laser-Assisted In Situ Keratomileusis

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Objective: To further investigate the hypothesis that epithelial ingrowth in human corneas after laser-assisted in situ keratomileusis (LASIK) correlates with basement membrane remodeling, as suggested by the presence of matrix metalloproteinase 9 around epithelial cells in the lamellar scar.

Methods: Immunohistochemical analysis and transmission electron microscopy were applied to human post-mortem corneas with post-LASIK epithelial ingrowth.

Results: Epithelial ingrowth into the flap margin was observed in 8 of 18 corneas (44%). Matrix metalloproteinase 9 immunolocalized around ingrown epithelium in 6 of these 8 corneas (75%). There was a positive correlation between the presence of matrix metalloproteinase 9 at the wound margin and discontinuities in the basement membrane, as determined by laminin and β1 integrin immunofluorescence. Transforming growth factor β2 was present into the stroma of some corneas with epithelial ingrowth and interrupted basement membrane, suggesting some degree of epithelial-stromal interaction. Transmission electron microscopy confirmed large areas of remodeled basement membrane along ingrown epithelial cells.

Conclusions: The neo–basement membrane components underlying the ingrown cells in human corneas with epithelial ingrowth after LASIK appear to be partially disassembled. Epithelial-stromal interaction over time may be related to prolonged wound healing remodeling, which calls into question the stability of the flap.

the ingrown cells can continue to grow, affecting vision as the cells encroach on the visual axis and cause distortion at the flap surface. Aggressive ingrowth can be associated with flap edge melting.1,2,4

Members of the matrix metalloproteinase (MMP) family are involved in normal and pathologic tissue repair processes, including epithelial regeneration and failure to heal, fibrotic repair and scar remodeling, infection, and angiogenesis.6-26 In a previous study, our group reported immunolocalization of MMP-9 (also known as gelatinase B) around epithelial cells trapped in the lamellar scar of post-LASIK corneas with epithelial ingrowth.27 Matrix metalloproteinase 9 catalyzes cleavage of all types of denatured collagens as well as components of the native basement membrane.28 It is produced in the corneal epithelium, migrating across the intact basement membrane after abrasion injury, and then disappears once regeneration is complete.1,4 However, in situations involving chronic epithelial defects, MMP-9 levels remain elevated, causally contributing to failure to heal.10,31 Similarly, a number of different MMPs are produced by the fibrotic repair tissue deposited in response to keratectomy that penetrates through the epithelium and into the stroma.9 A causal relationship between overexpression of MMP-9 in the corneal epithelium and basement membrane dissolution/failure to heal has been demonstrated in experimental models.10,31 In these studies, the corneal epithelium gave the appearance of an invading front, dissolving basement membrane and subsequently penetrating the underlying stroma in its path.

Our group recently suggested that MMP-9 immunolocalization around ingrown epithelium in post-LASIK corneas may correlate with basement membrane interruption or irregularities.27 Previous studies10,31 emphasized overexpression of MMP-9 in the corneal epithelium in cases in which epithelial-stromal interactions were observed. We undertook the present study to determine whether epithelial ingrowth in human post-LASIK corneas correlates with basement membrane remodeling at the flap margin. To address this question, we performed analyses using immunohistochemistry and transmission electron microscopy.

**METHODS**

**POST-LASIK TISSUE SAMPLES**

After approval by the Emory University Institutional Review Board, 18 postmortem corneoscleral buttons from 10 corneal eye bank donors with a history of LASIK surgery were obtained from various eye banks in North America. A number was assigned to each cornea according to the order of inclusion. The specimens were received in corneal storage medium (Optisol-GS; Bausch & Lomb Surgical, Irvine, California) within 6 days of death (mean [SD] time of preservation, 3.51 [1.40] days). Preoperative, intraoperative, and postoperative clinical records were reviewed when available. As controls, 4 postmortem normal corneas from 2 patients stored in corneal storage medium (mean time of preservation, 2.95 [0.35] days) were obtained from the Georgia Eye Bank (Atlanta) and the Lions Eye Bank (Miami, Florida).

The corneoscleral buttons were evaluated for signs of previous LASIK surgery by identifying a gray hazy semicircular ring evident at the LASIK flap margin. The corneoscleral buttons were oriented with the hinge in the superior position and then dissected. The central portion was immediately snap frozen in liquid nitrogen, embedded in optimal cutting temperature compound (Tissue-Tek-ll; Miles Inc, Elkhart, Indiana), and stored at −70°C. Frozen specimens were sectioned in a cryostat microtome (Leica 1850 cryostat; Leica Microsystems Inc, Deerfield, Illinois) and mounted on adhesive-coated glass slides for conventional and immunofluorescent histologic processing. The remaining frozen portion was processed for analysis by transmission electron microscopy (TEM). Normal control corneas were processed identically to the LASIK corneas.

