Corneal Femtosecond Laser Keratotomy Results in Isolated Stromal Injury and Favorable Wound-Healing Response

Christian Meltendorf,1,2 Guido J. Burbach,2 Jens Bühren,1,5 Reinbold Bug,4 Christian Ohrloff,3 and Thomas Deller2

PURPOSE. To examine the corneal repair response after intrastromal femtosecond (fs) laser keratotomy.

METHODS. Twelve rabbits underwent monocular intrastromal keratotomy performed with an fs laser at a preoperatively determined corneal depth of 160 to 200 μm. The fs laser-induced corneal repair response was compared with that of nonoperated control eyes and eyes treated with photorefractive keratectomy (PRK). Follow-up examinations were performed 1, 3, 7, and 28 days after surgery. Corneas were evaluated using slit lamp, in vivo confocal microscopy, and light microscopy. The extracellular matrix components fibronectin and tenasin were located using immunofluorescence staining. Anti-Thy-1 and anti-α-SMA antibodies and phalloidin were used to identify repair fibroblasts. Cell proliferation and nuclear DNA fragmentation were detected using an anti–Ki-67 antibody and the TUNEL assay, respectively.

RESULTS. Intrastromal fs keratotomy resulted in a hypocellular stromal scar discernible as a narrow band of increased reflectivity on slit lamp examination. Deposition of fibronectin and tenasin as well as death and subsequent proliferation of keratocytes were observed. No differentiation of keratocytes into Thy-1- or α-SMA–positive fibroblasts could be detected. In contrast, after PRK, which causes epithelial and stromal wounding, all markers for repair fibroblasts were found in subepithelial stromal layers. On slit lamp examination, a fibrotic scar and a corneal haze were revealed.

CONCLUSIONS. Isolated stromal injury using an fs laser avoids epithelial injury and is associated with a favorable wound-healing response preserving corneal transparency. Thus, fs laser keratotomy is a highly selective laser treatment that can be useful for the treatment of refractive errors. (Invest Ophthalmol Vis Sci. 2007;48:2068–2075) DOI:10.1167/iovs.06-1150

From the 1Department of Ophthalmology, the 2Institute of Clinical Neuroanatomy, Dr. Senckenberg Anatomy, and the 3Institute of Pathology, Johann Wolfgang Goethe-University, Frankfurt am Main, Germany; and the 4Department of Ophthalmology, University of Rochester Medical Center, Rochester, New York.

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Corneal wound healing after refractive surgery is essential for maintaining the structural integrity of the cornea. Two histopathologic types of stromal repair responses were described after refractive surgery. One is the fibrotic stromal scar, which is characterized by an increased density of stromal cells, the occurrence of repair fibroblasts (e.g., myofibroblasts), and disorganized collagen fibrils.1 This repair response is disadvantageous because it causes light scattering and may alter corneal shape.2 The fibrotic type of scar can be observed after photorefractive keratectomy (PRK) and is commonly referred to as haze. The other is the primitive stromal scar, which is characterized by a decreased density of stromal cells.3 This type of scar occurs in the central portion of the lamellar wound after laser in situ keratomileusis (LASIK) and is, in almost all cases, invisible to detection by clinical examination.

Because the clinical outcome of refractive surgery depends, at least in part, on the type of corneal repair response induced by the treatment, it is of considerable interest to understand the molecular and cellular events leading to the formation of either a fibrotic or a primitive stromal scar. Previously, it was proposed that the extent of epithelial injury plays a critical role in, and could determine the stromal response after, LASIK or PRK.4 It has been suggested that damage to the epithelium and its basement membrane could allow proinflammatory epithelial cytokines to enter the stroma.4 These cytokines and other epithelium-derived signaling molecules could then activate and differentiate keratocytes into unfavorable repair phenotypes,5–11 induce keratocyte death in the corneal stroma,12–14 and mediate the expression of the extracellular matrix (ECM) molecules fibronectin and tenasin.15–18 Because all these changes are seen in the fibrotic scar, the key to limiting corneal fibrosis may lie in keeping epithelial damage and proinflammatory epithelial–stromal interactions to a minimum.5,9,10,19

