

# Stimulatory Effect of Pseudomonal Elastase on Collagen Degradation by Cultured Keratocytes

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**PURPOSE.** The pathobiology of corneal ulceration induced by *Pseudomonas aeruginosa* was investigated by characterization of the pseudomonal pathogenic factors responsible for degradation of the collagen matrix.

**METHODS.** Three-dimensional gels of type I collagen containing (or not) rabbit keratocytes were incubated in the presence of either culture supernatant of *P. aeruginosa* strain PAO1 or pseudomonal pathogenic factors (elastase, lipopolysaccharide, or exotoxin A), and the extent of collagen degradation was assessed after 24 hours by measurement of released hydroxyproline. Activation of matrix metalloproteinases (MMPs) produced by keratocytes was also examined by gelatin zymography and immunoblot analysis.

**RESULTS.** In the absence of keratocytes, the PAO1-conditioned medium increased the extent of collagen degradation. The conditioned medium also promoted keratocyte-mediated collagen degradation. Of the pseudomonal pathogenic factors examined, only elastase degraded collagen directly as well as stimulated keratocyte-mediated collagen degradation. Culture supernatant of elastase-deficient *P. aeruginosa* (*lasR* or *lasB*) mutants had no effect on collagen degradation in the absence or presence of keratocytes. Elastase also induced the conversion of the inactive precursors of MMP-1, -2, -3, and -9 produced by keratocytes to the active forms of the enzymes.

**CONCLUSIONS.** These results suggest that pseudomonal elastase both degrades type I collagen directly and promotes collagen degradation mediated by keratocytes, the latter effect being likely attributable, at least in part, to the activation of proMMPs. (*Invest Ophthalmol Vis Sci.* 2001;42:1247-1253)

The opportunistic Gram-negative pathogen *Pseudomonas aeruginosa* is a causative agent of severe keratitis, corneoscleritis, and endophthalmitis. Pseudomonal keratitis progresses rapidly and results in damage to corneal tissue that is mediated by both pseudomonal and host factors. Pseudomonal pathogenic factors have been shown to cause destruction of corneal structure.<sup>1,2</sup> We have previously demonstrated that culture supernatant of *P. aeruginosa* both degrades collagen directly as well as stimulates collagen degradation by rabbit cultured keratocytes maintained in a three-dimensional gel of type I collagen.<sup>3</sup> We also showed that factors derived from *P. aeruginosa* activate the inactive pro-matrix metalloproteinases (proMMPs) produced by keratocytes.<sup>3</sup> However, little is

known of the pseudomonal pathogenic factors responsible for corneal matrix degradation.

The cornea is an avascular and transparent tissue. The transparency is primarily attributable to the architecture of corneal stromal collagen, which comprises mostly type I collagen. In the normal cornea, keratocytes are quiescent and inactive. However, corneal injury induces the activation of keratocytes at the wound edge. The activated keratocytes synthesize both extracellular matrix (ECM) proteins and MMPs,<sup>4</sup> the latter including interstitial collagenase (MMP-1), gelatinase A (MMP-2), stromelysin 1 (MMP-3), and gelatinase B (MMP-9).<sup>5-7</sup> Production of MMPs by these cells is regulated by various cytokines.<sup>7-10</sup> When the balance between MMP and collagen synthesis is tilted toward MMPs, the collagen matrix undergoes excessive degradation and corneal ulceration results. MMPs are synthesized and secreted as proenzymes that are activated by serine proteinases, such as plasmin, in the extracellular space.<sup>11-13</sup> Activated MMPs degrade the ECM of the stroma and basement membrane and thereby induce ulceration. It is therefore important to characterize the mechanism of activation of proMMPs to understand the pathophysiology of ulceration.

Culture of keratocytes in a three-dimensional collagen gel provides a useful model with which to elucidate the metabolism of the collagen matrix in corneal tissue. Incubation of such gels, as well as of gels not containing keratocytes, with either medium conditioned by *P. aeruginosa* or pseudomonal pathogenic factors allows study of the direct and indirect effects of bacterial products on collagen degradation. Pseudomonal elastase degrades corneal proteoglycans, resulting in the destruction of collagen.<sup>14</sup> Exotoxin A is a potentially lethal toxin<sup>15</sup> that inhibits mammalian protein synthesis by catalyzing the ADP-ribosylation of elongation factor 2.<sup>16</sup> Lipopolysaccharide (LPS) induces immune responses, including activation of the alternative complement cascade and the production of cytokines and is also implicated in modulation of the adherence of *P. aeruginosa* to the cornea.<sup>17</sup> To identify pseudomonal pathogenic factors responsible for collagen degradation, we have now examined the effects of culture supernatant of *P. aeruginosa* as well as of the pathogenic factors elastase, exotoxin A, and LPS on collagen degradation in the absence or presence of keratocytes. To clarify further the role of elastase, we examined the effect of culture supernatant of elastase-deficient *P. aeruginosa* mutants on collagen degradation. With the use of gelatin zymography and immunoblot analysis, we also investigated the effects of pseudomonal factors on activation of proMMPs produced by keratocytes.

