Regulation of MMP-9 Activity in Human Tear Fluid and Corneal Epithelial Culture Supernatant

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PURPOSE. To evaluate human corneal epithelial culture supernatant and tear fluid for the presence of activators and inhibitors of matrix metalloproteinase (MMP)-9, MMP-3, and tissue inhibitor of metalloproteinase (TIMP)-1, respectively, and to evaluate the effect of MMP-3 on the activation of MMP-9 in these specimens.

METHODS. Unstimulated tear fluid was collected from patients with ocular rosacea and normal control subjects. Levels of MMP-9, MMP-3, and TIMP-1 were determined by enzyme-linked immunosorbent assay (ELISA) and/or immunoblot analysis. Supernatants from primary human corneal epithelial cultures and human tear fluid were incubated with MMP-3. Cultured epithelial cells and their supernatants were also treated with doxycycline before MMP-3 was added. Gelatin zymography was used to identify activated 82-kDa MMP-9. MMP-9 activity was assessed with a commercial MMP-9 activity assay system.

RESULTS. MMP-9 and TIMP-1 were detected at significantly higher concentrations in rosacea-affected than in normal tear fluids. MMP-3 was detected exclusively in the tear fluid of patients with ocular rosacea who had corneal epithelial disease. Treatment of the supernatant and tear fluid with MMP-3 resulted in two bands with molecular weights of 92 kDa and 82 kDa, representing pro-MMP-9 and activated MMP-9, respectively. Doxycycline added to the conditioned media did not affect activation of MMP-9 by MMP-3. However, 24-hour treatment of corneal epithelial cultures with doxycycline resulted in a lower concentration and activity of MMP-9 in their supernatants.

CONCLUSIONS. MMP-9 and TIMP-1 are produced by the human corneal epithelium and are present in tear fluid. MMP-3 alone is sufficient to activate MMP-9 on the ocular surface. Doxycycline does not directly inhibit this activation by MMP-3, but it decreases MMP-9 activity when added to corneal epithelial cultures. (Invest Ophthalmol Vis Sci. 2000;41:1703–1709)

Matrix metalloproteinase (MMP)-9 (gelatinase B) is the primary matrix-degrading enzyme produced by the corneal epithelium.1 We have found significantly greater MMP-9 activity in the tear fluid of patients with the tear film disorder ocular rosacea than in normal control subjects.2 Most of this enzyme was detected in its 92-kDa pro form, although the active 82-kDa active form was also detected in half of the patients with rosacea and in none of the control subjects.2 MMP-9 activity in the tear fluid was directly correlated with tear fluid interleukin (IL)-1α concentration and was inversely correlated with tear fluorescein clearance.2

Ocular rosacea and other dry eye conditions are associated with an increased incidence of recurrent corneal epithelial erosion (RCEE) and sterile corneal stromal ulceration.3,4 RCEE has been reported to occur in up to 12% of patients with ocular rosacea.3,4 We have experienced clinical success in treating patients with RCEE associated with rosacea with doxycycline, a compound with documented anti-collagenolytic activity.5 MMP-9 produced by the corneal epithelium has been found to impede re-epithelialization of the cornea after experimental thermal injury in animal models.6 MMP-9 is secreted from cells as a 92-kDa proenzyme.7 Physiological mechanisms exist for suppressing and promoting activation of this enzyme. For example, tissue inhibitors of metalloproteinases (TIMPs) bind the proenzyme and inactivate it.8 In contrast, MMP-3 (stromelysin-1) has been reported to be an efficient activator of MMP-9.9

The purpose of this study was to evaluate human tear fluid from normal subjects and patients with ocular rosacea, with and without corneal epithelial disease, as well as conditioned media from primary human corneal epithelial cultures for the presence of TIMP-1 and MMP-3. We also evaluated the ability of MMP-3 to activate MMP-9 in these fluids and the effect of doxycycline on MMP-3 activation of MMP-9 in a corneal epithelial culture system.
**MATERIALS AND METHODS**

