Persistent Corneal Haze after Excimer Laser Photokeratectomy in Plasminogen-Deficient Mice

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PURPOSE. Excimer laser photorefractive keratectomy creates a nonvascular wound of the cornea. Fibrin deposition and resolution after excimer laser photokeratectomy were investigated in relation to corneal repair and restoration of clarity in mice with a genetic deficiency of plasminogen.

METHODS. A Summit Apex Laser (Summit, Waltham, MA) was used to perform 2-mm, 175-pulse, transepithelial photoablations that resulted in deep stromal keratectomies. Photokeratectomy was performed on the corneas of plasminogen-deficient (Plg<sup>−/−</sup>) mice and littermate control animals. Eyes were examined for re-epithelialization and clarity throughout the 21-day observational period. Histologic sections were taken during the observational period and fibrin(ogen) was detected immunohistochemically.

RESULTS. Re-epithelialization was rapid and complete within 3 days in both control and Plg<sup>−/−</sup> animals. Exuberant corneal fibrin(ogen) deposition was noted in Plg<sup>−/−</sup> mice and sparse fibrin(ogen) deposition in control mice on days 1 and 3 after injury. Fibrin(ogen) deposits resolved in control mice but persisted in Plg<sup>−/−</sup> mice (74% of eyes at 21 days; P < 0.004). Corneal opacification, scarring, and the presence of anterior chamber fibrin(ogen) occurred in plasminogen-deficient mice but not in control mice.

CONCLUSIONS. Fibrin(ogen) deposition occurs during corneal wound repair after photokeratectomy. Impaired fibrinolysis in Plg<sup>−/−</sup> mice caused persistent stromal fibrin deposits that correlated with the development of corneal opacity. (Invest Ophthalmol Vis Sci. 2000;41:67–72)

Excimer laser photorefractive keratectomy (PRK) is currently used for corneal reprofiling to create a refractive improvement in vision. The excimer laser creates a smooth stromal wound with minimal collateral damage. Re-epithelialization occurs within days of the procedure through migration of adjacent epithelial cells over a newly formed substratum rich in fibronectin and fibrin. Fibronec	

Fibronec	 and fibrin are deposited at the wound’s edge within hours after injury and persist for several days until re-epithelialization is complete. Diffuse penetration of fibrin and fibronectin into the anterior stroma occurs in injuries in which the basement membrane is disrupted. Fibronec	 and fibrin promote cell attachment and spreading in vitro and are likely to play a role in the migration of epithelial cells over the wound. The immediate deposition of fibrin after corneal injury precedes that of other more permanent matrix proteins involved in corneal wound repair, such as collagen IV and laminin, consistent with the concept that this protein serves as a provisional matrix supporting tissue repair. A balance between the creation of an appropriate fibrin/fibronectin substratum to facilitate re-epithelialization, and fibrinolytic enzymes to bring about the timely resolution of this matrix ensures optimal corneal repair. Plasmin, a serine protease capable of degrading fibrin and fibronectin, is detected in the tear fluid of injured corneas. Topical inhibition of serine protease activity with aprotinin successfully induces re-epithelialization over therapy-resistant ulcers and is currently used to treat persistent ulcers. However, prevention of the formation of the fibronectin/fibrin substratum over epithelial scrape wounds or superficial keratectomies in ex vivo rabbit eyes did not delay migration, indicating that the temporary matrix is not essential for re-epithelialization.

Plasmin is crucial for efficient wound repair and fibrin clearance. Plasminogen-deficient (Plg<sup>−/−</sup>) mice demonstrate impaired skin wound healing due to markedly reduced fibrinolytic capacity. Persistent fibrin and granulation tissue result in scarring and delayed wound resolution. Persistent fibrin in the cornea and conjunctiva of Plg<sup>−/−</sup> mice results in the development of pseudomembranous nodules on the tarsal or bulbar conjunctiva, identified as ligneous conjunctivitis, a condition that also occurs in plasminogen-deficient patients. In the present study, the role of plasminogen in corneal wound repair after excimer laser photokeratectomy was investigated. We report that plasminogen deficiency resulted in persistent corneal fibrin deposits and reduced corneal clarity after photokeratectomy.
METHODS

Generation of Mice
Plg−/− and littermate control mice of a mixed 129/Black Swiss background were generated as previously described.21 Mice were aged between 10 and 20 weeks at the initiation of experiments and showed no evidence of ligneous conjunctivitis. Genotypes were determined by polymerase chain reaction (PCR) analysis using DNA obtained from ear biopsy specimens14 and were reconfirmed after death.

