Cornea

Plasminogen-Dependent Collagenolytic Properties of *Staphylococcus aureus* in Collagen Gel Cultures of Human Corneal Fibroblasts

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**PURPOSE.** *Staphylococcus aureus* is a common cause of corneal ulceration, and staphylokinase (SAK) produced by this bacterium is a plasminogen activator. To investigate the pathogenesis of corneal ulceration induced by *S. aureus*, we examined the effects of bacterial culture broth and SAK on collagen degradation in a culture model in which human corneal fibroblasts are embedded in a collagen gel.

**METHODS.** Corneal fibroblasts embedded in collagen were exposed to *S. aureus* culture broth or SAK. Collagen degradation was assessed by measurement of hydroxyproline in acid hydrolysates of culture supernatants. Expression of pro–matrix metalloproteinase–1 (pro–MMP-1) was detected by immunoblot analysis as well as reverse transcription and real-time polymerase chain reaction analysis.

**RESULTS.** Both *S. aureus* culture broth and SAK markedly increased collagen degradation in the presence of corneal fibroblasts and plasminogen. This effect of the culture broth was dependent on cell number to a greater extent than was that of SAK. Whereas the culture broth also increased the expression of pro–MMP-1 in corneal fibroblasts at both mRNA and protein levels, SAK did not.

**CONCLUSIONS.** Our results suggest that *S. aureus* may promote collagen degradation both by upregulating pro-MMP1 expression in corneal fibroblasts, with pro–MMP-1 then being converted to active MMP-1 by plasmin, and by directing plasmin activity toward collagen in a SAK-dependent manner.

Keywords: *S. aureus*, staphylokinase, collagen degradation, corneal fibroblasts, plasminogen

**Staphylococcus aureus** is one of the most common causes of corneal ulceration worldwide.1 The clinical features of keratitis caused by *S. aureus* are variable, but the bacterium often induces corneal stromal melting and extensive inflammation that result in ulceration. A condition known as peripheral infiltrates or catarhal ulcer can also arise at the peripheral superficial cornea and is thought to reflect hypersensitivity or a toxic response to bacteria.2 with the presence of *S. aureus* having been detected at the lid margin in eyes affected by this condition.3–5 The various clinical features of ocular disease likely result from complex interactions among factors released from bacteria as well as humoral factors such as plasminogen and cellular components such as corneal fibroblasts and infiltrated inflammatory cells of the host.

Both bacterial factors and host components have been shown to contribute to the tissue destruction associated with the pathogenesis of bacterial keratitis.6 The production of collagen-degrading enzymes by both bacteria and host cells is thus thought to be relevant to the progression of bacterial corneal ulcers. Although collagen is resistant to most proteolytic enzymes, it is susceptible to degradation by bacterial collagenases and certain mammalian matrix metalloproteinases (MMPs).7 We have previously demonstrated the potential operation of both direct and indirect pathways for degradation of the corneal stroma in keratitis due to *Pseudomonas aeruginosa*.8 *Pseudomonas* bacteria thus release a collagenase that directly degrades stromal collagen and can give rise to ulceration, while another bacterial enzyme, elastase, activates collag enolytic pro-MMPs released from resident corneal stromal cells.8