**LIGHT MICROSCOPY**

Sections were fixed and stained with hematoxylin–cosin according to routine protocols. The peripheral lamellar wound at the flap margin, the central lamellar wound, and the overlying and underlying stroma were analyzed by light microscopy with an inverted microscope (Zeiss Axiovert 200M; Carl Zeiss Meditec, Jena, Germany) coupled to a camera (Zeiss AxioCam MRc5). Histopathologic findings were recorded. Epithelium was studied in detail with the use of step sections to focus on epithelial ingrowth underneath the flap.

**INDIRECT IMMUNOLOCALIZATION**

Slides to be stained were air-dried for 20 minutes at room temperature and then fixed for 20 minutes in 100% cold acetone (−20°C). They were washed with phosphate-buffered saline (PBS) 3 times for 5 minutes each time, then incubated for 1 hour in a humidified level chamber in 10% normal donkey serum (D9663; Sigma-Aldrich Corp, St Louis, Missouri) or goat serum (G9023; Sigma-Aldrich Corp) in PBS to block nonspecific staining. Primary antibodies were used at a dilution of 1:100 and incubated overnight at 4°C. The following antibodies were purchased: rabbit polyclonal antibody to gelatinase B (RP33MP9) (Triple Point Biologics, Forest Grove, Oregon), rabbit polyclonal antibody to transforming growth factor β2 (TGF-β2) (Santa Cruz Biotechnology, Santa Cruz, California); rabbit polyclonal antialtamin (L9393) (Sigma-Aldrich Corp); and mouse monoclonal antibody to mucin 16 (MUC16) and rat monoclonal antibody to β integrin (Abcam, Cambridge, Massachusetts). After 3 additional washes with PBS for 5 minutes each, secondary antibodies were applied for 1 hour. The secondary antibodies were conjugated goat anti–mouse IgG (A-11001), donkey anti–rat IgG (A-21208), and donkey anti–rabbit IgG (A-21206) (Alexa Fluor 488; Invitrogen Molecular Probes, Carlsbad, California). Samples were mounted with the use of mounting medium with 4′,6-diamidino-2-phenylindole (Vectashield; Vector Laboratories, Burlingame, California) for nuclear counterstaining. Negative control sections were processed identically but incubated with strain-specific IgG as the primary antibody. Rabbit IgG, rat IgG, and mouse IgG were purchased (Chemicon, Temecula, California).

Samples were examined with an inverted fluorescence microscope (Zeiss Axiovert 200M), and images were captured with a camera (Zeiss AxioCam MRc5) attached to the microscope. Camera and microscope settings were controlled by software (Axiovision version 4.1; Carl Zeiss Meditec). Four regions were evaluated: (1) the LASIK flap wound margin, (2) the corneal stroma in the LASIK flap, (3) the paracentral and central lamellar wound regions, and (4) the residual stromal bed. Normal control corneas were evaluated in central, paracentral, and peripheral regions. The LASIK flap wound margin was also examined with a confocal microscope (Leica TCS SP2; Leica Microsystems Inc, Bannockburn, Illinois).
Table. Demographics of Human Post-LASIK and Control Corneas From Eye Banks

<table>
<thead>
<tr>
<th>Patient No./ Age, y</th>
<th>No. of Corneas Harvested</th>
<th>Postoperative Interval, y</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/51</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>2/34</td>
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</tr>
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<td>9/62</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>10/48</td>
<td>1</td>
<td>8 (1st flap)/4 (2nd flap)</td>
</tr>
</tbody>
</table>

Control 1/51 2 NA
Control 2/55 2 NA

Abbreviations: LASIK, laser-assisted in situ keratomileusis; NA, not applicable.

TRANSMISSION ELECTRON MICROSCOPY

Corneal portions frozen in liquid nitrogen were fixed overnight in cold 3% glutaraldehyde and 2% paraformaldehyde in PBS. The specimens were then postfixed in 1% osmium tetroxide for 1 hour, rinsed in PBS, dehydrated in a series of ethanol and propylene oxide solutions, and embedded in epoxy resin. Semithin (0.5-1.0 µm) and ultrathin (70-80 nm) sections were cut on a microtome (Porter Blum MT-2; Sorvall, Newtown, Connecticut). Semithin sections were stained with toluidine blue and evaluated by light microscopy to identify regions of epithelial ingrowth or normal unaffected regions at the LASIK flap wound margin. The blocks were trimmed around these areas of interest and ultrathin sections were cut, placed in a copper grid, double-stained with uranyl acetate and lead citrate, and examined by TEM (CX-100; JEOL, Tokyo, Japan).