Photokeratectomy
Animal experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized with a sedative solution consisting of 66 mg/ml ketamine, 3.3 mg/ml xylazine, and 1.7 mg/ml acepromazine. This solution was administered intraperitoneally at 20 µl per 25 g body weight. Transepithelial photokeratectomies (2-mm diameter) were created on the corneas of both eyes of Plg−/− (n = 26) and littermate control (n = 27) mice using a Summit Apex Excimer Laser (Summit, Waltham, MA) on phototherapeutic settings. One hundred seventy-five pulses were delivered per cornea to create a stromal keratectomy with a depth of approximately 50%. Ofloxacin drops (0.3%; Ocuflox; Allergan, Irvine, CA) were instilled in the eyes immediately after surgery and twice daily until complete re-epithelialization occurred.

Assessment of Corneal Clarity and Re-epithelialization
Both corneal clarity and re-epithelialization were assessed 1, 3, 7, 14, and 21 days after laser injury. Fluorescein dye (Fluor-I-Strip; Ayerst Laboratories, Philadelphia, PA) was applied to the eyes of anesthetized mice, and re-epithelialization was assessed using a stereomicroscope. The size of the defect was estimated visually by an investigator unaware of the genotype of the mice and expressed as a percentage of the size of the original ablation area (2 mm). Stromal clarity was graded on a scale of 0 to 3, where grade 0 indicated complete clarity with no sign of haze, grade ½ indicated well-defined diffuse haze, grade 2 indicated obscuration of iris detail, and grade 3 indicated complete obscuration of the anterior chamber and iris. Loss of iris detail because of anterior chamber fibrin was not included in this stromal haze grading scale. Microcystic edema was also evaluated separately.

Collection of Tissue for Histologic Analysis
Five control and 5 Plg−/− mice were killed 1, 3, 7, 14, and 21 days after photokeratectomy, and 12 control and 11 Plg−/− mice were killed after 21 days, by anesthetic overdose. Whole eyes were enucleated with scissors after proptosis, fixed in neutral buffered formalin for 48 hours, and routinely processed and embedded in paraffin for histologic analysis. Tissue sections were stained with hematoxylin and eosin (H and E). Eyes were also taken from five control and five Plg−/− mice that were not treated with the excimer laser to investigate potential spontaneous corneal fibrin deposition.

Immunohistochemical Staining
Paraffin-embedded 4-µm sections were rehydrated, and an antigen-retrieval step was performed by microwaving for 4 minutes in citrate buffer. Slides were blocked with 10% goat serum in 5% bovine serum albumin. Sections were incubated with a polyclonal rabbit anti-mouse fibrinogen serum (preabsorbed against fibrinogen-deficient plasma). This antibody has previously been demonstrated to recognize both fibrin and fibrinogen but shows a stronger reaction with localized fibrin deposits in standard immunohistochemical staining of tissue sections. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. Bound primary antibody was detected using biotinylated goat anti-rabbit antibodies (Vector, Burlingame, CA) and a Vectastain ABC Kit (Vector). AEC (3-amino-9-ethylcarbazole; Vector) substrate was applied, and sections were counterstained with hematoxylin.

Statistical Analysis
Statistical analyses were performed using a nonparametric median scores test, the Mann-Whitney test, and a parametric χ² test for significance.

RESULTS

Gross Examination of Re-epithelialization and Clarity
Partial re-epithelialization was seen in all mice 1 day after surgery (range, 30%-80%) with no significant difference between control (n = 27) or Plg−/− (n = 26) mice (Fig. 1: P = 0.16). By day 3, all eyes were completely re-epithelialized except two control eyes and two Plg−/− eyes in which a 5% defect persisted. All eyes showed complete re-epithelialization by day 7. Some epithelial aberrations, including raised, punctate, and irregular areas, and/or microcystic edema were observed in mice in both groups at day 3 (control: 7/24 eyes, 29%; Plg−/−: 9/22 eyes, 41%) and day 7 (control: 2/24 eyes, 8%; Plg−/−: 4/22 eyes, 18%) but all irregularities were resolved by day 14. The occurrence of these epithelial aberrations was not significantly different between groups.

