Regulation of MMPs and TIMPs by IL-1β during Corneal Ulceration and Infection

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Purpose. This study was conducted to investigate the role of IL-1β in the regulation of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in a mouse model of experimental keratitis and corneal injury.

Methods. Mice were injected subconjunctivally with 10 μg of anti-mouse IL-1β antibody 2 hours before challenge with *Pseudomonas aeruginosa* (strain 6294). Control animals received an equal volume and concentration of isotype control antibody at the same time. Eyes were enucleated at 0, 8, 24, and 72 hours, after bacterial challenge and processed for histologic examination. Some eyes were homogenized and used to evaluate production of MMP-2, MMP-9, TIMP-1, and TIMP-2 protein, by zymography and reverse zymography.

Results. Injury without bacterial infection resulted in increases in both MMP-2 and -9 and a slight but significant downregulation of TIMP-1. Administration of anti-IL-1β just before injury and without bacterial infection resulted in a significant reduction in expression of MMP-2 (at 8 hours), MMP-9 (at 8 hours), TIMP-1 (at 8 and 72 hours), and TIMP-2 (at 8 hours). Mice treated with anti-IL-1β antibody, before bacterial challenge, demonstrated markedly reduced corneal damage compared with the severe corneal injury and massive neutrophil infiltration observed in infected mice treated with control antibody. Administration of the neutralizing anti-IL-1β antibody resulted in a significant reduction of MMP-9 and a change in the time course of TIMP-1 and -2 expression. The reduction in MMP-9 by anti-IL-1β during infection was much greater than the reduction without infection.

Conclusions. The results imply that IL-1β has a central role in corneal destruction during bacterial keratitis and suggests that targeting IL-1β may be a novel therapeutic strategy for microbial keratitis. (*Invest Ophthalmol Vis Sci.* 2003;44:2020–2025) DOI:10.1167/iovs.02-0565

The bacterium *Pseudomonas aeruginosa* can cause ulcerative microbial keratitis characterized by rapid corneal degradation.[1] Upregulation of proinflammatory cytokine expression and leukocytic infiltration at the site of infection are major host responses during corneal inflammation. Elevated levels of IL-1β have been observed during *P. aeruginosa*-induced infection of the cornea,[2–4] but relatively little is known about the specific role played by IL-1β in regulating corneal degradation during bacterial keratitis.[3–6]

The presence of IL-1β protein has also been correlated with the severity of various models of ocular inflammation, including herpes simplex virus–induced corneal infection[7] and corneal allograft rejection.[8] IL-1β is a potent proinflammatory cytokine and is involved in both the initiation and effector phases of inflammatory and immune responses, including upregulation and activation of vascular adhesion molecules,[9,10] such as intercellular adhesion molecule (ICAM)-1 and activation of the phagocytic and killing capacity of inflammatory cells. In addition, IL-1β induces expression of IL-8 and GROα potent neutrophil-attracting chemokines in many cell types to recruit polymorphonuclear leukocytes (PMNs) into the cornea.[11,12] In addition to facilitating bacterial clearance, PMNs are thought to play a role in tissue damage through activation and secretion of matrix metalloproteinases (MMPs).[13] Enhanced production and local release of IL-1β by infiltrating leukocytes during ocular infection with *P. aeruginosa* may amplify PMN recruitment. Our group[3] and others[4] have recently shown that neutralization of IL-1β activity can reduce the severity of corneal disease induced by *P. aeruginosa*.

