Differential Expression of MT1-MMP (MMP-14) and Collagenase III (MMP-13) Genes in Normal and Wounded Rat Corneas

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Purpose. Several members of the matrix metalloproteinase (MMP) group have been identified in the rat cornea during corneal wound healing. The aim of the present study was to identify additional members of the MMP gene family in the rat cornea and localize the expression of membrane type-1 matrix metalloproteinase (MT1-MMP; MMP-14) and collagenase III (MMP-13) in normal and wounded corneas.

Methods. Adult rats underwent laser keratectomy on the right eye. Unwounded left eyes were normal controls. Corneas were collected and processed at different times post-wounding. Reverse transcription–polymerase chain reaction (RT-PCR) and DNA sequencing were used to discover the MMP genes expressed in the corneas. In situ hybridization was performed to localize the mRNA expression of MMP-14 and MMP-13.

Results. MMP-13 mRNA was detected in epithelial cells of wounded corneas, but not in normal controls; MMP-14 was found in both normal and wounded corneas. MMP-14 mRNA was expressed predominantly in the stromal keratocytes and rarely in the basal epithelial cells in normal and wounded corneas. MMP-13 mRNA was localized exclusively to basal cells of the epithelium at the wounded area from 6 hours to 3 days after wounding.

Conclusions. MMP-14 and MMP-13 expression in rat corneas parallels that of gelatinases A and B, respectively. MMP-13 may play an important role in the gelatinase B–associated proteolytic cascade that allows rapid turnover of the extracellular matrix (ECM) components during corneal wound healing. MMP-14 may contribute to removing abnormal ECM components through activation of gelatinase A in rat corneas. (Invest Ophthalmol Vis Sci. 2000;41:2894–2899)
13), MT1-MMP (MMP-14), and metalloelastase (MMP-12), in the rat cornea. Then we focused on the study of MMP-13 and MMP-14 in the normal rat cornea and during the early stages of wound healing. Our data suggest a possible role for these MMPs in ECM modulation during corneal wound healing.

METHODS

Animal Models and Tissues

The right eyes of normal adult Sprague-Dawley rats, each weighing 225 to 250 g, underwent 3-mm-diameter excimer laser keratectomy. The untreated left eyes served as normal controls. 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 wt of a mixture of ketamine (100 mg/ml), xylazine (20 mg/ml), and promazine (35 mg/ml) and topical application of 1 drop of 0.5% proparacaine. A 3-mm trephine was used to demarcate the central cornea, followed by epithelial debridement using a No. 15 Beaver blade. The exposed corneal stroma was treated with 193-nm argon fluoride laser with the fluence set at 160 mJ/cm². The eye received 160 to 180 pulses, resulting in approximately 40 to 45 μm of stromal ablation. Immediately after the surgery, a 0.5% erythromycin ophthalmic ointment was applied.

The rats were killed at 6 and 18 hours and 1, 3, 5, 7, and 14 days after surgery. For in situ hybridization, the corneas were dissected at the scleral ring, flash-frozen in Optimum Cutting Temperature (OCT) compound (Miles, Elkhart, IN), and stored at −80°C. Cryostat sections, 8-μm-thick, were placed on Superfrost/Plus microscope slides (Fisher, Pittsburgh, PA), kept at room temperature for 15 to 20 minutes, and then stored in watertight boxes at −20°C. For RT-PCR, the corneal epithelium and superficial stroma were scraped off, flash-frozen in liquid nitrogen, and stored at −80°C in RNase-free tubes.

Reverse Transcription–Polymerase Chain Reaction

Messenger RNA was extracted from unwounded rat corneas and wounded rat corneas at 18 hours and 3 days post-wounding using QuickPrep Micro mRNA purification kit (Pharmacia, Piscataway, NJ). The first strand cDNA was reverse-transcribed in the presence of oligo(dT) primer and used for PCR.

