Cleavage and Activation of Corneal Matrix Metalloproteases by *Pseudomonas aeruginosa* Proteases

**Koki Matsumoto, Naveed B. K. Shams, Laila A. Hanninen, and Kenneth R. Kenyon**

**Purpose.** To examine the effect of *Pseudomonas aeruginosa* on the expression of corneal matrix metalloproteases and the effect of its proteases on activation of corneal matrix metalloproteases in vitro.

**Methods.** Rat corneas and human corneal fibroblasts were co-cultivated with two different strains (RPS & 599A) of *P. aeruginosa* and one strain of *Staphylococcus aureus*, and the conditioned media were analyzed for proteolytic activity by gelatin and casein zymography. Human corneal fibroblast-conditioned medium was incubated with that from either strain of *P. aeruginosa* and was analyzed in a similar manner.

**Results.** Normal rat corneas in organ culture produce a 65 kDa gelatinase (inactive matrix metalloprotease-2), whereas thermally injured rat corneas additionally produce gelatinases with molecular masses of 92 kDa (inactive matrix metalloproteases-9) and >200 kDa. Matrix metalloprotease-2 is also detected in human corneal fibroblast-conditioned medium.

Although these matrix metalloproteases are no longer detectable when rat corneas or human corneal fibroblasts are co-cultured with two strains of *P. aeruginosa* for 48 hr, a 58 kDa gelatinase fragment appears in earlier stages of co-culture. In contrast, *S. aureus* does not affect matrix metalloprotease-2.

The 58 kDa fragment is also evident by incubating human corneal fibroblast-conditioned medium with that from either strain of *P. aeruginosa*. Conditioned medium from the RPS strain, which produces both elastase and alkaline protease, is more effective in cleaving matrix metalloprotease-2 than that from the 599A strain, which expresses mainly alkaline protease.

**Conclusion.** The secreted inactive corneal matrix metalloprotease-2 is activated through limited proteolysis by pseudomonal proteases. Invest Ophthalmol Vis Sci 1993; 34:1945-1953.

**Corneal ulcers caused by *Pseudomonas aeruginosa* organisms are rapidly progressive and frequently resistant to even vigorous treatment.** Most *P. aeruginosa* strains produce extracellular proteases, which may be important pathogenic factors in destructive corneal ulceration. Recently pseudomonal elastase and alkaline protease have been shown to degrade proteoglycan, native type III and IV collagens, and laminin, most of which constitute the corneal extracellular matrix, as well as denatured collagens (gelatins) of all types. The substrate specificity of the pseudomonal proteases resembles that of the matrix metalloproteases (MMP) including collagenase, stromelysin, and gelatinase (type IV collagenase).

Recently two distinctive gelatinases, both MMP, have been shown to be synthesized and secreted by rabbit and rat corneal cells in serum-free organ culture. These gelatinases with approximate molecular masses of 72 and 92 kDa, termed MMP-2 and MMP-9, respectively, are the products of separate
Animals
Male Sprague-Dawley rats (Charles River Labs, Wilmington, MA), weighing 350-400 g were used according to the ARVO Resolution on the Use of Animals in Research and PHS policy on Humane Care and Use of Laboratory Animals.

Thermal Burn and Organ Culture
Immediately after sacrifice by intraperitoneal sodium pentobarbital overdose, a 3 mm thermokeratophore probe (Frigitronics, Shelton, CT) at 190°C was applied twice to the central cornea of both eyes for 1 sec. The enucleated eyes, under sterile condition, were immediately immersed in RPMI-1640 medium (Sigma, St. Louis, MO) and whole corneas, about 4 mm in diameter excluding the limbus, were excised with Vannas scissors. The corneas were then placed in RPMI-1640 medium and cultivated at 37°C in a 5% CO₂-95% air atmosphere for 48 hr. Normal corneas as well as thermally injured corneas treated with 10% glutaraldehyde (GA) in phosphate-buffered saline for 2 hr at room temperature were similarly cultivated.

Cell Culture of Human Corneal Fibroblasts
Human corneal fibroblasts were obtained from corneo-scleral rims of corneas obtained from an eye bank. After removing the epithelium by scraping, 6–8 stromal fragments from each cornea were placed in RPMI-1640 medium supplemented with 10% fetal bovine serum, 6000 units/ml penicillin, 5000 μg/ml streptomycin, 4 mM L-glutamine and 1 mM sodium pyruvate. Cells were subcultured by harvesting cells in trypsin-ethylenediaminetetraacetic acid (EDTA; Sigma) and splitting 1:3 into new wells (6 well clusters) containing the medium. For this study cells were used between passages 4 and 6. Before use, serum-containing medium was replaced with plain unsupplemented RPMI-1640 medium to remove any effects of protease inhibitors and albumin contained in the serum.

