Matrix Metalloproteinase-1 Localization in the Normal Human Uveoscleral Outflow Pathway

Dan D. Gaton, Takeshi Sagara, James D. Lindsey, and Robert N. Weinreb

PURPOSE. To determine the distribution of matrix metalloproteinase-1 (MMP-1) in the uveoscleral outflow pathway and other anterior segment tissues of normal human eyes.

METHODS. Normal human eyes were fixed in methacarn and sectioned and immunostained using a specific polyclonal antibody to MMP-1. Immunoreactivity was visualized using diaminobenzidine. To compare the staining intensity in various tissues, the mean optical density within the ciliary body, mid-iris stroma, iris root, uveal trabecular meshwork, cornea, and sclera was determined using imaging densitometry. To determine the cellular distribution of MMP-1 in ciliary muscle, additional sections were double-immunostained using antibodies to MMP-1 and calponin. These sections were examined by confocal laser scanning microscopy. Specificity of the antibody to MMP-1 in ocular tissues was confirmed by western blot analysis with uveal tract homogenates.

RESULTS. Moderate-to-strong MMP-1 immunoreactivity was observed in ciliary muscle, iris, sclera, corneal endothelium, and ciliary nonpigmented epithelium. Lighter immunoreactivity was observed in corneal epithelium, blood vessels, trabecular meshwork, Schlemm's canal, and associated collector channels. Confocal microscopy showed that ciliary muscle MMP-1 was primarily inside ciliary muscle cells. Densitometry showed that net optical density was approximately fivefold greater in ciliary muscle, iris root, and sclera than in trabecular meshwork.

CONCLUSIONS. MMP-1 was prominently identified in regions of the anterior segment of normal human eyes associated with the uveoscleral outflow pathway and in the iris, corneal endothelium, and ciliary nonpigmented epithelium. These data support the hypothesis that MMP-1 activity is involved in regulating uveoscleral outflow facility. (Invest Ophthalmol Vis Sci. 1999;40:363–369)

Matris metalloproteinase-1 (MMP-1), also known as interstitial collagenase, is a neutral zinc-dependent enzyme capable of hydrolyzing specific peptide sequences found in structural components of the extracellular matrix (ECM). MMP-1 is secreted as an inactive proenzyme (zymogen) and becomes activated by proteolytic truncation. It has been shown to mediate normal ECM turnover in a number of different cell systems, including synoviocytes and vascular smooth muscle cells.

In the eye, MMP-1 has been found in cultured human retinal pigment epithelium and trabecular meshwork. MMP-1 also has been detected in the aqueous humor. Recent evidence suggests that MMP-1 may be involved in regulating uveoscleral outflow. Tracer studies have shown that the uveoscleral outflow passes through the extracellular spaces among ciliary muscle fibers. These spaces contain collagen types I and III, which can be degraded by MMP-1.

Increased MMP-1 secretion and reduction of collagen types I and III in cultured ciliary smooth muscle cells occur after exposure to prostaglandins. Certain prostaglandins lower intraocular pressure by increasing uveoscleral outflow. Hence, it has been proposed that MMP-1-mediated reduction of ECM within the ciliary muscle may reduce hydraulic resistance around the fibers and thereby increase uveoscleral outflow.

Unknown, however, is the distribution of MMP-1 within the ciliary muscle or within other anterior segment tissues. In view of its potential role in regulating the uveoscleral outflow, we investigated the distribution of MMP-1 in sections of normal human anterior segment tissue, including the uveoscleral outflow pathway.

MATERIALS AND METHODS

Human Tissue

Five human eyes from five donors 47 to 80 years of age were obtained from the San Diego Eye Bank. Donors had no known history of glaucoma or other eye diseases. The anterior segments of the enucleated eyes were isolated by a circumferential cut made 4 mm posterior to the limbus. The tissue was then fixed in methacarn (60% methanol, 30% chloroform, and 10% glacial acetic acid) for 3 hours. Next, the tissue was embedded in paraffin by conventional protocol, and 6-μm-thick sections were collected on slides precoated with Vectabond (Vector Laboratories, Burlingame, CA).
Peroxidase Immunohistochemistry

Different dilutions of the anti-MMP-1 primary antibody were compared to optimize the immunostaining, and a ratio of 1:25 was found to produce subsaturating results. The sections were deparaffinized in xylene, rehydrated through graded ethanol, and treated with antigen retrieval solution (AR-10; BioGenex, San Ramon, CA) heated to 95°C for 5 minutes. After cooling, the sections were rinsed in PBS, preincubated with 3% H₂O₂ to suppress endogenous peroxidase activity, and blocked for 30 minutes with 0.1% bovine serum albumin (Sigma). The sections were incubated with the primary antibody for 30 minutes, rinsed in PBS, and incubated with donkey anti-sheep IgG (Biotin-SP-Conjugated Affipure; Jackson ImmunoResearch; diluted to 1:500) for 30 minutes. After they were rinsed in PBS, the slides were exposed to horseradish peroxidase-conjugated streptavidin (HRP Label; BioGenex) for 20 minutes, rinsed in PBS, developed with diaminobenzidine chromogen solution (BioGenex) for 10 minutes, washed with PBS, and mounted with gelvatol. Antigen-antibody reaction

