Expression of Gelatinases A and B, and TIMPs 1 and 2 during Corneal Wound Healing

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PURPOSE. To determine the expression of gelatinases A and B and the localization of tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 during the early stages of corneal wound healing in rats.

METHODS. One eye each of 30 adult Sprague-Dawley rats was treated with excimer laser keratectomy; the other eye was untreated. Frozen sections of corneas obtained 6, 18, and 24 hours and 3 and 7 days after wounding were used to perform immunofluorescent microscopy and in situ hybridization.

RESULTS. Gelatinase B was immunolocalized to the basement membrane zone and superficial stroma, and its mRNA was exclusively localized to basal epithelial cells migrating across the wound at 18 and 24 hours and 3 days after wounding. Tissue inhibitor of metalloproteinase-1 was also immunolocalized to the basement membrane zone and superficial stroma at the same time points. Gelatinase A was immunolocalized to the epithelium and stroma of normal corneas and was predominant in the basal epithelium and superficial stroma at 3 and 7 days after wounding. In situ hybridization confirmed gelatinase A expression by the epithelial cells and the stromal keratocytes. Tissue inhibitor of metalloproteinase-2 was immunolocalized to the epithelium in normal and wounded corneas. Intense TIMP-2 labeling of the basement membrane zone was noted 3 days after wounding.

CONCLUSIONS. Unlike gelatinase A, gelatinase B is expressed exclusively by migrating basal epithelial cells after wounding. The matrix metalloproteinase–tissue inhibitor of metalloproteinase (MMP)/TIMP systems may play an important role in the early stages of corneal wound healing after excimer laser keratectomy. (Invest Ophthalmol Vis Sci. 1998;39:913-921)

Gelatinases A and B are important members of the MMP family. They are zinc-binding proteolytic enzymes that participate in degrading and remodeling of the extracellular matrix (ECM) in various physiological and pathologic conditions including tumor progression and metastasis, inflammatory diseases, and corneal wound healing.\(^1\)\(^-\)\(^5\) MMPs are classified into three major groups according to substrate specificity.\(^6\) Collagenases (MMP-1, MMP-8, and MMP-13) are responsible for the cleavage of interstitial collagens types I, II, and III. Stromelysins (MMP-3, MMP-10, and MMP-11) and matrilysin (MMP-7) are important in degrading a variety of ECM components, including proteoglycans, fibronectin, and laminin. Gelatinases (MMP-2 and MMP-9), also known as type IV collagenases, are involved in cleaving collagen types IV, V, VII, and X, fibronectin, laminin, elastin, and collagen degradation products (gelatins).\(^6\) Gelatinase B (MMP-9) is involved in the early stages of corneal epithelial wound healing, and gelatinase A (MMP-2) may be important in remodeling of the ECM in the later stages of corneal wound healing.\(^7\)\(^-\)\(^8\)

Tissue inhibitors of MMPs (TIMPs) are produced in conjunction with MMPs. They regulate MMPs by interfering with active MMP enzyme and MMP proenzyme activation.\(^9\) Four types of TIMPs have been identified: TIMP-1, TIMP-2, TIMP-3, and TIMP-4.\(^10\)\(^-\)\(^13\) All inhibit MMPs, but with different affinities. TIMP-1 shows high affinity to gelatinase B, and TIMP-2 is normally bound to gelatinase A.\(^6\) Human progelatinase A can form a specific stoichiometric complex with TIMP-2 by interaction with the carboxy-terminal domain of the enzyme.\(^14\) We hypothesize that the gelatinase B–TIMP-1 and the gelatinase A–TIMP-2 systems exist in the rat cornea and may play an important role in the early stages of corneal wound healing after excimer laser surgery.

