Healing of Corneal Epithelial Defects in Plasminogen- and Fibrinogen-Deficient Mice

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PURPOSE. The local deposition of fibrinogen and other plasma products from tears within corneal wounds and the expression of plasminogen activator by corneal epithelial cells suggest that the coagulation and fibrinolytic systems play an important role in corneal wound healing. The authors used mouse lines deficient in plasminogen (Plg), fibrinogen (Fib), or both to elucidate the roles of these key fibrinolytic and coagulation factors in the healing of corneal epithelial defects.

METHODS. Mice were anesthetized, and corneal epithelial defects (3 mm) were created with a blade. The authors conducted histologic examination and immunohistochemical analysis on the healing of injured corneas.

RESULTS. The corneal epithelial defects of wild-type mice with transparent corneas healed quickly in 7 days, whereas the healing of plasminogen-deficient mice was impaired and complicated by severe and persistent inflammatory responses, the formation of retrocorneal fibrin deposits, corneal cloudiness caused by scar-tissue formation, and often stromal neovascularization. To determine whether these defects in corneal wound repair were specifically related to an impediment in fibrinolysis, corneal wound healing was compared in mice with a combined deficiency in plasminogen and fibrinogen. The loss of fibrinogen in mice lacking plasminogen resulted in the restoration of normal healing with transparent corneas in 7 days, similar to that of wild-type mice.

CONCLUSIONS. These results provide direct evidence that hemostatic factors play a crucial role in corneal wound repair despite the lack of local hemorrhage. Furthermore, they demonstrate that the essential role of plasmin in corneal wound healing is fibrinolysis. It prevents the adverse inflammatory responses caused by prolonged fibrin and fibrinogen deposition in injured corneas. (Invest Ophthalmol Vis Sci. 1998;39:502-508)

Fibrin deposition1 and fibrinolysis are two hallmark events at the early stage of wound healing. The local formation of fibrin provides a provisional matrix and chemo- tectants, which support the adhesion and migration of inflammatory cells (that is, polymorphonuclear neutrophils and macrophages), fibroblasts, and other cells to the wounds.1-6 The cellular invasion of inflammatory cells into fibrin and other extracellular collagenous matrix components through the plasminogen activation system, matrix metalloproteases, and other protease systems is a critical early feature of tissue repair.2-6-12 In addition, cytokines secreted by inflammatory cells can modulate cellular functions in the synthesis and remodeling of extracellular matrix components by epithelial cells and stromal fibroblasts during wound healing.13-16 It has been demonstrated that fibrin and fibrinogen can mediate acute inflammation.17,18 Thus, the prolonged fibrin deposition in injured tissues may have severe adverse effects on the health of animals, such as persistent inflammation, a condition that frequently leads to undesirable scar tissue formation, ulceration, and other abnormalities.8,9,19

Recently, plasminogen deficiency in mice20 and humans21 has been shown to be compatible with development and growth to adulthood, but it results in severe thrombosis, widespread organ damage, wasting, and reduced life expectancy. Fibrin deposition was commonly found in multiple organ systems, including liver, rectum, stomach, lung, and eyes of Plg−/− mice.20 It has also been reported that the healing of skin wounds is severely impaired in the Plg−/− mice.22 It is of interest to note that the multiple abnormalities associated with plasminogen deficiency can be effectively corrected by the simultaneous loss of fibrinogen.23 These observations imply that an important interplay of fibrin-plasmin exists in the process of wound repair. Thus, fibrinolysis by plasmin may serve to minimize the potential undesirable consequences of persistent fibrin deposition after tissue injury. However, the underlying mechanism in modulating wound repair by these two molecules remains largely unknown.