In recent years, effort has been devoted to developing new lasers for refractive surgery that reduce or avoid injury to the corneal epithelium. Troutman et al.20 first reported that isolated intrastromal tissue ablation can be achieved using a modified excimer laser. Later, the picosecond Nd:YLF laser was used for intrastromal ablation in laboratory investigations.21 Initial intrastromal corneal procedures were not successful, however, because of the limited precision of these lasers. Recently, femtosecond (fs) lasers were developed.22–24 These lasers generate microplasmas inside the corneal stroma and achieve a stromal ablation effect while leaving the anterior and posterior epithelial layers of the cornea intact.25

In the present study, we have made use of fs laser technology to study the role of epithelial injury in stromal scar formation. We hypothesized that in the absence of damage to the corneal epithelium, a primitive stromal scar should develop. We compared this experimental setting with an experimental one in which the corneal epithelium receives a clearly defined injury and in which robust fibrotic scarring occurs (PRK). We paid particular attention to stromal cell phenotypes, to their activation, proliferation, and death, and to the expression of the ECM molecules tenasin and fibronectin. Differentiation of
quiescent keratocytes (fibrocytes) into repair phenotypes\textsuperscript{8,9,26} was monitored using stress fiber labeling and fibroblast markers, such as Thy-1\textsuperscript{27,28} and α-smooth muscle actin (αSMA), a myofibroblast marker.\textsuperscript{29,30} We report that intrastromal fs keratotomy leads to a hypacellular stromal scar of mildly elevated reflectivity. Activation, proliferation, and death of putative keratocytes and deposition of fibronectin and tenasin occur without differentiation of keratocytes into repair fibroblasts. These observations indicate that an isolated stromal injury results in a favorable wound-healing response. Furthermore, our data strengthen the hypothesis that damage to the corneal epithelium determines the stromal response after corneal injury.

**METHODS**

**Animals**

Twenty-four New Zealand White rabbits (3–4 kg body weight) were obtained from Harlan Winkelmann Laboratories (Borchen, Germany) and housed under standard laboratory conditions. One eye of each rabbit, selected at random, was subjected to surgery. Postoperative survival times were 1, 3, 7, and 28 days. Both procedures—fs laser keratotomy and PRK—were performed on three eyes for each time point. Contralateral eyes served as unoperated controls.

Animals were anesthetized with xylazine hydrochloride (5 mg/kg intramuscularly; Rompun; Bayer, Leverkusen, Germany) and ketamine hydrochloride (50 mg/kg intramuscularly; Ketavet; Pharmacia, Erlangen, Germany). In addition, preservative-free oxybuprocain hydrochloride (Benozinat SE 0.4%; Alcon Pharma, Freiburg, Germany) was applied to each eye just before surgery. Animals treated with PRK received buprenorphine 0.05 mg/kg subcutaneously after surgery. The animals were humanely killed under anesthesia by intracardiac injection of 5 mL embutramine 0.2 g/mL, mebezonium 0.05 g/mL, and tetracaine hydrochloride 0.005 g/mL, (T61; Intervet, Unterschleissheim, Germany). All animals were treated in accordance with German law regarding the use of laboratory animals, the tenets of the Declaration of Helsinki, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Intrastromal Femtosecond Laser Keratotomy**

Preoperative corneal thickness was measured with an ultrasound pachymeter (SP 100; Tomey, Erlangen, Germany). A lamellar intrastromal cut was performed (Fig. 1A) on 12 rabbits at a preoperatively determined depth of 50% of the central corneal thickness (160–200 μm; diameter, 6.5 mm) using an fs laser (FEMTEC; 20/10 Perfect Vision, Heidelberg, Germany). This pulsed solid-body (Nd:glass) laser, with a repetition rate of 10 kHz, emits light with a wavelength of 1059 nm and a pulse duration of 600 to 800 fs. Laser energy of 2.8 mJ at a fluence of 160 mJ/cm² with an excimer laser (VISX Star S3; Santa Clara, CA). An optical zone of 6.0 mm was selected. The preoperatively determined ablation depth was 150 μm, corresponding to a sphere correction of –13.50 D. At the end of the procedure, ofloxacin ointment (Floxa; Alcon Pharma, Freiburg, Germany) was applied.