This study was conducted according to a protocol approved by the Institutional Review Board of the University of Miami School of Medicine and in accordance with the tenets of the Declaration of Helsinki. Informed consents were obtained from volunteers and patients after the nature and possible consequences of the study were explained to them. Tear fluid samples were collected from two groups of subjects. The first group consisted of patients with the diagnosis of meibomian gland disease associated with rosacea and a Schirmer 1 test score less than 10 mm. The diagnostic criteria for rosacea have been reported. The second group consisted of control subjects of similar age and gender distribution with no history of eye disease or ocular surgery, use of eye drops, or symptoms of ocular irritation. For detection of TIMP-1 and pro-MMP-9, tear fluid was collected from 15 patients with ocular rosacea and 8 normal control subjects. For detection of pro-MMP-3, tears were collected from 11 eyes of six patients with ocular rosacea and from 5 eyes of five normal control subjects. In the rosacea group, two eyes had epithelial basement membrane dystrophy, two eyes had RCEEs, and one eye had a nonhealing epithelial defect and sterile stromal thinning. For gelatin zymography to evaluate MMP-3 activation, tear fluid was taken from two normal control subjects who had detectable pro-MMP-9 in their tears.

**Tear Collection**

Tear fluid was collected from the inferior tear meniscus, causing the least irritation possible, using a preweighed polyester wick (Transorb rods; American Filtrona, Richmond, VA) to obtain the sample as previously described. Wicks were then placed into the end of a micropipette tip located within a 0.5-ml tube (Eppendorf, Fremont, CA). The volume of collected tears was determined by reweighing the tubes containing the least irritation possible, using a preweighed polyester wick (Transorb rods; American Filtrona, Richmond, VA) to obtain the sample as previously described. Wicks were then placed into the end of a micropipette tip located within a 0.5-ml tube (Eppendorf, Fremont, CA). The volume of collected tears was determined by reweighing the tubes containing the rods immediately after tear collection (model GA110 scale; Ohaus, Florham Park, NJ). The tear samples were then stored at −80°C until they were used for enzyme-linked immunosorbent assay (ELISA) or zymography.

**Sample Extraction**

Immediately before the tears were used for ELISA or zymography, the samples were maintained at room temperature for 30 minutes. The tears were then extracted from the saturated wicks by centrifuging at 12,000 rpm for 5 minutes within the pipette tip, after adding a volume of buffer (ELISA buffer from manufacturer; zymography buffer: 50 mM Tris/HCl, 0.15 M NaCl, 10 mM CaCl₂, 0.005% Brij 35 (23 lauryl ether; Sigma, St. Louis, MO), 0.02% sodium azide [pH 7.5]). The volume of buffer added to each tube brought the final volume of tears plus buffer to 20 µl. For example, if the volume of collected tears was 2 µl, 18 µl of buffer was added. The rods and pipette were carefully removed, and the tear fluid aspirated.

**Corneal Epithelial Cell Culture**

Human corneal limbal epithelium was cultured from explants of human donor corneoscleral rims preserved in modified McCoy–Kauffman medium that was provided by the Florida Lions Eye Bank. Each corneoscleral rim was trimmed, the endothelial layer and iris remnants removed, and the tissue treated with dispase for 15 minutes. Each rim was dissected into 12 equal parts. Each piece of limbus was placed in a single well of a six-well plastic culture plate and covered with a drop of fetal bovine serum overnight. The explants were cultured in medium containing equal amounts of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium, enriched with 5% fetal bovine serum, 1% ITS (insulin, transferrin, selenium), hydrocortisone, epidermal growth factor, and cholera toxin. After 10 to 14 days of incubation, the cells were trypsinized and seeded onto 24-well plates at a density of 10⁵ cells/well. More than 95% of these passaged cells stained positively for acidic cytokeratins with monoclonal antibody AE-1 (ICN, Costa Mesa, CA), confirming that they were epithelial cells. First-passaged epithelial cells were cultured until confluence and then switched to serum-free medium for 24 hours before the supernatant was harvested. The viability of these cultured cells was confirmed with an MTS [(3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium] inner salt] assay (CellTitier 96 Aqueous; Promega, Madison, WI).

**Pro-MMP-3, Pro-MMP-9, and TIMP-1 ELISA**

ELISAs for pro-MMP-3, pro-MMP-9, and TIMP-1 were performed using commercial kits (Oncogene, Cambridge, MA). Tear fluid samples were further diluted in the supplier-provided assay buffer to a final volume of 50 µl for pro-MMP-3 and 100 µl for pro-MMP-9 and TIMP-1. Human corneal epithelial cell supernatants were also assayed for pro-MMP-3 and TIMP-1 with a final volume of 200 µl per well. Samples were incubated for 2 hours at room temperature for 18 hours at 4°C. Assays were performed in duplicate and completed according to the manufacturer’s instructions.

