Tear Fluid Gelatinase B Activity Correlates with IL-1α Concentration and Fluorescein Clearance in Ocular Rosacea

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PURPOSE. To correlate tear fluorescein clearance with interleukin-1α (IL-1α) concentration and gelatinase B (matrix metalloproteinase [MMP]-9) activity in the tear fluid of patients with ocular rosacea and normal control subjects.

METHODS. Gelatinase activity was evaluated by gelatin zymography in tear fluid obtained from 13 patients with ocular rosacea (including 1 patient with recurrent epithelial erosion, 2 with recurrent peripheral corneal infiltrates and vascularization, and 2 patients with epithelial basement membrane dystrophy) and 13 normal control subjects with normal aqueous tear production and no irritation symptoms. Tear fluorescein clearance was evaluated by measuring fluorescence in tear fluid collected from the inferior meniscus 15 minutes after instillation of 5 μl of 2% Na–fluorescein with a CytoFluor II fluorometer. Pro–MMP-9 and IL-1α concentrations in the tear fluid were measured by enzyme-linked immunosorbent assay (ELISA).

RESULTS. Compared with normal control subjects, patients with ocular rosacea had a greater delay of tear fluorescein clearance (P < 0.001), a higher tear IL-1α concentration (P < 0.001), and a greater pro–gelatinase B (92 kDa) activity (P < 0.001) in their tear fluid. The 84-kDa active form of gelatinase B was observed in 46% of the rosacea tear samples and none of the controls. The zymographic results were confirmed by ELISA that showed a significantly greater concentration of pro–MMP-9 (92 kDa) in the tear fluid of rosacea patients than controls. Delayed tear clearance was correlated with elevated tear IL-1α concentration (p=0.67, P < 0.001) and increased tear gelatinase B activity (p=0.84, P < 0.001). Tear IL-1α concentration was correlated with tear gelatinase B activity (p=0.58, P < 0.002).

CONCLUSIONS. Gelatinase B (MMP-9) activity is greater in patients with ocular rosacea than in normal eyes. The majority of this activity is due to 92-kDa proform of this enzyme. This activity is correlated with delayed tear clearance and tear fluid concentration of interleukin-1α, a proinflammatory cytokine that has been reported to stimulate gelatinase B production. Elevated gelatinase B activity in ocular rosacea may be involved in the pathogenesis of the irritation symptoms, recurrent epithelial erosions, vascularization, and epithelial basement membrane dystrophy that develops in the cornes of patients with this condition. (Invest Ophthalmol Vis Sci. 1999;40:2506–2512)

Meibomian gland disease is recognized as a common cause of ocular irritation.1–3 This condition has been reported to occur in 50% to 93% of patients with the inflammatory skin condition, rosacea.4,5 In addition to meibomian gland disease, patients with rosacea also develop vascular telangiectasia and irregularity of their lid margins, conjunctival hyperemia, corneal vascularization, peripheral corneal infiltrates and ulcers, corneal epithelial basement membrane dystrophy, and recurrent corneal epithelial erosions.4,6–8 Indeed, recurrent corneal epithelial erosions have been reported to occur in 5% to 12% of patients with ocular rosacea.4,8 Meibomian gland disease has also been implicated as a risk factor for developing floppy eyelid syndrome.9,10

The exact mechanism for the irritation symptoms and the ocular surface disease that develops in patients with ocular rosacea is unknown. We reported that patients with ocular rosacea have a significantly elevated concentration of the proinflammatory cytokine interleukin-1α (IL-1α) in their tear fluid that correlates with a delay in tear fluorescein clearance.11 Interleukin-1 is known to increase the production and activity of certain enzymes of the matrix metalloproteinase (MMP) family, including collagenases and gelatinases, that degrade extracellular matrix and could contribute to the development of the eyelid and ocular surface disease in rosacea.12–16 Certain of these enzymes have the potential to break down the corneal...