Keratocytes are the resident mesenchyme-derived cells of the corneal stroma, and they play a key role in collagen degradation in the infected cornea.9–13 These cells thus produce and release pro-MMPs in response to infectious microorganisms.14–16 Among MMPs, MMP-1 plays a major role in collagen degradation. MMP-1 is produced and secreted by keratocytes and contributes to destruction of the corneal stroma by mediating the digestion of collagen fibrils.14,15 We have previously shown that plasminogen present in the extracellular environment is converted to plasmin by urokinase-type plasminogen activator (uPA) that is also synthesized and released by keratocytes, with this synthesis and release being stimulated by the proinflammatory cytokine interleukin (IL)-1β.16 Furthermore, IL-1β-induced collagen degradation by
these cells was found to be strictly dependent on uPA expression and mediated by a uPA-plasmin-MMP pathway. The plasmin generated from plasminogen thus mediates the proteolytic activation of released pro-MMPs, rendering collage- nolytic MMPs competent to degrade extracellular collagen. In the clinical setting, the expression of uPA was detected in keratocytes of a patient with corneal ulcer by immunofluorescence analysis. S. aureus releases various factors—including coagulase, staphylokinase (SAK), extracellular proteases (aureolysin, V8 protease, staphopain), and α-, β-, and γ-toxins—that subvert host homeostasis. In contrast to P. aeruginosa, the factors derived from S. aureus do not directly mediate extracellular degradation of collagen. However, SAK has been found to act as a plasminogen activator (PA). Although the function of SAK appears identical to that of uPA in terms of the conversion of plasminogen to plasmin, the activation mechanisms of SAK and uPA differ. Whereas uPA directly converts plasminogen to plasmin enzymatically, SAK does not possess proteolytic activity. Instead, SAK forms a complex with plasmin (but not with plasminogen), with this association rendering the active site of plasmin reactive toward plasminogen. With regard to the pathobiology of corneal stromal melting caused by S. aureus, it is highly unlikely that S. aureus directly degrades collagen. The cellular components of the corneal stroma, including the resident stromal cells and infiltrated inflammatory cells, might therefore play an essential role in collagen degradation through the release of uPA and pro-MMPs. We also hypothesized that SAK released by S. aureus might contribute to collagen degradation in the corneal stroma. To provide insight into the mechanism of collagen degradation associated with S. aureus infection, we have therefore now examined the effects of S. aureus culture broth and purified SAK on collagen degradation in a culture model in which human corneal fibroblasts (activated keratocytes) are maintained in a collagen gel.

METHODS

Cell Isolation

Corneal fibroblasts were isolated from the corneoscleral rim of tissue obtained for corneal transplantation surgery from The Eye-Bank for Sight Restoration (New York, NY, USA). The tissue was used in accordance with the tenets of the Declaration of Helsinki. The cells were prepared and maintained as described previously. In brief, the endothelial layer of the cornea was removed mechanically, and the remaining tissue was incubated for 1 hour at 37°C with dispase (2 mg/mL; Sigma-Aldrich Corp., St. Louis, MO, USA) in minimum essential medium (MEM). After removal of the epithelial sheet, the tissue was incubated for 5 hours at 37°C with collagenase A of Clostridium histolyticum (2 mg/mL in MEM; Sigma-Aldrich Corp.) in order to obtain a single-cell suspension. The isolated corneal fibroblasts were maintained under 5% CO₂ at 37°C in MEM supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA), and they were harvested for experiments after four to six passages.

Bacteria and Culture

S. aureus 6538P (ATCC, Manassas, VA, USA) was cultured for 16 hours at 37°C on brain heart infusion agar plates (Eiken, Tochigi, Japan). Single colonies were then cultured for 18 hours at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) to late log phase. Growth of the bacteria was monitored by measurement of optical density at 600 nm with the culture being terminated when the optical density reached a value of 2.0, corresponding to 2 × 10⁶ colony forming units (CFU)/mL. The cell suspension was centrifuged at 6000g for 10 minutes, and the resulting supernatant was passed through a 0.22-µm syringe filter to remove bacteria before addition to collagen gel cultures of corneal fibroblasts. DMEM without bacteria was used as a control (0% culture broth). The maximum concentration of culture broth tested in the present study was 10%, corresponding to a bacterial load of 2 × 10⁸ CFU/mL. A previous study found that S. aureus at 1 × 10⁷ CFU/mL did not cause evident corneal infection in rabbits. A bacterial load of 5 × 10⁶ CFU/mL was found to be required for corneal infection in an organ culture system. The concentrations of culture broth examined in our experiments therefore appear to be relevant to the pathogenesis of S. aureus infection of the cornea.