## METHODS

### Bacterial Strains and Culture

*P. aeruginosa* strains used in the present study are listed in Table 1. Culture supernatant of *P. aeruginosa* was prepared by growing bacterial cells with shaking for 24 hours at 37°C in Mueller-Hinton broth supplemented with calcium (50 µg/ml) and magnesium (20 µg/ml) to increase protease production. The culture was then centrifuged, and the supernatant was sterilized by filtration through a filter with

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TABLE 1. *P. aeruginosa* Strains Used in this Study

Strain	Genotype	Phenotype	References
PAO1	Parental strain	Wild type	28
PAO-R1	Str <sup>r</sup> , $\Delta lasR::tet$ of PAO1	LasR deficient	23, 29
PAO-B1	$lasB::\Omega$ of PAO1	Elastase deficient	30

0.45- $\mu$ m pores and stored at  $-80^{\circ}\text{C}$  until use. The protein concentration of the culture supernatant was determined.

### Keratocyte Culture

Rabbit keratocytes were prepared as described previously.<sup>18</sup> The cells were cultured under a moist atmosphere containing 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$  in TCM-199 medium supplemented with 5% fetal bovine serum. They were used for experiments after five or six passages. The phenotype of the passaged cells was examined by immunocytochemical analysis for the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), as previously described.<sup>19</sup> The passaged keratocytes used in this study expressed  $\alpha$ -SMA, indicating that they are transformed myofibroblasts.

### Three-Dimensional Culture

Collagen gels were prepared as described previously.<sup>20</sup> In brief, acid-solubilized porcine collagen type I (3 mg/ml, Cell Matrix Type Ia; Nitta Gelatin, Yao, Japan),  $5\times$  Dulbecco's modified Eagle's medium, reconstitution buffer (0.05 M NaOH, 0.26 M  $\text{NaHCO}_3$ , and 200 mM HEPES [pH 7.3]), and keratocyte suspension ( $2.2 \times 10^6$  cells/ml in minimum essential medium) were mixed in a ratio of 7:2:1:1 in an ice bath. The mixture (0.5 ml) was then added to each well of a 24-well culture plate and allowed to solidify in an incubator under 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ , after which minimum essential medium (0.5 ml) containing test agent, pseudomonal culture supernatant, elastase (provided by Kazuyuki Morihara, Institute of Applied Life Sciences, Graduate School, University of East Asia, Shimonoseki, Japan), LPS (Sigma, St. Louis, MO), or exotoxin A (Sigma), was overlaid. Pseudomonal culture supernatant was diluted  $\sim 1:10$  with minimum essential medium to achieve a protein concentration of 10 mg/ml. The resulting cultures were incubated for 24 hours.

### Assay of Collagenolytic Activity

The medium from collagen gel incubations was collected, and native collagen fibrils with molecular sizes of  $>100$  kDa were removed by ultrafiltration. The filtrates were then subjected to hydrolysis by concentrated HCl in a heat block at  $110^{\circ}\text{C}$  for 24 hours, after which the amount of hydroxyproline was measured spectrophotometrically.<sup>21</sup> The extent of collagen degradation was expressed as micrograms of hydroxyproline per well.

### Gelatin Zymography

The medium from collagen gel incubations was mixed with nonreducing SDS sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 2% SDS, 0.002% bromophenol blue) and fractionated by SDS-polyacrylamide gel electrophoresis at  $4^{\circ}\text{C}$  on 10% gels containing 0.1% gelatin, as previously described.<sup>22,20</sup> The gels were then washed in 2.5% Triton X-100 for 1 hour, to promote recovery of protease activity, before incubation for 18 hours at  $37^{\circ}\text{C}$  in a reaction buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM  $\text{CaCl}_2$ , and 1% Triton X-100. The gels were finally stained with Coomassie brilliant blue.