RESULTS

DEMOGRAPHICS OF LASIK AND CONTROL CASES

The demographics of the donors of the LASIK and control specimens are given in the Table. The average (SD) age of the 10 LASIK donors was 50.3 (7.15) years (range, 34-62 years), with an average time after LASIK of 4.7 (1.8) years (range, 2-8 years). Patient 10 underwent LASIK with 2 enhancements (flap relifting) at 6 and 12 months after the initial surgery, and a third enhancement 4 years after the initial surgery (flap recutting). The average age of the 2 control donors was 53.0 (2.8) years (range, 51-55 years). For patients whose clinical records were available, we determined that epithelial ingrowth was not clinically significant. No visual disturbances were reported, and none of the patients required treatment.

INCIDENCE OF EPITHELIAL INGROWTH IN POST-LASIK CORNEAS

Our group previously reported an incidence rate of 33.3% for epithelial ingrowth in this series.27 In the present study, we reviewed these data by increasing the number of serial sections examined. By means of this procedure, epithelial ingrowth into the flap margin was observed in 8 of the 18 corneas examined (44%). Epithelial ingrowth was limited to the peripheral edge of the flap in all cases. The amount of epithelial ingrowth into the lamellar wound varied between corneas and also between different parts of the same cornea. In 5 corneas, epithelial ingrowth was connected with the outer epithelial layer (Figure 1A and B). In 1 cornea, microscopic foci or islands of epithelial cells lay in the scar, and there was no evidence of a connection to the surface (Figure 1C). Two corneas showed both epithelial ingrowth patterns. These data show that epithelial ingrowth is a frequent pattern of healing at the margin of a LASIK flap.

MMP-9 IMMUNOLOCALIZATION

Our group previously localized MMP-9 in human post-LASIK corneas with epithelial ingrowth.27 To verify our previous findings, we repeated our experiments with the use of serial sections. The presence of MMP-9 was detected around epithelial cells trapped in the lamellar scar in 6 of 8 corneas (75%) with epithelial ingrowth. Normal control corneas and post-LASIK corneas without epithelial ingrowth did not stain for MMP-9 (not shown). Within the epithelial ingrowth subgroup, 6 of 7 corneas (86%) with epithelial ingrowth connected to the surface were immunoreactive for MMP-9 (Figure 2A-C). The 1 patient with epithelial ingrown not obviously connected to the surface showed no MMP-9 staining (Figure 2D). Within the 2 corneas with both epithelial ingrowth patterns, only 1 had MMP-9 staining around foci or islands of epithelial cells (Figure 2E). Therefore, staining for MMP-9 around epithelial cells trapped within the scar but not connected to the surface was observed in 1 of 3 corneas (33%). These results indicate that MMP-9 typically immunolocalizes around ingrown epithelium.

BASEMENT MEMBRANE DISCONTINUITIES

Studies using experimental models have suggested a causal relationship between overexpression of MMP-9 in the corneal epithelium and basement membrane dissolution/failure to heal.10,11 We investigated the distribution of basement membrane markers at the wound margin of post-LASIK corneas showing epithelial ingrowth with or without MMP-9 immunoreactivity. Corneas were stained with a polyclonal antibody against laminin and a monoclonal antibody against β4 integrin subunit and viewed by immunofluorescence microscopy.

Laminin is a component of the basement membrane, whereas the heterodimeric complex of α6β4 integrin associates with hemidesmosomes, which serve as the cells’ main anchor to the basement membrane.29,30 In undamaged corneas and post-LASIK corneas devoid of MMP-9 staining, the corneal epithelial basement membrane appeared as a continuous distinct line between the basal surface of the epithelial basal cells and the anterior stroma (Figure 3A-F). We consistently observed discontinuities or an apparent absence of localization of basement membrane markers in regions showing MMP-9 staining around ingrown epithelium (Figure 3G-L). At some sites we saw nonuniform staining, defined as a diffuse irregular staining; this was different from the patchy staining...
characterizing the absence of staining in some areas. These results suggest that MMP-9 immunolocalized around ingrown epithelial cells positively correlates with discontinuities in the basement membrane.