Three kinds of primer sets were designed for PCR amplifications: degenerate, degenerate/specific, and specific. A degenerate primer set for PCR was designed from two highly conserved sequences of known MMPs: the cysteine switch (PRCGPVD) and the zinc binding site (AAHELGH). Taking into account the codon usage in the rat, two 20-mer oligonucleotides were synthesized: sense primer CCIMGITGTG-GGTTCCWGA (cysteine switch) and antisense primer TGIC- GIGTICCWGA (cysteine switch) and antisense primer TGIC-GIGTICCWGA (cysteine switch). The sequences were as follows: for MMP-13, sense primer 5′-GAACACAGATAAAGGGAAT-T′ (1831-1850), antisense primer 5′-GCAGGGAAGGGGCTAATGAA-3′ (2414-2439); for MMP-14, sense primer 5′-AAAGGGAACAAATCTGGA-3′ (1607-1626), antisense primer 5′-ATGTAGGTAGGGGAGT-GAA-3′ (2202-2183). The PCR conditions were 95°C for 5 minutes followed by 35 cycles at 95°C for 45 seconds, 50°C for 30 seconds, and 72°C for 1 minute, with a final 7-minute extension at 72°C. The PCR products were separated by electrophoresis on 1% agarose gels. The corresponding DNA bands were cut off the gel, purified, and sequenced. The DNA fragments (confirmed by sequencing) were used for RNA probe synthesis for in situ hybridization.

RNA Probes for In Situ Hybridization

The sequencing-confirmed PCR fragments were individually ligated to T7 adapter using the Lig’n Scribe RNA polymerase promoter addition kit (Ambion, Austin, TX) and served as templates for RNA probe synthesis. The RNA probes were labeled with a digoxigenin-11-UTP using a DIG RNA labeling kit (Boehringer–Mannheim, Indianapolis, IN) and hydrolyzed to around 200 bp, as recommended by the manufacturer. The size and integrity of the DIG RNA probes were checked using standard Northern blot analysis and DIG probe detection methods as described previously.

In Situ Hybridization

In situ hybridization was performed as described, with modification. 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 then incubated in 1 μg/ml proteinase K in TE (10 mM Tris, 1 mM EDTA) at 37°C for 30 minutes, washed in PBS/0.2% glycine for 5 minutes to quench the proteinase K, and then fixed in 4% paraformaldehyde for 10 minutes. The slides were rinsed in PBS, then in 0.1 M triethanolamine (TEA), and treated with freshly prepared 0.25% acetic anhydride in 0.1 M TEA 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. The hybridization results were analyzed using the online National Center for Biotechnology Information (NCBI) BLAST program.

The degenerate/specific second set of primers consisted of the above degenerate 5′ primer (cysteine switch; sense) paired with a specific antisense primer for MMP-13, 5′-GGTT-GGGGCTTCTACTCTG-3′ (809–789, Accession No. M60616), or MMP-14, 5′-TAGGCATAGGCACTTCTCG-3′ (669–649, Accession No. X83537). The PCR conditions were 95°C for 5 minutes followed by 35 cycles at 95°C for 45 seconds, 50°C for 30 seconds, and 72°C for 1 minute, with a final 7-minute extension at 72°C. The PCR products were revealed by electrophoresis on 1% agarose gels. The corresponding DNA bands were cut off the gel, purified, and sequenced.

