Expression of Membrane-Type Matrix Metalloproteinases 4, 5, and 6 in Mouse Corneas Infected with *P. aeruginosa*

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Purpose. To investigate the expression and regulation of membrane-type matrix metalloproteinases (MT-MMPs) 4, 5, and 6 in the mouse corneas infected with *Pseudomonas aeruginosa*.

Methods. C57BL/6J mice were intracorneally infected with *P. aeruginosa*. The expression of MT4-, MT5-, and MT6-MMP was detected at both the mRNA and protein levels by RT-PCR and immunoblot analysis. Immunohistochemical staining was performed to localize the expression of MT4- and MT5-MMP in the mouse corneas.

Results. Expression of MT4- and MT5-MMP was detected in the normal (uninfected) cornea by RT-PCR and immunoblot analysis. When infected with *P. aeruginosa*, the corneas showed significant induction of each MT-MMP. Localization of MT4- and MT5-MMP revealed that the expression of MT5-MMP was restricted to the epithelial tissue in the normal cornea, whereas the induced expression of MT4- and MT5-MMP was predominantly in the substantia propria, which contained most of the infiltrating cells. MT6-MMP expression was not detected in the uninfected cornea but was upregulated in the infected corneas.

Conclusions. Expression of MT4-, MT5-, and MT6-MMP was induced in corneas infected with *P. aeruginosa*. Immunohistochemistry showed predominant immunoreactivity of MT4- and MT5-MMP in the substantia propria. Previous histologic studies have revealed different patterns of inflammatory cell infiltration with an increased number of polymorphonuclear neutrophils (PMNs) during the early stage of inflammation and increased macrophages during the late stage. These results indicate a good correlation between the overexpression of the MT-MMPs in the infected corneas and the inflammatory response—that is, leukocyte infiltration—indicating that inflammatory cells such as macrophages and PMNs may play a role in the upregulation of MT-MMPs during corneal infection, which in turn can cause the destruction of corneal tissue. (*Invest Ophthalmol Vis Sci.* 2001;42:3223–3227)

Recently, studies in our laboratory showed that membrane-type matrix metalloproteinases (MT-MMPs) 1, 2, and 3 were all upregulated in mouse corneas infected with *P. aeruginosa* and the peak of the induction correlated to the inflammatory events, suggesting the important role of MT-MMPs in corneal infection and destruction.[1]

MMPs are a group of structurally related and zinc-dependent enzymes that play a crucial role in physiologic and pathologic processes, such as embryonic development, endometrial cycling, wound healing, rheumatoid arthritis, tumor invasion, and metastasis.[2] MMPs have been classified into five broad categories based on their substrate specificity and domain organization. They include collagenases (MMP-1, -8, -13, and -18), stromelysins (MMP-3, -10, and -11), gelatinases (MMP-2 and -9), MT-MMPs (MMP-14, -15, -16, -17, -24, and -25), and other MMPs (MMP-7, -12, -19, -20, -21/22, and -26).[3–7] Since the first member was identified by Sato et al. in 1994,[8] the MT-MMP subgroup has grown rapidly and now has six members named after the transmembrane domains that anchor the molecules on the plasma membrane.[3,5,6] MT-MMPs are important in turnover of the extracellular matrix (ECM), because of their dual functions. Similar to other MMPs, they directly cleave ECM proteins, including type I and III collagens, fibronectin, and laminin. In addition, they activate other MMPs such as pro-MMP-2 (gelatinase A) and pro-MMP-13 (collagenase 3) and form an enzymatic cascade for regulation of ECM degradation.[9,10]

Although classified in the same subgroup, MT4-, MT5-, and MT6-MMP are different from the other three MT-MMPs in some respects. For example, MT4-MMP has the least sequence identity with other MT-MMPs, except for MT6-MMP.[11,12] Both MT4-MMP and MT6-MMP are predominantly expressed in leukocytes, and both are glycosylphosphatidylinositol-anchored MMPs.[12–15] In addition, MT4-MMP also shows TNF-α convertase activity and can potentially process pro-TNF-α to the 17-kDa form.[11] These characteristics of MT4-MMP and MT6-MMP suggest that they play a role in inflammation. However, the normal functions of MT4-, MT5-, and MT6-MMP remain unknown. Their expression and regulation in the cornea have not been reported.