Preparation of Bacteria
Three strains of bacteria were used: P. aeruginosa-RPS (an isolate from human corneal ulcer, untypeable by the Fisher method, which produces pyocyanine), P. aeruginosa-599A (an isolate from vitamin A-deficient rat eye, immunotype I) and Staphylococcus aureus (an isolate from human corneal ulcer, immunotype V). Bacteria were cultivated in nutrient broth for 24 hr at 37°C. One-milliliter samples of the culture were centrifuged at 10,000 rpm for 10 min at room temperature, and the precipitated bacteria were washed twice with sterile phosphate-buffered saline. Bacteria were then suspended in 1 ml RPMI-1640 medium and used as the live bacteria. Alternatively, bacterial cells were treated with 10% GA in phosphate-buffered saline for 2 hr at room temperature, and the cell density was adjusted to 0.3 of absorbance at 590 nm. Before use, GA-treated bacteria were washed with sterile phosphate-buffered saline containing 2% bovine serum albumin. These live and GA-treated bacteria were used to study their protease production and effects on the expression of MMPs by rat cornea in organ culture and human corneal fibroblasts in cell culture.

Effect of Live and GA-treated Bacteria on the Expression of Gelatinases by Rat Corneas in Organ Culture
The excised corneas were subjected to organ culture in RPMI-1640 medium (2 ml) with or without each (0.1 ml) of the three live or GA-treated bacterial strains at 37°C for 48 hr. These bacteria were similarly cultivated. The conditioned media were tested by gelatin and casein zymography.

Effect of Live Bacteria on the Expression of Gelatinase by Human Corneal Fibroblasts
Confluently grown human corneal fibroblasts in serum-containing medium were then cultivated in unsupplemented RPMI-1640 medium with or without each of the three live bacteria at 37°C for 24, 48, and 72 hr. These conditioned media were tested for pro-
Activation of Corneal Proteases

Similarly the three strains of bacteria were also cultivated in RPMI-1640 medium and were used as controls. To see the early effect of *P. aeruginosa* on the expression of gelatinase by human corneal fibroblasts, aliquot portions (100 µl) of co-culture media were taken every 30 min from 2 to 4 hr after co-cultivation in the case of the RPS strain and every 3 hr up to 12 hr in the case of the 599A strain. The conditioned media were tested by gelatin and casein zymography.

**Effect of Pseudomonal Conditioned Media on the Gelatinase from Human Corneal Fibroblasts**

Medium (800 µl) conditioned by human corneal fibroblasts for 72 hr was mixed with the conditioned medium (200 µl) from *P. aeruginosa* (RPS and 599A strains), and the mixtures were incubated at 37°C. Aliquot portions (50 µl) taken every 10 min up to 50 min were analyzed by gelatin and casein zymography. Hamster corneal fibroblast-conditioned medium (200 µl) was mixed with various volumes (5 µl-100 µl) of *P. aeruginosa* (RPS and 599A strains)-conditioned medium and incubated at 37°C for 60 min. These reaction mixtures were analyzed by both gelatin and casein zymography.

**Zymography**

Zymography was performed using the method of Heussen and Dowdle with some modifications. Gelatin (from bovine skin; Sigma) and alpha-casein (from bovine milk; Sigma) were used as substrates at the final concentration of 0.1%. The sodium dodecyl sulfate (SDS)-gels (10%) were prepared using 37.5:1 stock solution of acrylamide to bis-acrylamide (Boehringer Mannheim, Indianapolis, IN). Samples were diluted 1:2 or 1:4 in sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.002% bromphenol blue) and 20 µl of each sample was loaded on each lane. After electrophoresis at 4°C, the gels were shaken in a 2.5% solution of Triton X-100 (with or without 100 mM EDTA) for 1 hr at room temperature to remove SDS and then incubated in reaction buffer (50 mM Tris-HCl, pH 6.8, 10 mM CaCl₂) with or without 100 mM EDTA overnight at 37°C. We used EDTA, a well-known inhibitor of metalloprotease, to confirm whether or not the detected protease was metalloprotease. The positions of enzymatic species possessing either gelatinolytic or caseinolytic activity could be easily identified after the gel was stained with Coomassie brilliant blue R-250 (Sigma) as clear bands in the stained gelatin or casein background. Prestained SDS-PAGE protein standard (low range; Bio-Rad Laboratories, Richmond, CA) was used as the molecular mass marker.