### Immunoblot Analysis

The medium from collagen gel incubations was subjected to SDS-polyacrylamide gel electrophoresis on 12.5% gels under reducing conditions, and the separated proteins were then transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). After blocking of nonspecific sites, the mem-

branes were incubated with sheep antibodies to either rabbit MMP-1 or rabbit MMP-3. Immune complexes were detected with the use of an ECL kit (Amersham, Little Chalfont, UK).

### Assays of Proteolytic and Elastolytic Activities

The proteolytic and elastolytic activities of culture supernatant of *P. aeruginosa* were measured as previously described<sup>23,24</sup> with hide powder azure and elastin Congo red (Sigma), respectively, as substrates.

### Cytotoxicity Assay

Cytotoxicity was determined by measurement of lactate dehydrogenase (LDH) release. The medium from collagen gel incubations was assayed for LDH with an enzymatic assay based on the conversion of a tetrazolium salt to a red formazan product (LDH kit; Nippon Shoji, Osaka, Japan).

### Statistical Analysis

Data are expressed as means  $\pm$  SEM, and statistical analysis was performed with the Dunnett multiple comparison test or Student's *t*-test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Effect of Pseudomonal Culture Supernatant on Collagen Degradation

In the absence of cultured keratocytes, the addition of 4-aminophenyl mercuric acetate, an activator of proMMPs, to collagen gels did not induce collagen degradation (data not shown), indicating that zymogen forms of MMPs were not present as contaminants in the collagen preparation.

The effect of culture supernatant of elastase-producing *P. aeruginosa* strain PAO1 on collagen degradation was examined in the absence or presence of keratocytes. In the absence of the culture supernatant, collagen degradation was negligible either in the absence or presence of keratocytes. In the absence of keratocytes, collagen degradation was induced by the culture supernatant in a concentration-dependent manner (Fig. 1). In the presence of keratocytes, the concentration-dependent effect of the culture supernatant on collagen degradation

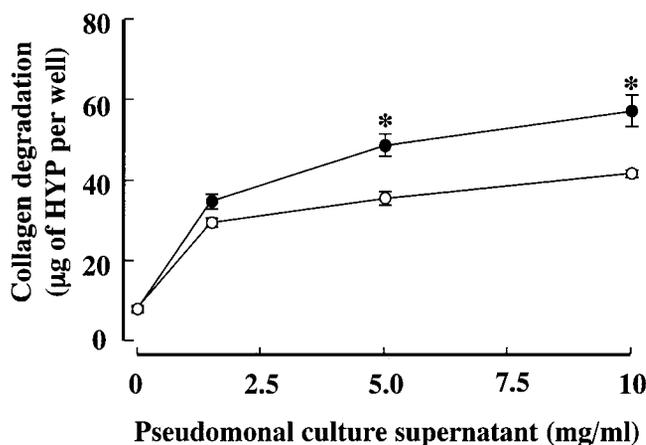
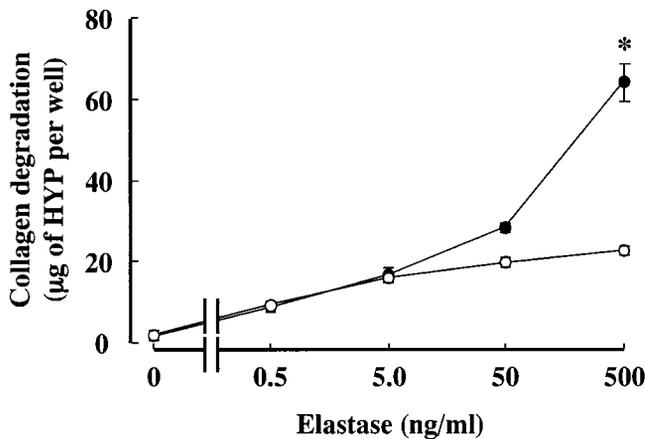


FIGURE 1. Effect of culture supernatant of *P. aeruginosa* PAO1 on collagen degradation. Collagen gels containing (●) or not containing (○) keratocytes were incubated for 24 hours in the presence of the indicated protein concentrations of culture supernatant of *P. aeruginosa* strain PAO1. The extent of collagen degradation was then determined and expressed as micrograms of hydroxyproline (HYP) per well. Data are means  $\pm$  SEM of three samples. \* $P < 0.05$  (Student's *t*-test) vs. the corresponding value for culture without keratocytes.