In the present study, we explored expression of newly identified MT4-, MT5-, and MT6-MMP in the corneas of naïve C57BL/6J mice infected with *P. aeruginosa*. RT-PCR, immunoblot analysis, and immunohistochemical staining revealed the significant induction of MT4- and MT5-MMP expression at both the mRNA and protein levels in naïve mice over an 8-day period. Similar results were obtained for MT6-MMP gene expression using only RT-PCR due to the absence of available antibody to mouse MT6-MMP.

Methods

Bacteria

Stock cultures of *P. aeruginosa* 19660 (ATCC, Rockville, MD) were stored at 4°C on tryptose agar slants (Difco Laboratories, Detroit, MI) and were used for the inoculation of 50 to 75 ml of broth medium containing 5% peptone (Difco Laboratories) and 0.25% trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD). Strain 19660 is hemolytic and lecithinolytic and produces exotoxin A, alkaline protease, and elastase under appropriate culture conditions. Cultures were grown on a rotary shaker at 37°C for 16 to 18 hours, centrifuged at

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Supported by National Eye Institute Grant EY-11757 and Grant P30 EY-04068 from the National Institutes of Health, Bethesda, Maryland.

Submitted for publication January 26, 2001; revised August 10, 2001; accepted August 13, 2001.

Commercial relationships policy: N.

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8000 rpm at 4°C for 10 minutes, washed with normal saline (Travenol Laboratories, Cambridge, MA), and diluted to a concentration of 2×10^{10} colony-forming units per milliliter. A standard curve was developed to relate viable counts to optical density at 440 nm.

Infection of Animals and Corneal Sample Collection

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Age-matched C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), each weighing 18 to 22 g, were infected at 14 weeks of age. Before infection, they were lightly anesthetized with ether and placed beneath a stereoscopic microscope. The corneal surface was then gently incised with three 1-mm incisions using a sterile 26-gauge needle, taking care not to penetrate the anterior chamber or to damage the sclera. A bacterial suspension (5 μl) containing 10^8 colony-forming units was topically delivered onto the wounded cornea using a micropipette with a sterile disposable tip. Mice were examined 24 hours later to verify infection. Controls consisted of scratched and unscratched mice that were uninfected.

At selected time points after infection, mice were killed and corneas were excised. Individual samples consisted of 12 pooled corneas per time point for RT-PCR and immunoblot analysis. Single corneas from different time points were used for immunohistochemical staining and histologic study. Immediately after isolation, corneas were rinsed in sterile saline and then processed for the purpose of the different assays. Control mice were treated similarly.

Semiquantitative RT-PCR

Total RNA was extracted from the harvested corneas with extraction reagent (TRIzol; Gibco, Grand Island, NY) according to the manufacturer's instruction. The total RNA was dissolved in water treated with diethylpyrocarbonate (DEPC), and the concentration was measured by a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan). The samples were treated with DNase I (0.2 U/μl; Ambion, Austin, TX) to remove possible DNA contamination. All the reagents needed for RT-PCR were purchased from Perkin Elmer (Norwalk, CT) except *Taq* DNA polymerase and the dNTP mixture, which were purchased from Gibco.