MMPs are zinc-binding, proteolytic enzymes that act collectively to degrade or modify most components of the extracellular matrix (ECM).[14] MMPs are classified into four major groups according to their substrate specificity.[15] Collagenases (MMP-1, -8, and -13) are responsible for cleavage of interstitial type I, II, and III collagens. Stromelysins (MMP-3, -7, -10, and -11) have a broad substrate specificity and are active in degrading a variety of ECM components. Gelatinases (MMP-2 and -9) are known as type IV collagenases and are involved in cleaving collagen types IV, V, VII, and X, as well as fibronectin, laminin, and elastin. MMP activity is regulated at multiple levels, including gene transcription, proenzyme activation, and inhibition by tissue inhibitors of matrix metalloproteinases (TIMPs).[16,17] The TIMPs can be produced by the same cells that express MMPs. Although TIMPs are multifunctional proteins, they are principally involved in the inhibition of MMP activity.[18] Four species of inhibitors have been identified in humans, including TIMP-1, -2, -3 and -4. TIMP-1 shows high affinity for MMP-9 and TIMP-2 for MMP-2.[18] MMPs participate in physiological and pathologic processes, including wound healing, angiogenesis, embryo implantation, rheumatoid arthritis, and neoplastic and ocular diseases.[19–23] MMP-9 is involved in early stages of corneal epithelial wound healing, and MMP-2 may be important in remodeling of ECM in the later stages of corneal ulceration.[24,25] Kernacki et al.[26] showed that MMP-9 expression is upregulated and that the increased production of TIMP-1, a natural antagonist of MMP-9, is associated with protection against *P. aeruginosa*–induced corneal ulceration in mice.

Because the mechanisms of host-mediated corneal destruction in bacterial keratitis are not clear, we propose that IL-1β induces the expression of MMPs, leading to an imbalance in the

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level of MMPs and TIMPs, thereby causing destruction of corneal connective tissue. In the present study, a murine model of keratitis and corneal injury was used to investigate the role of IL-1β in the regulation of MMPs and TIMPs during corneal ulceration and infection.

Methods and Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

Preparation of Bacteria

P. aeruginosa 6294, an invasive bacterial strain, isolated from a human corneal ulcer was used in all experiments. The inoculum was prepared by incubating bacterial cells in 10 mL of tryptone soya broth (TSB; Oxoid, Ltd., Sydney, Australia) at 35°C for 18 hours. Cells were centrifuged at 3000 rpm for 10 minutes and washed three times with phosphate-buffered saline (PBS; pH 7.4), and the bacteria were resuspended in PBS at a concentration of 1×10^9 colony-forming units (CFU)/mL (optical density at 660 nm [OD_{660}] = 1.0).

Infection of Mice

Eight- to 10-week-old inbred BALB/c mice (at least 5/group) were used. Mice were anesthetized with tribromoethanol (125 mg/kg, intraperitoneally) and given 0.01 mg/kg buprenorphine subcutaneously. The central corneal surface of the left eye was incised (two parallel incisions approximately 2 to 3 mm long and penetrating only the epithelium) with a sterile 27-gauge needle under a stereomicroscope. For infection, 5 μL of bacterial suspension (5×10^6 CFU/mL) was applied topically to the wounded cornea. Some corneas were scarified but not inoculated with *P. aeruginosa* and instead served as the injured but uninfected control. Three independent sets of experiments were performed. Mice were monitored during each experiment, and all protocols for animal use were approved by the Animal Care and Ethics Committee, University of New South Wales, Sydney, Australia. The research conformed to the guidelines embodied in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Administration of Anti-mouse-IL-1β Antibody

Anesthetized animals were injected with 10 μL (2 μg/μL) goat anti mouse-IL-1β antibody (R&D Systems, Minneapolis, MN) subconjunctivally 2 hours before corneal infection. Control mice received an equal volume and concentration of control goat IgG antibody (R&D Systems). Both the anti mouse-IL-1β antibody and the control antibody had less than 10 ng endotoxin/mg of antibody. These neutralization experiments were repeated three times.

Administration of Anti-MMP9 Antibody

Anesthetized animals were injected with 25 μg of mouse anti-MMP-9 antibody (Chemicon International, Temecula, CA) subconjunctivally 7 hours after corneal infection. Control mice received an equal volume and concentration of control antibody (R&D Systems). These neutralization experiments were repeated two times.

Collection of Corneas for Histologic Examination

Eyes were enucleated at 8, 24, and 72 hours after bacterial challenge. Eyes were immersed in cold 2.5% (vol/vol) glutaraldehyde and fixed at 4°C for 2 days. Fixed tissues were dehydrated in serial ethanol solutions: 30%, 50%, 70%, and 95% (vol/vol) for 15 minutes each, embedded in resin (Leica, Heidelberg, Germany), and kept at 4°C overnight. Tissue was sectioned (1 μm), stained with 0.1% (vol/vol) toluene blue for 7 minutes, mounted, and observed with a light microscope. At least two eyes were examined at each time point by a masked observer.