Gelatinase B (92 kDa) is expressed in many tumor tissues and is thought to play an important role in tumor progression and metastasis.\(^15\) Human T cell migration through a model basement membrane is MMP-9 dependent.\(^16\) Gelatinase B is also expressed by cultured rabbit corneal fibroblasts and epithelial cells.\(^5\)\(^,\)\(^7\) More recently, we have used zymography to demonstrate that gelatinase B is expressed in excimer laser-wounded rat and rabbit corneas.\(^8\) It was noted in the stroma and epithelium as early as 6 hours after wounding, peaked at 1 to 3 days, and was no longer detectable at 7 days and later. Gelatinase A (72 kDa) is widely expressed in various normal tissues and is upregulated in several diseases and pathologic conditions, such as tumor metastasis and ECM remodeling during wound healing.\(^5\)\(^,\)\(^15\) The human gelatinase A promoter has characteristics of a housekeeping gene.\(^18\)

In this study, we performed immunofluorescent microscopy and in situ hybridization to determine the expression of gelatinases A and B and their inhibitors during wound healing of
Figure 1. Immunoconfocal localization of gelatinase B in rat corneas 18 hours (A, B), 24 hours (C, D), and 3 days (E, F) after wounding, and in unwounded control corneas (G). (A, C, E) Wound edge (solid upright arrow) indicates junction between wounded and unwounded tissue. (B, D, F) Central area of the wound. (A) Gelatinase B localized to basal cell–basement membrane zone in wounded areas (right of arrow). No detectable fluorescence was noted in unwounded areas (left of arrow). Open arrows indicate superficial margin of epithelium. (B) Immunolocalization of gelatinase B to the leading edge (open triangle) of the epithelium 18 hours after wounding (before complete closure of the epithelial defect). (C, D) Twenty-four hours after wounding, immunolocalization of gelatinase B peaked and shows the strongest labeling. (E, F) Labeling started to diminish 3 days after wounding. (G) No detectable fluorescence in unwounded control. Scale bar, 10 μm.
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Figure 2. Immunolocalization of TIMP-1. (A) Patchy immunolocalization to the basement membrane zone of epithelial leading edge (epi) 18 hours after wounding. TIMP-1 is not seen in the stroma (s). (B) Twenty-four hours after wounding. Note intense immunolocalization to the basement membrane zone (open arrow) in the wounded area (right of arrow). (C) Three days after wounding. Intense immunolocalization to the superficial stroma (ss) in the wounded area. Arrow, wound edge. Open triangle, leading edge of epithelium. TIMP, tissue inhibitor metalloproteinase. Scale bar, 10 μm.

excimer laser-treated rat corneas. By combining immunoconfocal microscopy and nonradioactive in situ hybridization, we localized the expression of gelatinases A and B mRNA to specific cells in the rat cornea during the early stages of corneal wound healing.

Materials and Methods

Animal Models and Tissues

Thirty normal adult Sprague-Dawley rats (Taconic, Germantown, NY) each weighing 225 to 250 g, underwent excimer laser keratectomy. All animals were cared for and used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Anesthesia was achieved by intramuscular injection of 0.5 to 0.7 ml/kg body weight of a mixture of 100 mg/ml ketamine, 20 mg/ml xylazine, and 33 mg/ml promazine, and topical application of 1 drop 0.5% proparacaine. A 3-mm trephine was used to demarcate the central cornea, followed by epithelial debridement, using a number 15 Beaver blade. The exposed corneal stroma was treated with 193-nm argon fluoride laser with the fluence set at 160 mJ/cm² and repetition rate at 5 Hz. One hundred sixty to 180 pulses were administered to the right eye of each animal, resulting in approximately 40 to 45 μm of stroma ablation. Immediately after the surgery, 0.5% erythromycin ophthalmic ointment was applied. The left eyes served as unwounded controls.

The rats were killed at 6, 18, and 24 hours and 3 and 7 days after surgery (six rats per time point). The corneas were dissected from the eye at the scleral ring, were flash frozen in (O.C.T. compound, Miles, Elkhart, IN), and were stored at -80°C before sectioning. Cryostat sections 8 μm thick were placed on superfrosted-plus slides (Fisher Scientific, Pittsburgh, PA), were kept at room temperature for 15 to 20 minutes, and were stored in watertight boxes at -20°C before use.