RT-PCR was performed as previously described.[1] Briefly, RT was performed in 0.65-ml RNase-free tubes under optimized conditions in a DNA thermal cycler (model 480; Perkin Elmer). Equal amounts of total RNA (500 ng) from each sample were used for this reaction. The whole product of RT in each tube was amplified by PCR. Cycle parameters were generally a 1-minute melting step at 95°C, a 1-minute annealing step at 55°C, and a 2-minute extension step at 72°C. Thirty cycles were selected for amplification of all target genes, based on results of the experiments that tested the linear range of amplification with different cycles. The following specific primers for MT4-, MT5-, and MT6-MMP were designed and prepared according to the available information for these specific genes (GenBank, provided in the public domain by the National Center for Biotechnology information, Bethesda, MD, and available at http://www.ncbi.nlm.nih.gov/genbank). Because the mRNA sequence for mouse MT6-MMP has not been reported, the primers for human MT6-MMP were prepared and used, assuming there is high homology between human and mouse MT6-MMP. The primers of mouse MT4-MMP (5′-TGG TCT GAT GGT GCA TCC TA-3′ and 5′-TGC AGG AAC ATA CTG CCA GA-3′) amplified a 205-bp product (gene sequence 1550-1754); the primers of mouse MT5-MMP (5′-GTG ACA GCT CCC CAT TTG AT-3′ and 5′-TAG AGT GCT CCA AGC CCA GT-3′) amplified a 192-bp product (682-873); and the primers of human MT6-MMP (5′-ATG GCC TGC AGC AAC TCT AT-3′ and 5′-AGG GGC CTT TGA AGA AGA AA-3′) amplified a ~200-bp product. There were two negative controls: one without reverse transcriptase and the other one without specific primers. The housekeeping gene 18S RNA (Ambion) was also amplified and used as an internal control for the comparison of all time point target genes. Finally, the amplified genes were resolved by 1% agarose gels and revealed by ethidium bromide staining. RT-PCR for each MT-MMP was repeated at least twice.

To confirm the specificity of the primers, the amplified DNA sequences were subjected to restriction enzyme digestion. The 205-bp PCR product was treated with *Sma*I (Gibco), which recognizes the sequence CCC∧GGG. The 192-bp product was treated with *Mbo*I (Gibco), which recognizes the sequence ∧GATC. Each of them was incubated with respective enzyme (5 U) for 90 minutes, with different reaction buffer and at different temperature, according to the manufacturer's instruction. The results were revealed by 3% agarose gel electrophoresis.

The ~200-bp PCR product was purified with a kit (MinElute; Qiagen, Valencia, CA) according to the manufacturer's instruction. The concentration of the purified DNA was measured by the spectrophotometer at 260 nm. The purified DNA at a concentration of 10 ng/μl and human MT6-MMP primers at a concentration of 1 pM/μl were then prepared. The DNA samples were analyzed in the DNA Sequencing Facility at the Center for Molecular Medicine and Genetics (Wayne State University, Detroit, MI).

Immunoblot Analysis

Cornea samples were immediately frozen with liquid nitrogen after isolation from mice. To enrich the extracts with membrane-bound proteins, TBS-CM buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM $CaCl_2$, and 1.5% Triton X-114) was used. Samples were homogenized with TBS-CM buffer on ice and then centrifuged at 12,000 rpm at 4°C for 2 minutes. The supernatants were collected and incubated at 37°C for 3 minutes, followed by centrifugation at 12,000 rpm for 2 minutes. The detergent phase, which contained enriched membrane-bound proteins, was saved for immunoblot analysis. The concentrations of the total protein were measured with a bicinchoninic acid (BCA) protein assay. Equal amounts of individual samples (5 μg) were mixed with 5 μl of 4× sample loading buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 40% glycerol, and 0.02% bromphenol blue) containing β-mercaptoethanol and boiled for 5 minutes. The samples and a prestained molecular weight marker (Bio-Rad, Cambridge, MA) were electrophoresed on 12% SDS gels and subsequently transferred to nitrocellulose membranes. The membranes were blocked for 30 minutes in blocking reagent (Blotto; Santa Cruz Biotechnology, Santa Cruz, CA; TBS, containing 0.5% Tween 20, 3% nonfat milk, and 2% bovine serum albumin) and then incubated with a polyclonal anti-human MT4-MMP (AB854,1 μg/ml; Chemicon, Temecula, CA) and a polyclonal anti-human MT5-MMP antibody (AB924, 0.2 μg/ml; R&D, Minneapolis, MN), respectively, on a rocker at room temperature for 2 hours. The species reactivity for MT5-MMP is positive to both human and mouse according to the information from R&D. In addition, alignment of mouse MT4- and MT5-MMP protein sequences with that of human revealed 82.0% and 92.6% of homology, respectively. Samples treated with nonimmune (normal) rabbit IgG or without primary antibody treatment were processed as the negative control. Afterward, the blot was incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (0.5 μg/ml; Roche Molecular Biochemicals, Indianapolis, IN) at room temperature for 1 hour. Finally, the blot was developed by a chemiluminescence kit (Amersham, Arlington Heights, IL), and MT4- and MT5-MMP were visualized as dark bands with corresponding molecular weights. Immunoblot analysis of each MT-MMP was repeated at least twice.