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epithelial basement membrane and to dissolve collagen and ground substance in the corneal stroma, processes that could lead to corneal epithelial erosion or frank corneal ulceration.\textsuperscript{12,18–20} The corneal epithelium and stromal keratocytes have been reported to be capable of producing certain MMPs.\textsuperscript{19,21} Specifically, the corneal epithelium produces gelatinase B (MMP-9), an enzyme that participates in the wound healing process that follows experimental mechanical, thermal, or laser injury to the cornea, by degrading the corneal epithelial basement membrane.\textsuperscript{19,22} Furthermore, gelatinase B has been detected in the basal corneal epithelial cells at the edges of nonhealing corneal ulcers in humans.\textsuperscript{20}

We hypothesized that patients with ocular rosacea have increased gelatinase B activity in their tear fluid as a result of their delayed tear clearance and increased tear fluid IL-1\textalpha{} concentration. The purpose of this study was to evaluate gelatinase activity in the tear fluid of patients with symptomatic ocular rosacea and in normal control subjects and correlate the results with tear fluorescein clearance and the concentration of IL-1\textalpha{} in their tear fluid.

\section*{METHODS}

This study was conducted according to a protocol approved by the University of Miami School of Medicine Institutional Review Board and in accordance with the Tenets of the Declaration of Helsinki. Informed consent was obtained after the nature and possible consequences of the study were explained.

Two groups of subjects were evaluated. One group consisted of patients presenting to the Ocular Surface and Tear Center of the Bascom Palmer Eye Institute with chief complaints of ocular irritation, redness, or both that was diagnosed as ocular rosacea by previously reported criteria.\textsuperscript{11} The medical records of these patients were reviewed, and patients were questioned for a history of corneal ulceration, peripheral infiltrates, corneal vascularization, recurrent erosion symptoms (stabbing pain, foreign body sensation occurring during sleep or upon awakening, or both), or clinically documented recurrent erosion.

The other group consisted of “ideal” normal control subjects who had no history of eye disease or ocular surgery, or use of eye drops or symptoms of ocular irritation. All these subjects had a Schirmer 1 test score of >15 mm, normal meibomian glands, and no corneal fluorescein staining. In experiments correlating tear fluid 92-kDa gelatinase activity with tear fluorescein clearance, and IL-1\textalpha{} concentration, 13 ocular rosacea patients and 13 normal controls were evaluated. To evaluate the concentration of pro–MMP-9 in tear fluid by enzyme-linked immunosorbent assay (ELISA), a separate group of 13 ocular rosacea patients and 11 normal controls were evaluated.

\textbf{Tear Collection}

A sample of the 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.\textsuperscript{23} The volume of collected tears was determined by reweighing the rods immediately after tear collection with an OHAUS model GA110 scale (OHAUS, Bern, Switzerland). Wicks were then placed into the end of a micropipette tip located within a 0.5 ml Eppendorf tube as described by Jones and colleagues.\textsuperscript{23}

\textbf{Extraction of Sample}

Tears were extracted from the saturated wicks by centrifuging at 12,000 rpm for 5 minutes within the pipette tip after adding a volume of buffer (50 mM Tris–HCl, 0.15 M NaCl, 10 mM CaCl\textsubscript{2}, 0.005% Brij 35, 0.02% sodium azide, pH 7.5) 10 times greater than the original volume of the tear sample. This resulted in a final dilution factor of 1:11 for the gelatinase assay.