**Photorefractive Keratectomy**

A wire lid speculum was positioned, and transepithelial laser ablation (Fig. 1B) was performed. Tissue ablation was performed with a fluence of 160 mJ/cm² with an excimer laser (VIXX Star S3; Santa Clara, CA). An optical zone of 6.0 mm was selected. The preoperatively determined ablation depth was 150 μm, corresponding to a sphere correction of –13.50 D. At the end of the procedure, ofloxacin ointment (Floxa; Alcon Pharma, Freiburg, Germany) was applied.

**Slit Lamp Examination and In Vivo Confocal Microscopy**

In vivo imaging with slit lamp and confocal microscopy was performed before surgery and on days 1, 3, 7, and 28 after surgery. Slit lamp micrographs were taken with a digital camera (MVC-CD400; Sony Corporation, Tokyo, Japan) attached to the slit lamp (model 100/16; Carl Zeiss, Oberkochen, Germany). At each time, images with diffuse and slit illumination were taken.

Confocal microscopy was performed with a white light slit-scan-microscopy (Confoscan 4; Nidek Technologies, Erlangen, Germany). The objective used was a 40X water-immersion objective (Achromat; Carl Zeiss) that provided a field of view of 460 × 345 μm. A carborner gel (Visdics; Mann Pharma, Berlin, Germany) was used as immersion fluid. All corneas were examined centrally. The endothelium was imaged first to ensure an exact alignment parallel to the corneal surface. After that, at least 3 z-axis scans from endothelium to epithelium were taken.

**Tissue Fixation and Sectioning**

For histologic analyses, whole globes were embedded in liquid tissue freezing medium (Leica Microsystems, Nuussloch, Germany). The tissue was rapidly frozen in 2-methylbutane at ~80°C. Frozen tissue blocks were stored at ~80°C until sectioning. Serial sagittal corneal sections (14-μm thick) were cut using a cryostat (CM 5050S; Leica Microsystems). Sections were placed on microscope slides (SuperFrost Plus; Menzel, Braunschweig, Germany) and air dried.

**Light Microscopy**

Cryostat sections were stained with hematoxylin-eosin according to routine protocols. Histopathologic findings from light microscopic examinations were recorded (Microscope IX50 [Olympus, Tokyo, Japan]; SPOT RT Color Digital Camera [Diagnostic Instruments, Sterling Heights, MI]).

**Immunofluorescence Microscopy Studies**

Cryostat sections were incubated with primary antibodies overnight in a humidified chamber at room temperature. The following antibodies and dilutions were used: mouse anti-human cardiac cell ED-A fibronectin (EDA-Fn, clone DH1, 1:400; Biohit, Helsinki, Finland); mouse anti-human tenascin-cytotactin (TN-C, clone EB2, 1:200; Biohit); mouse anti-human α-smooth muscle actin (αSMA, clone 1A4, 1:50; DakuCyto- tion, Glostrup, Denmark); mouse anti-human Ki-67 (clone B11, prediluted; Zymed, San Francisco, CA); and polyclonal goat anti-human Thy-1 antibody (CDw90, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA). An Alexa Fluor 488–conjugated phallolidin probe (Molecular Probes, Eugene, OR) was used to detect filamentous actin. Secondary antibodies (Alexa 488 donkey anti-mouse, Alexa 568 donkey anti-goat, Alexa 568 goat anti-mouse, 1:1000; Molecular Probes) were applied for 90 minutes at room temperature. A counterstain for cell...
nuclei was performed using Hoechst 33258 (1 μg/mL; Sigma-Aldrich, Munich, Germany). Finally, sections were coverslipped using anti-fading mounting medium (Fluorescent Mounting Medium; Dako, Hamburg, Germany). To verify the specificity of the antibodies, separate incubations were performed with or without primary or secondary antibodies. Sections were investigated with a microscope (IX50; Olympus) equipped with a color digital camera (SPOT RT; Diagnostic Instruments). Figures were prepared digitally using commercially available graphics software (Adobe Photoshop CS 8.0.1). Single fluorescent images of the same section were digitally superimposed. Images were adjusted for contrast, brightness, and sharpness.