**Western Blot Analysis**

Samples, including purified TIMP-1 (Oncogene) incubated with purified pro-MMP-9 for 3 hours at 37°C, untreated human corneal epithelial supernatant, 16× concentrated untreated supernatant, supernatant incubated with purified TIMP-1 for 3 hours at 37°C, and supernatant incubated with 0.33 ng/µl human neutrophil elastase (Sigma) for 3 hours at 37°C, were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), using a 4% to 15% gradient, 0.75 mm thick gels (Mini-ready; Bio-Rad, Richmond, CA) at a constant 200 V for 45 minutes, in an electrophoresis apparatus (Mini-Protean, Bio-Rad). Prestained (7.5–203 kDa) molecular weight protein markers (Bio-Rad) were run simultaneously with the samples. Resolved proteins were transferred to nitrocellulose membranes (BioTrace NT, Ann Arbor, MI) using a minitank blot apparatus (Bio-Rad). Membranes were blocked in 3% fat-free milk for 45 minutes. After a 1-hour incubation with the primary antibody (diluted to 1 µg/ml in 50 mM Tris-HCl and 150 mM NaCl [pH 7.4], containing, 1% bovine serum albumin and 0.5% Tween 20), the membranes were incubated with 0.5 µg/ml IgG-horseradish peroxidase–conjugated goat anti-mouse IgG (Pierce, Rockford, IL). Specifically bound antibody was detected using an immunodetection kit (Renaissance Enhanced Chemiluminescence; duPont NEN, Boston, MA), and then exposed to x-ray film (Eastman Kodak, Rochester, NY) from 30 seconds to 3 minutes.
Activation of Pro-MMP-9 in Conditioned Media and Tear Fluid

Corneal epithelial culture supernatant was thawed to room temperature for 1 hour. Ten microliters of the supernatant were added to each of six tubes (Eppendorf). Different amounts of the catalytic domain of MMP-3 were added in duplicate: 0.63 ng/μl, 1.5 ng/μl, and 2.3 ng/μl. One tube of each MMP-3 concentration was incubated at 37°C for 4 hours, and the other tube was incubated at 37°C for 8 hours. Untreated supernatant samples were used as normal control subjects.

Two tear samples from normal control subjects were incubated with MMP-3 for 4 hours at 37°C. Untreated tear samples from the same individuals were assayed as untreated control samples.

Doxycycline Treatment of Corneal Epithelial Cell Culture and Conditioned Media

Ten microliters of corneal epithelial supernatant was added to each of four tubes (Eppendorf). Each of the tubes was incubated for 4 hours at 37°C with the following reagents: 50 μg/ml doxycycline (Sigma), 50 μg/ml doxycycline + 1.5 ng/μl MMP-3, and 100 μg/ml doxycycline + 1.5 ng/μl MMP-3.

In addition, cultured human corneal epithelial cells were treated with 10 μg/ml doxycycline for 24 hours at 37°C. Ten microliters of supernatant from these cell cultures was added to each of three tubes (Eppendorf). Two of the tubes were incubated for 4 hours at 37°C with 1.5 ng/μl MMP-3. The third tube was incubated without MMP-3. This set of experiments was performed using both purified MMP-3 catalytic domain and human neutrophil elastase-activated MMP-3 (whole enzyme). These experiments were performed three times. The protein concentration in control and doxycycline-treated cultures was measured with a microprotein assay kit (BCA; Pierce).

Gelatin Zymography and MMP-9 Activity

Gelatinase activity in the corneal epithelial supernatant and tear fluid was measured by gelatin zymography. Culture-conditioned media (10 μl) and diluted tear samples were incubated with SDS gel sample buffer for 30 minutes at room temperature and electrophoresed on a 10% SDS-polyacrylamide gel containing gelatin (1 mg/ml). After electrophoresis, the proteins were renatured by washing the gel twice in 0.25% Triton X-100 (20 minutes per wash) followed by an 18-hour incubation at 37°C in the digestion buffer (50 mM Tris-HCl [pH 7.4] containing 0.15 M NaCl, 10 mM CaCl2, 2 μM ZnSO4, 1 mM phenylmethylsulfonyl fluoride, 0.005% Brij35, and 0.02% sodium azide).