**POLARITY OF TRAPPED EPITHELIAL CELLS IN EPITHELIAL INGROWTH**

Basal cells of the corneal epithelium secrete the components necessary to form the basement membrane. The presence of the basement membrane between the basal epithelium and the underlying stroma determines the polarity of epithelial cells. Therefore, loss of polarity in the trapped ingrown cells may disrupt the basement membrane. Previous reports have localized the membrane-associated mucin MUC16 along the apical membrane of the apical and subapical cells in human ocular surface epithelium. Apical and subapical cells, however, show low metabolic activity. On the other hand, basal cells are not embedded with MUC16 mucin but secrete the components necessary to form the basement membrane. We investigated the epithelial distribution of MUC16 mucin to determine whether ingrown cells express basal or apical patterns. In all cases, MUC16 mucin immunolocalized around the apical and subapical epithelial cells (Figure 4). No staining for MUC16 mucin was found around trapped cells, suggesting that ingrowing epithelium retains its polarity and appears to comprise basal or intermediate cells.

One major role that integrins fulfill as cell membrane receptors is to ensure anchorage dependence; this means that, under normal conditions, most cells must remain in their precise location because their detachment from the extracellular matrix usually leads to apoptosis. Staining for the β1 integrin subunit is seen only in basal cells, where the αβ complex interacts with hemidesmosomes. No staining was seen for β1 integrin around ingrown cells not bound to the basement membrane. The staining for β1 integrin localized along the basal membrane of basal cells in contact with the basement membrane (Figure 3B, D, F, H, J, and L).

Taken together, these data suggest that the polarity of ingrown cells is maintained and that basement membrane discontinuities do not correlate with a loss in the ability of epithelial cells to secrete the components necessary for formation of the basement membrane.
CORRELATION OF STROMAL STAINING FOR TGF-β2 WITH EPITHELIAL STAINING FOR MMP-9 AND WITH DISCONTINUITIES OF EPITHELIAL BASEMENT MEMBRANE

Our group previously determined that TGF-β2, the major cytokine that mediates fibrotic repair in corneas, is confined to the epithelium in the presence of basement membrane. In the absence of basement membrane, however, the stroma stains strongly for TGF-β2, meaning that basement membrane prevents fibrotic marker expression by inhibiting TGF-β2 release into the stroma. Therefore, we hypothesized that the presence of TGF-β2 in the stroma is further evidence of epithelial-stromal interaction with basement membrane discontinuities. In unwounded corneas and post-LASIK corneas devoid of MMP-9 staining, TGF-β2 was localized to the epithelium (Figure 5A-C). In 6 corneas with epithelial ingrowth as well as MMP-9 staining around ingrown epithelium and discontinuities of the basement membrane, the stroma stained for TGF-β2 directly beneath the epithelium (Figure 5D and E). Staining was absent in the adjacent stroma and remained associated with the anterior stromal region along the apparently disassembled basement membrane. These data indicate localized epithelial-stromal interactions at sites where the basement membrane shows discontinuities.

TEM ANALYSIS

The immunofluorescence data suggest that the basement membrane may be lost in some post-LASIK corneas with epithelial ingrowth. To examine this question more carefully, TEM studies were conducted. The TEM data shown come from areas with epithelial ingrowth. An apparently intact neo–basement membrane could be visualized along most of the interface between epithelial ingrown cells and the overlying stroma. Neo–basement membrane underlying the ingrown cells demonstrated a basement membrane–like structure with a pale layer (the lamina lucida) immediately posterior to the cell membrane of the epithelial basal cells and an electron-dense layer (the lamina densa). Numerous hemidesmosomes anchor the epithelial cells to the basement membrane (Figure 6). The Bowman layer did not regenerate even though, in some regions, a thin, acellular, membranelike zone separated the basement membrane from the collagen fibers (Figure 6). When epithelial ingrowth was connected to the surface, in-
grown cells looked viable. No pyknotic nuclear alterations were observed, whereas small vacuoles were observed within the cytoplasm. Cells were generally compressed against each other. Viable foci or islands of epithelial cells in the scar were close to the margin of the flap. In corneas that showed epithelial ingrowth together with MMP-9 staining around ingrown epithelium and basement membrane discontinuities observed by immunofluorescence, the disappearance of specific components of basement membrane was visualized with TEM with different patterns. In some corneal areas, the lamina lucida was highly disrupted or completely absent, and the spacing between the basal cell membrane and the apparent lamina densa (Figure 6A and C) was increased. Hemidesmosomes were absent, and the electron-dense lamina densa exhibited occasional focal disruptions. The disassembly of the basement membrane was substantial but incomplete: remnants of the lamina densa were observed at some locations. In other areas, the lamina lucida showed significant discontinuities and the lamina densa was almost entirely absent. In some places, no components of the basement membrane were distinguishable, and in its place were spaces filled with an electron-dense granular material between ingrown cells and the adjacent stroma (Figure 7F). No apparent lamina lucida and densa were visible. Despite the absence of mature hemidesmosomes, the epithelial cells appeared to be associated with the underlying stroma through a fibrous capsule around the trapped cells. Thus, ultrastructural analysis of the basement membrane at the wound margin of post-LASIK corneas with epithelial ingrowth confirms large areas of remodeling of basement membrane.