TUNEL Assay

To detect fragmentation of DNA, a fluorescence-based TUNEL assay (ApopTag Red In Situ Apoptosis Detection Kit S7165; Chemicon, Temecula, CA) was used according to the manufacturer’s instructions.

RESULTS

Slit Lamp Microscopy Detects Intrastromal Femtosecond Laser Keratotomy as a Narrow Band of Increased Reflectivity

On slit lamp examination before fs laser keratotomy or PRK, all corneas appeared normal. One day after fs laser keratotomy, slightly elevated light scattering was observed at the keratotomy zone. The reflectivity of the keratotomy zone seemed to be comparable to that of the epithelial and endothelial basement membranes. After 28 days, the site of keratotomy was still detectable in all animals as a narrow band of increased reflectivity (Fig. 2A).

One day after PRK, corneas showed circular epithelial defects and discoid edematous opacities of the anterior two thirds of the corneal stroma. In all animals, complete epithelial closure was reached after 28 days, and a severe circular subepithelial haze was observed in the corneal center at that time (Fig. 2B).

In Vivo Confocal Microscopy Reveals Reactive Changes of Keratocytes and the Deposition of an Acellular Substance in the Corneal Stroma after Fs Laser Keratotomy

One day after fs keratotomy, the epithelium showed no alterations. In the stromal layer directly anterior and posterior to the keratotomy zone, keratocytes with highly reflective nuclei and cell bodies were observed (Fig. 3A). Many of the nuclei showed a pattern of reflective granules. In contrast, keratocytes of nonoperated corneas showed only moderate light scattering of their nuclei (Fig. 3, control). The keratotomy itself was characterized by regularly spaced dots (arrow) that corresponded most likely to the impact of the fs laser. (B) Three days after surgery, highly reflective fragmented keratocyte nuclei (arrow; inset) were present. (D) Reactivity of the dots at the level of keratotomy decreased. (E) Seven days after surgery, the number of highly reflective fragmented nuclei decreased, (F) whereas diffuse background reflectivity at the keratotomy level increased. (G) After 28 days the appearance of keratocytes returned to almost preoperative status. (H) The acellular substance showed further increased reflectivity and appearance of a wavelike pattern. Scale bars, 50 μm.

FIGURE 3. In vivo confocal microscopy of stromal cells adjacent to the keratotomy zone (left) and of corneal stroma at the level of fs laser keratotomy (right). (A) One day after surgery, activated keratocytes (arrow) with highly reflective nuclei and processes were present directly anterior to the keratotomy. The anterior corneal stroma of nonoperated eyes showed quiescent keratocytes with nuclei of intermediate reflectivity. (B) The keratotomy itself was characterized by distinct regularly aligned dots (arrow) that corresponded most likely to the impact of the fs laser. (C) Three days after surgery, highly reflective fragmented keratocyte nuclei (arrow; inset) were present. (D) Reactivity of the dots at the level of keratotomy decreased. (E) Seven days after surgery, the number of highly reflective fragmented nuclei decreased, (F) whereas diffuse background reflectivity at the keratotomy level increased. (G) After 28 days the appearance of keratocytes returned to almost preoperative status. (H) The acellular substance showed further increased reflectivity and appearance of a wavelike pattern. Scale bars, 50 μm.
surgery, the number of highly reflective fragmented nuclei adjacent to the keratotomy had decreased (Fig. 3E) in all eyes, and a concomitant increase in diffuse background reflectivity at the level of keratotomy was observed (Fig. 3F). After 28 days, the reflectivity of keratocytes adjacent to the zone of keratotomy was almost comparable to that of control eyes (Fig. 3G). At this time, the density of the reflective acellular substance at the level of the keratotomy increased and exhibited a wavelike pattern (Fig. 3H).