After incubation, the gel was briefly rinsed in distilled water and stained with 0.25% Coomassie brilliant blue R250 in 40% isopropanol solution for 2 hours. The gel was destained with 7% acetic acid. Gelatinase activity in the gel was visible as a clear area on a blue background, indicating where the gelatin had been digested. The molecular weights of the gelatinases in the samples were determined from protein markers (Bio-Rad) and purified 92-kDa pro-MMP-9 that were run in separate lanes on the gel. Gels were then photographed (Polaroid, Cambridge, MA). For experiments comparing band densities, the photographs were scanned (Scan Jet 4C; Hewlett-Packard, Palo Alto, CA). Relative band densities in the digitized images were determined by computer with a gel analysis software program (Gel-Pro Analyzer; Media Cybernetics, Silver Springs, MD).

MMP-9 activity in culture conditioned media (100 μl) was assessed with an MMP-9 activity assay system (Biotrak; Pharmacia Biotech, Piscataway, NJ).

Statistical Analysis

The t-test was used to compare statistical levels of MMP-9 and TIMP-1 concentrations in human tear fluid samples, MMP-9 concentrations in untreated and doxycycline-treated cultures, and protein concentrations in culture supernatants.

RESULTS

Concentration of Pro-MMP-3 in the Tear Fluid by ELISA

The concentrations of pro-MMP-3 in the tear fluid samples from patients with ocular rosacea and normal subjects are presented in Table 1. Pro-MMP-3 was detected in four of the five patients with rosacea with RCEEs and/or corneal ulceration but was below the level of detection of the ELISA in the tear fluid samples obtained from all the patients with rosacea without corneal epithelial disruption or from the normal control subjects. Pro-MMP-3 was not detectable by ELISA in human corneal epithelial cell culture supernatant.

Immunodetection of TIMP-1 and Pro-MMP-9 in Tear Fluid and Culture-Conditioned Media

The concentrations of TIMP-1 and pro-MMP-9 in the tear fluid of patients with ocular rosacea and in that of normal subjects are shown in Figure 1. The mean concentrations of TIMP-1 and pro-MMP-9 detected by ELISA in the tear fluid samples from patients with ocular rosacea and normal control subjects are presented in Table 2. Both TIMP-1 and pro-MMP-9 were detected at statistically significant higher levels in rosacea-

<table>
<thead>
<tr>
<th>Tear Sample</th>
<th>Diagnosis</th>
<th>Pro-MMP-3 (ng/ml)</th>
<th>Corneal Disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MGD</td>
<td>31.27</td>
<td>RCEE</td>
</tr>
<tr>
<td>2</td>
<td>MGD</td>
<td>32.82</td>
<td>RCEE</td>
</tr>
<tr>
<td>3</td>
<td>MGD</td>
<td>43.93</td>
<td>Stromal ulcer</td>
</tr>
<tr>
<td>4</td>
<td>MGD</td>
<td>19.76</td>
<td>EBMD</td>
</tr>
<tr>
<td>5</td>
<td>MGD</td>
<td>&lt;3.75</td>
<td>EBMD</td>
</tr>
<tr>
<td>6</td>
<td>MGD</td>
<td>&lt;3.75</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>MGD</td>
<td>&lt;3.75</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
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<td>&lt;3.75</td>
<td>None</td>
</tr>
<tr>
<td>9</td>
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<td>&lt;3.75</td>
<td>None</td>
</tr>
<tr>
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<td>&lt;3.75</td>
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</tr>
<tr>
<td>11</td>
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<td>&lt;3.75</td>
<td>None</td>
</tr>
<tr>
<td>12</td>
<td>NL</td>
<td>&lt;3.75</td>
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</tr>
<tr>
<td>16</td>
<td>NL</td>
<td>&lt;3.75</td>
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</tr>
</tbody>
</table>

MGD, meibomian gland disease (associated with ocular rosacea); NL, normal volunteer; EBMD, epithelial basement membrane dystrophy.
affected than in normal tear fluids. However, there was no statistically significant difference between the TIMP-1-to-pro-MMP-9 ratio in the two groups. TIMP-1 was also detected in culture-conditioned supernatant at a mean concentration of 43.54 ± 10.15 μg/ml.