FIGURE 1. Re-epithelialization of eyes from control (shaded bars) and Plg−/− (open bars) mice at days 1, 3, and 7 after photokeratectomy (mean ± SEM). Fluorescein dye and a stereomicroscope were used to assess re-epithelialization as a percentage of the original defect.
Severe corneal haze developed and persisted throughout the observational period in Plg$^{−/−}$ eyes but not in control eyes. Examples of graded haze are shown in Figure 2 to demonstrate the classification system used. One day after photokeratectomy, minimal corneal haze (median score: 1/2) was observed in 3 of 24 (13%) control eyes, whereas, at this time point, 7 of 22 (32%) of Plg$^{−/−}$ eyes had some degree of haze, with most achieving a score of 1 (Fig. 3). The maximum number of eyes with haze occurred at day 3 in both control and Plg$^{−/−}$ mice; 7 of 24 (29%) control eyes had low grades of haze (median score: 1/2); 11 of 22 (50%) Plg$^{−/−}$ eyes had visible haze at this time point, and 10 of these eyes had a haze grading of 2. At day 14, only a single control mouse exhibited haze (one eye with a score of 1), which was resolved by day 21. In contrast, significant haze persisted in 11 of 22 (50%) Plg$^{−/−}$ eyes through days 7 and 14 (median score: 3) and in 9 of 22 (41%) eyes at day 21 (Fig. 3; control versus Plg$^{−/−}$ eyes, day 7: $P < 0.04$; day 14: $P < 0.003$; day 21: $P < 0.0004$). Minor resolution of haze occurred in some Plg$^{−/−}$ eyes by day 21 with a reduction of the median score from 3 to 2, but as a group, this improvement was not significantly different from day 14.

Eyes of Plg$^{−/−}$ mice showed mild inflammatory complications during the 21-day period, such as anterior chamber fibrin (Fig. 2C), retrocorneal membranes, iridocorneal adhesions, and/or pupillary membranes (Fig. 4H; 8/22 Plg$^{−/−}$ eyes, 36%). These aberrations were never seen in control eyes ($n = 24$).

**Histologic Examination and Fibrin Immunohistochemistry of Corneal Sections**

At 1 and 3 days after photokeratectomy, hypertrophied and irregular epithelium was forming over thinned stroma with no microscopically appreciable difference in re-epithelialization between control and Plg$^{−/−}$ mice. Inflammatory cells were seen at day 1 after photokeratectomy, in both control and Plg$^{−/−}$ mice but inflammatory cells were rarely seen at days 3, 7, or 21 in either group of mice. Although fibrin was neither present in untreated eyes from control or Plg$^{−/−}$ mice nor in eyes taken from mice killed within 1 hour of photokeratectomy, fibrin deposits were easily detected at 1 and 3 days after
photokeratectomy. Fibrin was deposited predominantly sub-epithelially and diffusely throughout the anterior stroma (Fig. 4). At day 7 after photokeratectomy, although normalization of the epithelium was occurring in mice of both genotypes, control mice had only subtle fibrin deposits in some corneas (2/10 eyes), whereas corneas of Plg$^{-/-}$ mice contained moderate to intense fibrin in the stroma and anterior chamber (7/10 eyes; Fig. 4). At day 21, control eyes had no evidence of stromal or anterior chamber fibrin. However, stromal fibrin was detected in 14 of 19 (74%) Plg$^{-/-}$ eyes in a diffuse pattern throughout both the anterior and posterior stroma (Fig. 4; control versus Plg$^{-/-}$: $P < 0.0001$). Anterior chamber fibrin was detected in 9 of 19 (47%) Plg$^{-/-}$ eyes in the form of pupillary, retrocorneal, and transchamber membranes; anterior synechiae; and iridocorneal adhesions (Fig. 4H; control versus Plg$^{-/-}$: $P = 0.013$). Mild corneal edema was present in 8 of 19 (42%) Plg$^{-/-}$ eyes and in 0 of 22 control eyes (control versus Plg$^{-/-}$: $P = 0.003$) at day 21. Early scar formation was occasionally seen in Plg$^{-/-}$ eyes (Fig. 4G).

**DISCUSSION**

In this study, photokeratectomy was performed on corneas of both Plg$^{-/-}$ and littermate control mice resulting in a 2-mm epithelial defect and a deep stromal ablation. Although the rate of re-epithelialization was similar between groups, fibrin deposition, which occurred in the greatest number of eyes in both groups at 3 days after injury, was soon resolved in control mice but persisted in 74% of Plg$^{-/-}$ eyes.
Plasmin activity is upregulated in tear fluid after corneal injury\textsuperscript{10,11} and specifically after PRK and anterior keratectomy.\textsuperscript{23,24} Inhibition of serine proteases with aprotinin has been demonstrated to promote re-epithelialization of corneal ulcers, previously resistant to healing. In normal corneal wound repair, plasmin may be beneficial to re-epithelialization by facilitating cell migration at the leading edge by either directly degrading the fibrin-fibronectin substrate or by activation of epithelial metalloproteinases, or both. However, similar re-epithelialization rates in Plg\textsuperscript{−/−} and control corneas, in the present study, suggest that plasmin activity is not strictly required for epithelial cell migration after photokeratectomy injury. Given that inhibition of matrix metalloproteinase activity markedly delays re-epithelialization,\textsuperscript{29} metalloproteinase activity, independent of plasmin, may make a more dominant contribution to corneal re-epithelialization.