**Light Microscopy Reveals a Primitive Stromal Scar after fs Laser Keratotomy**

One day after fs laser keratotomy, conventional light microscopy revealed an eosinophilic acellular band (thickness, 50–70 μm) in the fs keratotomy zone. Neighboring collagen fibrils were not disrupted but showed reduced eosin staining, which might have been regarded as a sign of weak edema (Fig. 4A). No cavitations were observed at any time. Nuclear or cellular debris was not detectable by conventional light microscopy. After 28 days, specimens still revealed the acellular zone that became smaller with time in the sagittal diameter (Fig. 4B). Infiltration of inflammatory cells was not found during the postoperative follow-up period. The fs-laser treated corneas did not show any alterations of the epithelium, basement membranes, or endothelium.

In contrast, PRK resulted in the destruction of the outer corneal layers with a complete loss of the central epithelium. Superficial stromal collagen fibrils of the thinned central stroma showed increased eosin staining 1 day after treatment. Dense infiltration of polymorphonuclear leukocytes was found in the anterior half of the central cornea that extended to subepithelial stromal layers underneath the epithelium at the wound edge. The surface of the defect was covered with cellular debris rich in polymorphonuclear leukocytes (Fig. 4C). After 3 days, the regenerating epithelium had a spongy texture, and the epithelial basal cell layer appeared pseudostratified. Twenty-eight days after surgery, the regenerated epithelium was partially thickened and showed a regular stratification. We observed a high density of fibroblasts in the subepithelial and slightly edematous stroma, corresponding to a fibrotic stromal scar. The leukocyte infiltration had disappeared (Fig. 4D).

**Thy-1–Positive Fibroblasts and Myofibroblasts Do Not Appear after Intrastromal Femtosecond Laser Keratotomy**

After intrastromal fs keratotomy, we observed only slightly stronger phalloidin staining around the keratotomy zone compared with staining in untreated controls (Fig. 5A). Thy-1- and α-SMA-positive cells were not detectable in the stroma between days 1 and 28 after fs laser keratotomy (Figs. 5B, 5C). In contrast to intrastromal keratotomy, all markers used to detect different fibroblast phenotypes were markedly expressed after PRK. By 1 day after treatment, the anterior half of the corneal stroma showed a prominent and clumpy assembly of filamentous actin. After 28 days, we observed an increase of phalloidin staining in the subepithelial stromal layers (Fig. 5D). Thy-1- and α-SMA–positive stromal cells were observed at day 3. These cells were typically found beneath the corneal epithelium at the wound edge. After 7 days, Thy-1 and α-SMA were expressed in stromal cells located in the subepithelial layers beneath the regenerating epithelium. After 28 days, the strongest expression of these markers was observed. At this time, staining of Thy-1 and α-SMA was found in the subepithelial stromal layers (Figs. 5E, 5F). Thy-1 and α-SMA expression were compared using double-immunofluorescence labeling. Immunoreactivity for Thy-1 and α-SMA was colocalized in some stromal layers. Interestingly, α-SMA expression was only observed in layers in which Thy-1 was also expressed. In contrast, Thy-1 expression was also observed without α-SMA (Figs. 5G–5I).

**Deposition of Provisional ECM Components Does Not Depend on the Differentiation of Keratocytes into Repair Fibroblasts**

Tenascin and fibronectin expression were observed by 1 day after fs laser keratotomy. Tenascin was deposited in a characteristic pattern: a 50- to 70-μm-wide nearly acellular zone around the keratotomy showed no tenascin deposition. Above this zone, a small band of tenascin immunoreactivity was seen in the anterior stroma. A more pronounced reaction was found in the adjacent area below the laser injury. Three days after intrastromal keratotomy, the intensity of the tenascin immunoreaction increased (Fig. 6A). Up to 28 days after surgery, the location of tenascin was unchanged but staining intensity decreased (Fig. 6B). Fibronectin immunoreactivity was present 1 day after surgery in the stroma and did not show a detectable change in intensity until 28 days after surgery. The distribution pattern differed between tenascin and fibronectin. Whereas tenascin was found around the keratotomy zone and had a diffuse and clumpy appearance, fibronectin immunolabeling was more restricted to the direct site of laser injury. It could be detected as a thin line parallel to the corneal surface (Figs. 6E, 6F). Interestingly, fibronectin depositions also delineated vertical fiber offshoots (Fig. 6E). In these areas, a broadened expression of tenascin was found (Fig. 6A). Thus, depositions of tenascin and fibronectin were observed in the corneal stroma after fs laser keratotomy in the absence of any Thy-1- and α-SMA–positive fibroblast phenotypes.

