Matrix Metalloproteinases in Epithelia from Human Recurrent Corneal Erosion

Randa M. R. Garrana,1 James D. Zieske,1 Michael Assouline,2 and Ilene K. Gipson1

PURPOSE. To assay for the presence of matrix metalloproteinases (MMPs) in human corneal epithelium affected by recurrent erosion compared with that in normal corneal epithelium.

METHODS. Corneal epithelial débridement samples were obtained from 13 patients with recurrent epithelial erosion. For control specimens, epithelia were obtained from healthy patients undergoing photorefractive keratectomy. Zymography was performed on all samples to identify MMPs. Immunolocalization of MMP-2, laminin, and collagen type VII was determined in two samples with human recurrent epithelial erosion and compared with that in control epithelium.

RESULTS. Twelve of 13 erosion samples showed MMP-2 enzymatic activity; one of the 12 also showed MMP-9 activity. Only one erosion sample showed no MMP enzymatic activity. All normal control specimens were negative for MMP. Immunohistochemical analysis of two recurrent erosion samples showed MMP-2 presence in basal cells, whereas, in normal epithelium it was not detected. One sample with epithelial erosion showed laminin localization in basal epithelial cells and basal lamina. Type VII collagen localized in basal epithelial cells only in this sample. A second erosion sample showed localization of laminin and type VII collagen in basal epithelial cells only. Normal corneal epithelium showed presence of laminin and type VII collagen in basal epithelium and basal lamina.

CONCLUSIONS. Matrix metalloproteinase-2 expression is upregulated in human epithelia affected by recurrent erosion compared with that in normal control samples. Immunolocalization studies suggest that this enzyme is concentrated in basal epithelial cells where it may play an important role in degradation of the epithelial anchoring system and the recurrent epithelial slippage and erosion observed in these patients. (Invest Ophthalmol Vis Sci. 1999;40:1266–1270)

Recurrent epithelial erosion is a clinical entity characterized by the abrupt transition from one with a variable degree of distress. Clinical symptoms include recurrent attacks of acute ocular pain, tearing, and redness, typically occurring when the person awakens. Besides epithelial defects, slit lamp examination may reveal a variable combination of epithelial microcysts (dots), basal lamina folds (fingerprints), and geographic thickening of the epithelial basal lamina (map). The most common predisposing process in this condition is ocular surface trauma.

Defective epithelial anchoring has been hypothesized to be the cause of epithelial erosions.1,2 Anchoring of the epithelium is mediated by integrins, type VII collagen, and laminin, which form a complex that attaches the basal epithelial cells to the underlying connective tissue. Anchoring proteins, which are protease sensitive, may also be involved in successful epithelial migration.6-9

Matrix metalloproteinases (MMPs) are a family of protein-cleaving enzymes that degrade extracellular matrix and basement membrane components.10,11 They are ordinarily secreted as proenzymes and are activated by proteolytic cleavage of the N-terminal region in the extracellular compartment. The latent proenzymes may undergo conformational rearrangement and subsequent change in molecular weight to attain collagenolytic activity.11,12 The family of MMPs continues to grow as more protein-cleaving enzymes are identified. Currently, the 20 known MMPs are divided into five major subclasses, according to their substrate specificity.13,14 Collagenases are responsible for the cleavage of interstitial collagen types I, II, and III. Stromelysins and matrilysins are important in degrading a variety of extracellular matrix components. Gelatinases (MMP-2 and MMP-9) are involved in cleaving collagen types IV, V, VII, and X, fibronectin, laminin, elastin, and gelatins. Membrane-type MMPs are important in the process of degradation of, among other things, collagens I, II, III, gelatin, fibronectin, laminin, and MMP-2.

Matrix metalloproteinase expression has been reported in cultured rabbit cornea, normal human cornea, and keratoconic cornea.15,16 In the rabbit, MMP-2 proenzyme is produced mostly by stromal fibroblasts, with the corneal epithelium producing little MMP-2 proenzyme or MMP-9 enzyme.15,17 During rabbit corneal wound healing, the expression of MMP-2 is increased in the stroma, and MMP-9 is expressed in the epithelial layer.17,18

As shown by zymography, normal human corneas and keratoconic corneas contain MMP-2, primarily in the proenzyme form in the corneal stroma and the endothelial layer, including Descemet's membrane.16 In the same human study,16 a minor amount of a 92-kDa gelatinase, corresponding to the proenzyme form of MMP-9, was found in 50% of normal human corneas examined. The 92-kDa gelatinase was not localized to any specific layer of the normal cornea.