SDS-PAGE Gelatin Zymography and Reverse Zymography

MMPs were separated on the basis of molecular weight by electrophoresis through a sodium dodecyl sulfate (SDS)-polyacrylamide gel copolymerized with gelatin. The gelatinases were visualized as lytic bands in the gelatin substrate. Both the latent and active forms of MMPs, denatured by the SDS in the gel during electrophoresis, when renatured (after removal of SDS from the gel) in an active form, can be visualized by zymography. At 0 and 8 hours and 1 day and 3 days after infection, mice were killed and eyes were removed and homogenized in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% [wt/vol] Nonidet P-40, and 0.1% [wt/vol] SDS). Samples were incubated with proteinase inhibitors (2 μg/mL leupeptin, 2 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) for 30 minutes on ice. The homogenate was centrifuged at 14,000*g* for 10 minutes at 4°C. Protein concentrations in the supernatants were measured using the Bradford assay (Bio-Rad, Sydney, New South Wales, Australia). Approximately 10 μg of each sample was loaded per lane into the wells of a 10% (wt/vol) polyacrylamide gel containing 0.1% (wt/vol) gelatin. Electrophoresis was performed in Tris-glycine running buffer at 125 V constant voltage for 1.5 to 2 hours. The gels were incubated in 100 mL of substrate buffer (50 mM Tris base, 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 0.2% Brij35) at 37°C for 14 to 18 hours. Gels were subsequently stained in 100 mL of 0.5% (wt/vol) Coomassie blue (G-25; Bio-Rad) in 30% (vol/vol) methanol and 10% (vol/vol) acetic acid for 1 hour and destained. Semiquantitative data were generated by scanning each gel on a densitometer (Bio-Rad) with multianalyst software (Bio-Rad) and comparing the density to that from a standard curve of MMP-9 and -2. Each time gels were run, 10 ng of MMP-9 was run on the gel to standardize the gels between runs. Reverse zymography was performed as described previously.[26,27] Briefly, 10% polyacrylamide gels were impregnated with both 0.1% gelatin and recombinant mouse-gelatinase B (1.6 μg/gel; R&D Systems). Approximately 10 μg of each sample was loaded per lane into the wells of each gel, electrophoresed, and semiquantified as just described, using TIMP-2 as a standard. Each time gels were run, 10 ng TIMP-2 was run on the gel to standardize the gels between runs.

Statistical Analysis

Results were analyzed by Student's *t*-test and ANOVA to assess the differences in MMP and TIMP protein expression in anti-IL-1β antibody–treated versus control-treated corneas.

Results

Histopathological Changes after Injury or *P. aeruginosa* Infection

Histopathological examination of injured, but uninfected mouse corneas at 8 hours after injury showed mild leukocytic infiltrates and partial healing of the scarified sites. At 24 hours after injury, the corneal epithelium had healed, but some infiltrating leukocytes were still present (Fig. 1A). By 72 hours, the cornea appeared normal.

Corneas of mice that received control antibody before challenge with *P. aeruginosa* showed a prominent PMN infiltration extending from the limbus to the central cornea at 8 hours after challenge. Bacteria were present in the anterior corneal stroma (data not shown). At 24 hours after challenge, a massive recruitment of PMNs into the central stroma was evident. By this time point, bacteria were evident throughout the stroma, with a complete stromal denudation of the epithelium in the central cornea. The whole corneal stroma was markedly edematous, and a moderate anterior chamber response was evident (Fig. 1B). By 72 hours, the corneal histology remained essentially the same as at 24 hours, with numerous PMNs still evident in the corneal stroma and anterior chamber, although