The injury of avascular corneal tissue results in an apparently bloodless wound field that heals without neovascularization. Nevertheless, the local deposition of fibrinogen and other plasma products from tears within corneal wounds and the expression of plasminogen activators by...
Healing of Corneal Epithelial Defects

Table 1. Summary of Corneal Wound Healing in Plg and Fib Knockout Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of Eyes Healed/No. of Eyes Injured</th>
<th>No. of Eyes with Neovascularization/No. of Eyes Examined*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plg Fib</td>
<td>3 days</td>
<td>7 days</td>
</tr>
<tr>
<td>+/+ +/+</td>
<td>16/28 (57%)</td>
<td>20/24 (83%)†</td>
</tr>
<tr>
<td>+/- +/+</td>
<td>18/32 (56%)</td>
<td>20/24 (83%)†</td>
</tr>
<tr>
<td>+/- +/-</td>
<td>16/32 (50%)</td>
<td>20/22 (91%)†</td>
</tr>
<tr>
<td>-/- +/+</td>
<td>4/24 (17%)</td>
<td>3/22 (14%)‡</td>
</tr>
<tr>
<td>-/- +/-</td>
<td>2/60 (5%)</td>
<td>4/58 (7%)‡</td>
</tr>
<tr>
<td>-/- -/-</td>
<td>16/20 (80%)</td>
<td>18/20 (90%)‡</td>
</tr>
</tbody>
</table>

Corneal epithelial defects (3 mm in diameter) were created at the centers of experimental animals. The sizes of epithelial defects were measured with a stereomicroscope as described in Figure 5. Eyes were considered to be fully healed when the corneas became transparent and failed to stain with fluorescein and no retrocorneal deposits were detected. Some of the animals were killed on early days for histologic examination.

* Neovascularization was scored in those injured corneas that had healed for more than 2 weeks.
† Epithelial defects completely healed in all the animals after 14 days of injury, and corneas became transparent.
‡ Epithelial defects healed after 14 days of injury. The corneas were cloudy and never became transparent 21 days after injuries.

Methods

Animal Experiments

Animal experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and...
Vision Research. Age-matched littersmates were used as controls in all experiments. Mice were of a mixed 129-Black Swiss genetics background or a mixed 129-Black Swiss-CF-I genetics background. No differences were observed between the two mouse backgrounds with respect to healing of epithelial defects. Mice were genotyped by using multiplexed polynucleotide chain reaction analysis of the DNA from a tail biopsy as described previously. Adult mice were anesthetized by intraperitoneal injections of 70 mg/kg sodium pentobarbital. Under a stereomicroscope, a partial corneal epithelial defect (3 mm in diameter) was created in both eyes by scraping the corneal surface using a blade (Beaver, number 69; Becton-Dickinson, Franklin Lakes, NJ). Neomycin ointment was applied on the eyes immediately after surgery. Eyes were examined using a stereomicroscope (Olympus, Melville, NY) every other day, beginning on the first day after wounding, to evaluate reepithelialization and to detect any signs of infection. The animals were killed in a CO2 chamber, and the corneas were removed. Corneas were embedded in paraffin for histologic examination and immunohistochemical staining as described below.

**Histologic Examination and Immunohistochemical Staining**

The excised corneas were fixed in 4% paraformaldehyde in phosphate-buffered saline at 4°C overnight and embedded in paraffin as previously described. Sections (5 µm thick) were mounted on slides (Super Frost; Fisher Scientific, Pittsburgh, PA). Histologic examination was performed after Harris hematoxylin and eosin staining. Immunohistochemical staining was performed using goat anti-collagen IV (Southern Biotechnology, Birmingham, AL), rabbit anti-mouse fibrinogen antiserum, and rabbit anti-mouse vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology, Santa Cruz, CA). Paraffin sections of injured corneas, which had healed for various times, were treated with trypsin (1 µg/ml) for 10 minutes at room temperature and then incubated with goat anti-collagen IV antibodies (1 µg/ml) at 4°C overnight. The tissue sections were incubated with rabbit antigoat immunoglobulin G (IgG)-peroxidase conjugate (500X dilution) at room temperature for 2 hours. Paraffin sections of experimental corneas were incubated with rabbit anti-fibrinogen antibodies (5 µg/ml) at 4°C overnight and further incubated with goat anti-rabbit IgG-peroxidase conjugates (500X dilution). The antibody reactions were visualized with diaminobenzidine-hydrochloride as previously described.