In the follow-up period after PRK, the expression patterns of tenascin and fibronectin were almost similar. One day after PRK, tenascin and fibronectin deposits were observed directly
within the anterior and midstromal layers, under the zone of ablation. Three days after surgery, the intensity of both ECM components increased slightly (Figs. 6C, 6G). After that, tenascin and fibronectin deposits became restricted to the subepithelial stromal layers, and the reaction in the posterior regions decreased (Figs. 6D, 6H). Interestingly, immunolabeling for tenascin was reduced in the newly grown epithelium after PRK (Fig. 6D).

**Death and Proliferation of Stromal Cells Occur in the Absence of Epithelial Damage**

After fs laser keratotomy, TUNEL assay detected DNA fragmentation adjacent to the keratotomy site. The zone of TUNEL-positive cells was observed on day 1 and decreased with time (Figs. 7B, 7C). No TUNEL-positive cells were seen on day 28 in the stroma. The resultant reduction of cell density was still detectable by Hoechst staining in the keratotomy zone after 28 days (Fig. 7D). Ki-67-positive cells first appeared in the keratotomy zone at day 3 (Fig. 7F). These proliferating cells in the stroma were exclusively localized to the area immediately adjacent to the area of cell death. Few proliferating cells were also found after 7 days (Fig. 7G). On day 1 and day 28, no Ki-67-positive cells were seen in the stroma.
Keratocyte Differentiation into Repair Fibroblasts Can Be Monitored with α-SMA and Thy-1 Immunofluorescence

The reaction of keratocytes, especially the differentiation of keratocytes to repair fibroblasts, determines the extent and type of corneal scarring after injury.5 The differentiation of keratocytes to myofibroblasts can be induced by epithelial-derived TGF-β.19,29,30 The myofibroblast is characteristic of the fibrotic scar. It is a highly contractile cell type with reduced transparency, and it appears to be responsible for the formation of haze.31–33 In most studies, α-SMA, a myofibroblast marker, was used for the immunohistochemical detection of repair phenotypes in corneal wound healing.34–38 Recent in vitro studies indicate, however, that the expression of the cell surface protein Thy-1 (CDw90) can also be used to distinguish quiescent keratocytes from reactive phenotypes.27 Thy-1–positive cells express higher levels of interstitial collagen39 and exhibit profibrogenic properties.39 Koumas et al.28 suggested that only Thy-1–positive fibroblasts are able to differentiate to myofibroblasts. To clarify whether Thy-1 can be used as a marker for fibroblasts after corneal injury in vivo, we stained corneal tissue after PRK with an antibody against Thy-1. Our results confirmed the in vitro data: after PRK, we observed Thy-1 immunoreactivity in α-SMA-positive subepithelial stromal cells. Furthermore, our double-labeling experiments demonstrated that α-SMA-positive stromal layers were always Thy-1 positive. Thus, we demonstrate here for the first time that Thy-1 is a useful marker to label fibroblasts in corneal tissue after injury in vivo.

The prominent assembly of intracellular actin filaments into phalloidin-stained stress fibers in the subepithelial layers after PRK confirmed the differentiation of quiescent keratocytes to repair phenotypes.

Type of Corneal Injury Determines Keratocyte Differentiation

Given that the presence of reactive fibroblasts, especially the presence of myofibroblasts, indicates fibrotic scarring after corneal injury,5 we studied the injured cornea after fs laser keratotomy and PRK and looked for signs of keratocyte differentiation under both conditions. Immunofluorescence for α-SMA and Thy-1 were used to identify repair phenotypes in tissue sections, and in vivo confocal imaging of keratocytes was performed after fs laser keratotomy to analyze keratocytes in the living cornea. Whereas a strong keratocytic response and the differentiation of keratocytes to repair fibroblasts was observed after combined epithelial and stromal injury (PRK), Thy-1–positive fibroblasts and α-SMA–positive myofibroblasts did not appear after isolated intrastromal keratotomy (fs laser keratotomy). In addition, frontal sections obtained by in vivo confocal microscopy did not reveal any cells with the spindle-shaped morphology described for fibroblasts.5 Thus, keratocytes differentiated to myofibroblasts after PRK but not after fs laser keratotomy. We concluded from these observations that keratocyte differentiation to Thy-1– or α-SMA–positive fibroblasts does not occur in the absence of epithelial damage to the cornea.