Figure 5. Finding of stromal staining for transforming growth factor β2 (TGF-β2) only in corneas treated with laser-assisted in situ keratomileusis (LASIK) exhibiting matrix metalloproteinase 9 (MMP-9) staining around ingrown epithelium and immunofluorescence basement membrane discontinuities. Immunoreactive protein for TGF-β2 was confined to the epithelium in control corneas (A) and in post-LASIK corneas devoid of MMP-9 staining (B [patient 3, right eye] and C [patient 9, right eye]). In post-LASIK corneas with MMP-9 staining around ingrown epithelium and basement membrane discontinuities detected by immunofluorescence, TGF-β2 was localized in both the epithelium and the stroma adjacent to the ingrown cells (arrowheads) (D [patient 7, left eye], E [patient 9, left eye]) (original magnification ×40 [A–C] and ×63 [D and E]).

Figure 6. Ultrastructural transmission electron micrograph studies show a basement membrane–like structure (A–C) beneath the epithelial ingrown cells (EP) with hemidesmosomes (arrowheads), a lamina lucida (LL), and a lamina densa (LD). The Bowman layer did not regenerate but, in some regions, a thin acellular membrane-like zone (*) separated the basement membrane from the collagen fibers of the underlying stroma (S) (A–C). Some areas showed increased spacing between the LL and the LD and the absence of hemidesmosomes (white arrows) (A and C) (original magnification ×5200 [A] and ×8900 [B and C]).
MMP STAINING IN AREAS OF FIBROSIS

The corneal epithelium controls fibrotic activation. When the basement membrane is preserved, changes in corneal stromal cells associated with fibrotic activation do not occur. In the absence of a basement membrane, TGF-β2 is released into the stroma: keratocytes become active with a fibrotic phenotype and accumulation of extracellular matrix. Therefore, we hypothesized that the epithelial-stromal interaction during epithelial ingrowth, which induces stromal exposure to TGF-β2, may lead to fibrosis. The TEM confirmed fibrotic foci near the ingrown epithelial cells, with deposition and remodeling of the extracellular matrix with disorganized stromal lamellae (Figure 8A and B). There was also clear evidence of a breakdown in some stromal lamellae (Figure 8C and D) and of spaces between the stromal lamellae in some cases (Figure 8C).

To verify our previous findings, we investigated the distribution of fibrotic MMPs. In addition to depositing extracellular matrix, activated keratocytes and contiguous epithelium turn on new synthesis of enzymes that can degrade extracellular matrix, demonstrating that this new stroma is being actively remodeled. Matrilysin (MMP-7) and stromelysin 1 (MMP-3) are members of the stromelysin family of MMP enzymes. These contribute to extracellular matrix degradation and may play a role in the stromal degradation and matrix remodeling after excimer keratectomy. In all cases with epithelial ingrowth, MMP-7 was immunolocalized to the ingrown epithelial cells (Figure 9A and C). Normal control corneas and post-LASIK corneas without epithelial ingrowth did not stain for MMP-3 (not shown). Normal and post-LASIK corneas without epithelial ingrowth displayed little staining for MMP-7 (not shown).

Abnormal accumulation of fibrotic extracellular matrix components and MMPs near epithelial ingrowth suggests ongoing lysis and remodeling of corneal stroma.

COMMENT

Our previous study focused on the long-term MMP-9 localization around epithelial cells trapped in the lamellar scar of post-LASIK corneas with epithelial ingrowth. In this article, we report the absence of continuous epithelial basement membrane around ingrown epithelial cells. With LASIK, the epithelium, basement membrane, and Bowman layer remain intact except for the area in which the flap is cut. Advantages are quicker recovery and less postoperative haze because of the lack of epithelial-stromal interactions. One limitation of this study is that it was performed in a cadaver eye model, specifically, in eyes with no clinical evidence of visually or functionally significant epithelial ingrowth. However, even in these infraclinical cases, LASIK with epithelial ingrowth appears as a human postsurgical model featuring epithelial-stromal interaction during healing.