RNA Probes for In Situ Hybridization

A cDNA probe p112Z92, which included a pGEM-11Zf vector and a 1.3 kb insert encoded for rat gelatinase B coding region, and a cDNA probe p4Z72, which included a pGEM-4Zf vector and a 222 bp insert encoded for rat gelatinase A coding region, were kindly gifts from Dr. Lili Feng (Scripps Research Institute, La Jolla, CA). Digoxigenin (DIG)-labeled RNA probes were generated by in vitro transcription using a DIG RNA labeling kit (Boehringer Mannheim, Indianapolis, IN). The antisense probe of gelatinase B was transcribed by SP6 RNA polymerase on an EcoRI linearized p112Z92 DNA template, and the sense probe was transcribed by T7 RNA polymerase on a p112Z92 DNA template linearized with XbaI. The antisense probe of gelatinase A was transcribed by T7 RNA polymerase on an EcoRI-linearized p4Z72 DNA template, and the sense probe was transcribed by SP6 RNA polymerase on a p4Z72 DNA template linearized with BamHI. The RNA transcripts were labeled with DIG-11-uridine triphosphate, according to the manufacturer’s recommendations. The size and integrity of the probes were checked using standard northern blot analysis and DTG probe detection methods. Briefly, blotted and baked membranes were detected by antidigoxigenin antibody conjugated with alkaline phosphatase, and then by alkaline phosphatase-catalyzed nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate color reaction.

To allow the probe to diffuse in or out of the tissue easily during hybridization, the 1.3 kb DIG-labeled MMP-9 RNA probes were hydrolyzed to approximately 200 bp. One microgram 1.3 kb RNA was hydrolyzed by adding an equal volume diethyl carbonate-treated water and two volumes carbonate buffer (60 mM Na₂CO₃, 40 mM NaHCO₃; pH 10.2) incubated at 60°C for 40 minutes. The reaction was stopped by adding an equal volume of hydrolysis-neutralizing buffer (200 mM sodium acetate; 1% acetic acid; pH 6). A standard ethyl alcohol precipitation technique was used to purify the probe. The size and integrity of the probe were checked again, as described earlier.
FIGURE 3. Immunolocalization of gelatinase A. (A, B) Unwounded corneas. Cells in epithelium (epi) and stroma (s) are positive for matrix metalloproteinase-2. (C, E) Wound edge. (D, F) Wounded central area. (C, D) Three days after wounding. Note increased immunostaining of superficial stroma (ss) of wounded area (right of arrow). (E, F) Seven-day-old wound shows intense immunolocalization of gelatinase A to the superficial stroma (ss). Scale bar, 10 μm.

In Situ Hybridization

In situ hybridization was performed using a previously described technique, with modifications. Briefly, slides were warmed to room temperature, washed in phosphate-buffered saline (PBS) for 5 minutes, and fixed with 4% paraformaldehyde in PBS for 20 minutes. The slides were incubated in 1 μg/ml proteinase K in 10 mM Tris and 1 mM EDTA at 37°C for 30 minutes, were washed in PBS-0.2% glycine for 5 minutes to quench the proteinase K, and were fixed in 4% paraformaldehyde for 10 minutes. The slides were rinsed in PBS, and then in 0.1 M triethanolamine, and were treated with freshly prepared 0.5% acetic anhydride in 0.1 M triethanolamine for 10 minutes for background reduction. Before air drying, the slides were rinsed with 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate).

Hybridization was performed in a buffer containing 50% formamide, 1× Denhardt's solution, 5× SSC, 5 mM EDTA, 500 μg/ml yeast tRNA, and 8% dextran sulfate. Twenty-five microliters per section of hybridization mixture containing 0.2 to 0.6 ng/μl of DIG-labeled probe was applied to the tissue sections, which were covered by plastic coverslips (PGC Scientific, Frederick, MD) made for in situ hybridization and were incubated in a closed, moist chamber at 42°C to 45°C for 16 hours. After hybridization, the coverslips were removed gently after washing in 4× SSC at 42°C to 45°C for 15 minutes, and then the slides were washed in fresh 4× SSC. The slides were incubated in 20 μg/ml RNase A in RNase digestion buffer (0.5 M sodium chloride, 10 mM Tris-HCl, 1 mM EDTA) at 37°C for 30 minutes. After digestion, the slides were washed in 2× SSC, 1× SSC, and 0.5× SSC for 15 minutes each. After a 30-minute wash in 0.1× SSC at 55°C, the slides were brought to room temperature by washing in 0.1× SSC.