Extracellular Matrix Molecules Are Deposited within the Corneal Stroma in the Absence of Epithelial Injury

As shown previously for anterior and photorefractive keratectomy and for uncomplicated LASIK, fs laser keratotomy resulted in the deposition of tenasin and fibronectin.39–41 Since fs laser keratotomy leaves the corneal epithelium intact, this demonstrates that neither epithelial debridement with subse-

**FIGURE 7.** DNA fragmentation detected by TUNEL assay (left) and cell proliferation detected by Ki-67 antibodies (right) after fs laser keratotomy. (A) The maximum of DNA fragmentation in the keratotomy zone (arrowheads) was detected by TUNEL assay 1 day after fs laser keratotomy. (B, C) The number of TUNEL-positive cells decreased with time. (D) No TUNEL-positive cells were seen on day 28 in the stroma. (E, H) No proliferating cells in the stroma could be detected by Ki-67 labeling on day 1 and day 28. (F) Ki-67–positive cells (arrow) first appeared in the keratotomy zone at day 3. (G) Few proliferating cells (arrow) were also found after 7 days. Sections are counterstained with Hoechst for nuclei (blue). Scale bars, 100 μm.

cells were seen (Figs. 7E, 7H). The number of Ki-67–positive epithelial cells was not affected by intrastromal fs keratotomy.

**DISCUSSION**

In the present study, we showed that an isolated stromal injury (fs laser keratotomy) leads to the formation of a hypocellular primitive stromal scar. In addition, we compared this experimental setting, in which epithelial damage is avoided, to an experimental situation in which the corneal epithelium receives a defined and reproducible lesion (PRK). We concluded from our findings that the type of repair response after corneal injury is largely determined by the integrity of the corneal epithelium and its basement membrane. Because fs laser keratotomy completely avoids epithelial damage, fs laser keratotomy could be a promising approach for the treatment of refractive errors.
quent epithelial proliferation nor injury of the epithelial base-
ment membrane is required for the production of tenasin and
fibronectin by stromal cells.16,41–43 The distribution pattern of
tenasin and fibronectin after intrastromal keratotomy suggests
that stromal cells are the source of these provisional ECM
components. Several studies identify stromal keratocytes as the
source of fibronectin and tenasin.16,44,45 Interestingly, fi-
bronectin was deposited into the acellular space after intra-
stromal keratotomy, whereas tenasin was missing. One expla-
nation for this difference in the distribution of the two
molecules could be that fibronectin, in contrast to tenasin, is
secreted around the keratotomy site before cell death occurs,
consistent with previous findings that fibronectin is deposited
before tenasin after corneal and dermal wounding.46,47
Regardless of the cause, however, the deposition of fibronectin
can be used to evaluate the accuracy of the fs laser cutting
process.

The deposition of tenasin and fibronectin after fs laser
keratotomy could be the cause of elevated light scattering
restricted to the keratotomy zone, as seen by slit lamp exami-
nation.48,49 Through confocal in vivo microscopy, the newly
deposited cell-free amorphous mater at the keratotomy level,
which may be regarded as deposited ECM, showed profoundly
increased reflectivity. However, as known from previous stud-
ies, deposition of tenasin and fibronectin is time limited and
might, therefore, not be the cause of persistent light scatter-
ing.50,45 Based on observations that tenasin and fibronectin
accumulate more rapidly and at higher concentrations in in-
jured tissues, where wounds heal perfectly without scarring
(e.g., in fetal wound healing), some authors postulated that
tenasin and fibronectin could direct wound-healing processes
toward a favorable wound-healing response.51,52 Nevertheless,
the function of tenasin and fibronectin in the corneal repair
response remains to be elucidated.53