Basal cells of the corneal epithelium secrete the components necessary for formation of the basement membrane. New basement membrane components such as laminin and type IV collagen are deposited underneath migrating corneal epithelial cells and around corneal

Figure 7. Ultrastructural transmission electron micrograph studies confirming that the basement membrane–like structure separating epithelial ingrown cells (EP) from the adjacent stroma (S) exhibits areas of various discontinuities. The disassembly of the basement membrane took several features. Some increased spacing (*) between the lamina lucida and the lamina densa were observed (A–E) with (B and E) or without (A, C, and D) disappearance of hemidesmosomes (arrowheads). Lamina lucida was highly disrupted (A–D) or completely absent (E). Lamina densa (black arrows) exhibited areas of chaotic arrangements with scalloped features (A, B, C, and E) or interruptions (C and D). In some places, no components of the basement membrane were distinguishable, and an electron-dense granular material between ingrown cells and the adjacent stroma formed a fibrous capsule (white arrows) (F) (original magnification ×8900).
Matrix metalloproteinase 9 is produced in the corneal epithelium as it regenerates across the intact basement membrane after an abrasion injury, and then it disappears once regeneration is complete. In situations involving chronic epithelial defects, however, MMP-9 levels remain elevated and contribute to the failure to heal. Matrix metalloproteinase 9 has the capacity to cleave epithelial basement membrane components such as collagen types IV and VII and laminin. Experimental models suggest a causal relationship between overexpression of MMP-9 in the corneal epithelium and basement membrane dissolution/failure to heal.

In these studies, the corneal epithelium gave the appearance of an invading front, dissolving basement membrane and subsequently penetrating the underlying stroma lying in its path. The presence of MMP-9 associated with a disrupted basement membrane around ingrowing epithelium suggests a similar long-term, ongoing process of remodeling and invasion.

Both TEM and immunofluorescence microscopy were used in the present study to evaluate the basement membrane in post-LASIK corneas. The disappearance of specific components of basement membrane was visualized by indirect immunolocalization methods. The probes were chosen to detect components localized to 2 areas in the basement membrane: anti-laminin to detect the lamina lucida and anti-β1 integrin subunit, which together with α6 integrin subunit associates with hemidesmosomes. Both of these components are considered the cells’ main anchors to the basement membrane. Both laminin and β1 integrin exhibit immunostaining discontinuities. This is consistent with the capacity of MMP-9, when overexpressed, to cleave epithelial basement components and reduce adhesion complex integrity.

Our research group has previously shown that, in the presence of basement membrane, TGF-β2 is absent from the stroma and is found only in the epithelium; in contrast, in the absence of basement membrane, the stroma stains strongly for TGF-β2. Therefore, stromal staining for TGF-β2 in areas of post-LASIK corneas with epithelial ingrowth constitutes further evidence of the existence of basement membrane discontinuities. Because we observed staining discontinuities of basement membrane markers in only some areas around ingrown cells, additional ultrastructural studies were conducted. This analysis showed basement membrane-like structures in some places. Other areas were devoid of such structures, confirming the basement membrane discontinuities. Although some places appeared to lack specific components of the basement membrane, other places lacked any basement membrane-like structures or even showed a capsule of connective tissue, similar to scar tissue, that separated the cells from the healthy stroma. These data may explain differences in immunostaining findings that show epithelial-stromal interactions in some cases and their absence in others. This variability could be caused by a basement membrane-like structure or a capsule acting as a fibrous cocoon around the epithelium itself.

**Figure 8.** Ultrastructural transmission electron micrograph studies showing fibrotic spots (F) within the stroma (S) adjacent to the epithelial ingrown cells (EP). Fibrotic areas exhibit a disorganized pattern of collagen fiber deposition (*). Keratocyte (K) activation is observed, as evidenced by increased keratocyte density and adjacent collagen deposition (*). Spaces between the stromal lamellae have formed in some cases (arrowheads). Disorganized collagen deposition (black arrows) separates the neo–basement membrane from the collagen fibers, exhibiting clear evidence of a breakdown in the stromal lamellae (C), whereas others do not show any spacing (white arrows) between the neo–basement membrane and the stromal lamellae (D) (original magnification ×8900 [A, C, and D] and ×5200 [B]).