The slides were incubated with blocking buffer for 30 minutes and with anti-DIG-alkaline phosphate for 1 hour and
washed three times in Tris buffer 1 (100 mM Tris, 150 mM sodium chloride; pH 7.5) for 5 minutes each wash. Then the slides were equilibrated in alkaline reaction buffer twice. Last, the slides were incubated in 1:50 nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate stock for 2 to 16 hours, washed in distilled water, and mounted with 80% glycerol in 10 mM Tris and 1 mM EDTA.

Immunofluorescence Confocal Microscopy

The frozen sections were warmed to room temperature for 20 minutes, immersed in chilled acetone for 10 minutes, and then air dried. After washing in PBS for 5 minutes, the slides were incubated with primary antibody for 1 hour. The primary antibodies (all obtained from Oncogene Science, Cambridge, MA) were mouse anti-human MMP-9 (Ab-1), working dilution 1:150; mouse anti-human MMP-2 (Ab-3), working dilution 1:100; rabbit anti-rat TIMP-2 (Biosource, Camarillo, CA), working dilution 1:50; and mouse anti-human TIMP-1 (Ab-1), working dilution 1:20.

After washing in PBS, the slides were incubated with secondary antibody conjugated with fluorescein isothiocyanate or rhodamine for 30 minutes. Fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (1:50) and lissamine-conjugated donkey anti-mouse IgG (1:50) (Jackson ImmunoResearch, West Grove, PA) were used against corresponding primary antibodies. The manufacturer's product specifications indicated that the donkey anti-mouse antibodies showed minimal cross-reactivity to rat. After washing with PBS, the slides were mounted with antifading medium (Vectorshield; Vector, Burlingame, CA) and were examined using a laser scanning confocal microscope (Leica TCS 4D; Leica, Deerfield, IL). Slides incubated with PBS instead of primary antibody served as negative controls (secondary antibody controls).

RESULTS

Immunolocalization of Gelatinase B

Unwounded control cornneas showed no detectable gelatinase B immunolocalization. After keratectomy wounds were imposed, gelatinase B immunolocalization was limited to the wounded areas (central 3 mm, Fig. 1). In the 18- and 24-hour
Expression of gelatinase B mRNA by in situ hybridization with digoxigenin-labeled RNA probe. (A, C, E, G) Wound edge. (B, D, F, H) Central area of the wound. Localization of gelatinase B mRNA is seen predominantly in the basal epithelial cells in the wounded area (right of arrow) 18 hours (A, B), 24 hours (C, D), and 3 days (E, F) after wounding, when hybridized with antisense probe. No significant staining is seen in the unwounded areas (left of arrow) and stroma. Note that the basal epithelial cells at the leading edge (open triangle) and central cornea are positive for gelatinase B mRNA and correspond to the immunolocalization patterns seen in Figure 1. (D) Strong signals are noted 24 hours after wounding. (F) Signals are less intense 3 days after wounding. (G) No detectable signal is seen 7 day after wounding with antisense hybridization. (H) Sense probe control cornea 18 hours after wounding. Scale bar, 10 μm.

and 3-day excimer-treated sections, the wounded area showed an intense band of fluorescence encompassing the epithelial basement membrane and the superficial stroma (Figs. 1B, 1D, 1F). Fluorescence intensity and bandwidth were greatest 24 hours after wounding (Fig. 1D); they decreased at 3 days, and the immunolocalization disappeared at 7 days. There was no detectable immunolocalization of gelatinase B in the stromal keratocytes, unwounded peripheral epithelium, and unwounded control eyes (Fig. 1G).