Three-Dimensional Culture of Corneal Fibroblasts in a Collagen Gel Matrix

Culture of corneal fibroblasts in a collagen gel was performed as previously described. In brief, type 1 collagen (Nitta Gelatin, Osaka, Japan) was mixed with 10× MEM and neutralized with 0.2 M NaOH before the addition of corneal fibroblasts to a final density of 1 × 10⁵ cells/mL and a final collagen concentration of 2 mg/mL. Portions (300 µL) of the cell suspension were transferred to the wells of a 24-well tissue culture plate, which was then incubated for 1 hour at 37°C to allow gel formation. MEM (300 µL) with or without test agents—including human plasminogen (Hyphen BioMed, Paris, France), GM6001 (Calbiochem, La Jolla, CA, USA), α2-antiplasmin (Molecular Innovations, Novi, MI, USA), the selective uPA inhibitor uPA-STOP (American Diagnostica, Stamford, CT, USA), SAK (Cell Sciences, Newburyport, MA, USA), S. aureus culture broth, recombinant human uPA (R&D Systems, Minneapolis, MN, USA), or recombinant human IL-1β (R&D Systems) was then overlaid on each collagen gel.

Sample Preparation

Culture supernatants from cells cultured in three-dimensional collagen gels were collected for assay of collagen degradation. Cell lysates were prepared from corneal fibroblasts cultured in collagen gels as previously described. In brief, the cells embedded in each gel were pulverized in 200 µL extraction buffer (10 mM sodium phosphate buffer [pH 7.2], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.2% NaN₃), and the samples were centrifuged at 17,000g for 5 minutes at 4°C. The resulting supernatants (10 µg protein) were then examined by fibrin zymography and immunoblot analysis.

Assay of Collagen Degradation

The amount of collagen degradation products in culture supernatants was measured as previously described. In brief, nondegraded collagen was first removed from the supernatants by ultrafiltration, the filtrates were then subjected to hydrolysis for 24 hours at 110°C with 6 M HCl, and the amount of hydroxyproline in the hydrolysates was measured by spectrophotometry.

Fibrin Zymography

PA activity of corneal fibroblasts was examined by fibrin zymography as previously described. In brief, cell lysates of corneal fibroblasts cultured in collagen gels as well as molecular markers were subjected to electrophoresis on a
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**RESULTS**

**Effect of *S. aureus* Culture Broth on Collagen Degradation**

We first examined the effect of *S. aureus* culture broth on collagen degradation in the absence or presence of corneal fibroblasts and plasminogen (Fig. 1). In the absence of plasminogen, collagen degradation induced by *S. aureus* culture broth was negligible regardless of the absence or presence of corneal fibroblasts. In the presence of plasminogen, however, the extent of collagen degradation induced by *S. aureus* culture broth increased in a concentration-dependent manner up to a concentration of 1%. Moreover, the additional presence of corneal fibroblasts resulted in a further marked increase in the extent of collagen degradation in the presence of plasminogen, and this effect was also dependent of the...
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Collagen gels with or without embedded corneal fibroblasts were incubated for 24 hours in the absence or presence of plasminogen (plg, 50 \(\mu\text{g/mL}\)) and with the indicated concentrations of SAK. The amount of degraded collagen was then determined. Data are mean \(\pm\) SEM from three separate experiments. *\(P < 0.05\) versus the corresponding value for the absence of SAK; †\(P < 0.05\) versus the corresponding value for each of the other three groups (Dunnett’s multiple comparison test).

![Figure 2](image)

**Figure 2.** Effects of SAK, corneal fibroblasts, and plasminogen on collagen degradation. Collagen gels with or without embedded corneal fibroblasts were incubated for 24 hours in the absence or presence of plasminogen (plg, 50 \(\mu\text{g/mL}\)) and with the indicated concentrations of SAK. The amount of degraded collagen was then determined. Data are mean \(\pm\) SEM from three separate experiments. *\(P < 0.05\) versus the corresponding value for the absence of SAK; †\(P < 0.05\) versus the corresponding value for each of the other three groups (Dunnett’s multiple comparison test).