**Figure 9.** Immunolocalization of fibrotic matrix metalloproteinases (MMPs) around epithelial ingrowth with fibrotic spots. MMP-3 was localized around keratocytes in the stroma adjacent to the ingrown cells (arrowheads) (A and C). MMP-7 was detected around epithelial cells at the surface of the edge of the laser-assisted in situ keratomileusis and around epithelial cells trapped in the lamellar scar (B and D) (original magnification ×63). L next to patient number indicates left eye.
In addition to the fibrotic environment, the presence of stromelysins is further evidence of an ingrown epithelial-stromal interaction and that this adjacent stroma is being actively remodeled. Matrix metalloproteinase 3 was only immunolocalized to some keratocytes around epithelial cells trapped in the lamellar scar of post-LASIK corneas. This is consistent with the capacity of activated keratocytes to upregulate the synthesis of MMP-3, whereas MMP-3 is not synthesized by stromal cells in the uninjured cornea. Matrix metalloproteinase 7 was immunolocalized to the ingrown epithelial cells. This protein is expressed in epithelial-derived dividing cells. The corneal epithelium is one of the few nontumor sites of MMP-7 expression, whereas stromelysin 1 may facilitate the corneal fibrosis through its natural substrates are proteoglycans, elastin, and glycoproteins such as fibronectin and laminin. Increased epithelial-stromal interactions have been implicated as a cause of scarring after removal of a basement membrane layer. Matrilysin may play a role in basement membrane degradation in the cornea and in facilitation of epithelial-stromal interactions, whereas stromelysin 1 may facilitate the corneal fibrosis through keratocyte activation. Girard et al. found more expression of stromelysin 1 in the repair-adjacent stroma than in the repairing stroma 1 week after injury. In addition, stromelysin 1 may play a role in reactivating collagenase. Stromelysin 1 may thus help clear the subepithelial scarring, a process that is often observed several months after injury.

Two reasonable mechanisms can be proposed to explain pathologic epithelial growth under a corneal flap. The first suggests that the cells are implanted under the flap during the surgery. The implantation may be secondary to the microkeratome blade mechanically dragging the cells into the lamellar interface during keratectomy. Epithelial cells from the corneal surface may also float into the interface during irrigation of the stromal bed after the ablation. It is believed that such cells have a very low potential to spread because they do not have mitotic potential, inasmuch as they are disconnected from the limbal stem cells and transient amplifying cells. These epithelial clusters, which are unconnected to the flap edge, may therefore resolve within months. Indeed, in the present study, corneae had an isolated colony of epithelial cells located away from the edge of the flap. Ingrown cells may experience the corneal stroma as a “hostile” hypoxic environment, lacking basic nutrients from the tear film. This may explain the presence of epithelial cell remnants within the interface at the edge of the flap and the absence of MMP-9 release or basement membrane markers. We hypothesize that enzymes are likely to be released soon after LASIK surgery but may not have a strong influence on the normal epithelial-stromal cytokine interaction because the amounts released are probably limited.

The second theory states that the corneal epithelium invades the interface through a defect in the flap or in areas where the flap borders adhere poorly to the stroma. In most cases, continuity between the epithelium in the interface and the surface epithelium suggests that postoperative invasion is the most likely cause.

It is appropriate to view epithelial ingrowth as an epithelial fistula underneath the flap. Majo et al. have shown that the epithelial cells in the interface communicate with the limbal stem cells. Asano-Kato et al. in a histopathologic study of 5 specimens of epithelial ingrowth, reported that early epithelial ingrowth consists of multilayered squamous epithelial cells resembling the differentiated corneal epithelium; late epithelial ingrowth, in contrast, was made up of clumps containing amorphous materials with scarce cellular elements. As in our present study, those authors observed basement membrane–like structure in some places and the absence of basement membrane in other places. Thus, we probed further to test whether loss of basement membrane in some places may be due to a lack of its formation by epithelial cells or to its excessive degradation by enzymatic proteolysis.

The data presented herein and in the literature show that a basement membrane–like structure surrounds epithelial ingrown cells. In addition, it has been reported that cells involved in epithelial ingrowth after LASIK have short generation times with stem cell characteristics and a pluristratified appearance. It may be that the loss of polarity of ingrown cells acts to reduce basement membrane synthesis. We failed to detect the membrane-associated mucin MUC16 constitutively expressed by the human ocular surface epithelium. Taken together, these data suggest that cells within the lesion may differentiate with polarity as in the corneal surface epithelium, which would explain their ability to secrete basement membrane components.

In the normal cornea, epithelial cells undergo relatively rapid mitosis with differentiation toward the apical side and exfoliation of superficial flattened squamous cells. In the case of epithelial ingrowth, however, differentiated cells cannot exfoliate and consequently accumulate. These physically limited conditions may explain the observed local cell degeneration, phagocytosis with local detorsion of cell debris, and accumulation of amorphous material. Lack of oxygen and various nutrients from the tear film necessary for maintaining cellular metabolism may also explain some metabolic changes and the inability of ingrown cells to synthesize basement membrane.