**RESULTS**

**Clinical Observations of Corneal Epithelial Defects in Mice Deficient in Plg, Fib, or Both**

To examine the healing of epithelial defects in mouse lines lacking plasminogen or fibrinogen, experimental mice were anesthetized and epithelial defects (3 mm in diameter) were created at the centers of corneas with a number 69 Beaver blade as described in Methods. Corneal wound healing was evaluated 1 day after surgery, then every other day for the first week, and twice a week thereafter. Epithelial defect healing in experimental animals is shown (see Fig. 5). Corneal epithelial defects in wild-type, Plg+/–Fib+/+, Plg+/–Fib+/−, and Plg−/−Fib+/− mice heal with transparent corneas within 7 days, whereas in Plg−/−Fib−/− and Plg−/−Fib+/− mice, the healing is impaired and complicated by stromal cloudiness, neovascularization, and retrocorneal fibrin deposition. Figure 1 demonstrates the delayed reepithelialization of epithelial defects (as determined by fluorescein staining) in Plg−/−Fib−/− mice compared with that of Plg+/−Fib+/− mice. The epithelial defects of wild-type mice healed at the same rate as in Plg+/−Fib+/+ mice (data not shown). One day after injury, the epithelial defect reduced to approximately 25% of the original size of the defect in Plg+/−Fib+/+ mice. In 3 and 7 days, more than 50% and 80% of injured eyes completely reepithelialized and became transparent in wild-type, Plg+/−Fib+/+ mice.
Histologic Examination of Injured Corneas

Plg~/*-Fib+/~ mice, approximately 10% to 15% of epithelial defects remained 3 days after injury (Fig. 1), but corneal haze and retrocorneal fibrin deposition were found. In less than 5% and 17% of Plg~/*-Fib+/~ and Plg~/*-Fib+/~ mice, respectively (Table 1) injured eyes healed properly and corneal transparency was restored. Many of the corneas of Plg~/*-Fib+/~ mice (62%) became neovascularized as judged under a stereomicroscope (Table 1) and confirmed by histology (see below), whereas a smaller percentage of Plg~/*-Fib+/~ corneas (15%) were vascularized. Plg~/*-Fib+/~ mice healed normally, and in most of them (approximately 80%) epithelial defects were restored with transparent corneas in 3 days (see Fig. 5 and Table 1), and no neovascularization was noted.

**Histologic Examination of Injured Corneas**

To examine the histologic changes during the healing of corneal epithelial defects, the sections of injured corneas were examined using a light microscope (Labophot; Nikon, Melville, NY). The healing in wild-type mice was characterized by a moderate inflammatory response 1 day after injury (Fig. 2A). Relatively normal results of histologic examination showed stratified corneal epithelium and minimal or no inflammatory cells in the corneas, which healed for 3 days (Fig. 2B). No histologic abnormality could be seen in the corneas that had become transparent beyond 3 days of injury (data not shown). Similar histologic changes were found in mouse lines with at least one allele of the functional Plg gene, for example, Plg~/*-Fib+/~ and Plg~/*-Fib+/~ mice. To further examine whether the long-term healing in heterozygous Plg~/*-Fib+/~ differs from that of the wild type, the injured corneas healed for 21 and 33 days were also examined with hematoxylin and eosin staining. The micrographs shown in Figures 2C and 2D for injured corneas healed for 21 and 33 days of Plg~/*-Fib+/~ do not reveal any significant pathologic changes. There is little inflammatory response.

The histologic examinations of injured corneas revealed severe and persistent inflammatory responses in Plg~/*-Fib+/~ mice. There were numerous polymorphonuclear neutrophils invading the corneas after 1 and 3 days of injury as shown in Figures 3A and 3B. Figure 3C shows the micrographs of an injured Plg~/*-Fib+/~ cornea, which has healed for 7 days. Inflammatory cells can still readily be seen beneath the epithelium. Figure 3D is a micrograph of unusual severe inflammation and neovascularization seen in an injured Plg~/*-Fib+/~ cornea that healed for 21 days. Nevertheless, persistent inflammation and neovascularization are usually noted in most Plg~/*-Fib+/~ mice. At 33 days of injury, blood vessels can still be seen in corneas of Plg~/*-Fib+/~ mice as shown in Figure 3E. Occasionally, retrocorneal fibrin deposition can still be seen even in an injured Plg~/*-Fib+/~ cornea that healed for 33 days (Fig. 3F). The impairment of the healing of Plg~/*-Fib+/~ and Plg~/*-Fib+/~ is similar.

No apparent histologic changes could be noted in injured Plg~/*-Fib+/~ corneas compared with those of wild-type mice (compare Fig. 4 with Fig. 2). A moderate inflammatory response was observed at 1 day of injury as judged by the numbers of inflammatory cells in the stroma. The corneas look normal after 8 and 33 days of injury.