![Figure 3](image)

**Figure 3.** Effects of plasminogen and \textit{S. aureus} culture broth on collagen degradation by corneal fibroblasts. Collagen gels embedded with corneal fibroblasts were incubated for 24 hours in the absence or presence of \textit{S. aureus} culture broth 1% and with the indicated concentrations of plasminogen. The amount of degraded collagen was then determined. Data are mean \(\pm\) SEM from three separate experiments. *\(P < 0.05\), **\(P < 0.01\) versus the corresponding value for the absence of plasminogen; †\(P < 0.05\) versus the corresponding value for the absence of \textit{S. aureus} culture broth (Dunnett’s multiple comparison test).

**Effect of Plasminogen on Collagen Degradation by Corneal Fibroblasts**

We examined the effect of plasminogen concentration on collagen degradation by corneal fibroblasts (Fig. 3). In the absence of plasminogen, S. aureus increased collagen degradation by corneal fibroblasts in a concentration-dependent manner up to a concentration of 50 \(\mu\text{g/mL}\). Moreover, this concentration-dependent effect of plasminogen was markedly more pronounced in the presence of culture broth.

**Effects of GM6001, \(\alpha2\)-Antiplasmin, and a uPA Inhibitor on Collagen Degradation in the Presence of \textit{S. aureus} Culture Broth and Corneal Fibroblasts**

In our culture system, the conversion of exogenous plasminogen to plasmin by endogenous uPA and the activation of pro-MMPs by plasmin is a primary pathway for collagen degradation by corneal fibroblasts.\textsuperscript{14,15} To elucidate the mechanism by which \textit{S. aureus} culture broth might stimulate collagen degradation by corneal fibroblasts, we examined the effects of the broad-spectrum MMP inhibitor GM6001 (10 \(\mu\text{g/mL}\)), \(\alpha2\)-antiplasmin (50 \(\mu\text{g/mL}\)), and a uPA inhibitor (0.1 \(\mu\text{g/mL}\)) on collagen degradation in the presence of culture broth (1%), corneal fibroblasts, and plasminogen (Fig. 4A). The extent of collagen degradation was significantly, but not completely, attenuated by GM6001, with the residual collagen degradation being essentially prevented by the further removal of plasminogen. These results suggested that the residual collagen degradation was mediated by \textit{S. aureus} culture broth in a manner independent of MMPs. On the other hand, \(\alpha2\)-antiplasmin and the uPA inhibitor each completely blocked collagen degradation in the presence of culture broth, corneal fibroblasts, and plasminogen. These inhibitory effects of GM6001, \(\alpha2\)-antiplasmin, and the uPA inhibitor were concentration dependent (Fig. 4B). Together, these data suggested that \textit{S. aureus} culture broth promotes collagen degradation both directly by plasmin generated from plasminogen as well as...
indirectly via the plasminogen-dependent activation of pro-MMPs released from corneal fibroblasts.

Effects of GM6001, α2-Antiplasmin, and a uPA Inhibitor on Collagen Degradation in the Presence of SAK and Corneal Fibroblasts

Whereas α2-antiplasmin and a uPA inhibitor each prevented collagen degradation in the presence of SAK (1 μg/mL), corneal fibroblasts, and plasminogen, GM6001 had no effect on the amount of collagen degraded (Fig. 5). These results suggested that the stimulatory effect of SAK on collagen degradation in the presence of corneal fibroblasts is dependent on the uPA-plasminogen system but not on the activation of MMPs.

Effects of Corneal Fibroblast Density on Collagen Degradation in the Presence of S. aureus Culture Broth or SAK

We next examined the effects of corneal fibroblast density on collagen degradation in the presence of plasminogen and either S. aureus culture broth (1%) or SAK (1 μg/mL) (Fig. 6). In the absence of corneal fibroblasts, the addition of S. aureus culture broth, but not that of SAK, induced collagen degradation, indicating that SAK by itself does not possess collagenolytic activity and that culture broth contains various factors released from the bacteria that promote collagen degradation. In the presence of SAK, the addition of corneal fibroblasts significantly increased the amount of degraded collagen, but this effect was not dependent on cell density above 1 × 10^3 cells per well, suggesting that the effect of SAK is dependent on corneal fibroblasts or cellular factors but that this dependence is saturated at low cell densities. On the other hand, in the presence of culture broth, the addition of corneal fibroblasts increased the amount of degraded collagen in a manner dependent on cell density. Together, these results thus indicated that S. aureus culture broth and SAK may promote collagen degradation through different mechanisms.