**FIGURE 2.** Effect of elastase on collagen degradation. Collagen gels containing (●) or not containing (○) keratocytes were incubated for 24 hours in the presence of the indicated concentrations of elastase, after which the extent of collagen degradation was determined. Data are means  $\pm$  SEM of three samples. \* $P < 0.01$  (Student's *t*-test) vs. the corresponding value for culture without keratocytes.

was significantly increased compared with that in their absence. Thus, the culture supernatant of *P. aeruginosa* strain PAO1 not only degraded collagen fibrils directly but also promoted keratocyte-mediated collagen degradation.

### Effect of Elastase on Collagen Degradation

To identify the factor (or factors) in the *P. aeruginosa* culture supernatant responsible for the effects on collagen degradation, we first examined the ability of pseudomonal elastase to mimic these effects. In the absence or presence of keratocytes, elastase increased the extent of collagen degradation in a dose-dependent manner; the amount of collagen degraded in the presence of elastase at a concentration of 500 ng/ml was significantly greater in the presence of keratocytes than in their absence (Fig. 2).

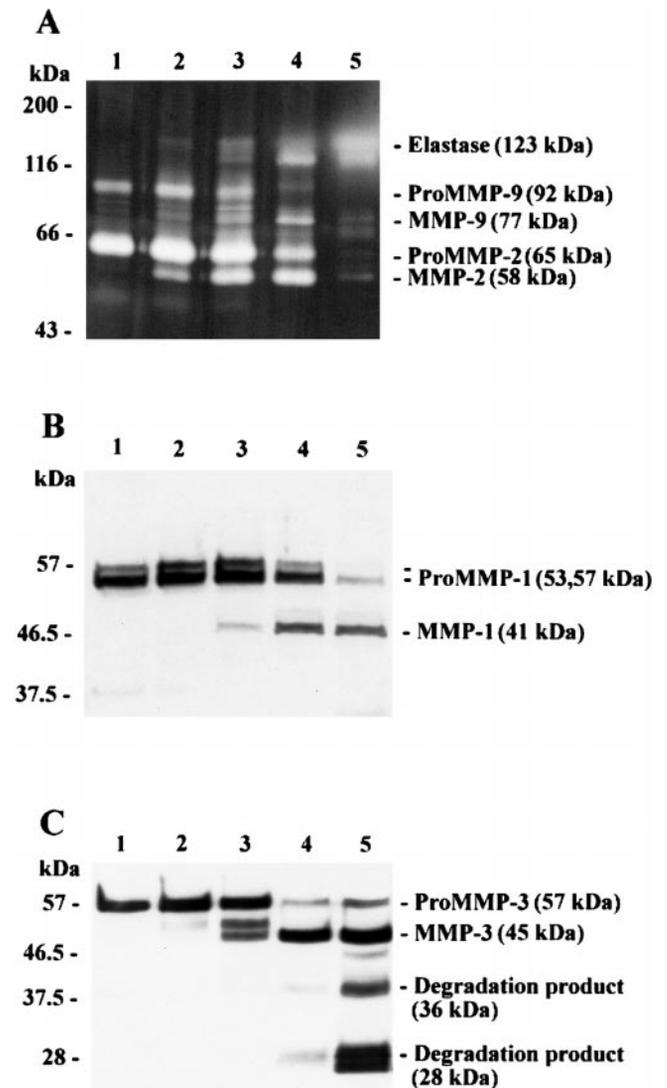
Gelatin zymography of the medium obtained after incubation of collagen gels containing keratocytes for 24 hours revealed two major bands of 92 and 65 kDa (Fig. 3A), corresponding to proMMP-9 and proMMP-2, respectively.<sup>25</sup> When incubations were performed in the additional presence of elastase, the bacterial protease induced a concentration-dependent decrease in the intensity of these two bands and the appearance of gelatinolytic bands at 77, 58, and 123 kDa, corresponding to active MMP-9, active MMP-2, and elastase, respectively. These results suggested that the proMMPs were activated by elastase. The gelatinolytic bands corresponding to proMMPs and active MMPs had virtually disappeared when incubations were performed in the presence of elastase at a concentration of 500 ng/ml, probably as a result of further proteolysis of MMPs by elastase.