Immunolocalization of Tissue Inhibitor Metalloproteinase-1

Immunofluorescent microscopy showed faint immunolocalization of TIMP-1 to the intercellular spaces in the epithelium at
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**Figure 7.** Expression of gelatinase A mRNA by in situ hybridization with digoxigenin-labeled antisense (A, C, D) or sense (B) RNA probe. (A) Three days and (B) 7 days after wounding. There is positive localization of gelatinase A mRNA to epithelial cells and keratocytes. (B) Sense control cornea 7 days after wounding. (D) Eighteen hours after wounding. No specific staining in the tissue hybridized with antisense probe. epi, epithelium; s, stroma. Scale bar, 10 μm.

all postoperative time points examined (Fig. 2) and in the unwounded controls. However, at 24 hours, a bright band of immunofluorescence appeared in the basement membrane zone of the wounded areas (Fig. 2B). Three days after wounding, intense, irregular immunofluorescence was seen in the superficial stroma but was not immunolocalized to specific cells (Fig. 2C). Seven days after wounding, there was no detectable staining in the basement membrane zone or in the superficial stroma.

**Immunolocalization of Gelatinase A**

Gelatinase A staining was positive in epithelial cells and keratocytes in unwounded control corneas and all wounded corneas between 6 hours and 3 days (Figs. 3A, 3B, 3C, 3D). At 7 days after surgery, strong gelatinase A immunolocalization was seen in the basal epithelial cells and the superficial stromal region (Figs. 3E, 3F). Other cell layers of the cornea showed equally moderate immunolocalization.

**Immunolocalization of Tissue Inhibitor Metalloproteinase-2**

TIMP-2 was immunolocalized at all time points to the intercellular spaces of epithelial cells in the central and peripheral areas of corneas of wounded and unwounded controls (Figs. 4A, 4C). The most intense immunolocalization to the basal epithelial cells in the wounded area were seen at 3 days (Fig. 4B). There was no specific immunolocalization to the stromal keratocytes (Figs. 4A, 4B, 4C) and secondary antibody control (Fig. 4D).

**Integrity of RNA Probe**

The integrity of DIG-RNA probes for gelatinases A and B was confirmed by northern blot analysis. A labeled 760 bp control RNA marker was loaded with the gelatinase probes on formamide gel. Sense and antisense probes for gelatinase B formed single bands at 1.3 kb positions, and the sense and antisense gelatinase A probes formed single bands at the 222 bp positions (Fig. 5).

**Gelatinase B mRNA Expression**

Antisense gelatinase B probe was most exclusively hybridized with basal epithelial cells in the wounded area at 18 and 24 hours and 3 days after surgery, indicated by the dark purple staining around the nuclear area (Fig. 6). In contrast, the corresponding sense probe did not hybridize to any specific cells (Fig. 6H). The basal epithelial cells located at the leading edge or in the wounded center were intensely labeled (Figs. 6B, 6D, 6F). The labeling extended to the wound periphery and disappeared in the unwounded tissue (Figs. 6A, 6C, 6E). No detectable signal was seen in the 6-hour- and 7-day-old wounds with antisense probe hybridization (Fig. 6G). Under our experimental conditions, epithelial cells in the wounded area were the only cells obviously stained positively at 18 and 24 hours and 3 days.

**Gelatinase A mRNA Expression**

Three and 7 days after wounding, epithelial cells and keratocytes in the wounded area showed evidence of hybridization...
with the gelatinase A antisense probe. Heavy labeling appeared in the basal epithelium and in the superficial stroma (Figs. 7A, 7C). Under our hybridization conditions, there was no detectable signal in the unwounded areas of wounded corneas at 6, 18, and 24 hours and in the unwounded controls, when hybridized with antisense gelatinase A probe (Fig. 7D). The sense control sections that were analyzed along with the antisense sections showed no significant staining (Fig. 7B).