**RESULTS**

**Proteases Production by Both Strains of *P. aeruginosa***

The RPS strain of *P. aeruginosa* produced 53 kDa, 70 kDa, and 110 kDa proteases (Figs. 1A and B, lane 1), whereas the 599A strain released mainly 53 kDa protease (Figs. 1A and B, lane 2). However, the *S. aureus* strain did not produce any proteases (Figs. 1A and B, lane 3). The pseudomonal proteases possessed both gelatinolytic and caseinolytic activities (Figs. 1A and B, lanes 1 and 2). GA-treated *P. aeruginosa* did not produce any proteases (data not shown).

**FIGURE 1.** Gelatinase production by rat corneas and effects of three strains of bacteria. Rat corneas with or without thermal burn were cultivated in the presence or absence of either *P. aeruginosa*-RPS, *P. aeruginosa*-599A strain and *S. aureus* in serum-free RPMI-1640 medium at 37°C in a 5% CO₂-95% air atmosphere for 48 hr. Glutaraldehyde (GA)-treated corneas were also cultivated. Conditioned media were tested by gelatin (A) and casein (B) zymography. Lanes 1-3: *P. aeruginosa*-RPS(1), *P. aeruginosa*-599A(2), and *S. aureus* (3)-conditioned media. Lanes 4 and 5: Conditioned media from normal and thermally injured rat corneas, respectively. Lane 6: Conditioned medium from thermally injured rat cornea treated with GA before cultivation. Lanes 7-9: Conditioned media from thermally injured rat cornea cultivated with *P. aeruginosa*-RPS(2), *P. aeruginosa*-599A(8) and *S. aureus* (9) strains. Lanes 10 and 11: Conditioned media from thermally injured rat cornea cultivated with GA-treated RPS and 599A strains of *P. aeruginosa*, respectively.
Gelatinase Production by Rat Corneas in Organ Culture and the Effect of Thermal Burn

Normal rat corneas produced a 65 kDa gelatinase (Fig. 1A, lane 4) but did not show any detectable caseinolytic activity (Fig. 1B, lane 4). A 3 mm thermal wound to the cornea before organ culture led to the appearance of 92 kDa and >200 kDa gelatinases in addition to the 65 kDa gelatinase (Fig. 1A, lane 5). GA-treated corneas did not produce any gelatinase or caseinase (Figs. 1A and B, lane 6).

Effects of Live and GA-treated Bacteria on the Expression of Rat Corneal MMPs

Rat corneal gelatinases could no longer be detected when corneas were co-cultured with the RPS strain of P. aeruginosa for 48 hr (Fig. 1A, lane 7). However, co-culture with the 599A strain resulted in the appearance of a new gelatinase with an approximate molecular mass of 58 kDa (Fig. 1A, lane 8). The 58 kD gelatinase seemed to rise from the corneal gelatinase(s) as it was detected by only gelatin zymography (Figs. 1A and B, lane 8). Live S. aureus showed almost no effect on the expression of the 65 kDa gelatinase (Fig. 1A, lane 9). GA-treated P. aeruginosa did not affect these corneal gelatinases (Fig. 1A, lanes 10 and 11).

Effect of Live Bacteria on the Expression of Gelatinase by Human Corneal Fibroblasts

Human corneal fibroblasts also produced a 65 kDa gelatinase (Figs. 2A and B, lane 5), which disappeared...
when fibroblasts were co-cultured with the RPS strain of P. aeruginosa for 24 hr (Fig. 2A, lane 6) or with the 599A strain for 48 hr (Fig. 2B, lane 7). However, a limited cleavage fragment (58 kDa) of the 65 kDa gelatinase was detected in the sample of co-cultivation for 24 hr with the 599A strain (Fig. 2A, lane 7). Moreover, a 40 kDa protease fragment was detected (Figs. 2A–D, lanes 6 and 7). The 40 kDa fragment seemed to rise from the pseudomonal proteases as it possessed both gelatinolytic and caseinolytic activities. A live strain of S. aureus did not show any effect on the expression of the 65 kDa gelatinase (Fig. 2A, lane 8). The 58 kDa gelatinase fragment was detected 9 hr after co-cultivation with the 599A strain (Fig. 3A, lane 3) after the production of the 53 kDa protease (Figs. 3A and B, lane 2) and increased in amount thereafter (Fig. 3A, lane 4). The 58 kDa fragment was also detected 4 hr after co-cultivation with the RPS strain (Fig. 4A, lane 6) slightly later than the production of the pseudomonal proteases (Figs. 4A and B, lanes 4 and 5).