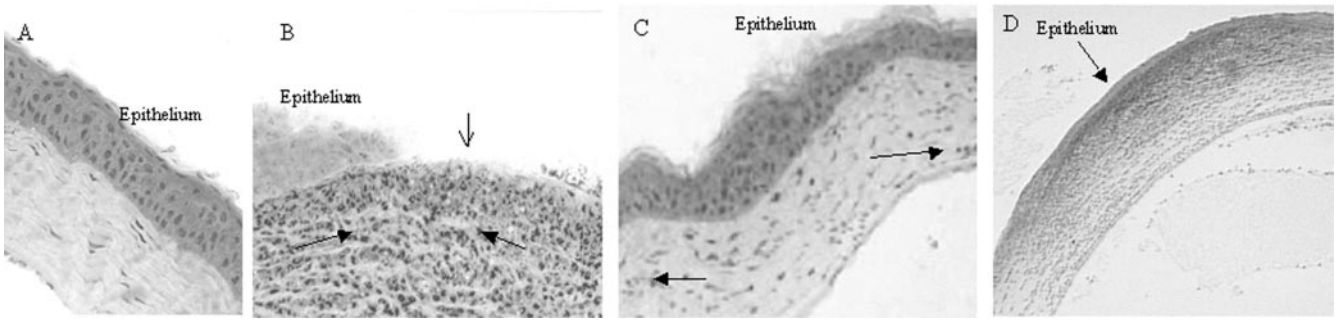

FIGURE 1. Histologic examination of mouse eyes injured, or injured and challenged with *P. aeruginosa* 6294 treated with anti-IL-1β or control antibody at 24 hours after challenge. (**A**) Eyes injured but not infected showed healing of the epithelium with no evidence of PMN infiltration at 24 hours. (**B**) Control antibody–treated eyes infected with the invasive bacterial strain (6294) showed massive PMN infiltration (*horizontal arrows*) at 24 hours after challenge. The epithelium in the central cornea was completely destroyed (*vertical arrow*). (**C**) Eyes treated with anti-IL-1β antibody before bacterial challenge showed fewer infiltrates in the corneal stroma (*arrows*) at 24 hours after challenge, and no corneal epithelial defect was observed. (**D**). Eyes treated with anti-MMP9 7 hours after challenge showed reepithelialization (*arrow*) over the entire central cornea. There was extensive infiltration into the central cornea. Magnification: (**A–C**) ×250; (**D**) ×100.

the epithelium had partially healed at the periphery of the defect (data not shown). By contrast, eyes that had been treated anti-IL-1β antibody had substantially reduced cellular infiltration at 24 hours (Fig. 1C) after challenge, when compared with eyes treated with control antibody. Slight edema was present in the corneas of these animals, but no anterior chamber reaction or epithelial defect was observed. At 72 hours, the corneas of the animals treated with anti-IL-1β antibody appeared normal with only few PMNs in the corneal stroma (data not shown).

Effect of IL-1β on MMP and TIMP Production in Mouse Corneas

Uninfected Injured Corneas. Corneal injury alone significantly induced MMP-9 ($P < 0.03$) and -2 ($P < 0.04$) at 8 hours after injury (Figs. 2A, 2B) when compared with normal corneas examined by zymography. Prior administration of anti-IL-1β antibody significantly reduced the induction of MMP-9 ($P < 0.05$) and -2 ($P < 0.04$) at 8 hours. Injury had no significant effect on the induction of TIMP-1 or -2 (Figs. 2C, 2D). Prior treatment with anti-IL-1β slightly but significantly ($P < 0.05$) reduced the induction of TIMP-1 at 8 and 72 hours and TIMP-2 at 8 hours only.

Infected Corneas. Corneal infection with *P. aeruginosa* reduced the level of MMP-2 protein significantly ($P < 0.03$) at 8 hours compared with injured but uninfected corneas (compare Figs. 3A, 2A). Infection prolonged the induction of MMP-9, and MMP-9 was significantly increased at 72 hours after infection compared with injured but uninfected corneas ($P < 0.01$; compare Figs. 3B, 2B). Infection caused a significant

