

Evaluation of matrix metalloproteinase concentrations in precorneal tear film from dogs with *Pseudomonas aeruginosa*-associated keratitis

Li Wang, DVM, PhD; Qingshan Pan, DVM; Qin Xue, DVM; Jun Cui, DVM; Changming Qi, DVM, PhD

Objective—To evaluate the changes in concentrations of matrix metalloproteinase (MMP)-2 and MMP-9 in the precorneal tear film of dogs with *Pseudomonas aeruginosa*-associated keratitis during corneal healing and stromal remodeling.

Animals—10 dogs with unilateral *P aeruginosa*-associated keratitis and 10 clinically normal dogs.

Procedures—Precorneal tear film samples were collected from both eyes of 10 dogs with unilateral *P aeruginosa*-associated keratitis on the day of admission to the hospital and then at various time points until complete healing of the cornea was achieved. Precorneal tear film samples were also collected from both eyes of 10 clinically normal adult dogs (control group). Concentrations of MMP-2 and MMP-9 in precorneal tear film samples from each group were determined via gelatin zymography for comparison.

Results—The proteolytic processes in the ulcerated eyes decreased as corneal healing progressed. On the day of admission, concentrations of latent and active forms of MMP-2 and MMP-9 in ulcerated eyes were significantly higher than values in the contralateral unaffected eyes in dogs with *P aeruginosa*-associated keratitis; concentrations of latent MMP-2 and MMP-9 were also greater than control group values. Concentrations of latent and active forms of MMP-2 and MMP-9 in the healed eyes of dogs with *P aeruginosa*-associated keratitis were significantly lower than concentrations in the ulcerated eyes on the day of admission.

Conclusions and Clinical Relevance—Results suggest that reduction of precorneal tear film concentrations of MMPs by use of proteinase inhibitors may be effective in the treatment of dogs with *P aeruginosa*-associated keratitis. (*Am J Vet Res* 2008;69:1341–1345)

In various studies, *Pseudomonas* spp were recovered from the conjunctival sac of 1%¹ and 14%² of healthy rabbits and dogs and from the conjunctival sac of 9.4%³ of dogs with external ocular disease. Although the frequency of isolation of *Pseudomonas* spp is low, keratitis associated with *Pseudomonas aeruginosa* infection is often difficult to treat, progresses rapidly, and may lead to extensive corneal perforation and loss of vision.^{4,5} The corneal stroma consists of a few keratocytes and fibrocytes; multiple contiguous layers of thin and uniformly positioned collagen microfibrils surrounded by an extracellular matrix of collagen types I, III, V, VI, and XII; stromal glycosaminoglycans; and glycoproteins.⁶ Proteinases of the precorneal tear film and cornea provide mechanisms for the detection, repair, and removal of damaged corneal epithelial cells, altered corneal stromal collagen, and abnormal components of the extracellular matrix that are caused by normal wear and tear of the cornea.^{7,8} Proteinases are classified

ABBREVIATION

MMP Matrix metalloproteinase

into the following categories: MMPs, serine proteases, aspartic proteinases, and cysteine proteinases.⁹ The MMPs and serine proteinases seem to predominate in the disease processes and wound healing of the cornea in horses and dogs, and MMP-2 (72 kd) and MMP-9 (92 kd) are of major importance in terms of remodeling and degradation of the corneal stromal collagen.^{9,10} The MMPs are excreted as inactive precursors (proMMPs). These latent forms of the MMPs are not biologically active; however, they are of important consideration because they can be activated by various factors and can be involved in physiopathologic processes.¹¹ Once activated, MMPs lose approximately 10 kd in molecular weight and require Ca²⁺ for their stability and Zn²⁺ as a cofactor.^{12,13}

Results of multiple studies^{7,13–16} have indicated that concentrations of proteinases are abnormally high in tears from humans and other animals with so-called melting ulcers. The changes in concentrations of various MMPs in the precorneal tear film as well as expression of MMPs in the cornea during wound healing in rats, dogs, rabbits, and horses have also been

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From the Department of Clinical Veterinary Medicine, College of Veterinary Medicine, China Agriculture University, No. 2 Yuanming Yuan Xi Lu, Beijing 100094, China.