Effect of Exogenous uPA on Collagen Degradation in the Presence of SAK and Plasminogen

To examine whether uPA produced by corneal fibroblasts might be necessary for the stimulatory effect of SAK on collagen degradation in the presence of these cells and plasminogen, we tested the effect of exogenous uPA on collagen degradation in the presence of SAK (1 μg/mL) and plasminogen but in the
absence of corneal fibroblasts. We found that the addition of uPA at 0.001 μg/mL markedly increased the extent of collagen degradation under these conditions, but that higher concentrations of uPA did not have a further stimulatory effect, with uPA at 1 μg/mL actually having no effect at all (Fig. 7). These results suggested that the stimulatory effect of SAK on collagen degradation in the presence of corneal fibroblasts and plasminogen may be dependent on uPA produced by these cells. The reason for the lack of effect of uPA at the highest concentration tested remains unclear, although it is possible that the high level of uPA sequesters plasmin and thereby prevents its association with SAK.

Effects of SAK, S. aureus Culture Broth, and IL-1β on uPA Production by Corneal Fibroblasts

We then examined the effects of SAK (1 μg/mL) and S. aureus culture broth (1%) on uPA production by corneal fibroblasts cultured in a collagen gel. Given that we previously showed that IL-1β increases uPA production by corneal fibroblasts in collagen gel cultures,16 we also examined the effect of IL-1β as a positive control. Cell lysates were prepared and analyzed for PA activity by fibrin zymography. The expression of uPA in corneal fibroblasts was significantly increased in the presence of S. aureus culture broth or IL-1β but not by the addition of SAK (Figs. 8A, 8B). RT and real-time PCR analysis also revealed that S. aureus culture broth and IL-1β each induced a marked
increase in the amount of uPA mRNA in corneal fibroblasts, whereas SAK had no such effect (Fig. 8C).

**DISCUSSION**

We have shown here that SAK mediates an important pathway of collagen degradation elicited by *S. aureus* infection, likely through formation of a complex with plasmin and activation of its collagenolytic activity (Fig. 10). This pathway appears to require uPA released from corneal fibroblasts to convert plasminogen to plasmin, and it operates in addition to a pathway by which factors released by *S. aureus* upregulate the expression of pro–MMP-1 at the mRNA and protein levels in corneal fibroblasts, with the released pro–MMP-1 protein being activated in the presence of plasmin. Furthermore, factors released by *S. aureus* directly mediate collagen degradation in the presence of plasmin in a manner independent of corneal fibroblasts.

In the present study, we examined the effects of *S. aureus* culture broth at a maximal concentration of 10%, which corresponds to a bacterial load of $2 \times 10^8$ CFU/mL. A previous study found that *S. aureus* at $1 \times 10^7$ CFU/mL did not cause evident corneal infection in rabbits, whereas $5 \times 10^8$ CFU/mL *S. aureus* were required for infection of the cornea in an organ culture model. The concentrations of culture broth examined in our experimental model thus appear to be relevant to the pathogenesis of corneal infection by *S. aureus*. As far as we
are aware, the concentration of SAK in tear fluid or in corneal ulcers caused by *S. aureus* infection has not been measured. However, *S. aureus* is an indigenous bacterium in nasal passages, with 67% of healthy individuals having been found to possess detectable levels of SAK (0.4–3.7 μg/mL) in nasal secretions. The maximal concentration of SAK in culture supernatants of *S. aureus* at 2 × 10^9 CFU/mL was found to be ∼1.0 μg/mL. Given that a concentration of *S. aureus* culture broth of 10% likely corresponds to a SAK concentration of ∼0.1 μg/mL and that culture broth likely contains additional factors that affect collagen degradation in our system, we examined the effects of purified SAK at concentrations of 0.1 to 10 μg/mL and detected a significant effect on collagen degradation at 0.1 μg/mL.