We hypothesize that the gelatinases MMP-2 and MMP-9 are upregulated in human recurrent epithelial erosion and thus affect the normal anchoring of the epithelium to the basement membrane and underlying connective tissue. Our data show that MMP-2 is present in these epithelia.

MATERIALS AND METHODS

All subjects were treated in accordance with the tenets of the Declaration of Helsinki. Human epithelial samples were collected immediately before phototherapeutic keratectomy from 13 patients with history and slit lamp findings consistent with...
posttraumatic recurrent epithelial erosions and from 6 normal patients undergoing photorefractive keratectomy (Table 1). Samples were frozen immediately on harvesting and stored at −80°C. Of the collected samples, two were frozen in ornithine carbamoyltransferase for immunofluorescence microscopy. The epithelial samples were collected by gentle scraping of the corneal surface from the limbus to the center with a blade. In patients with recurrent erosion, the entire epithelial layer could be readily detached from Bowman’s layer, using simple traction, whereas energetic scraping was required in normal control patients.

**Gelatin Zymography**

Gelatin zymography was performed on recurrent erosion epithelia and normal epithelia, as previously described. Samples were thawed at room temperature and the proteins immediately extracted by homogenizing the tissue in a solution of 1% sodium dodecyl sulfate (SDS). An equal volume of SDS was used in all samples, resulting in equal dilution of extracted gelatinases. Samples of homogenized human epithelium in 1% SDS solution were diluted in phosphate-buffered saline (PBS) before loading on a gel. Equal amounts of protein from recurrent erosion epithelium and normal epithelium were loaded after assay of protein with a commercial kit (Bio-Rad, Hercules, CA). After electrophoresis of the samples, the gel was shaken in a 2.5% solution of Triton X-100 for 30 minutes and then incubated in reaction buffer (50 mM Tris [pH 7.5], 10 mM CaCl₂) overnight at 37°C. After the gel was stained with Coomassie brilliant blue (Sigma, St. Louis, MO), the positions of enzymatic species could be easily identified as clear bands in the stained gelatin background. In each gel, a positive MMP-2 and MMP-9 control sample obtained from a sarcoma cell culture supernatant, HT1080, (ATCC, Rockville, MD) and a molecular weight standard were used to characterize and identify the enzymatic bands.

**Immunofluorescence Microscopy**

Frozen sections were air dried for 90 minutes. After they were washed in PBS, the slides were blocked in 1% bovine serum albumin in PBS and incubated with primary antibody for 1 hour. The primary antibodies were monoclonal mouse anti-human MMP-2 (1:10; Calbiochem; Cambridge, MA), rabbit anti-laminin polyclonal antibody (1:500; Chemicon; Temecula, CA), and monoclonal mouse IgG anti-collagen type VII (1:500; Sigma). The MMP-2 antibody recognizes proenzyme and active enzyme forms, according to the manufacturer’s data.

After washing in PBS, the slides were incubated with secondary antibody for 60 minutes. Fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (1:50), and fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (1:50; both from Jackson ImmunoResearch, West Grove, PA), were used against corresponding primary antibodies. Slides were then washed with PBS, mounted, and examined using a confocal microscope (model TCS 4D; Leica, Deerfield, IL).

**RESULTS**

**Zymography**

Using zymography, we observed enzymatic activity in a 72-kDa band in all but one of the samples with human recurrent epithelial erosion (Table 1). One sample showed a 72-kDa band and a 92-kDa proteinase band (Table 1). By comparing zymography bands produced by sarcoma cell culture supernatant HT1080, which served as a positive control for MMP-2 and MMP-9, we were able to confirm that the 72-kDa band was MMP-2 and the 92-kDa band was MMP-9. Normal control epithelial samples showed no detectable enzymatic activity (Fig. 1).