\begin{table}
\centering
\begin{tabular}{|l|l|l|l|l|l|l|l|}
\hline
Number & Age & Gender & Schirmer 1 & MG Metaplasia & %MG Acinar Dropout & Irregular Marx’ Line & Conjunctival Redness & Corneal FI Staining & Corneal Signs/ Symptoms \\
\hline
1 & 79 & F & 10 & + & 75 & + & 2 & 1 & RCEE, CV, PI, PU \\
2 & 76 & F & 18 & + & 75 & + & 1 & 0 & CV, Hx RCEE, PI, EBMDF \\
3 & 56 & F & 25 & + & 75 & + & 1 & 2 & EBMD \\
4 & 60 & F & 30 & + & 100 & + & 0 & 1 & EBMD \\
5 & 74 & F & 9 & + & 75 & + & 1 & 3 & \\
6 & 65 & F & 4 & + & 75 & + & 3 & 3 & \\
7 & 77 & F & 8 & + & 100 & + & 2 & 8 & \\
8 & 60 & F & 10 & + & 100 & + & 1 & 2 & \\
9 & 24 & F & 5 & + & 75 & + & 2 & 1 & \\
10 & 23 & F & 9 & + & 100 & + & 2 & 0 & \\
11 & 78 & M & 16 & + & 50 & + & 1 & 3 & \\
12 & 77 & M & 30 & + & 50 & + & 1 & 4 & \\
13 & 80 & M & 19 & + & 100 & + & 2 & 3 & \\
\hline
\end{tabular}
\caption{Clinical Features of Ocular Roseaca Patients}
\end{table}

\textsuperscript{*} MG (meibomian glands, ductal orifice squamous metaplasia, and percentage of acinar dropout were evaluated and graded as previously reported.

\textsuperscript{‡} Redness of bulbar conjunctiva graded with a standard photographic plate: 0 = no redness, 1 = mild, 2 = moderate, 3 = severe redness.

\textsuperscript{§} Conjunctival redness graded 0–12 using a previously reported scale.

\textsuperscript{†} As defined by Norn.\textsuperscript{17}
Normal Controls

92 kDa

Ocular Rosacea

92 kDa
84 kDa
72 kDa

Subjects

C
1
2
3
4
5
6
7
8
9
10
11
12
13

FIGURE 1. Gelatin zymograms of tear samples collected from 13 normal controls (upper gel) and 13 patients with ocular rosacea (lower gel). Purified 92-kDa pro–gelatinase B (0.1 ng/lane) is in the first lane designated C.

For example, if the volume of collected tears was 2 μl, 20 μl of buffer was added. The rods and pipette were carefully removed and the tear fluid aspirated. Tear samples were placed in numbered 500 μl Eppendorf tubes and stored at −80°C for 3 to 7 days until they were used for pro–MMP-9 or IL-1α ELISA or for gelatin zymography. Samples were evaluated from the eye where the greatest volume of tear fluid was collected.

IL-1α and Pro–MMP-9 ELISA

Interleukin-1α concentration was determined with a commercial kit (R&D Systems, Minneapolis, MN). The tear sample was diluted in ELISA buffer (supplied by the manufacturer) to a final volume of 200 μl. The assay was performed by a previously reported protocol.11 ELISA for pro–MMP-9 was performed using a commercial kit from Oncogene Research Products (Cambridge, MA). The standard curve for this kit ranged from 0.05 to 32 ng/ml.

Gelatin Zymography

Gelatinase activity in the tear fluid was measured by gelatin zymography. Diluted tear samples (10 μl of tears diluted 1:11) were incubated with sodium dodecyl sulfate (SDS)–gel sample buffer for 30 minutes at room temperature and analyzed by electrophoresis on a SDS–polyacrylamide gel (10%) containing gelatin (1 mg/ml). After electrophoresis, the proteins were renatured by removing SDS from the gel using two washes of 0.25% Triton X-100 (30 min/wash). This was followed by an 18-hour incubation at 37°C in the digestion buffer consisting of 50 mM Tris–HCl (pH 7.4) containing 0.15 M NaCl, 10 mM CaCl₂, 2 μM ZnSO₄, 1 mM phenylmethylsulfonyl fluoride, 0.005% Brij 35, and 0.02% sodium azide. After this incubation, the gel was briefly rinsed in distilled water and stained with 0.25% Coomassie brilliant blue R250 prepared in 40% isopropanol solution for 1 hour. The gel was destained with 7% acetic acid. Gelatinase activity in the gel was visible as a clear area in the blue background, indicating an area where the gelatin had been digested. The minimum sensitivity of this technique for detecting gelatinase B is 0.05 ng/lane. The molecular weight of gelatinases in the tear fluid was determined from molecular weight standards (prestained broad range standards; Bio-Rad, Hercules, CA) and purified rabbit 92-kDa pro–gelatinase B (0.1 ng; Oncogene Research, Cambridge, MA) that were run in separate lanes on the gel. These gels were photographed with a Polaroid camera, and the photographs were scanned with an HP Scanjet 4c scanner (Hewlett-Packard, Palo Alto, CA). The optical densities of bands in the digitized images were determined with the Gel-Pro Analyzer gel analysis software program (Media Cybernetics, Silver Spring, MD). The ratio of the optical density of the 92-kDa gelatinase band in each tear fluid sample to the optical density of the pro–gelatinase B positive control band (0.1 ng) was used for statistical analysis.