Address correspondence to Dr. Qi.

described.^{7,8,14,17–20} To the authors' knowledge, only 1 study²⁰ has evaluated changes in concentrations of various MMPs in precorneal tear film of dogs, and changes in MMP-2 and MMP-9 concentrations in the precorneal tear film of dogs with *P aeruginosa*-associated keratitis during corneal healing and stromal remodeling have not been reported. The purpose of the study reported here was to evaluate the changes in concentrations of MMP-2 and MMP-9 in the precorneal tear film of dogs with *P aeruginosa*-associated keratitis during corneal healing and stromal remodeling. Based on current knowledge, we hypothesized that precorneal tear film concentrations of MMPs would decrease as the corneal ulcer epithelialized and remodeled.

Materials and Methods

Dogs with ulcerative keratitis—Ten client-owned dogs with unilateral ulcerative keratitis were included in the study. To determine the diagnosis, results of bacteriologic culture of swab specimens from the ulcerated eyes were assessed. Sterile swabs were passed back and forth directly on the corneal ulcer of each affected eye and were then transferred immediately to a laboratory for evaluation. *Pseudomonas aeruginosa* was identified by use of standard biochemical tests. The affected dogs were admitted to the China Agricultural University Veterinary Teaching Hospital (December 2004 through June 2007) and had not previously been treated for this condition. Owners provided informed consent before collection of specimens for research purposes.

Monitoring—In the 10 affected dogs, precorneal tear film MMP-2 and MMP-9 concentrations were assessed serially via quantitative gelatin zymography until complete recovery from *P aeruginosa*-associated keratitis. Corneal ulcers were confirmed by the use of fluorescein stain strips. The progression of corneal disease in the dogs was monitored via daily ophthalmic examination including slit-lamp biomicroscopy and fluorescein staining of the cornea. Complete recovery from ulcerative keratitis was confirmed by negative results for corneal retention of fluorescein dye. After collection of bacteriologic specimens and precorneal tear film samples, treatment of the 10 affected dogs on the day of admission included topical administrations of autoserum, antimicrobials, and tropicamide and oral administration of NSAIDs. Surgical treatment was performed in addition to medical treatment for 8 dogs. The surgical treatments included a conjunctival pedicle flap procedure in 7 dogs and corneal primary closure in 1 dog.

Control dogs—In addition, MMP-2 and MMP-9 concentrations in the precorneal tear film of both eyes of 10 university-owned clinically normal adult Beagles (control group) were assessed. There was no evidence of external ocular disease in any control dog as determined via routine ophthalmic examination (including tonometry, indirect ophthalmoscopy, Schirmer tear testing, and slit-lamp examination), and these dogs were housed in individual runs and were separated from the affected dogs throughout the study.

Collection of precorneal tear film samples—Precorneal tear film samples were collected from both eyes

of the 10 dogs with unilateral *P aeruginosa*-associated keratitis on the day of admission to the hospital and at various time points (depending on response to treatments) until complete healing of the cornea. Precorneal tear film samples from both eyes of the 10 control dogs were also collected prior to performing each electrophoresis. In each dog, tear fluid specimens were collected from the lower fornix of each eye with capillary force by use of 20- μ L glass capillary tubes with an atraumatic tip, as previously described.²¹ Briefly, to avoid trauma to the conjunctiva, the capillary tip was blunted with a flame and carefully checked. The capillary was held gently in place, touching the tear fluid meniscus but avoiding any contact with the eyelid skin. This step was repeated 3 times for each eye with a 5-minute interval between each collection. The total sample volume collected from each eye was 40 to 50 μ L. All samples were immediately transferred into Eppendorf polypropylene microcentrifuge tubes, centrifuged, and stored at -80°C until analysis.