Immunoblot analysis with antibodies to MMP-1 of the medium obtained after incubation of collagen gels containing keratocytes revealed a typical doublet of 57- and 53-kDa immunoreactive bands corresponding to proMMP-1 (Fig. 3B); the 57-kDa protein is a glycosylated form of the 53-kDa proMMP-1.<sup>26,27</sup> Elastase induced a dose-dependent decrease in the amount of proMMP-1 and the appearance of a 41-kDa band corresponding to active MMP-1. Similar immunoblot analysis with antibodies to MMP-3 revealed that keratocytes produced a 57-kDa immunoreactive protein corresponding to proMMP-3 (Fig. 3C). Elastase reduced the intensity of the 57-kDa band and induced the appearance of immunoreactive bands at 45, 36, and 28 kDa. The 45-kDa band corresponds to active MMP-3, and the bands at 36 and 28 kDa represent degradation prod-

ucts. These results thus suggested that both proMMP-1 and proMMP-3 produced by keratocytes were activated by elastase.

### Effect of Culture Supernatant of Elastase-Deficient *P. aeruginosa* on Collagen Degradation

To clarify further the role of elastase in collagen degradation induced by *P. aeruginosa*, we examined the effects of culture supernatant of the elastase-deficient mutants PAO-R1 and PAO-B1, in which *lasR* and *lasB*, respectively, are disrupted. Elastin Congo red and hide powder azure assays verified that the elastolytic and proteolytic activities, respectively, of culture supernatant of each of these mutants were greatly reduced compared with those of the parent strain PAO1 (Table 2), consistent with the original characterization of these mutants.<sup>23,28-30</sup> The collagenolytic activity assay revealed that, in contrast to the marked effects of PAO1-conditioned medium,



**FIGURE 3.** Effect of elastase on activation of proMMPs produced by keratocytes. Collagen gels containing keratocytes were incubated for 24 hours in the absence (lane 1) or presence of elastase at concentrations of 0.5, 5.0, 50, or 500 ng/ml (lanes 2 to 5, respectively). The culture medium was then analyzed by gelatin zymography (A) and immunoblot analysis either with antibodies to MMP-1 (B) or with antibodies to MMP-3 (C). The positions of molecular size standards are indicated (in kilodaltons) on the left, and those of elastase and MMPs on the right.

**TABLE 2.** Elastolytic and Proteolytic Activities by Elastin Congo Red Assay and Hide Powder Azure Assay, Respectively, of Culture Supernatant of *P. aeruginosa* Strains

Strain	Elastolytic Activity*	Proteolytic Activity†
PAO1	0.83 ± 0.01	10.28 ± 0.69
PAO-R1	0.11 ± 0.00	0.28 ± 0.03
PAO-B1	0.05 ± 0.01	0.42 ± 0.14

Data are means ± SEM of three samples.

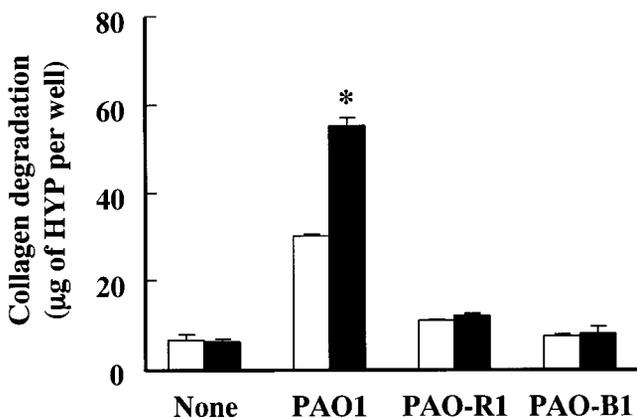
\* Absorbance at 492 nm per mg of protein.

† Absorbance at 570 nm per mg of protein.

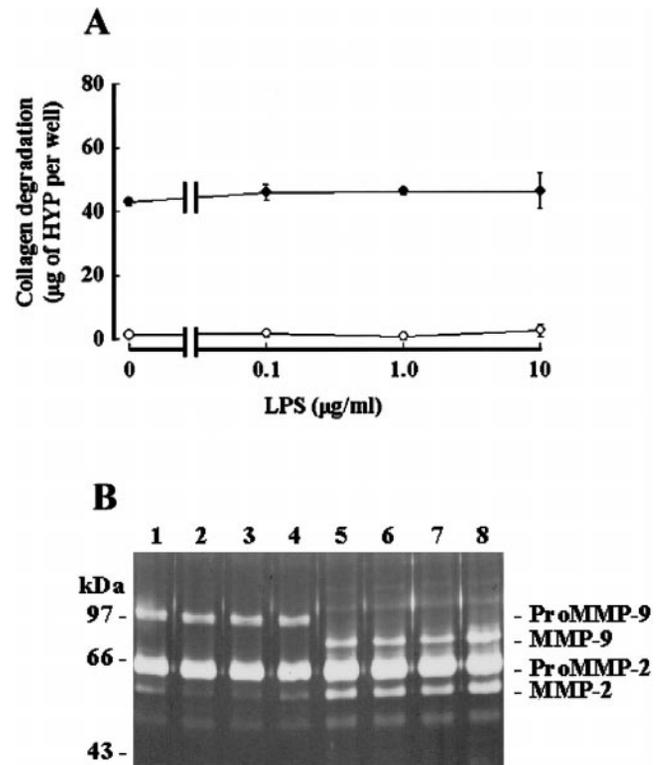
the medium conditioned by either PAO-R1 or PAO-B1 had no effect on collagen degradation in the absence or presence of keratocytes (Fig. 4).