Effect of EDTA on Gelatinases and Pseudomonal Proteases

Gelatinases from rat corneas (Fig. 5A, lanes 1 and 2), human corneal fibroblasts (Fig. 5A, lane 3), and pseudomonal proteases (Figs. 5A and C, lanes 4 and 5) were almost completely inactivated by 100 mM EDTA (Figs. 5B, lanes 1–5 and 5D, lanes 4 and 5) indicating that they are metalloproteases.

Identification of Pseudomonal Proteases

Pseudomonal 53 kDa and 110 kDa proteases were identified as alkaline protease and elastase, respectively, from the result of gelatin and casein zymography where each pair showed the same electrophoretic mobility (Figs. 6A and B).

Effect of Pseudomonas-Conditioned Media on the 65 kDa Gelatinase from Human Corneal Fibroblasts

The 58 kDa fragment was detected together with the 65 kDa gelatinase immediately after incubation of the corneal fibroblast-conditioned medium with that from...
the RPS strain (Fig. 7A). The 58 kDa fragment increased steadily in amount during the incubation time for 50 min (Fig. 7A). The 58 kDa fragment was also detected when the corneal fibroblast-conditioned medium was incubated with that from the 599A strain although it required longer incubation time (data not shown). This limited proteolysis of the 65 kDa gelatinase by Pseudomonas-conditioned media from both strains exhibited a dose-dependent pattern (Fig. 8A), whereas the 40 kDa fragment of pseudomonal protease was detected after mixing the corneal fibroblast-conditioned medium with that from P. aeruginosa (Figs. 7A and B, lanes 2-10, 8A and B, lanes 11-17).

DISCUSSION

Two distinctive gelatinases (type IV collagenases; members of MMP family) have been produced and secreted by corneal cells under physiologic and pathologic conditions.17,18 These gelatinases have been shown to be important in tissue repair, wound healing, and stromal ulceration.17,18 However, most MMPs are secreted as an inactive proenzyme and have to be cleaved and activated before performing their functions.

In this study, we have demonstrated, for the first time, that corneal gelatinase (MMP-2) can be cleaved and activated by pseudomonal protease(s) in vitro. We have also confirmed that both rat corneas and human corneal fibroblasts produce gelatinase with an approximate molecular mass of 65 kDa, which is completely inactivated by EDTA. It has been shown that the 65 kDa gelatinase visualized on gelatin zymography corresponds to the 72 kDa gelatinase (MMP-2) determined by its electrophoretic mobility under sulfhydryl-reducing condition.17 Therefore, the 65 kDa gelatinase...
tinase can be classified as MMP-2. Thermally-injured rat corneas additionally produced gelatinases with molecular masses of about 92 kDa and >200 kDa of which 92 kDa gelatinase can be classified as the inactive proenzyme form of MMP-9.\textsuperscript{17,18} Keratocytes are the most likely source of MMP-2, whereas MMP-9 is produced by corneal epithelial cells.\textsuperscript{17}

Co-culture experiments of rat corneas with two different strains of \textit{P. aeruginosa} showed a biologically active fragment with a molecular mass of 58 kDa. It is obvious that the 58 kDa fragment was derived from the corneal MMP-2 because it was detected only by gelatin zymography completely different from the pseudomonal proteases, which have both gelatinolytic and caseinolytic activities. Emergence of the fragment might have resulted from a limited cleavage of MMP-2 by extracellular proteases produced by \textit{P. aeruginosa} because the RPS and 599A strains of \textit{P. aeruginosa} can produce at least 1 and 3 proteases, respectively, whereas GA-treated \textit{P. aeruginosa} or live \textit{S. aureus} do not produce any proteases.