**Collagen IV in Stroma of Injured Corneas**

To visualize the neovascularization in injured Plg~/*-Fib+/~ corneas, tissue sections were exposed to goat anti-collagen IV antibodies, which labeled the basement membrane of blood vessels. Figure 4A shows a negative control with nonimmune goat IgG. Figures 4B and 4C show the presence of blood vessels labeled by the anti-collagen IV antibodies in Plg~/*-Fib+/~ corneas that healed for 7 and 21 days, respectively. Figure 4D shows the moderate neovascularization seen in a Plg~/*-Fib+/~ mouse. No blood vessels reacted by the antibodies could be seen in the injured Plg~/*-Fib+/~ (Fig. 4E) and Plg~/*-Fib+/~ (Fig. 4F) corneas that healed for 21 days and 33 days, respectively. The antibodies did not label the corneal endothelial basement membrane and Descemet membrane of Plg~/*-Fib+/~ (Fig. 4D) and Plg~/*-Fib+/~ (Fig. 4F) corneas. It should be noted that the antibodies also did not label the epithelial basement membrane and Descemet membrane of injured or uninjured corneas of wild-type mice (data not shown). It is likely that epitopes recognized by the antibodies are masked in wild-type, Plg~/*-Fib+/~ and Plg~/*-Fib+/~ corneas, similar to what has been demonstrated in human cornea.

**Fibrin and Fibrinogen Deposition in Corneas**

To evaluate the accumulation of fibrin deposition in stroma after deep epithelialization, corneal sections were incubated with...
FIGURE 5. Corneal epithelial wound repair in mice with single and combined deficiencies in plasminogen and fibrinogen. Epithelial defects (3 mm in diameter) were produced at the centers of corneas using a blade (number 69; Beaver) as described in Methods. The size of the epithelial defects was measured at various days after injury by fluorescein staining under a stereomicroscope. The numbers in each panel indicate days after injury. The healing of corneal wounds of Plg-deficient, Fib-positive (Plg+/~ Fib+/+ and Plg~~/~-Fib+/~) mice was impaired and complicated by corneal cloudiness. The corneal epithelial defects in wild-type, heterozygous Plg+/~ and double-deficient Plg~~/~-Fib~~/~ mice healed with transparent corneas within 6–10 days.

FIGURE 6. Immunohistochemical analysis of corneal sections with antibodies against collagen IV. (A) Negative control with nonimmune goat immunoglobulin G (1 μg/ml). (B) Injured Plg~~/~-Fib+/+ cornea, which healed for 7 days. Note that the basement membrane of blood vessels can be seen under the epithelium. (C) Injured Plg~~/~-Fib+/+ corneas healed for 21 days. Note that many blood vessels in the stroma were labeled by the antibody. (D) One blood vessel labeled by the antibody can be seen in an injured Plg~~/~-Fib+/+ cornea healed for 33 days. (E, F) No blood vessels were seen in the injured corneas of Plg~+/~ and Plg~~/~Fib+, which healed for 21 and 33 days, respectively.

FIGURE 7. Immunohistochemical analysis of injured corneas of wild-type, heterozygous Plg+/~ Fib+/+, and homozygous Plg~~/~ Fib~~/~ mice with anti-fibrinogen antibodies. (A) Negative control of an injured wild-type cornea healed for 1 day. (B) Weak positive reactions are seen in the stroma of an injured wild-type cornea healed for 1 day. (C) Little, if any, fibrinogen deposition can be detected in an injured wild-type cornea healed for 5 days. (D) A weak positive reaction can be seen in the stroma of an injured Plg~~/~ Fib~~/~ cornea healed for 21 days. (E) A negative reaction is seen in injured Plg~~/~ Fib~~/~ corneas healed for 1 day.