**Immunolocalization of MMP-2, Laminin, and Collagen Type VII**

To determine the cellular source of the MMP-2 present in recurrent erosion samples, MMP-2 was immunolocalized in two epithelial samples. In both samples, MMP-2 was localized predominantly in basal cells of the epithelium (Fig. 2). Matrix metalloproteinase-2 was not detected in any of the cell layers of normal epithelium (Fig. 2).

Laminin and type VII collagen, both known substrates of MMP-2, were localized to determine the integrity of the anchoring complex in recurrent erosion samples. In one sample, laminin was localized to the basal epithelial cells and in the area adjacent to the basal lamina, indicating adherence (Fig. 3A). In the same sample, type VII collagen was localized in the basal epithelial cells but was not detected in the basal lamina (Fig. 3B). A second human epithelial erosion sample did not show laminin localization in the area of the basal lamina, indicating absence of anchoring structures (Fig. 3C). There was clear localization of laminin in the basal epithelial cells. Type VII collagen was localized within basal epithelial cells but, as in the first sample, was absent in the basal lamina zone (Fig. 3D).

Secondary antibody control samples were negative for MMP-2 (Fig. 2C) and for laminin and type VII collagen (data not shown). Immunolocalization was also performed in the normal human corneal epithelium that served as positive control samples. Laminin and collagen type VII localized in basal epithelial cells and in the basal lamina (data not shown).

---

**Table 1. Expression of MMP in Samples with Human Recurrent Epithelial Erosion Compared with That in Normal Control Samples**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>Eye</th>
<th>MMP-2</th>
<th>MMP-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recurrent epithelial erosion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>38</td>
<td>F</td>
<td>OS</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>M</td>
<td>OD</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>F</td>
<td>OS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>54</td>
<td>F</td>
<td>OD</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>M</td>
<td>OS</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>37</td>
<td>M</td>
<td>OD</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>F</td>
<td>OD</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>8</td>
<td>31</td>
<td>M</td>
<td>OD</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>9</td>
<td>38</td>
<td>M</td>
<td>OS</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>10</td>
<td>37</td>
<td>M</td>
<td>OD</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>11</td>
<td>48</td>
<td>M</td>
<td>OD</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>12</td>
<td>38</td>
<td>F</td>
<td>OS</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>13</td>
<td>50</td>
<td>F</td>
<td>OS</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Normal control samples

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>Eye</th>
<th>MMP-2</th>
<th>MMP-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>F</td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>31</td>
<td>M</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>M</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>F</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
<td>M</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>M</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>
FIGURE 1. Gelatin zymography of a positive MMP-2 and MMP-9 control sample obtained from a sarcoma cell culture supernatant HT1080 (lane 1 ATCC; Rockville, MD); normal control samples (lanes 2, 3); and recurrent erosion samples (lanes 4, 5). In lanes 2 and 4, 8 µg protein was loaded; in lanes 3 and 5, 4 µg was loaded. The erosion samples show a clear band (arrow), corresponding to the clear band in the sarcoma cell sample, representing MMP-2 with a molecular mass of 72 kDa. The higher molecular weight clear band in the sarcoma cell line has an approximate molecular mass of 92 kDa and represents MMP-9. Molecular weight standards (in kilodaltons) are shown at left.

DISCUSSION

To our knowledge, this is the first report of the presence of matrix metalloproteinases in human epithelium affected by recurrent erosion. We have observed, using gelatin zymography, that 12 of 13 of the epithelial erosion samples contained MMP-2, whereas only one sample contained MMP-9. None of the normal human epithelia samples contained MMP-2 or MMP-9. These results suggest that the expression of MMP-2 is upregulated in epithelia with recurrent erosion. The immunolocalization data show that the cellular source of MMP-2 is the epithelium with the most intense localization of the enzyme in the basal epithelium. This indicates that MMP-2 is actively produced in the erosion epithelium but does not rule out the possibility that some of the MMP-2 activity is derived from a stromal cell source.