Gelatin zymography—Gelatin zymography and measurement of optical density were used to evaluate MMP-2 and MMP-9 concentrations in tear fluids. Ten microliters of the tear fluid samples was mixed with an equal volume of 2X nonreducing buffer. Fifteen microliters of the mixture was loaded into each well. Prestained molecular weight standards^a and gelatinases zymography standards^b for human active and latent forms of MMP-2 and MMP-9 were also run on each gel. Gels underwent electrophoresis with 125 V at 4°C until the bromophenol blue marker dye reached the bottom of the gel.

Following electrophoresis, SDS was removed from each gel by washing it 3 times (10 minutes' duration each) in 2.5% Triton X-100 solution.^c This allowed the MMPs to renature and digest the surrounding substrate when incubated overnight (approx 12 hours) at 37°C in zymogram incubation buffer.^d After incubation, each gel was stained with a solution of 0.25% Coomassie blue,^e 40% methanol, and 10% acetic acid for 2 hours at room temperature (approx 20°C) and destained with 40% methanol and 10% acetic acid until the bands of lysis became clear.

Image analysis—Concentrations of MMP-2 and MMP-9 were measured via optical density scanning of the gelatin zymograms of the tear fluid samples. The photographs of stained gelatin zymograms were scanned and digitized by use of a scanner,^f and the images were analyzed by use of an image analysis program.^g Bands of proteolytic activity appeared uncolored against a dark blue background. The identities of the putative proteases were determined via analysis of the distance that the bands migrated on the gels versus the distance for migration of molecular weight and protease standards. Higher optical density readings were indicative of lower levels of staining, greater amounts of gelatin substrate digestion, and higher levels of proteolytic activity. Individual proteinase bands within each image were represented as positive waveforms. Measurement of the area under the curve of each waveform provided a numeric score or densitometry reading, which allowed an ac-

curate estimation of proteolytic activity in relative standard units (Figure 1).

Statistical analysis—The densitometry readings obtained for the eyes of clinically normal dogs and for the contralateral unaffected eyes (on the day of hospital admission), contralateral unaffected eyes (on the day of complete healing of the affected eyes), ulcerated eyes (on the day of hospital admission), and healed eyes of dogs with keratitis were compared by use of an ANOVA. The densitometry readings of latent and active forms of MMP-2 and MMP-9 within the eyes of clinically normal dogs and the contralateral unaffected eyes (on the day of hospital admission), contralateral unaffected eyes (on the day of complete healing of the affected eyes), ulcerated eyes (on the day of hospital admission), and healed eyes of dogs with keratitis were compared by use of a *t* test. The statistical analysis was performed by use of computer software^h; significance was set at a value of $P < 0.05$.

Results

Dogs with unilateral *P aeruginosa*-associated keratitis—Ten dogs with unilateral *P aeruginosa*-associated keratitis were included in the study. The age of these dogs ranged from 28 to 108 months. There were 5 females and 5 males. Breeds included 7 Pekingese and 3 mixed. The duration of clinical signs before the first evaluation at the hospital was 1 to 6 days. The dogs received various combinations of medical and surgical treatments. Treatment of these 10 dogs included topical administrations of autoserum, antimicrobials, and tropicamide and oral administration of NSAIDs. Surgical treatment was performed in addition to medical treatment in 8 dogs. The surgical treatments included a conjunctival pedicle flap procedure in 7 dogs and corneal primary closure in 1 dog. The follow-up period for the 10 affected dogs ranged from 20 to 80 days.

For the eyes of the clinically normal control dogs, analysis of precorneal tear film samples revealed the presence of latent forms of MMP-2 and MMP-9, whereas the

active forms were not detected. There was no significant difference in mean concentrations of MMP-2 ($P = 0.417$) and MMP-9 ($P = 0.158$) between the right eye and the left eye in the control dogs' eyes.

The latent and active forms of MMP-2 and MMP-9 were detected in ulcerated eyes of affected dogs. The total MMP concentrations (ie, MMP-2 and MMP-9 concentrations combined) in the ulcerated eyes were significantly higher than values in the contralateral unaffected eyes of dogs with *P aeruginosa*-associated keratitis and in the control eyes on the first day of admission. However, active MMP-2 was detected in the contralateral unaffected eyes of dogs with *P aeruginosa*-associated keratitis, whereas it was not detected in eyes of the control dogs.