Epithelial ingrowth soon after surgery may also cause melting of the flap edge. This peripheral loss of flap tissue has been connected to the release of proteases in the area of epithelial ingrown cells. We did see MMP localization. Matrix metalloproteinase 9 is capable of degrading components of the basement membrane. Such degradation may account for the partial loss of basement membrane over time, and for the alterations in both its structure and its function. Immunolocalization of TGF-β2 in the region directly beneath the epithelium is further evidence of the epithelial-stromal interaction. Fibrosis occurred as a result of abnormal extracellular matrix remodeling and is thought to be dependent on specific cytokines such as TGF-β2. Extracellular matrix deposits accumulated during wound healing are often removed over time by specific proteases expressed in the affected areas. We found cells overexpressing MMP-9, MMP-3, and MMP-7, which can degrade extracellular ma-
trix and basement membrane components. These data suggest the occurrence of ongoing proteolysis, possibly as an attempt of corneal stromal and epithelial cells to locally remodel the stroma and remove fibrotic deposits. Fibrosis resorption by extracellular proteolysis may take several months to several years. This process does not seem to require myofibroblasts. Myofibroblasts are necessary at the early stages of fibrotic scar formation; at later stages and in chronic scars, active stromal remodeling may be continued by activated keratocytes in the fibrotic scar area. These results suggest that epithelial ingrown cells after LASIK initiate a stromal wound healing response consisting of an acute response in the early stages and a more chronic, progressive response in later stages. A new extracellular matrix may develop in some places and grow to form a fibrous capsule around the trapped cells. A basement membrane–like structure or this fibrous cocoon may thus explain why most cases of epithelial ingrowth are self-limiting and asymptomatic over time. Nevertheless, our data suggest an ongoing, low level of remodeling around some ingrown epithelial cells.

Therefore, either hypothesis—lack of synthesis or excessive degradation—may account for the observed loss of basement membrane components. Even though a synthesis defect is possible in light of the data, the presence of MMP-9 favors the degradation hypothesis.

The pathogenesis and progression of epithelial ingrowth are unknown. Studies do show, however, that LASIK enhancement by flap relifting increases the risk of epithelial ingrowth. Even if most patients are asymptomatic, progressive epithelial ingrowth can encroach on the central visual axis or can result in flap melting and irregular astigmatism. Treatment in these cases consists of lifting the flap and scraping the epithelial cells.

Most cases of epithelial ingrowth are mild and self-limiting and require only careful observation. The diagnosis of epithelial ingrowth is not necessarily an indication for immediate removal. Many isolated nests of ingrown cells will regress within a few months with no adverse consequences. However, some epithelial ingrowth may persist over time. Schmack et al studied the cohesive tensile strength of human LASIK corneal wounds and found that the weakest wound margin scars showed epithelial cell ingrowth. Even if there is no clinical evidence of an increased risk of flap displacement in patients with epithelial ingrowth after LASIK, the presence of MMP-9, which is associated with a disrupted basement membrane around ingrown epithelium, suggests an ongoing remodeling process. This fact may help explain the previous findings that identified the weakest wound margin scars as those with epithelial ingrowth. The potential consequences of such long-term remodeling on the edge of the flap remain unknown.

We cannot say with certainty whether epithelial ingrowth and the ongoing repair process it involves are the cause or the consequence of the presence of MMP. However, we propose that minor defects in the surgically created flap prevent perfect alignment with the underlying cornea after surgery. This would make slippage likely, creating space for epithelial ingrowth and a long-term requirement for fibrotic repair. As in other long-term repair processes, MMP expression may become excessive because of ever-amplifying feedback loops, and this could gradually contribute to the failure to heal. In such situations, judicious and timely use of appropriate MMP inhibitors may be beneficial by inhibiting epithelial ingrowth and enabling fibrotic repair tissue to accumulate sufficiently to “tack” the flap in place.

In the present study, we showed immunolocalization of MMP-9 and basement membrane discontinuities around ingrown epithelial cells in post-LASIK corneas. The epithelial-stromal interaction observed after LASIK over time, together with chronic epithelial ingrowth, may be related to the prolonged remodeling of the adjacent stroma and calls into question the potential long-term consequences for the edge of the flap. A more complete understanding of epithelial cell–matrix interactions after LASIK may enable us to prevent or treat significant epithelial ingrowth more successfully.

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