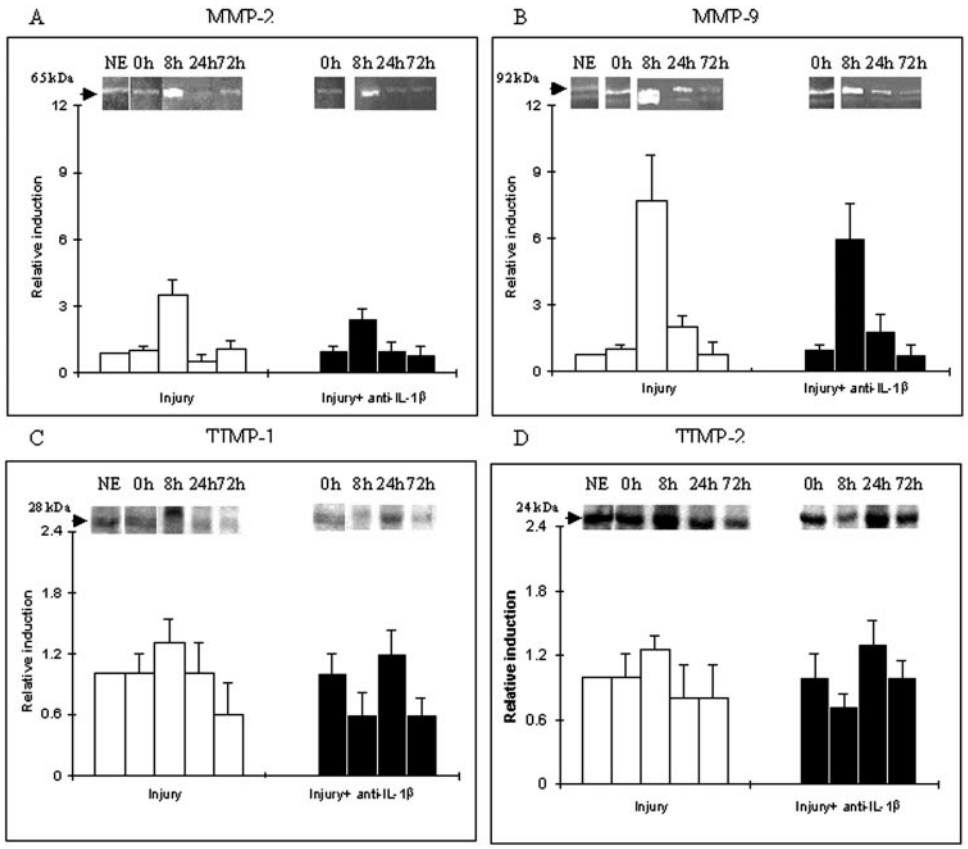

FIGURE 2. Relative intensities of (**A**) MMP-2, (**B**) MMP-9, (**C**) TIMP-1, and (**D**) TIMP-2 proteins analyzed by zymography or reverse zymography in control antibody– or anti-IL-1β antibody–treated injured (scratched), but uninfected, corneas at 0, 8 , 24, and 72 hours after injury. Representative zymograms and reverse zymograms of ocular homogenates of injured mouse eyes are also depicted. NE, normal eye (unscratched and uninfected).