Immunohistochemical Staining

The corneas, harvested on days 0 (uninfected) and 6 after infection (peak inflammatory response), were fixed with 10% buffered formalin overnight, dehydrated with increased concentrations of ethanol and 100% xylene, infiltrated with paraffin overnight, and embedded in fresh paraffin. The tissues were sectioned (4 μm) to prepare slides, and the slides were deparaffinized, rehydrated, and treated with proteinase K (Sigma, St. Louis, MO). Normal horse serum and 3% hydrogen peroxide were applied separately to reduce nonspecific staining and to

remove endogenous peroxidase. The slides were treated with specific primary antibodies recognizing mouse MT4- and MT5-MMP (the same as used for immunoblot analysis) in a humidified chamber at room temperature for 2 hours, followed by subsequent treatment with a biotinylated anti-rabbit secondary antibody and the ABC reagents (Vectastain; Vector Laboratories, Burlingame, CA). Positive staining was exhibited by diaminobenzidine (DAB; Vector) treatment appearing as brown granules.

Results

Expression of MT4-, MT5-, and MT6-MMP in Mouse Cornea

Semiquantitative RT-PCR was performed to determine whether normal mouse corneas contain mRNA for MT4-, MT5-, and MT6-MMP and whether the MT-MMP expression is modulated by corneal infection. The corneas were harvested on days 0 (control), 1 through 5, and 8 after infection and total RNA was prepared. As shown in Figure 1, the gene expression for MT4- and MT5-MMP was found in normal (uninfected) mouse corneas, whereas MT6-MMP was not detectable by human MT6-MMP primers in uninfected corneas. When mice were infected with *P. aeruginosa*, the MT4-MMP mRNA expression in the cornea remained the same on the first 4 days and was then induced on day 5, remaining high through day 8. The MT5-MMP mRNA expression in the cornea infected with *P. aeruginosa* did not change for the first few days and then showed slight reduction until day 8, when significant induction of the MT5-MMP mRNA expression was observed. In contrast, RT-PCR results showed a ~200-bp mRNA segment that was amplified by human MT6-MMP primers and peaked at day 4. DNA sequencing and alignment with human MT6-MMP revealed 46.6% of gene homology of this DNA segment when compared with human MT6-MMP (data not shown). In addition, there was no significant similarity between the amplified DNA and other known genes.

To verify the amplified MT-MMPs, the amplified DNA sequences were subjected to restriction enzyme digestion. The amplified MT4-MMP product was cleaved by *Sma*I at the site between base pairs 78 and 79, which generated two smaller pieces (78-bp and 127-bp bands). The amplified MT5-MMP product was hydrolyzed by *Mbo*I at the site between bp 64 and 65, which generated two bands of 64 bp and 128 bp (data not shown).

There was no difference in any MT-MMP mRNA expression between the scratched corneas and the unscratched corneas on day 0, indicating that corneal abrasion did not affect the mRNA expression (data not shown). In addition, negative control samples (those not treated with reverse transcriptase or amplified without specific primers) did not exhibit any MT-MMP expression (data not shown).

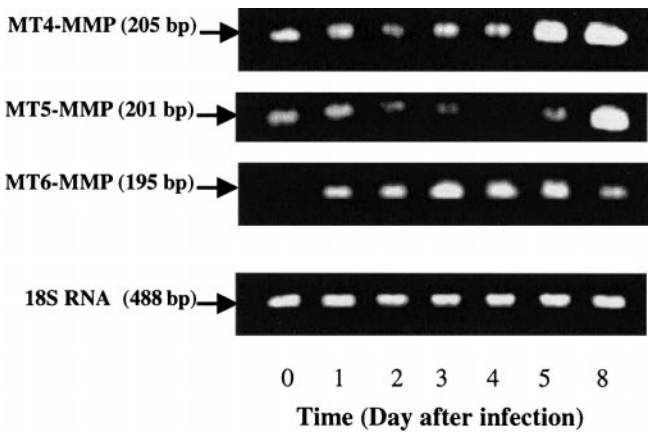

Figure 1. mRNA expression of MT4-, MT5-, and MT6-MMP in mouse corneas infected with *P. aeruginosa*.