Role of an Intact Corneal Epithelium for Stromal
Cell Death

Detection of DNA fragmentation after fs laser keratotomy
showed that epithelial debridement is not an essential prereq-
usite for kerocyte death. Kerocyte death seen after epithelial
debridement was first observed by Dohlman et al.54 and
was later confirmed by others55 for PRK. Kerocytes also
disappear after LASIK from an area adjacent to the microker-
toma cut, forming an acellular zone.55Investigators have fo-
cused on possible epithelium–stroma interactions underlying
this phenomenon, and it has been suggested that cytokines
such as interleukin-1114 and tumor necrosis factor,52 as well as
Fas ligand,53,54 may be released from the injured epithelium and
may induce apoptosis in the underlying kerocytes. It is
 speculated by some authors that these cytokines from the
injured epithelium could diffuse after LASIK along the lamellar
interface or that epithelial debris dropped into the interface by
the microkeratome could trigger apoptosis.7,10,11,4,55 Given that
fs laser keratotomy also induces kerocyte cell death in the
corneal stroma in the absence of epithelial damage or epithelial
displacement, other mechanisms may also play a role. Laser-
induced reactive oxygen radicals may be a causative factor.56
Alternatively, gap junction–mediated cell coupling between
kerocytes could contribute to the expansion of injury, as
previously shown for astrocytes.57

Clinical Perspectives

In the present study, we analyzed the corneal wound-healing
response after intrastromal fs laser keratotomy. We showed
that fs laser keratotomy induces a primitive stromal scar similar
to the favorable scar type described for the central regions of
the cornea after LASIK. We did not observe histologic signs of
infection or inflammation after fs laser keratotomy, even
though no anti-inflammatory or antibiotic treatment was given.
Thus, fs laser keratotomy seems to be a corneal tissue ablation
technique that is accompanied by minimal corneal fibrosis and,
probably, low risk for infection. Previous studies have demon-
strated that the desired effect of central corneal thinning and
flattening can be achieved by intrastromal ablation21 and that
refractive changes resulting from intrastromal ablation are of
significant magnitude and remain stable.53 Studies on the clin-
ical potential of selective intrastromal ablation techniques for
the treatment of refractive errors and a systematic comparison
of the fs laser technique with LASIK may now be warranted.

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References

1. Dawson DG, Edelhauser HF, Grossniklaus HE. Long-term histopathologic findings in human corneal wounds after refractive
2. Möller-Pedersen T, Cavanagh HD, Petroll WM, Jester JV. Stromal
wound healing explains refractive instability and haze develop-
ment after photorefractive keratectomy: a 1-year confocal micro-
cornea: a review of refractive surgery complications and new
growth factor to normal and neovascularized rabbit cornea. Invest
5. Nakamura K, Kurosaka D, Bissen-Miyajima H, Tsubota K. Intact
corneal epithelium is essential for the prevention of stromal haze
6. Iversen A, Laurberg T, Möller-Pedersen T. Characterisation of cor-
neal fibrotic wound repair at the LASIK flap margin. Br J Ophthal-
ithelial tissue within the stroma on kerocyte apoptosis, mitosis,
8. Jester JV, Ho-Chang J. Modulation of cultured corneal kerocyte
phenotype by growth factors/cytokines control in vitro contractil-
ity and extracellular matrix contraction. Exp Eye Res. 2003;77:
581–592.
9. Fini ME. Kerocyte and fibroblast phenotypes in the repairing cor-
10. Wilson SE, Mohan RR, Ambrosio R Jr, Hong J, Lee J. The corneal
wound healing response: cytokine-mediated interaction of the
epithelium, stroma, and inflammatory cells. Prog Retin Eye Res.
portance in wound healing and maintenance of transparency of the
12. Dohlman CH, Gasset AR, Rose J. The effect of the absence of
corneal epithelium or endothelium on the stromal kerocytes.
different methods of de-epithelialization. Ophthalimology. 1994;
101:890–894.
cyte apoptosis: hypothesized role for the interleukin-1 system in
the modulation of corneal tissue organization and wound healing.
15. Ignozzi RA, Massague J. Transforming growth factor-beta stimulates
the expression of fibronectin and collagen and their incorporation
16. Gipson IK, Watanabe H, Zieske JD. Corneal wound healing and
Corneal Femtosecond Laser Keratometry