The specific primers were designed from corresponding sequences obtained from GenBank under Accession No. M60616 for MMP-13 and Accession No. X83537 for MMP-14. The sequences were as follows: for MMP-13, sense primer 5′-GAACACAGATAAAGGGAAT-3′ (1831-1850), antisense primer 5′-GCAGGGAAGGGGCTAATGAA-3′ (2414-2439); for MMP-14, sense primer 5′-AAAGGGAACAAATCTGGA-3′ (1607-1626), antisense primer 5′-ATGTAGGTAGGGGAGT-GAA-3′ (2202-2183). The PCR conditions were 95°C for 5 minutes followed by 35 cycles at 95°C for 45 seconds, 50°C (MMP-13) or 54°C (MMP-14) for 30 seconds, and 72°C for 1 minute, with a final 7-minute extension at 72°C. The PCR products were separated by electrophoresis on 1% agarose gels. The corresponding DNA bands were cut off the gel, purified, and sequenced. The DNA fragments (confirmed by sequencing) were used for RNA probe synthesis for in situ hybridization.
mixture (25 μl per section) 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 Scientifics, Frederick, MD) made for in situ hybridization and incubated in a closed moist chamber at 42°C to 45°C for 16 hours. After immersion in 4× SSC and incubation at 42°C to 45°C for 15 minutes, the coverslips were removed gently, and the slides were washed in fresh 4× SSC. The slides were then 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 sequentially 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 blocked with 1% BSA (bovine serum albumin) in PBS, and then incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase. Finally, the slides were developed in NBT/BCIP (nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate) mixture for 2 to 16 hours, washed in distilled water, and mounted with 80% glycerol.

RESULTS

MMP Family Members Detected in Rat Cornea: MMP-12, -13, and -14

With the use of a set of degenerate PCR primers to determine which MMP transcripts are expressed in normal and wounded rat corneas, we expected the initial PCR product to contain a mixture of DNAs reflecting all the MMP transcripts capable of hybridizing to the primers. We observed a 450-bp amplified band from corneas 3 days after wounding but not from normal control corneas (Fig. 1A). No visible band in normal corneas may be due to lower amounts of MMP amplified. We obtained and sequenced 38 positive clones, 30 from wounded and 8 from normal corneal scrapings. Sequence analysis with BLAST revealed that 3 clones from wounded corneas matched MMP-13, and 23 matched macrophage metalloelastase (MMP-12). One clone from normal corneal scraping matched MMP-14. The localization of expression of these MMPs in the rat cornea has not been reported previously.

Messenger RNA Expression of MMP-14 and MMP-13

Detection of MMP-14, MMP-13, and MMP-12 by degenerate PCR primers suggests the expression of these MMPs in the cornea. To confirm the results of degenerate MMP primer-based PCR, PCR amplification of rat corneal cDNA was performed using degenerate/specific primers. This also allowed us to compare gene expression in control and wounded corneas (Fig. 1B). A 227-bp band was amplified when the sense degenerate primer and MMP-14 antisense primer were used; this band was found in both normal and wounded corneas. A 544-bp band amplified with MMP-13-specific antisense primer was detected in the wounded but not in the stationary corneas.

The PCR product sequencing results indicated indeed that the 544 bp (266–809) is MMP-13 cDNA fragment and the 227 bp (443–669) band is MMP-14 cDNA.

A pair of gene-specific primers was also synthesized from MMP-13 or MMP-14 cDNA and used for PCR amplification, followed by DNA sequencing. Similar results were obtained. These PCR fragments (MMP-13, 584 bp, 1831–2414, Accession No. M60616; and MMP-14, 596 bp, 1607–2202, Accession No. X83537) were amplified from nonconserved regions of MMPs and used as probes for in situ hybridization. To exclude the possibility of sequence homology between two hybridization probes, computing sequences homology checks were performed between MMP-13 and gelatinase B. No significant sequence homology was found.
mRNA in situ hybridization analysis was performed at various time points during wound healing. MMP-13 mRNA hybridization was localized to the basal cell layers in the wounded area at 6 hours, 1 day, and 3 days after surgery (Fig. 2). No detectable signal was seen in normal corneas or at 7 days after wounding. The elevated expression was evident at the early stages of corneal wounding. When hybridized with the sense probe, the corresponding sections showed no significant staining.

MMP-14 expression was detectable in the basal epithelial cells and stromal keratocytes in the unwounded corneas and 6 hours after wounding (Fig. 3). Elevated mRNA expression was observed at 1, 3, and 14 days after wounding, as evidenced by strong labeling of the stromal keratocytes.

Taken together, these data show a distinct pattern of temporal and spatial expression of MMP-13 and MMP-14 during corneal wound healing, similar to the expression of gelatinases B and A, respectively.