The Western blot for TIMP-1 in culture conditioned supernatant is presented in Figure 2. TIMP-1 (28 kDa) was detected in this supernatant, and its immunoreactivity was destroyed by human neutrophil elastase (Fig. 2, lane 6). TIMP-1–MMP-9 complex was not detected in undiluted (Fig. 2, lanes 2 and 5) or in 16× concentrated supernatant (Fig. 2, lane 4).

Gelatinase Activity of MMP-3–Activated Samples

The gelatin zymogram of MMP-3–activated corneal epithelial culture supernatant is presented in Figure 3. Untreated supernatant (Fig. 3, lane 2) produced two bands of molecular weights 92 kDa and 72 kDa representing pro-MMP-9 and pro-MMP-2, respectively. In contrast, MMP-3–treated supernatant samples (Fig. 3, lanes 3 through 8) showed an additional band with a molecular weight of 82 kDa, which represents the activated form of MMP-9. Incubation with MMP-3 (0.65 ng/μl) produced subtotal activation of MMP-9 (Fig. 3, lanes 3 and 6), with some still remaining in its latent form. With higher concentrations of MMP-3 (1.5 and 2.3 ng/μl; Fig. 3, lanes 4, 5, 7, and 8), there was total conversion of MMP-9 to the active form. A longer incubation time of 8 hours (Fig. 3, lanes 6, 7, and 8) decreased the amount of MMP-9 detected, possibly because of enzymatic degradation or autolysis.

The gelatin zymogram of MMP-3–activated human tear fluid is presented in Figure 4. When compared with untreated samples (Fig. 4, lanes 2 and 4), the tear fluid treated with MMP-3 (Fig. 4, lanes 3 and 5) contains an additional 82-kDa band, representing activated MMP-9.

Gelatinase Activity of Doxycycline–Treated Samples

The gelatin zymogram of doxycycline-treated corneal epithelial supernatant is presented in Figure 5. Supernatant treated with doxycycline alone produced a single 92-kDa band (Fig. 5, lane 2). In the two samples that were incubated with doxycycline and MMP-3 simultaneously (Fig. 5, lanes 3 and 4), there was a complete conversion of pro-MMP-9 to its 82-kDa active form, indicating that doxycycline did not interfere with the activation of MMP-9 by MMP-3. Supernatants from cultured corneal epithelial cells that were treated with doxycycline for 24 hours (Fig. 5, lane 5) showed a greater than 70% (doxycycline treated/untreated activity, 0.298392 ± 0.000517; P < 0.005) decrease in pro-MMP-9 activity compared with untreated cultures. When MMP-3 was added to this supernatant, again there was a complete conversion to the 82-kDa active form of MMP-9 (Fig. 5, lane 6). Compared with untreated cultures, MMP-9 activity, evaluated with an MMP-9 activity assay system (Biotrak; Pharmacia Biotech), decreased 81% ± 27.8% in doxycycline-treated cultures. There was no significant difference in supernatant protein concentrations in doxycycline-treated cultures compared with control cultures (media alone). No change in MMP-9 activity was noted when doxycycline was directly added to the culture supernatant.

DISCUSSION

Our study demonstrates that human tear fluid contains factors capable of inhibiting or activating MMP-9. TIMP-1, an inhibitor of MMP-9 activity, was detected in both tear fluid and corneal epithelial culture-conditioned medium. An increase in the concentration of both these proteins has been reported in joint fluid from inflammatory disorders. The increased concentrations of pro-MMP-9 and TIMP-1 were detected in the tear fluid of patients with the external ocular inflammatory disorder rosacea could be induced by elevated levels of inflammatory cytokines. Indeed, several cytokines, including IL-1β and transforming growth factor-β1, have been shown to increase the concentrations of these proteins in nonocular tissues. We have observed an increase in the concentration of both IL-1α and IL-1β in the tear fluid of patients with ocular rosacea. Furthermore, we have recently reported an increased level of MMP-9 (both latent and activated forms) in tear fluid of eyes with ocular rosacea and RCEEs. Barro et al. noted significantly elevated levels of MMP-9, both latent and active forms, in the tear fluid of patients with corneal transplant failure compared with those with successful grafts.