Stromal haze was detected in almost half the corneas examined in both control and Plg\textsuperscript{−/−} mice between days 3 and 7. Haze did not correlate with moderate stromal hypercellularity occurring at these early time points in both control and Plg\textsuperscript{−/−} eyes. Neither is it likely that haze was a result of mild edema (detected by histology) in some Plg\textsuperscript{−/−} eyes at day 21. Prominent fibrinogen staining was always detected in corneas with obvious haze, suggesting that significant corneal fibrin deposition may be associated with the development of clinically apparent haze. Based on the close correlation between clinically apparent haze and persistent fibrin deposition, one hypothesis is that haze is a result of stromal fibrin deposits (either directly or indirectly). This hypothesis can be directly explored by photokeratectomy studies in fibrinogen-deficient mice. Unlike the collagen-dependent haze occurring after long-term healing in patients who undergo PRK, early haze in mice (day 21), appeared to be associated with exaggerated fibrin-matrix deposition. Previous studies have also demonstrated diffuse patterns of fibrinogen and fibronectin at these earlier stages of corneal wound repair.\textsuperscript{14} In control animals, fibrin clearance was complete in most corneas by day 14 after injury and in the remainder by day 21. Impaired fibrinolysis in Plg\textsuperscript{−/−} animals resulted in persistent haze that often worsened during the observational period. The continued presence of fibrin in corneas of Plg\textsuperscript{−/−} mice may result in prolonged or aberrant stromal wound repair processes that ultimately lead to further fibrin deposition and persistent haze.

Inflammation was seen only in the corneas of mice 1 day after photokeratectomy. This inflammatory infiltrate occurred in both control and Plg\textsuperscript{−/−} mice and was resolved in both groups of mice by day 3. Unlike epithelial scrape injuries in mice, in which inflammatory cells were seen at day 21 in some control mice,\textsuperscript{28} excimer laser ablation of epithelial cells caused only brief inflammation. The antibiotic used in our studies, Ofloxacin, may have anti-inflammatory properties,\textsuperscript{27,28} which may account for less inflammation in our study. PRK in patients, however, is associated with only low-grade, postoperative intraocular inflammation that is quickly resolved. Early inflammation after photokeratectomy was quickly resolved in all mice, in the present study but initiated transchamber membrane formation in Plg\textsuperscript{−/−} mice.

Delayed corneal re-epithelialization and persistent stromal fibrin has been demonstrated in Plg\textsuperscript{−/−} mice after epithelial scrape injury.\textsuperscript{28} The delayed re-epithelialization was brief and may be consistent with a possible trend toward delayed re-epithelialization in some Plg\textsuperscript{−/−} mice in the present study, although this was not statistically significant. Impaired epithelial cell migration in Plg\textsuperscript{−/−} mice may be highly dependent on the nature and extent of the corneal injury.

Although prominent corneal haze and immunohistochemically detectable fibrin predominated in Plg\textsuperscript{−/−} mice after photokeratectomy, it is notable that neither haze nor persistent fibrin was seen in 26% of Plg\textsuperscript{+/−} mice. The basis for the incomplete penetrance of this phenotype is uncertain but may result from our inability to detect minute amounts of fibrin immunohistochemically. Alternately, appreciable fibrin may not be present in some of these corneas either, because it has been resolved by a plasmin-independent mechanism or because it was never deposited. Stochastic differences in corneal wound healing between individual mice may avoid significant fibrin deposition in a small percentage of animals. Differences in corneal wound repair processes are apparent in patients, after excimer laser photoablation, resulting in variability in corneal scarring.\textsuperscript{29}

In this study, Plg\textsuperscript{−/−} mice showed an impaired ability to resolve corneal fibrin and clinically apparent haze after photokeratectomy. Fibrin deposition resulting from photokeratectomy resolved in control mice within several days, resulting in corneal clarity. Re-epithelialization occurred at a similar rate in Plg\textsuperscript{−/−} and control mice indicating that the absence of plasmin activity neither enhances nor markedly delays this process in a defect of this type and size. These findings indicate that plasminogen plays an important role in fibrin clearance and the achievement of corneal clarity after PRK. A more detailed understanding of the role of plasminogen and fibrinogen in wound repair may allow the development of strategies that reduce or prevent haze in patients after PRK. Further studies of photokeratectomy in mice with a simultaneous deficiency of plasminogen and fibrinogen will further elucidate the nature and occurrence of haze and the role of fibrin in corneal wound repair.

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References