### Effect of LPS on Collagen Degradation

We have previously shown that plasminogen, the precursor of plasmin, promotes collagen degradation by keratocytes in culture and that a plasminogen activator-plasmin system contributes to this process.<sup>20</sup> We next examined the effect of LPS on collagen degradation in the absence or presence of plasminogen (60 µg/ml). In the absence of keratocytes, the addition of LPS in the absence or presence of plasminogen had no effect on collagen degradation (data not shown). Although plasminogen increased the extent of collagen degradation mediated by keratocytes, LPS (at concentrations of up to 10 µg/ml) had no effect on keratocyte-mediated collagen degradation in the absence or presence of plasminogen (Fig. 5A). We also subjected the culture media from these various incubations to gelatin zymography. LPS alone had no effect on the zymographic pattern (two major bands corresponding to proMMP-9 and proMMP-2) observed with keratocytes (Fig. 5B). In the presence of plasminogen, the proMMP-9 band disappeared, and new bands appeared at 77 kDa (active MMP-9) and 58 kDa (active MMP-2); the further addition of LPS had no effect on this pattern of gelatinolytic activity. These results demonstrated that LPS alone had no effect on collagen degradation or gelatinolytic activity.



**FIGURE 4.** Effects of culture supernatant of elastase-deficient *P. aeruginosa* strains PAO-R1 or PAO-B1 on collagen degradation. Collagen gels containing (■) or not containing (□) keratocytes were incubated for 24 hours in the absence (none) or presence of culture supernatant of *P. aeruginosa* strains PAO1, PAO-R1, or PAO-B1 (protein concentration, 10 mg/ml). The extent of collagen degradation was then determined. Data are means ± SEM of three samples. \* $P < 0.01$  (Student's *t*-test) vs. the corresponding value for culture without keratocytes.



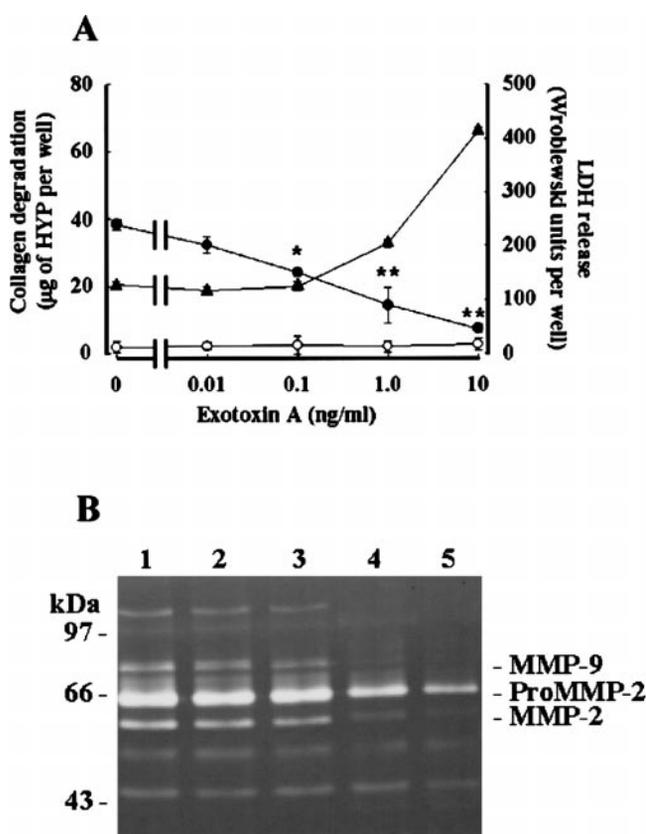
**FIGURE 5.** Effects of LPS on collagen degradation by and gelatinolytic activities of keratocytes. (A) Collagen gels containing keratocytes were incubated for 24 hours in the presence of LPS at the indicated concentrations either alone (○) or in the additional presence of plasminogen at a concentration of 60 µg/ml (●). The extent of collagen degradation was then determined. Data are means ± SEM of three samples. (B) Collagen gels containing keratocytes were incubated for 24 hours in the absence (lanes 1 to 4) or presence (lanes 5 to 8) of plasminogen (60 µg/ml) as well as in the absence (lanes 1 and 5) or presence of LPS at concentrations of 0.1 ng/ml (lanes 2 and 6), 1.0 ng/ml (lanes 3 and 7), or 10 ng/ml (lanes 4 and 8). The culture medium was then analyzed by gelatin zymography.