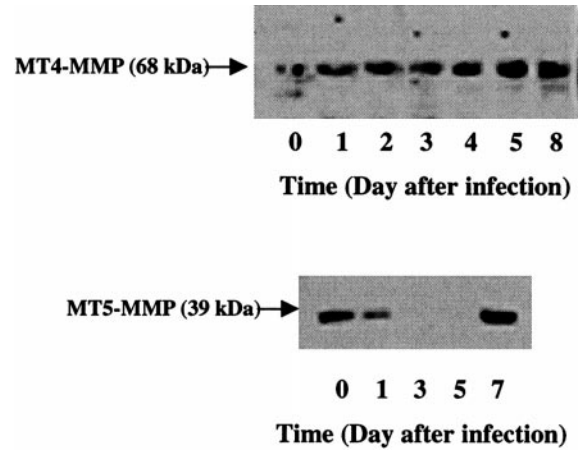

Figure 2. Protein expression of MT4- and MT5-MMP in mouse corneas infected with P. aeruginosa.

To confirm MT-MMP expression at the protein level and to compare MT-MMP expression in the corneas at different time points during the infection, immunoblot analysis of corneal extracts was performed. As shown in Figure 2, MT4-MMP with a molecular weight of ~68 kDa was detected in the uninfected mouse corneas (day 0 sample), which had a molecular weight similar to human MT4-MMP identified in brain extracts.[13] In corneas infected with *P. aeruginosa*, the expression of MT4-MMP was gradually induced and remained at a high level on day 8. MT5-MMP expression in corneas was different from that of MT4-MMP. MT5-MMP with a molecular weight of ~39 kDa, probably the soluble species, was found in normal corneas.[5] The expression was reduced from day 1 through day 5 after infection. There was a significant induction of MT5-MMP expression on day 7 after infection. These results were consistent with the gene expression of MT5-MMP during the infection.

MT-MMP expression at the protein level in the scratched corneas was comparable to that of the unscratched corneas on day 0. No signals were detected in any negative control samples (data not shown).

Localization of MT4- and MT5-MMPs in Mouse Corneas

To localize MT-MMP expression in the mouse corneas, immunohistochemical staining was performed using the corneal samples of normal and infected mice. As shown in Figure 3, no

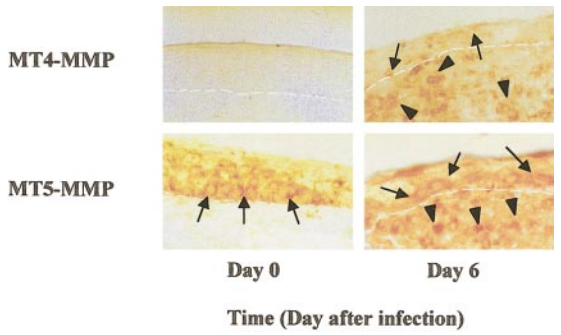

Figure 3. Localization (*arrows*) of MT4- and MT5-MMP expression in mouse corneas infected with P. aeruginosa.

positive staining of MT4-MMP was found in the normal cornea, although immunoblot analysis showed a weak band of MT4-MMP on day 0 (normal cornea), perhaps because there is much less sensitivity in immunohistochemical staining than in immunoblot analysis. On day 6, MT4-MMP expression was localized in both epithelial tissue (weak positive staining) and substantia propria (strong positive staining). MT5-MMP expression was observed in corneas of normal mice, mainly localized in the epithelium. On the sixth day after infection, the distribution of MT5-MMP was not restricted to the epithelium but was also detected in the substantia propria.