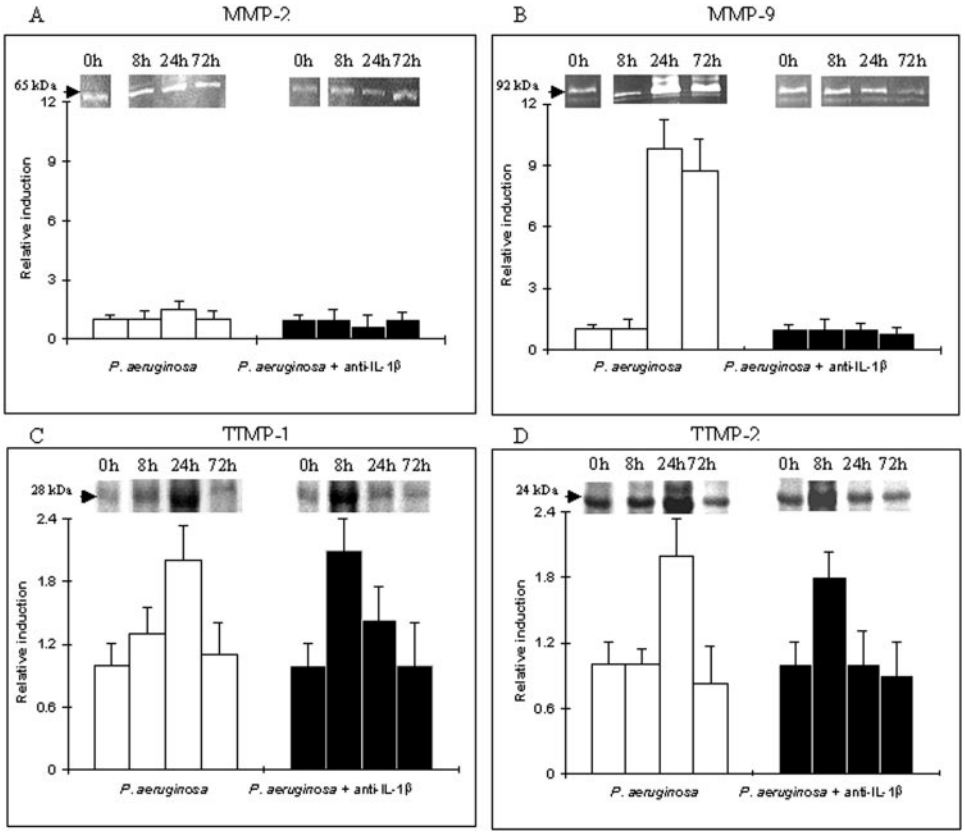

FIGURE 3. Relative intensities of of (**A**) MMP-2, (**B**) MMP-9, (**C**) TIMP-1, and (**D**) TIMP-2 analyzed by zymography or reverse zymography in control antibody– or anti-IL-1β antibody–treated corneas infected with *P. aeruginosa* at 0, 8, 24, and 72 hours after infection. Representative zymograms and reverse zymograms of ocular homogenates of injured mouse eyes are also depicted.

induction of TIMP-1 ($P < 0.05$; Fig. 3C) and TIMP-2 at 24 hours ($P < 0.02$; Fig. 3D). Application of IL-1β antibody significantly reduced the induction of MMP-9 protein at 24 ($P < 0.002$) and 72 hours ($P < 0.01$) after infection (Fig. 3B). MMP-2 was not altered by anti-IL-1β (Fig. 3A). Anti-IL-1β altered the time course of induction of TIMP-1 and -2 during infection, with these TIMPs being maximally induced earlier, 8 hours versus 24 hours (Figs. 3C, 3D).

Effect of Anti-MMP-9 in Infected Mouse Corneas

Mouse corneas treated with anti-MMP-9 showed reepithelialization or progression to reepithelialization 24 hours after challenge with *P. aeruginosa* 6294 (Fig. 1D). A magnification of 100× was used to show both central and peripheral regions of the corneal epithelium. In contrast, control animals (Fig. 1B) showed extensive epithelial defects at this time. However, anti-MMP-9 corneas had extensive infiltration with predominantly neutrophils at levels comparable to those in control animals.

DISCUSSION

In this study, MMPs were upregulated after injury or infection of the cornea, and administration of anti-IL-1β antibody reduced the expression of MMP proteins, altered the temporal sequence of TIMP protein expression, and abrogated corneal damage. The presence of IL-1β has been correlated with the severity of ocular inflammation.[4,8] The *P. aeruginosa* strain 6294 caused extensive ulceration in conjunction with induction of high levels of IL-1β.[3] Such dramatically elevated levels of IL-1β appear to represent an uncontrolled and adverse inflammatory response in this model.[3,4,8] IL-1β has been shown to induce the expression of MMPs in cell culture systems,[28] including corneal epithelial cells[29] and fibroblasts isolated from corneal stroma[30] and conjunctiva.[31] MMPs have also been implicated in tissue degradation associated with inflammatory diseases of the eye, including uveitis, scleritis, and pterygium.[32–34]