**DISCUSSION**

In a previous study using zymography, we demonstrated the presence of the activated and proenzyme forms of gelatinase B in the early stages of wound healing in excimer laser-treated rat and rabbit corneas. In the present study, we used immunofluorescent microscopy to localize gelatinase B to the epithelial basement membrane and the superficial stroma in the wounded area 18 and 24 hours and 3 days after wounding. In addition, we used in situ hybridization to localize the gelatinase B mRNA to the basal epithelial cells in the same areas but not in unwounded controls. This is the first study in which these techniques have been used to establish that the migrating basal epithelial cells are responsible for gelatinase B synthesis during corneal wound healing. Previous studies predicted this finding.

We detected gelatinase A mRNA only in the wounded area of rat corneas, 3 and 7 days after excimer laser keratotomy wounds were imposed. The stromal cells expressing gelatinase A were probably activated myofibroblasts that replaced superficial keratocytes that underwent apoptosis. The re-epithelialization and the anterior stroma, where it underwent rapid degradation. The short turnover time of gelatinase B is illustrated by the in vitro artifacts and the possibility that the role of the epithelium is to activate latent MMPs produced by concordant corneal fibroblasts known to produce MMPs in culture.

Immunofluorescent microscopy was valuable in studying the immunolocalization of TIMP-1 and TIMP-2 and gelatinases A and B at various time points after laser corneal wounding. By correlating these findings with the in situ hybridization results, we observed that the turnover of gelatinase A differed from that of gelatinase B in that it was constitutively present in the unwounded corneal stroma and was upregulated after wounding. In contrast, gelatinase B was expressed by regenerating epithelial cells and was secreted into the extracellular space and the anterior stroma, where it underwent rapid degradation. The short turnover time of gelatinase B is illustrated by the temporal congruity of gelatinase B mRNA expression (Fig. 6) and its gene product (Fig. 1). Additional investigations of the factors contributing to the rapid turnover of gelatinase B may be important in furthering our understanding of the exact role of gelatinase B in corneal wound healing.

TIMP-1 and TIMP-2 inhibit the various MMPs with different affinities. TIMP-1 has high affinity to gelatinase B and is commonly bound to gelatinase B at a 1:1 molar ratio. TIMP-2 shows high affinity to gelatinase A and normally is bound 1:1 to gelatinase A at its C-terminus. We demonstrated that both TIMPs were expressed in normal and peripheral epithelial during excimer wounding. The upregulation of TIMP after excimer wounding was shown in wounded epithelia and in superficial stroma, suggesting that MMP and TIMP coexist in the laser-wounded cornea.

The use of the excimer laser to study wound healing after keratectomy has distinct advantages in comparison with other means of studying corneal wounding, such as those of chemical and thermal burns and mechanical keratectomy. These include achievement of predictable wound depths and sizes, reproducibility, minimal leukocyte infiltration, ease of tissue handling for histologic analyses, and the ability to compare the time and pattern of wound healing in different species.

Previous investigators suggest that gelatinase B expression is important in impeding corneal epithelialization. Clinically, corneal wound healing after excimer laser surgery has rarely been associated with corneal vascularization, opacification, inflammation, melting, or persistent epithelial defects; yet, our gelatinase localization data do not seem to differ from biochemical and cell culture data reported by others concerning the production of gelatinases after severe and mild burns, alkali injuries, and persistent epithelial defects. This may indicate an exaggeration of the "normal" process of gelatinase expression in these pathologic conditions. Alternatively, the role of gelatinases A and B in the pathogenesis of persistent epithelial defects and stromal melting in these conditions may be limited. We are investigating the hypothesis that gelatinases may play an important role in maintaining corneal clarity after nonpathologic wounds and in unwounded corneas, but their expression may be insufficient to maintain corneal transparency in severely inflamed corneas.

In summary, our study demonstrated that gelatinase B is expressed by migrating and regenerated basal epithelial cells and that gelatinase A is expressed by regenerated epithelial cells and superficial stromal keratocytes after keratectomy in rat corneas. It also suggested that gelatinase B–TIMP-1 and gelatinase A–TIMP-2 systems in the rat cornea after excimer laser surgery may play important roles in the early stages of wound healing and ECM remodeling.

**References**

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