Discussion

Corneal damage caused by *P. aeruginosa* infection starts with inflammatory reactions such as edema, leukocyte infiltration, and angiogenesis. With continuation of the inflammation, corneal ulceration may occur, and corneal perforation may eventually take place because of the stromal dissolution.[16–18] One of the possible mechanisms responsible for corneal destruction is that both host and bacterial proteases contribute to the degradation of Bowman's membrane and the ECM, which leads to severe corneal damage. Evidence for this hypothesis is based on the observations that purified proteases are able to cleave ECM proteins and that bacterial proteases can cause keratitis by activating host proteases.[19,20] In addition, the two gelatinases MMP-2 and -9 have been identified in corneal tissues and shown to play a role in corneal ulceration and tissue remodeling in a noninfectious disease model.[21–23]

Although a number of studies have begun to reveal the effects of some MMPs on corneal tissues under physiological and pathologic conditions, little is known about the expression and regulation of MT-MMPs in the cornea. Our recent work on MT1-, MT2-, and MT3-MMP showed that these three MT-MMPs are all present in the normal mouse cornea and that there is a significant induction of each of them in the corneas of naïve (unimmunized) mice intracorneally infected with *P. aeruginosa*, whereas the induction in the infected corneas of immunized mice reaches a much lower level and has a shorter duration.[1]

In the present study, we further investigated the expression and regulation of another three recently identified MT-MMPs—MT4-, MT5-, and MT6-MMP, in the mouse corneas infected with *P. aeruginosa*. MT4- and MT5-MMP were identified in the normal (uninfected) mouse cornea at both the mRNA and protein levels, whereas MT6-MMP was not detectable by RT-PCR. Similar to MT1-, MT2-, and MT3-MMP, expression of MT4-, MT5-, and MT6-MMP also showed a significant induction in corneas when mice were intracorneally infected with *P. aeruginosa*. However, the induction pattern was different from that previously observed (MT1-, MT2-, and MT3-MMP). MT4-MMP expression in the infected corneas did not change dramatically until day 5 after infection. It was reported that MT4-MMP was mainly expressed by leukocytes, especially by monocytes and macrophages.[11] Indeed, our data showed strong positive staining of MT4-MMP in the cornea on day 6 after infection. Moreover, our previous histologic study also showed increased macrophage infiltration of the corneas during days 6 and 9 after infection.[1] Therefore, the infiltrating macrophages seemed responsible for the induction of MT4-MMP expression in the cornea during the late stage of the inflammation. Although MT5-MMP was primarily identified in human brain tumor,[24] basal expression of MT5-MMP was also found in our corneal studies, primarily localized in the epithelial tissue.

Downregulation of MT5-MMP during the early stage of the infection (days 2–4) possibly resulted from injury of epithelial cells, because they seem to be the only source of MT5-MMP in the normal cornea, according to our studies of immunohistochemical staining. Later induction of MT5-MMP was probably also due to the infiltration of macrophages into the infected cornea. MT6-MMP, also called leukolysin,[12] was not detected in the normal cornea by RT-PCR, but with infection, there was a significant induction of MT6-MMP expression in corneas. It was reported that MT6-MMP was predominantly expressed in leukocytes.[6,12] Our data regarding MT6-MMP induction correlate well with our previous findings on corneal inflammation as quantified by bacterial numbers, PMNs measured by myeloperoxidase, arachidonic acid metabolites, and cytokines.[25–27] In addition, previous histologic studies showed that PMNs peak at day 6 after infection and then diminish gradually, which has good correlation with the MT6-MMP induction pattern.[1] Thus, induced expression of MT6-MMP in the cornea may be produced by PMNs.

As members of the MMP family, MT4- and MT5-MMP hydrolyze a variety of ECM components, such as fibronectin, gelatin, fibrinogen, fibrin, and proteoglycans.[11,28] The induced enzymatic activity during the inflammation may cause degradation of the Bowman membranes and ECM components, leading to severe corneal damage. Similar to other MT-MMPs, MT4-, MT5-, and MT6-MMP are also capable of activating pro-MMP-2.[5,6,29] Therefore, the induction of these MT-MMPs in the mouse corneas infected with *P. aeruginosa* may also be responsible for corneal damage caused by other proteases that are activated by these MT-MMPs.

Acknowledgments

The authors thank Julianna M. Berk for technical assistance.

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