**DISCUSSION**

Corneal wound healing is a complex process that relies on the interplay of proteinases and their inhibitors for proper ECM remodeling. MMPs are thought to play a major role in ECM remodeling during corneal development and wound healing. Several MMPs have been identified in the cornea, and their expression has been characterized during wound healing. However, the expression and potential role of newly discovered MMPs in the cornea remain largely unknown.

Using degenerate PCR primers, we observed that three members of the MMP gene family are expressed in rat corneal cells: MMP-14, MMP-13, and MMP-12. It is possible that other MMP gene transcripts expressed in these cells might not be detected if the degenerate PCR primers used for cDNA amplification did not sufficiently match their respective DNA sequences or if the concentration of certain MMPs is relatively low. To the best of our knowledge, this is the first report of in situ hybridization of MMP-13 and MMP-14 in the cornea. MMP-13 was expressed in the regenerating rat corneal epithelium 18 hours and 3 days after wounding but not in normal epithelium. In contrast, MMP-14 was expressed in both normal and wounded corneas. RT-PCR using gene-specific primers and sequencing confirmed these findings. In situ hybridization localized MMP-13 mRNA to migrating basal epithelial cells and MMP-14 predominantly to superficial stromal keratocytes in the wound area. Similar findings have been reported during rat skin wound healing.22 Rat MMP-15 (collagenase III), originally named rat collagenase,18,23 shares 86% identity with human MMP-13 at the amino acid level but not with human collagenase I.24 Collagenase I expression has been shown in fibroblasts, macrophages, chondrocytes, and certain tumor cells.25 In contrast, we found that collagenase III (MMP-13) is expressed in the corneal epithelium but not in the stroma. In addition to its corneal expression, MMP-13 has been detected in human malignant squamous epithelium of skin,26 fetal development,27 and pathologic conditions28 but not in normal adult tissues. As compared with MMP-15, the classic fibroblast collagenase I (MMP-1) is expressed by many mesenchymal and epithelial cell types, including a repair fibroblast in healing skin wounds and rabbit keratocytes.6,18,23 Fini et al. reported that in contrast to the results in rabbit and human corneal tissues, they could not find evidence for the synthesis or presence of MMP-1 in the injured rat cornea.18

We found that MMP-13 was localized to basal epithelial cells in the leading edge of wounded corneas at 6 hours and in the wound area at 1 and 3 days after surgery, but not in normal rat corneas. Under our experimental conditions, the corneal reepithelialization usually occurred 0 to 4 days after wounding. The temporal and spatial correlation between MMP-13 and corneal reepithelialization suggests that MMP-13 plays a role in reepithelialization after corneal wounding. Human gelatinase B is activated by MMP-13 in vitro.20 Our previous study7 localized
gelatinase B to the basal epithelial cells in the wounded area 18 and 24 hours and 3 days after wounding. The spatial and temporal correlation between gelatinase B and MMP-13 expression provided the first line of evidence that MMP-13 may activate gelatinase B in vivo.

MMP-14 plays a role in activating gelatinase A. In a previous study, we showed that gelatinase A was predominantly expressed by superficial stromal keratocytes 3 and 7 days after corneal excimer keratectomy and maintained at a low level of expression in wounded and normal epithelia and in normal stroma. Interestingly, MMP-14 shows a similar stromal expression pattern. This suggests that MMP-14 may activate pro-gelatinase A at the stromal cell surface and play a role in maintaining the normal balance between ECM synthesis and degradation during normal ECM turnover and connective tissue restoration during wound healing.

In summary, we have found three members of the MMP family (MMP-14, MMP-13, and MMP-12) that are expressed in the rat cornea. MMP-14 was expressed in normal and wounded corneas, but MMP-13 was expressed only in wounded corneas during reepithelialization. Our data suggest that MMP-14 may activate gelatinase A, and MMP-13 may activate gelatinase B in the cornea.

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References