The total MMP concentrations in affected eyes that had healed were significantly lower than values in the ulcerated eyes on the first day of admission. However, the latent MMP-2 concentration in the healed eyes was significantly ($P < 0.001$) higher than the corresponding concentration in the control eyes. In addition, active MMP-2 was unexpectedly detected in the healed eyes. No significant ($P = 0.169$) difference in mean MMP-9 concentration between healed and control eyes was detected (Table 1).

The concentration of latent MMP-2 was significantly higher than the concentrations of active MMP-2 in the

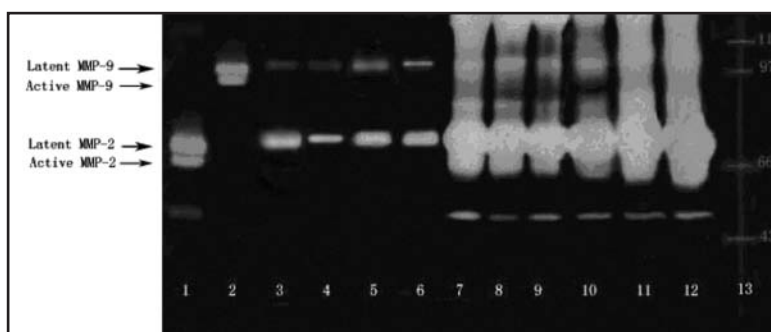


Figure 1—Photograph of representative results of gelatin zymography to detect active and latent forms of MMP-2 and MMP-9 performed on precorneal tear fluid samples collected from ulcerated eyes of 6 dogs with unilateral *Pseudomonas aeruginosa*-associated keratitis (lanes 7 to 12) on the day of admission to hospital and from 4 control eyes of 4 clinically normal dogs (lanes 3 to 6). Human standards of MMP-2 (lane 1) and MMP-9 (lane 2) and molecular weight markers (lane 13) were loaded on each gel.

Table 1—Mean \pm SD concentration of latent and active forms of MMP-2 and MMP-9 in both eyes of 10 clinically normal dogs (CNE) and in the contralateral unaffected eyes (on the day of hospital admission [CUE1] and the day of complete healing of the affected eyes [CUE2]), the ulcerated eyes (on the day of hospital admission [UE]), and the healed eyes (HE) of 10 dogs with unilateral *Pseudomonas aeruginosa*-associated keratitis.

MMP	CNE	CUE1	CUE2	UE	HE
Latent MMP-2	674.4 \pm 102.13*	659.6 \pm 20.18*	673.6 \pm 99.29*	1,989.4 \pm 244.34*,†	1,220.3 \pm 254.30*,‡
Active MMP-2	0	71.8 \pm 34.49	0	1,641 \pm 460.97†	296.5 \pm 89.65‡
Latent MMP-9	179.85 \pm 57.16§	202 \pm 24.22§	176.6 \pm 66.08§	733 \pm 130.30§,¶	208.3 \pm 39.02§
Active MMP-9	0	0	0	417.50 \pm 210.71¶	0

*Within an eye group, concentration of latent MMP-2 is significantly ($P < 0.05$) higher than the concentration of active MMP-2. †Value is significantly ($P < 0.05$) higher than corresponding concentration in CNE, CUE1, CUE2, and HE groups. ‡Value is significantly ($P < 0.05$) higher than corresponding concentration in CNE, CUE1, and CUE2 groups. §Within an eye group, concentration of latent MMP-9 is significantly ($P < 0.05$) higher than the concentration of active MMP-9. ¶Value is significantly ($P < 0.05$) higher than corresponding concentration in CNE, CUE1, CUE2, and HE groups.