Fluorescein Tear Clearance

This test was performed as previously described.3 Five microliters of 2% sodium fluorescein (IOLAB, Claremont, CA) was instilled into the inferior conjunctival sac, and the subject was instructed to carry on normal activities for 15 minutes. After that time, a sample of the tear fluid was collected from the lower meniscus, causing the least irritation possible, using a Transorb wick (American Filtrona, Richmond, VA). Wicks were then placed into the end of a micropipette tip located within a 0.5-ml Eppendorf tube as described above, and a volume of phosphate-buffered saline (100 μl, weight of rod in micrograms) was added to the end of the wick. The tubes were then spun at 12,000 rpm for 5 minutes and the fluid was transferred to wells of a 96 well polycarbonate microtiter plate (Corning 96; Corning, NY). Fluorescence was measured with a fluorescence multiplate reader (CytoFluor II, PerSeptive Biosystems, Framingham, MA).

TABLE 2. IL-1, Gelatinase B, and Fluorescein Concentration in Tear Fluid

<table>
<thead>
<tr>
<th>Group</th>
<th>Log Tear Fluorescein Concentration§</th>
<th>IL-1α Concentration (pg/ml)</th>
<th>Gelatinase B Activity‡</th>
<th>Pro–MMP-9 Concentration (ng/ml)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>1.81 ± 0.77</td>
<td>43.08 ± 60.23</td>
<td>0.16 ± 0.15</td>
<td>0.33 ± 0.15</td>
</tr>
<tr>
<td>Ocular rosacea</td>
<td>3.31 ± 0.42*</td>
<td>253.69 ± 325.36#</td>
<td>1.48 ± 1.62#</td>
<td>1.39 ± 1.12#</td>
</tr>
</tbody>
</table>

* 13 ocular rosacea patients and 13 normal controls were compared.
† 13 ocular rosacea patients and 11 normal controls were compared.
‡ Optical density of 92-kDa band on gelatin zymogram.
§ Fluorescence of tear fluid 15 minutes after fluorescein instillation as described in the Methods section.
# P < 0.0001 for gelatinase B activity and log tear fluorescein concentration; P = 0.002 for tear IL-1α concentration; P = 0.047 for tear pro–MMP-9 concentration compared with normal controls.
Statistical Analysis

Between-group comparisons for tear IL-1α and pro–MMP-9 concentrations, tear fluorescein clearance, and tear gelatinase activity were performed with the Wilcoxon two-sample test. The Spearman nonparametric correlation test was used for determining if there was correlation between study parameters.

RESULTS

Clinical Features

The average ages of the ocular rosacea patients and normal control subjects used for correlating 92-kDa gelatinase activity with tear fluid fluorescein and IL-1α concentrations were 64 years (range, 23–80 years) and 37 years (range, 24–50 years), respec-

![Pro-MMP 9 in Tear Fluid](image)

**Figure 2.** Results of pro–MMP-9 ELISA on tear samples collected from 11 normal subjects (samples 1 through 11) and 13 subjects with ocular rosacea (samples 12 through 25). Concentrations are expressed in nanograms per milliliter. The mean pro–MMP-9 concentration in the tear fluid was significantly higher in the rosacea samples (1.39 ± 1012 ng/ml) than in the controls (0.33 ± 0.15 ng/ml, P = 0.047).