The data suggest that synthesis of MMP-2 occurs in the basal epithelial layers and is induced by injury to the human corneal epithelium, because recurrent epithelial erosion is usually secondary to trauma. Animal studies have shown some similar and some differing results. Ye and Azar21 recently showed, using in situ hybridization and immunolocalization in a rat wound healing model, that although MMP-2 is present in basal epithelial cells and in the superficial stroma of unwounded corneas, the expression of MMP-2 is upregulated after wounding. Studies of rabbit cell culture models have shown that unwounded corneal epithelial cells synthesize a 92-kDa gelatinase corresponding to MMP-9, whereas a 72-kDa gelatinase corresponding to MMP-2 is synthesized mostly by stromal fibroblasts.15 In rabbits with keratectomy wounds, it has been shown that stromal fibroblasts remain a major producer of MMP-2, with little produced by the corneal epithelial cells.18 In this healing tissue, corneal epithelial cells synthesized predominantly MMP-9. That only one sample with human epithelial erosion expressed MMP-9 may be because of collection of this sample closer to the time of injury. In rat models, MMP-9 plays a role in epithelial regulation and migration early in corneal wound healing.21

Immunolocalization of laminin and type VII collagen in one erosion sample showed the presence of laminin in basal epithelial cells and the basal lamina zone. However, type VII collagen, an integral part of the anchoring complex, was only detected in basal epithelial cells and not in the basal lamina. This suggests that type VII collagen is degraded and removed from the basal lamina zone. The colocalization of MMP-2 to the basal epithelium and the fact that known substrates of this enzyme include type VII collagen suggest that it may be playing a key role in the breakdown of anchoring complex.

A second erosion sample revealed the absence of laminin and type VII collagen in the area of the basal lamina. Both of these proteins, however, were clearly present in the basal epithelial cell. This suggests that the epithelium in this sample...
FIGURE 3. Immunolocalization of laminin (A, C, E) and type VII collagen (B, D, F) in two samples of recurrent epithelial erosion (A+B, C+D) and normal control samples (E, F). In the first sample (A, B), laminin is detected in the basal epithelial cells where it is normally synthesized (A, arrows) and in the basal lamina area (A, arrow 2). Type VII collagen localized in basal epithelial cells (B, arrows); however, it was not detected within the basal lamina area. In the second sample (C, D), laminin was detected in basal epithelial cells, but not along the base of the epithelium (C). Type VII collagen was detected within basal epithelial cells (arrows), but not along the base of the epithelium (D). Bar, 50 μm.

In conclusion, the expression of MMP-2 appears to be upregulated in corneas affected by recurrent erosion compared with that in normal corneas. The enzyme may be responsible for the breakdown of the anchoring macromol-
Dorzolamide Effect on Ocular Blood Flow

Antonio Martinez,1 Francisco Gonzalez,2,3 Carmen Capeans,1,2 Roberto Perez,2 and Manuel Sanchez-Salorio1,2

PURPOSE. To evaluate the effect of dorzolamide on ocular blood flow in normal and glaucomatous eyes.

METHODS. Twenty-six eyes with documented open-angle glaucoma of 26 patients and 13 normal control eyes of 8 age-matched subjects were included in this study. All eyes underwent color Doppler imaging for measuring peak-systolic velocity, end-diastolic velocity, and resistance index in the ophthalmic and central retinal arteries and the maximal and minimal velocities in the central retinal vein. Eyes were grouped in control and initial and advanced glaucoma categories. Measurements were made in all groups before and after application of topical dorzolamide. Intragroup comparisons between baseline and dorzolamide conditions were made using paired Student's t-test. Intergroup comparisons under baseline conditions between normal and glaucomatous eyes were made by using the one-way ANOVA test. Statistical significance was set at P < 0.05.

RESULTS. The peak-systolic velocity of the central retinal artery in glaucomatous eyes and the end-diastolic velocity of the ophthalmic and central retinal arteries in all groups were significantly higher after application of dorzolamide. The minimal velocity of the central retinal vein showed significantly higher values after dorzolamide, whereas the maximal velocity remained unchanged. The peak-systolic velocity of the ophthalmic artery in all groups and the peak-systolic velocity of the central retinal artery in normal eyes also remained unchanged. The resistance index

References