### Effect of Exotoxin A on Collagen Degradation

We finally examined the effect of exotoxin A on collagen degradation by keratocytes in the absence or presence of plasminogen (60 µg/ml). In the absence of keratocytes, exotoxin A (in the absence or presence of plasminogen) had no effect on collagen degradation (data not shown). In the absence of plasminogen, exotoxin A had no effect on keratocyte-mediated collagen degradation (Fig. 6A). In the presence of plasminogen, exotoxin A inhibited keratocyte-mediated collagen degradation in a dose-dependent manner. Measurement of LDH release by keratocytes revealed that exotoxin A exerted a dose-dependent cytotoxic effect. Gel zymography of the medium obtained after incubation of keratocyte-containing collagen gels in the presence of plasminogen and various concentrations of exotoxin A revealed that the toxin reduced the intensity of all gelatinolytic bands (proMMP-2, MMP-2, and MMP-9; Fig. 6B). These results indicated that exotoxin A inhibited both collagen degradation and MMP synthesis by keratocytes but that these effects were due to cytotoxicity.

### DISCUSSION

We have shown that culture medium conditioned by the elastase-producing strain PAO1 of *P. aeruginosa* not only directly degraded collagen fibrils but also promoted collagen degrada-



**FIGURE 6.** Effects of exotoxin A on collagen degradation and LDH release by and gelatinolytic activities of keratocytes. **(A)** Collagen gels containing keratocytes were incubated for 24 hours in the absence (○) or presence (●, ▲) of plasminogen (60 µg/ml) as well as in the presence of the indicated concentrations of exotoxin A. The extent of collagen degradation (●) and the amount of LDH release (expressed in Wroblewski units per well; ▲) were then determined. Data are means ± SEM of three samples. \* $P < 0.05$ , \*\* $P < 0.01$  (Dunnett multiple comparison test) vs. the corresponding value for culture in the absence of exotoxin A. **(B)** Collagen gels containing keratocytes were incubated for 24 hours in the presence of plasminogen (60 µg/ml) as well as in the absence (lane 1) or presence of exotoxin A at concentrations of 0.01, 0.1, 1.0, or 10 ng/ml (lanes 2 to 5, respectively). Incubation media were then subjected to gelatin zymography.

tion mediated by keratocytes. Of the three pseudomonal pathogenic factors examined, only elastase was able to induce collagen degradation by both direct and keratocyte-mediated mechanisms. Culture supernatants of the elastase-deficient mutants PAO-R1 and PAO-B1 did not affect collagen degradation in the absence or presence of keratocytes. The stimulation of keratocyte-mediated collagen degradation by elastase is likely attributable, at least in part, to the activation of proMMP-1, -2, -3, and -9.

Experiments with variants of *P. aeruginosa* have demonstrated a correlation between the production of proteases and severe corneal infection in mice.<sup>31</sup> Both elastase and alkaline protease have been isolated from *P. aeruginosa*-conditioned culture broth, and both proteases contribute to corneal destruction by degrading corneal proteoglycans, resulting in the dispersal of collagen fibrils.<sup>14</sup> Alkaline protease-deficient, non-elastolytic mutants of *P. aeruginosa* exhibit a loss of virulence, and the visible corneal damage induced by these mutants is markedly reduced.<sup>32</sup> Matsumoto et al.<sup>33</sup> showed that purified pseudomonal elastase cleaves proMMP-2 to yield its active form. Okamoto et al.<sup>34</sup> showed that purified elastase activates proMMP-1, -8, and -9 by limited proteolysis. Our present results

are consistent with these previous observations, indicating that elastase is an important mediator of collagen matrix degradation. Elastase also inactivates  $\alpha 1$ -proteinase inhibitor in vivo during pseudomonal diseases.<sup>35</sup> The loss of  $\alpha 1$ -proteinase inhibitor activity may allow endogenous serine proteases to induce tissue destruction. Given that serine proteinases activate matrix-degrading enzymes (MMPs), such a mechanism may contribute to elastase-induced degradation of the corneal stroma.