**Figure 3.** Correlation between tear fluid gelatinase B activity and the concentration of IL-1α in the tear fluid (Spearman’s ρ = 0.576, P < 0.002). NL, normal; MGD, meibomian gland disease.

![Correlation Graph](image)
tively. The gender distribution of subjects in both groups was the same (10 women and 3 men). The clinical features of these ocular rosacea patients are presented in Table 1.

**Gelatinase Activity**

The gelatin zymograms of tear samples from rosacea patients and normal control subjects are presented in Figure 1. Patients with ocular rosacea had significantly greater pro–gelatinase B (92-kDa) activity than the normal controls ($P < 0.001$, Table 2). An 84-kDa band of digestion, representing the active form of gelatinase B, was noted in six of the tear samples from rosacea patients but in none of the controls. In addition to the 92- and 84-kDa bands, several high-molecular-weight bands ranging from 105 to 210 kDa were also found in samples from rosacea patients. These bands are likely to be dimeric complexes of MMP-9, MMP-9 complexed to tissue inhibitors of metalloproteinase (TIMPs), or both. Pro–gelatinase A (72-kDa) activity was observed in 11 of 13 rosacea tear samples and none of the controls.

**Concentration of Pro–MMP-9 in the Tear Fluid by ELISA**

The concentrations of pro–MMP-9 in tear fluid samples from ocular rosacea patients and normal controls are depicted in Figure 2. The mean pro–MMP-9 concentration in the tear fluid was significantly higher in the rosacea samples ($1.39 \pm 1012$ ng/ml) than the controls ($0.33 \pm 0.15$ ng/ml, $P = 0.047$).

**Correlation between IL-1$\alpha$ Concentration and Gelatinase Activity**

The patients with ocular rosacea had a higher concentration of IL-1$\alpha$ in their tear fluid than the normal group (Table 2). A positive correlation was observed between gelatinase B activity and the concentration of IL-1$\alpha$ in tear fluid obtained from both groups of subjects (Spearman’s $\rho = 0.576$, $P < 0.002$, Fig. 3).

**Correlation between IL-1$\alpha$ Concentration and Tear Fluorescein Clearance**

Patients with ocular rosacea showed a significantly greater delay in tear fluorescein clearance than in normal subjects (Table 2). An inverse correlation between the concentration of IL-1$\alpha$ in tear fluid and the tear fluorescein clearance was noted (Spearman’s $\rho = 0.665$, $P < 0.001$, Fig. 4). Those patients with the highest tear fluorescein concentrations at 15 minutes (indicating delayed tear clearance) had the highest concentrations of IL-1$\alpha$ in their tear fluid.

**Correlation between Gelatinase Activity and Tear Fluorescein Clearance**

A strong positive correlation between tear gelatinase B activity and the tear fluorescein clearance was observed (Spearman’s $\rho = 0.842$, $P < 0.001$, Fig. 5). The greater the concentration of fluorescein in the tear fluid, the greater the gelatinase B activity.
DISCUSSION

This study clearly demonstrates that there is increased 92-kDa pro–gelatinase B activity in the tear fluid of patients with ocular rosacea. The increased activity was associated with an increased concentration of this enzyme measured by ELISA. Furthermore, pro–gelatinase B activity was strongly correlated with the concentration of IL-1α in the tear fluid, as well as tear fluorescein concentration at 15 minutes, a measure of tear clearance. We have reported that delayed tear clearance is a feature shared by both aqueous tear deficiency and meibomian gland disease.3 A strong correlation between delayed tear clearance and decreased corneal and conjunctival sensitivity scores was observed in patients with these tear film disorders.3 The cause for the decrease in ocular surface sensation has not been established; however, the ocular surface inflammation that develops in patients with delayed tear clearance is a possible cause.24 Inflammatory factors, including interleukin-1, have been reported to alter sensory neural threshold.25 Regardless of the cause, it has been established that reduced ocular surface sensation results in decreased reflex stimulated tear production by the lacrimal glands.26,27 This in turn further decreases tear clearance and creates a viscous cycle.