In the present study, mice treated with the anti-IL-1β antibody, before bacterial challenge, showed significantly reduced MMP-9 protein along with the reduced severity of corneal damage when compared with mice treated with control antibody. This suggests that MMP-9 may contribute to stromal degradation after bacterial infection and inflammation. This was confirmed using anti-MMP-9 antibodies that showed increased healing of the epithelial defect. This finding that MMP-9 is likely to be regulated by IL-1β is supported by Li et al.[35] who demonstrated that IL-1β had a stimulatory effect on expression of MMP-9 in cultured corneal epithelial cells. Similarly, a *Pseudomonas* keratitis study demonstrated increased activation of MMP-9 and the possible role of bacterial proteases and/or lipopolysaccharide in this induction.[36] Other studies have shown that increased levels of activated MMP-9 may play a role in remodeling of new matrix components and regeneration of corneal tissue early during wound healing.[37,38] The greater effect of anti-IL-1β in the infection model compared with injury alone may indicate that regulation of gene expression of MMP-9 was different in these two situations. MMP-9 has been shown to possess motifs homologous to the binding sites for the transcription factors activator protein-1, nuclear factor-κB, and SP-1.[39] It is possible that these transcription factors respond differently to injury than to infection.

MMP-2 protein was present only in low levels early after infection or injury, when compared with levels of MMP-9 protein. However, there was a significant increase in MMP-2 after injury, and levels were reduced significantly in anti-IL-1β antibody–treated mice compared with control antibody–treated animals. The small reduction in MMP-2 after anti-IL-1β

administration in the injury model is consistent with other studies that have shown that MMP-2 appears to be constitutive in the cornea.[24] It has also been suggested that MMP-2 may appear later during the wound-healing process.[25]

The time course for induction and suppression of TIMP-1 and -2 was altered in anti-IL-1β antibody-treated corneas. These antiproteases play an important role in tissue repair and wound healing.[38,40–46] There is evidence that in ulcerative corneal disease, alteration of the ratio of MMPs to TIMPs plays a role in progressive stromal degradation.[27] In this murine model of keratitis, it was interesting that infection with *P. aeruginosa* induced a significant (fivefold) induction (at 24 hours), in MMP-9, whereas there was only a twofold induction of TIMP-1 or -2. Administration of anti-IL-1β antibody before infection showed a significant decrease in MMP levels and a significant change in the time course of TIMP induction. This reduction in MMP-9 may contribute to reduced corneal damage (as was indicated in the anti-MMP-9 experiments).

Corneal ulceration after *P. aeruginosa* infection has been associated with the prominent infiltration of PMNs in various corneal ulcerative models.[1,3,4,30] These studies have shown that areas where the corneal stromal matrix is undergoing remodeling are also the areas of greatest neutrophil accumulation.[31] In the present study, histologic examination showed fewer infiltrating cells in IL-1β–treated mice compared with control antibody–treated mice. One component of the reduced MMP-9 protein in anti-IL-1β–treated mouse corneas (infection model) may therefore relate to the reduction in infiltrating PMNs, because these cells are known to be a potential source of MMPs. It is likely that IL-1β upregulates other mediators, such as nitric oxide (NO) that may regulate MMP-9. Murrel et al.[47] for example, reported that NO induced an increase in MMP-9.

This study also demonstrated enhanced wound healing in the absence of infection, by administration of anti-IL-1β before the cornea was wounded. This effect was probably the consequence of the reduction in both MMP-9 and -2. Schultz et al.[48] have shown that the absence of IL-1β (in gene-knockout mice) or application of an IL-1 receptor antagonist reduces the inflammatory response during *Pseudomonas* pneumonia in mice. In addition, the number of bacteria in the IL-1β– knockout mice was lower. This may indicate that the regulation of MMP-9 by IL-1β prevents effective colonization of bacteria. Collectively, the results of the present study provide new insight into the role of IL-1β and MMPs-TIMPs in the pathogenesis of ulcerative keratitis. Future work will focus on blocking individual MMPs and the use of transgenic animals, because these models may provide detailed information regarding the role of MMPs in the pathogenesis of keratitis. These findings may support novel therapeutic strategies for the treatment of bacterial keratitis, notably to allow prevention of the key complications of corneal ulceration and scarring.

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