Although both latent-MMP-9 and TIMP-1 concentrations were elevated in tear fluids obtained from patients with rosacea compared with normal control subjects, we found no significant difference in the TIMP-1-to-pro-MMP-9 ratio between the two groups. It has been previously reported that in patients with ocular rosacea and normal control subjects.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Mean Pro-MMP-9 (ng/ml)</th>
<th>TIMP-1 (ng/ml)</th>
<th>TIMP-1/Pro-MMP-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.2930 ± 0.1497</td>
<td>4.4890 ± 5.7819</td>
<td>16.9358 ± 15.5588</td>
</tr>
<tr>
<td>Rosacea</td>
<td>1.1796 ± 1.2416</td>
<td>17.7853 ± 18.3715</td>
<td>26.1228 ± 26.9303</td>
</tr>
</tbody>
</table>

Data are means ± SD.

* For each subject, the TIMP-1 concentration was divided by the MMP-9 concentration, and the mean of these ratios was calculated for each group.
some systems, devoid of other MMP secretion, a ratio of TIMP-1 to MMP-9 less than 1 may promote the activation of MMP-9 by other proteinases, such as MMP-3. In the tear fluid of patients with rosacea, we found the TIMP-1-to-MMP-9 ratio to be more than 1, and yet we had detected the 82kDa activated form of MMP-9 in 50% of tear samples obtained from patients with ocular rosacea in earlier research.

The presence of active MMP-9 in the tear fluid of patients with ocular rosacea in our previous study, despite the high levels of TIMP-1 detected in our present study, suggests an alternate mechanism of activation of this enzyme in the ocular surface milieu. One such mechanism could be a concomitant increase in the levels of both MMP-3 and elastase. The latter protease is known to degrade TIMP-1 and activate MMP-3. Indeed, the concentration of MMP-3 in the tear fluid of patients with rosacea was also elevated, compared with that in normal volunteers, in whom this enzyme was undetectable (<3.75 ng/ml) in our assay. Consistent with this proposed mechanism is our finding that MMP-3 was undetectable in culture supernatants of corneal limbal epithelial cells where only pro-MMP-9 (92 kDa) activity was detected. These observations, together with our finding that TIMP-1 was degraded by neutrophil elastase, suggests the possibility that MMP-9 is activated by MMP-3 on the ocular surface of patients with rosacea. These enzymes may be secreted by the neutrophils and macrophages that have been reported to infiltrate the ocular surface in rosacea, or other conditions associated with delayed tear clearance. Although we did not attempt to measure elastase activity in our tear samples simultaneously (because of low sample volume), its accumulation in conditions of delayed tear clearance (e.g., during sleep) has been reported. Inflammatory leukocytes were observed to be the likely source of this elastase.

Human neutrophil elastase has been reported to be capable of degrading TIMP-1. We obtained a similar result, in that the immunoreactive epitope on TIMP-1 (essentially the intact TIMP-1 molecule) produced by the corneal epithelium was degraded by neutrophil elastase. Neutrophil elastase has also been reported to be the principal caseinolytic protease in tear fluid. Elevated activity of this enzyme has been observed in the closed-eye state, a condition during which most RCEEs occur. Once freed from TIMP-1, pro-MMP-9 is amenable to activation by MMP-3. Thus, it is possible that degradation of TIMP-1, by neutrophil elastase in the closed eye, leads to increased MMP-9 activity, dissolution of the corneal epithelium basement membrane, and epithelial detachment.

As stated before, MMP-3 was not produced by cultured human corneal epithelium. Similar findings were reported by Fini and Girard in rabbit corneal epithelial cultures. This metalloproteinase was also not detected in normal unstimulated human tear fluid. In contrast, MMP-3 was detected in tear fluid from subjects with delayed tear clearance, with the high-

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**Figure 2.** Western blot of human corneal epithelial cell supernatant for TIMP-1. Lane 1: Purified TIMP-1 standard (28 kDa); lane 2: 28-kDa purified TIMP-1 standard + 92-kDa purified pro-MMP-9 standard (1:1 molar ratio), 3 hours; lane 3: untreated corneal epithelial culture supernatant; lane 4: 16× concentrated untreated corneal epithelial culture supernatant; lane 5: supernatant + 28-kDa purified TIMP-1 standard, 3 hours; and lane 6: supernatant + 0.35 ng/μl human neutrophil elastase, 3 hours.