Minimal gelatinase B activity was observed in tear fluid samples obtained from normal controls who had no symptoms of ocular irritation, exhibited normal aqueous tear production and clearance, and showed no ocular surface disease. Based on this finding, it appears that homeostatic mechanisms on the ocular surface minimize the level and activity of gelatinase B. The biosynthesis and activation of this enzyme are regulated by its environment.12 Inflammatory cytokines, such as IL-1, have been reported to stimulate gelatinase B activity by a number of different cell types in vitro.13,14,28–41 Furthermore, elevated IL-1 concentration and increased gelatinase B activity have been observed in the gingival crevicular fluid of patients with periodontitis, an inflammatory condition in which the connective tissue attachments of teeth to their supporting articular bone are destroyed.32–34 Our group has previously found that the concentration of IL-1α is significantly increased in the tear fluid of patients with ocular rosacea.11 This finding provided the rationale for evaluating the correlation between tear IL-1α concentration and gelatinase B activity in the present study. As anticipated, a strong positive correlation was observed between IL-1α and gelatinase B activity. One explanation for the elevated concentration and activity of pro–gelatinase B in ocular rosacea is increased production of this enzyme by cells on the ocular surface, a process that could be stimulated by IL-1α. Another but not mutually exclusive possibility is that the increased concentrations of IL-1α and gelatinase B in the tear fluid could result from delayed clearance of these factors from the ocular surface.

The 84-kDa active form of gelatinase B was not observed in normal tear fluid, only in the tear fluid of rosacea patients. Proteolytic cleavage of the “cysteine-switch” covering the active site of gelatinase B has been reported to be the physiological mechanism of activation of this enzyme.12,55 Maximum activation of gelatinase B has been observed after sequential treatment with two proteases, neutrophil elastase to remove TIMP-1 and stromelysin (MMP-3) to expose the active site.36–37 Delayed tear clearance has been reported to be associated with increased activity of proteolytic enzymes in the tear fluid, including plasmin and neutrophil elastase, increased numbers of neutrophils on the ocular surface, and increased concentrations of neutrophil chemotactic factors such as complement components and IL-8.38–41 In fact, neutrophil-derived elastase has been reported to be the major source of tear fluid caseino-
lytic activity in the “closed eye,” a state associated with a marked decrease in tear clearance. Thus, it appears that the increased inflammation and proteolytic activity that accompany a decrease in tear clearance disrupts ocular surface homeostasis. These pathologic changes have the capability of promoting gelatinase B activation and could be responsible for the active form of gelatinase B in the tear fluid of ocular rosacea patients.

A number of the pathologic changes that have been reported to occur on the ocular surface and eyelids of patients with ocular rosacea could be attributed to increased local activity of matrix-degrading enzymes, such as gelatinase B. Indeed, gelatinase B has been implicated as a key causative factor in sterile corneal ulceration. As noted in Table 1, three ocular rosacea patients had a history of recurrent erosion or symptoms consistent with this condition, 2 had peripheral corneal infiltrates, and 1 had a history of a frank corneal ulcer. Tear samples from these patients showed the greatest gelatinase B activity in Figure 1 (lanes 5 through 8). This finding suggests that gelatinase B could be a potential therapeutic target to prevent some of these sight-threatening corneal complications that are associated with ocular rosacea.

References

27. van der Zee E, EGF and IL-1 alpha modulate the release of collagenase, gelatinase and TIMP-1 as well as the release of calcium by rabbit corvalar bone explants. J Periodontal Res. 1998;33:65–72.