To confirm this hypothesis, we tested the effect of conditioned media from two strains of \textit{P. aeruginosa} on MMP-2 from human corneal fibroblasts. As expected, MMP-2 was cleaved into a 58 kDa fragment in a time- and dose-dependent manner by the conditioned me-

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\caption{Effect of the conditioned media from two different strains of \textit{P. aeruginosa} on the 65 kDa gelatinase (MMP-2) from human corneal fibroblasts. Human corneal fibroblast-conditioned medium (200 \(\mu\)l) was mixed with various volumes (5–100 \(\mu\)l) of \textit{P. aeruginosa} (RPS and 599A strains)-conditioned media (total 300 \(\mu\)l) and incubated at 37\(^\circ\)C for 1 hr. Conditioned media from human corneal fibroblasts (200 \(\mu\)l) and bacteria (100 \(\mu\)l) were similarly incubated and served as controls. These samples were analyzed by gelatin (A) and casein (B) zymography. \textit{Lane 1:} Prestained SDS-PAGE protein standard. \textit{Lane 2:} Conditioned medium from human corneal fibroblasts. \textit{Lanes 3–9:} Reaction mixtures (20 \(\mu\)l) in which 0.17 (3), 0.33 (4), 0.67 (5), 1.33 (6), 2.00 (7), 2.67 (8), and 3.33 (9) \(\mu\)l of the RPS-conditioned media were used. \textit{Lane 10:} Conditioned medium (3.33 \(\mu\)l) from \textit{P. aeruginosa}-RPS strain. \textit{Lanes 11–17:} Reaction mixtures in which 0.17 (11), 0.33 (12), 0.67 (13), 1.33 (14), 2.00 (15), 2.67 (16), and 3.33 (17) \(\mu\)l of the 599A-conditioned media were used. \textit{Lane 18:} Conditioned medium (3.33 \(\mu\)l) from \textit{P. aeruginosa}-599A strain. Note dose-dependent effect on the 65 kDa gelatinase (MMP-2) and the appearance of the 58 kDa fragment by the conditioned media from both the RPS and 599A strains of \textit{P. aeruginosa}.}
\end{figure}
dia from both strains. The conditioned medium from the RPS strain was much more effective than that from the 599A strain in cleaving MMP-2. This difference may be attributable to different kinds or amounts of proteases produced by these strains. The RPS strain produced 53 kDa, 70 kDa and 110 kDa proteases, whereas the 599A strain produced mainly 53 kDa protease. These findings suggest that the cleavage of MMP-2 is caused by the pseudomonal protease(s).

Although the emergence of the 40 kDa protease fragment needs further investigation, it seems to arise from pseudomonal protease because it possesses both gelatinolytic and caseinolytic activities.

As pseudomonal alkaline protease and elastase showed identical mobilities on zymography to the 53 kDa and 110 kDa proteases, respectively, we conclude that the 53 kDa and 110 kDa proteases are alkaline protease and elastase, respectively. Therefore, the RPS strain of P. aeruginosa produces alkaline protease and elastase besides other proteases, whereas the 599A strain produces mainly alkaline protease.

Although the established molecular masses of secreted mature alkaline protease and elastase are 49.5 kDa and 33 kDa, respectively,26,27 elastase showed peculiar mobility on zymography with an apparent molecular mass of 110 kDa. This discrepancy may be explained by inadequate denaturing conditions particularly for elastase. To preserve proteolytic activity we did not boil the samples or use denaturants other than 1% SDS.

It has been shown that most MMPs are secreted in the inactive proenzyme form and may be activated in the extracellular space through proteolytic cleavage from the N-terminal of the protein.19,28 The limited cleavage fragment (58 kDa) of the corneal MMP-2 with a decrease in molecular mass of 7 kDa correspond to the active form of MMP-2.18,19,29 Therefore, we conclude that corneal MMP-2 can be cleaved and activated by the pseudomonal proteases through limited proteolysis. An uncontrolled activation of corneal MMPs might result in enhanced degradation of the corneal matrix leading to ulceration.

Although after infiltration of the cornea with massive polymorphonuclear leukocytes, lysosomal enzymes from polymorphonuclear leukocytes have been shown to be the main source of tissue destruction in experimental pseudomonal keratitis in rabbit,30 pseudomonal proteases are believed to be important for the establishment of corneal infection and severity of the lesion in the early stages.7,8,10,31–33

Our study suggests a new role for pseudomonal proteases in the initiation and progression of corneal ulceration as well as invasion of corneal tissue by P. aeruginosa. Thus, P. aeruginosa could contribute to corneal ulceration by releasing proteases and activating endogenous MMPs.

Key Words
gelatinase, corneal matrix metalloprotease, Pseudomonas aeruginosa, pseudomonal elastase, pseudomonal alkaline protease

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References

14. Berman MB. Collagenase and corneal ulceration. In
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