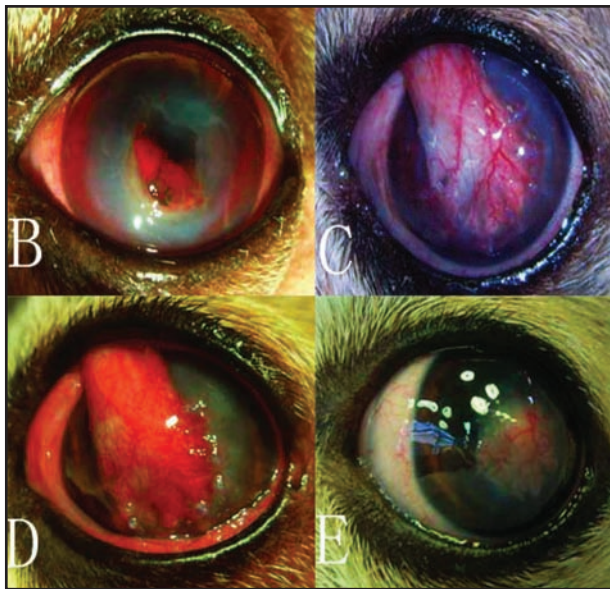
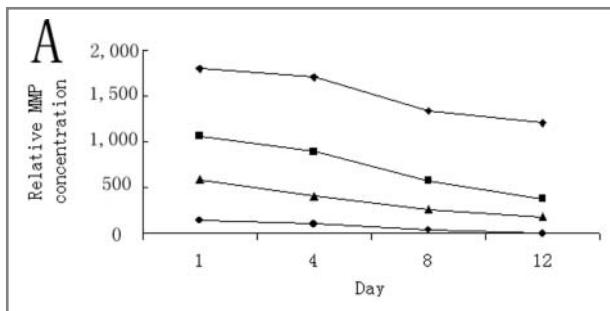


Figure 2—Representative changes in concentrations (relative standard units) of active and latent MMP-2 (squares and diamonds, respectively) and MMP-9 (circles and triangles, respectively) in precorneal tear film samples collected from the ulcerated right eye of a dog with unilateral *P aeruginosa*-associated keratitis on days 1 (day of admission), 4, 8, and 12 (A) and photographs of the ulcerated right eye on days 1 (B), 10 (C), 20 (D), and 48 (E). The dog received medical treatment of the ulcerated eye and a conjunctival pedicle graft on the day of admission.

eyes of control dogs and in the contralateral unaffected eyes, ulcerated eyes, and healed eyes of dogs with *P aeruginosa*-associated keratitis. Similar differences in the concentrations of latent MMP-9, compared with concentrations of active MMP-9, were detected (Table 1).

In dogs with *P aeruginosa*-associated keratitis, the total MMP concentrations in the ulcerated eyes significantly decreased as corneal healing progressed, compared with concentrations measured in those eyes on the first day of admission to the hospital (Figure 2).

Discussion

Among the various pathogens associated with bacterial keratitis, *P aeruginosa* causes the most devastating injury.^{4,22} This may be because most *P aeruginosa* strains produce extracellular proteases^{23–25} that may be important pathogenic factors in destructive corneal ulceration,^{26–28} even after the bacteria have been eliminated by topical antimicrobial treatments.²⁹ The pseudomonal elastase strongly activates proMMPs via limited proteolysis to generate active forms of MMPs,³⁰ although the exact mechanisms in vivo are not clearly

understood.⁷ The proteinases perform important physiologic functions in the slow turnover and remodeling of the corneal stroma. The activities of these proteolytic enzymes are normally balanced by inherent protease inhibitors, thereby preventing excessive degradation of healthy tissue.³¹ Thus, melting ulceration can be considered a disorder of proteinase homeostasis.^{9,21}

In our study, latent forms of MMP-2 and MMP-9 were detected in the eyes of the clinically normal control dogs, whereas active forms of these MMPs were not. In addition, total MMP concentrations were significantly higher in ulcerated eyes, compared with values in the control eyes. This difference is similar to findings in other species, which suggests that the increase in precorneal tear film proteinase concentrations is part of a fundamental response of the mammalian eye to corneal injury.^{32–34}