Both *S. aureus* culture broth and SAK increased collagenolytic activity in corneal fibroblast cultures in a manner dependent on the presence of plasminogen, with this effect of *S. aureus* culture broth being more pronounced than that of SAK. The combination of culture broth and plasminogen, with or without SAK, increased the production of pro-MMP-1 by corneal fibroblasts in the absence or presence of plasminogen, whereas SAK had no such effect. Moreover, *S. aureus* culture broth increased the production of uPA by corneal fibroblasts, whereas SAK again had no such effect. These observations suggest that *S. aureus* culture broth, which contains SAK, stimulates collagen degradation by increasing the production

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**Figure 9.** Effects of *S. aureus* culture broth, SAK, IL-1β, and plasminogen on the production and activation of pro–MMP-1 in corneal fibroblast cultures. (A, B) Cells were cultured for 24 hours in collagen gels and in the absence or presence of plasminogen (50 μg/mL, plg), SAK (1 μg/mL), *S. aureus* culture broth (1%), or IL-1β (5 ng/mL), as indicated, after which cell lysates were subjected to immunoblot analysis with antibodies to MMP-1 and to β-actin (loading control). Representative results (A) and quantitative data for the pro–MMP-1/β-actin ratio or active MMP-1/β-actin ratio (mean ± SEM) from three independent experiments (B) are shown. *P < 0.05* (unpaired Student’s t-test) versus the value for cells incubated without addition (top) or with plasminogen alone (bottom). (C) Cells were cultured for 24 hours in collagen gels and in the absence or presence of plasminogen (50 μg/mL), SAK (1 μg/mL), *S. aureus* culture broth (1%), or IL-1β (5 ng/mL), after which total RNA was isolated from the cells and subjected to RT and real-time PCR analysis of pro–MMP-1 mRNA. Data were normalized by the amount of β-actin mRNA and are mean ± SEM from three separate experiments. **P < 0.01** versus the value for cells incubated without addition (Student’s unpaired t-test).
and release of pro–MMP-1 by corneal fibroblasts, with the released protein then being activated by plasmin generated from plasminogen by cell-derived uPA. In addition to this MMP-dependent pathway, SAK released from S. aureus binds to plasmin generated from plasminogen by uPA and forms a complex that also catalyzes the conversion of plasminogen to plasmin.22,23 Our results also suggest that formation of the SAK-plasmin complex may promote the direct degradation of collagen by plasmin.

The clinical characteristics of bacterial corneal ulcers differ depending on the type of infecting bacteria.33 Such differences might be due in part to differences in the enzymes released from the bacteria. For example, P. aeruginosa releases proteases that degrade collagen.8,34 In contrast, although S. aureus secretes various proteases, none of these enzymes has been shown to possess collagenolytic activity in corneal ulcers.35 We have here shown that plasminogen was essential for collagen degradation associated with S. aureus in the absence or presence of corneal fibroblasts. Moreover, we found that the addition of S. aureus bacteria to the medium of empty collagen gels did not result in collagen degradation in the absence of plasminogen (data not shown), providing further support for the notion that S. aureus does not secrete collagenolytic agents.

In the clinical setting, SAK is a potential therapeutic target for the amelioration or prevention of corneal ulcer associated with S. aureus infection. The antimicrobial peptide α-defensin was shown to neutralize SAK activity.36 Topical application of α-defensin might thus attenuate the degradation of stromal collagen associated with S. aureus infection.

In summary, our results suggest the operation of two key pathways for collagen degradation associated with S. aureus infection of the cornea, one of which is dependent on MMP production by corneal fibroblasts and the other is mediated by SAK-dependent plasmin activation. We found that plasminogen was essential for the activity of both these pathways.

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