**Figure 3.** Gelatin zymogram of human corneal epithelial cell supernatant: activation with MMP-3. Lane 1: Purified pro-MMP-9 standard (92 kDa); lane 2: untreated corneal epithelial culture supernatant; lane 3: supernatant + 0.65 ng/μl MMP-3, 4 hours; lane 4: supernatant + 1.5 ng/μl MMP-3, 4 hours; lane 5: supernatant + 2.3 ng/μl MMP-3, 4 hours; lane 6: supernatant + 0.65 ng/μl MMP-3, 8 hours; lane 7: supernatant + 1.5 ng/μl MMP-3, 8 hours; and lane 8: supernatant + 2.3 ng/μl MMP-3, 8 hours.
est concentrations in the tear fluid obtained from patients with either RCEE or frank corneal stromal ulceration. These findings suggest that the pathologic alterations of the ocular surface that accompany delayed tear clearance may also contribute to the increased production of MMP-3 by cells on the ocular surface, such as infiltrating leukocytes. Delayed tear clearance may also lead to increased retention of this enzyme in the tear fluid and to an accumulation of plasmin, another known activator of MMP-9.

A 4-hour incubation in vitro with the catalytic domain of MMP-3 was found to completely convert 92-kDa pro-MMP-9 present in both tear fluids and corneal epithelial supernatants to its active 82-kDa form. This finding suggests that MMP-3 serves as an activator of pro-MMP-9 in human tear fluid in vivo. MMP-9 has been implicated as a causative factor in delayed corneal surface wound healing and re-epithelialization. A similar role for MMP-9 in rosacea and RCEE would have significant clinical implications. Activation of MMP-9 may be one of the key pathophysiological events in the corneal ulcers that develop in ocular surface disease. Some or many events in this cascade may be mediated by inflammatory cytokines. For example, we have found that tear fluid from patients with rosacea has greater concentrations of the 17-kDa mature form of IL-1β and lower concentrations of the biologically inactive 31-kDa precursor form than tear fluid from normal eyes. Activation of this important inflammatory cytokine from its precursor to mature form may be an important step in the inflammatory cascade. The mechanism of extracellular conversion of pro-IL-1β to its biologically active mature form is not completely understood; however, activated MMP-9 has been reported to be a key enzyme responsible for this conversion in the extracellular environment.

We report here for the first time, the effect of and the potential use of doxycycline on the activity of pro-MMP-9 released by human corneal epithelium, as well as its enzymatic activation by MMP-3. We found that doxycycline at a nontoxic concentration markedly decreased the activity of pro-MMP-9. However, doxycycline did not affect the activation of pro-MMP-9 by MMP-3, a finding consistent with a previously reported study that doxycycline does not affect MMP-3 activity. Our finding could explain the observed clinical efficacy of doxycycline in the treatment of RCEE and the prophylaxis of its recurrence.

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932906/)  
**FIGURE 4.** Gelatin zymogram of human tear fluid: activation with MMP-3. Lane 1: Purified pro-MMP-9 standard (92 kDa); lane 2: untreated human tear fluid, patient 1; lane 3: human tear fluid treated with MMP-3 (1.5 ng/µl), patient 1; lane 4: untreated human tear fluid, patient 2; and lane 5: human tear fluid treated with MMP-3 (1.5 ng/µl), patient 2.

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932906/)  
**FIGURE 5.** Gelatin zymogram of human corneal epithelial cell supernatant: doxycycline treatment and MMP-3 activation. Lane 1: Purified pro-MMP-9 standard (92 kDa); lane 2: corneal epithelial supernatant + 50 µg/ml doxycycline; lane 3: supernatant + 50 µg/ml doxycycline + 1.5 ng/µl MMP-3; lane 4: supernatant + 100 µg/ml doxycycline + 1.5 ng/µl MMP-3; lane 5: supernatant from cell culture pretreated with doxycycline; and lane 6: supernatant from cell culture pretreated with doxycycline + 1.5 ng/µl MMP-3.
Ocular rosacea and RCEE are serious eye conditions that cause discomfort and can threaten vision. An effective mechanism to inhibit MMP-9 activity in vivo, possibly by using a nontoxic inhibitor of MMPs, such as doxycycline, has the potential to bring measurable relief to patients who have these conditions.

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References