FIGURE 8. Immunohistochemical analysis of injured corneas of Plg~~/~ mice with anti-fibrinogen antibodies. Paraffin sections from injured corneas of Plg~~/~ mice were incubated with anti-fibrinogen antibodies as described in Figure 7. (A) Negative control with nonimmune rabbit immunoglobulin G. Fibrin deposition can be readily seen in stroma of injured Plg~~/~ Fib~+/+ corneas healed for various times. (B) 1 day; (C) 3 days; (D) 14 days; (E) 21 days; (F) 33 days after injury. Occasionally, retrocorneal fibrin deposition can be seen in F.
anti-fibrinogen antibody as described in Methods. Figure 7A shows a negative control of nonimmune IgG. Only weak positive reactions could be seen in injured wild-type corneas healed for 1 and 3 days, as shown in Figures 7B and 7C. A moderate positive reaction was found in an injured Plg"/-"-Fib"/-" cornea that healed for 21 days (Fig. 7D). Figure 7E shows a negative reaction of Plg"/-"-Fib"/-" corneas by anti-fibrinogen antibodies. Figure 8A shows a negative control of injured Plg"/-"-Fib"/-" corneas at 1 day with nonimmune rabbit IgG. Fibrin deposition in the stroma of injured corneas that healed for 1 day (Fig. 8B), 3 days (Fig. 8C), 14 days (Fig. 8D), 21 days (Fig. 8E), and 33 days (Fig. 8F) could be recognized by the antibodies. Retrocorneal fibrin deposition could be seen in an injured Plg"/-"-Fib"/-" cornea healed for 33 days, as shown in Figure 8F.

**Immunohistochemical Analysis Using Anti-Vascular Endothelial Growth Factor Antibodies**

Cornea sections were incubated with anti-VEGF antibodies to examine whether there was an upregulation of VEGF in injured corneas of Plg"/-" mice after deepithelialization. The presence of VEGF was noted in all injured corneas despite the genotypes of mice (data not shown).

**DISCUSSION**

The present study provides evidence that plasminogen plays a vital role in corneal wound healing and that this critical role is related to fibrinogen and, presumably, fibrinolysis. It has been previously suggested that, in addition to fibrinolysis, plasmin has important roles at the intersection of different inflammatory pathways that lead to the activation of cytokines, which can further modulate the synthesis of extracellular matrix by fibroblasts, endothelial cells, and epithelial cells.3,6,31-33 It has been demonstrated that plasmin can activate latent collagenase; thus, it participates in the cascade reactions of remodeling extracellular matrix in injured tissues.12 However, our data indicate that, in Plg"/-" mice, there is substantiated and persistent fibrin deposition in injured corneas. These corneas are characterized by prolonged inflammation, cloudiness, and neovascularization. These phenotypic changes of corneal wound healing were not observed in heterozygous Plg"/-" mice, implying that even approximately half the normal levels of plasminogen will support normal tissue repair without prolonged fibrin deposition and persistent inflammation. This observation is consistent with the normal development and health of Plg"/-" mice and humans.20,21 Protease levels play an important role in corneal wound healing. For example, topical application of serine protease inhibitors can slow down corneal wound healing.34 On the contrary, excessive protease activities can lead to an impaired healing process.35 In the present study, we have demonstrated that the loss of fibrinogen in the Plg"/-" mice results in a normal pattern of corneal wound healing after surgical removal of epithelium. These observations indicate that the impairment in corneal wound healing in Plg"/-" mice is mechanistically related to fibrinogen. The defects in corneal repair in Plg"/-" mice result from the absence of plasmin-mediated fibrin clearance, persistent fibrin deposition, and secondary adverse inflammatory reactions.

The upregulation of VEGF expression in Plg"/-" mice cannot account for neovascularization. Our experiment using immunohistochemical analysis with anti-VEGF antibodies failed to demonstrate any increase of VEGF in Plg"/-"-Fib"/-" mice compared with other mice examined. The neovascularization in corneas of Plg"/-" mice may be a result of the persistent inflammation in which the cytokines secreted by inflammatory cells can induce or augment the growth of blood vessels. In this regard, it is notable that fibrin and fibrinogen have been proposed as factors in the inflammatory response,17 particularly as mediators of the transendothelial migration of inflammatory cells.18 Furthermore, fibrin matrices prepared in vitro and implemented subcutaneously in rodents support robust angiogenesis,36-38 and purified proteolytic derivatives of fibrin have been shown to be angiogenic.39-40 Whatever the mechanism, it is clear that fibrinogen is crucial in establishing the proatherogenic setting in the injured corneas of Plg"/-" mice.

**References**


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