An unexpected finding in the present study was that concentrations of latent and active forms of MMP-2 in the healed eyes were significantly higher than concentrations in control eyes and in contralateral unaffected eyes on the day when the ulcer had completely healed and yet were significantly lower than concentrations in the ulcerated eyes on the day of admission to the hospital. In contrast, the mean MMP-9 concentration in healed eyes had returned to control eye values on the day that the ulcer had completely healed. One possible explanation is that MMP-2 and MMP-9 perform different roles during corneal healing and stromal remodeling. Matrix metalloproteinase-2 is synthesized by corneal keratocytes and performs a surveillance function in corneas, becoming locally activated to degrade collagen molecules that occasionally become damaged as a result of normal wear and tear.^{6–8} Alternatively, MMP-9 is suspected to participate in the degradation of the basement membrane in the early phase after the corneal injury.⁷ It appears that stromal ulceration does not occur until after the epithelial basement membrane disappears; it is the controlling step leading to stromal ulceration.^{7,10} Negative results for retention of fluorescein dye on ulcerated eyes indicate that the basement membrane has recovered and epithelialization is completed; at this time, the concentrations of latent and active MMP-9 should theoretically return to the values expected in healthy eyes. This was evident in the dogs of the present study. Given the presumed role of MMP-2, it is not surprising that the concentrations of latent and active forms of MMP-2 were significantly higher than those in the control eyes on the day when ulcerated eyes no longer retained fluorescein dye; nevertheless, the concentrations were decreased, compared with the findings on the day of admission. The concentration of latent MMP-2 or MMP-9 was significantly higher than the concentration active MMP-2 or MMP-9 in clinically normal control eyes, contralateral unaffected eyes, ulcerated eyes, and healed eyes. This also might be attributable to the different functions of MMP-2 and MMP-9. We speculated that concentrations of latent and active forms of MMP-2 would decrease gradually with recovery of corneal transparency; moreover, active MMP-2 would disappear in the healed eyes eventually.

It is also interesting to note that active MMP-2 was detected in contralateral unaffected eyes of dogs with *P aeruginosa*-associated keratitis on the day of admission to

the hospital. This suggests that the high concentrations of latent and active forms of MMP-2 induced by *P aeruginosa* infection in ulcerated eyes might contribute to activation of latent MMP-2 in contralateral unaffected eyes via the mucosal immune system of the ocular surface.

The results of our study are partially in accordance with findings of previously reported studies^{14,19,35} of corneal wound healing, although the latter also suggested that persistence of high levels of proteolytic activity in corneal wounds is responsible for failure to heal. Our data support the therapeutic use of proteinase inhibitors to rapidly reduce the activity of tear proteases in dogs with *P aeruginosa*-associated keratitis. Autoserum, which contains broad protease inhibitors such as α_2 -macroglobulin and α_1 -proteinase inhibitor, was used typically in the ulcerated eyes of several affected dogs. Various other antiprotease compounds (N-acetylcysteine, doxycycline, lloprost, and EDTA) that work via different mechanisms to inhibit different families of proteases present in canine tears are also available. However, further studies will be required to evaluate the effect of these synthetic protease inhibitors on clinical cases. Because *P aeruginosa*-associated keratitis in dogs appears to trigger initially high levels of proteolytic activity in precorneal tear film that decrease as the ulcers heal, reducing precorneal tear film concentrations of MMPs might represent a treatment strategy for the condition in dogs.

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- a. Blue prestained standard, Invitrogen, Carlsbad, Calif.
 - b. Chemicon, Billerica, Mass.
 - c. Novex zymogram renaturing buffer, Invitrogen, Carlsbad, Calif.
 - d. Novex zymogram developing buffer, Invitrogen, Carlsbad, Calif.
 - e. R250, Amresco Inc, Solon, Ohio.
 - f. HP ScanJet 5P, Hewlett-Packard, Palo Alto, Calif.
 - g. Alpha Ease, version 3.3, Alpha Innotech Co, San Leandro, Calif.
 - h. SPSS, version 10.0, SPSS Inc, Chicago, Ill.
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