Exotoxin A is a potentially lethal toxin.<sup>15</sup> Both exotoxin A and diphtheria toxin inhibit mammalian protein synthesis by catalyzing the ADP-ribosylation of elongation factor 2.<sup>16</sup> In the present study, exotoxin A inhibited keratocyte-mediated collagen degradation as a result of its cytotoxicity. Thus, exotoxin A is unlikely to contribute to the degradation of corneal collagen associated with *P. aeruginosa* infection. LPS induces immune responses, including activation of the alternative complement cascade and the production of cytokines, and exposure of the abraded cornea to LPS results in acute red eye.<sup>36</sup> LPS has also been implicated in modulation of the adherence of *P. aeruginosa* to the cornea.<sup>17</sup> However, LPS did not affect the degradation of collagen in the present study.

Pathogenic factors other than elastase, exotoxin A, and LPS have also been associated with *P. aeruginosa* infection; these additional factors include exoenzyme S, phospholipase C, alkaline protease, alkaline phosphatase, leukocidin, and alginate.<sup>37,38</sup> Alkaline protease is an important corneal virulence factor.<sup>25</sup> Pseudomonal protease IV has also been implicated as an important determinant of corneal virulence, in part by the observation that intrastromal injection of an elastase-deficient *P. aeruginosa* strain induced corneal ulceration.<sup>39-42</sup> Purified elastase also induces corneal ulceration. We therefore propose that not only elastase but other factors, such as protease IV, contribute to infectious corneal ulceration and that elastase may mediate subsequent perforation of corneal ulcers. The possibility that these various pathogenic factors participate in collagen matrix degradation therefore warrants further investigation.

In the normal cornea, keratocytes are quiescent and inactive. However, corneal damage such as that caused by infection or surgical incision results in the activation of keratocytes at the wound edge. The activated keratocytes have the ability to migrate to the acellular zone of the cornea, to proliferate, and to synthesize ECM proteins and MMPs.<sup>4</sup> During the contraction phase of wound repair, myofibroblasts that have differentiated from the activated keratocytes and express  $\alpha$ -SMA appear in the wound area.<sup>43</sup> Expression of  $\alpha$ -SMA is thought to confer contractile properties on the myofibroblasts.<sup>44</sup> Transforming growth factor  $\beta$  induces the differentiation of cultured stromal fibroblasts into myofibroblasts. Culture of keratocytes in serum-supplemented medium also induces differentiation into myofibroblasts.<sup>19,45</sup> We detected the expression of  $\alpha$ -SMA in the passaged keratocytes used in the present study, indicating that these cells comprised myofibroblasts.

Cultured keratocytes produce proMMP-1, -2, -3, and -9. ProMMPs are activated by serine proteases such as plasmin.<sup>11</sup> We have previously shown that plasminogen promotes collagen degradation by keratocytes in culture, suggesting that a plasminogen activator-plasmin system contributes to this process.<sup>20</sup> Our present data suggest that the stimulatory effect of elastase on collagen degradation by keratocytes is attributable, at least in part, to the activation of keratocyte-derived proMMP-1, -2, -3, and -9.

*P. aeruginosa* is an important cause of destructive ocular diseases. Pseudomonal keratitis progresses rapidly and is characterized by infiltration of inflammatory cells and tissue destruction. This infection can result in corneal perforation, iris destruction, lens opacification, and endophthalmitis.<sup>46</sup> In the

last 10 years or so, corneal abrasions induced by contaminated contact lenses or produced during the application of contaminated cosmetics have become a major cause of pseudomonal keratitis.<sup>47-49</sup> Trauma, herpes simplex virus infection, and immunosuppression also facilitate the establishment of pseudomonal keratitis.<sup>47,50,51</sup> Pathogenic factors secreted by *P. aeruginosa* induce the activation of proMMPs produced by keratocytes and polymorphonuclear leukocytes. Such protease activation and consequent collagen degradation may persist even after the bacteria have been eliminated. Thus, the administration of antibiotics at this stage is unlikely to affect corneal keratitis that persists as a result of proteases already released from bacteria and the consequent activation of proMMPs and degradation of the ECM. Instead, treatment with drugs able to inhibit pseudomonal elastase activity might prove therapeutically beneficial.

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