Western Blot Analysis

The primary antibody used for immunohistochemistry was polyclonal sheep anti-porcine collagenase (Chemicon International, Temecula, CA). This antibody can detect collagenase in human tissues and in porcine tissues. The specificity of this antibody for MMP-1 was assessed by Western blot analysis. Anterior segments were isolated from fresh human eyes by incising 5 mm posterior to the limbus. Uveal tract tissues, including the ciliary muscle and iris, were collected and then diced and mixed with an equal volume of extraction buffer containing 2.5% sodium dodecyl sulfate, 1 mM ethylene diamine tetraacetic acid, and 50 mM Tris, pH 8. After homogenization by 10 strokes in a dounce homogenizer, the homogenate was centrifuged at 12,000g for 20 minutes, and the supernatant was collected. Proteins in this supernatant were separated by electrophoresis on a 10% polyacrylamide gel (NuPage; Novex, San Diego, CA) and then transferred to nitrocellulose membranes for Western blot analysis. The membranes were blocked by a 1-hour exposure to phosphate-buffered saline (PBS) containing 0.2% Tween-20 and 30% fresh nonfat milk. Next, the membranes were incubated with sheep polyclonal anti-porcine MMP-1 antibody (diluted 1:200), washed, and incubated with biotinylated donkey anti-sheep IgG secondary antibody that had been preadsorbed to minimize cross reaction with human proteins (1:500; Jackson ImmunoResearch, West Grove, PA). After washing, the membranes were incubated with avidin-conjugated horseradish peroxidase (Sigma Chemical, St Louis, MO), washed again, and developed using 2.8 mM 4-chloro-1-napthol and 0.18% H₂O₂ in PBS.
FIGURE 3. High-power magnification images of immunostained ciliary muscle (A, C) and corresponding control stained ciliary muscle (B, D). Cellular distribution of calponin immunoreactivity (E) and MMP-1 (F) present in ciliary smooth muscle cells. Antibody binding detected using immunoperoxidase method (A, B, C, D) or using fluorescein-labeled secondary antibodies (E, F). Note that most of the MMP-1 immunoreactivity colocalizes with calponin immunoreactivity. Magnification, (A, B) ×60; (C, D) ×260; (E, F) ×420.

| Table 1. MMP-1 Immunoreactivity in Human Anterior Segment Tissues |
|-----------------|-------|-------|-------|-------|-------|-------|
| Location                     | 47 yo | 56 yo | 75 yo | 78 yo | 80 yo |
| Ciliary muscle              | ++    | ++    | ++    | ++    | ++    |
| Iris anterior border        | ++    | ++    | ++    | ++    | ++    |
| Iris stroma                 | ++    | ++    | ++    | ++    | ++    |
| Adjacent to iris pigment epithelium | +      | ++    | ++    | ++    | ++    |
| Iris sphincter smooth muscle | ++    | ++    | ++    | ++    | ++    |
| Iris root                   | +     | ++    | ++    | ++    | ++    |
| Sclera                      | +     | +     | ++    | ++    | ++    |
| Corneal epithelium          | +     | ++    | ++    | ++    | ++    |
| Corneal stroma              | +     | ++    | ++    | ++    | ++    |
| Corneal endothelium         | +++   | ++    | ++    | ++    | ++    |
| trabecular meshwork         | +     | ++    | ++    | ++    | ++    |
| Ciliary nonpigmented epithelium | + +   | ++    | ++    | ++    | +++  |
| Blood vessel endothelium    | ++    | ++    | ++    | ++    | ++    |
| Arteriole smooth muscle     | ++    | +     | ++    | ++    | ++    |

yo, years old; +, light staining; ++, moderate staining; ++++, intense staining.
FIGURE 4. MMP-1 immunoreactivity present in the mid-central iris (A) or in the iris root (C). Control sections prepared without primary antibody (B, D). Note that MMP-1 immunoreactivity is less in regions of the stroma that do not have clusters of pigment-containing cells (*). Magnification, ×120.

FIGURE 5. Distribution of MMP-1 immunoreactivity in sclera (A), corneal epithelium (C), and corneal endothelium (E). Corresponding control sections prepared without primary antibody showed minimal staining (B, D, F). Magnification, (A, B) ×150; (C, D) ×230; (E, F) ×280.

on the platen of a two-dimensional scanning densitometer (model GS-700 Imaging Densitometer; Bio-Rad, Hercules, CA). Transilluminating image scans of five parallel mid-sagittal-stained sections from each eye were collected at a resolution of 1200 dpi (50 μm). Data analysis was performed using Molecular Analyst software (Bio-Rad).

was signified by a brown precipitate. Controls for nonspecific secondary antibody binding were treated identically except that the primary antibody incubation was omitted.

Confocal Microscopy
To assess the cellular distribution of MMP-1 in the ciliary muscle, additional sections were double-immunostained using the same polyclonal sheep anti-porcine collagenase described above (diluted 1:25) and monoclonal anti-human calponin antibody (clone hCP, diluted 1:1000; Sigma). Specificity of the anti-calponin has been previously confirmed. Calponin is a cytoplasmic actin-binding protein present in the ciliary muscle cells. Secondary antibodies included Alexa 594-conjugated donkey anti-sheep IgG antibody (diluted at 1:200; Molecular Probes, Eugene, OR) and Alexa 488-conjugated goat-anti-mouse IgG (diluted at 1:200; Molecular Probes). These sections were examined using a confocal laser scanning microscope fitted with a krypton-argon laser (FLUOVIEW; Olympus America, Melville, NY).

Densitometry
To quantify MMP-1 immunoreactivity, human anterior segment tissue-stained sections were scanned by directly laying them on the platen of a two-dimensional scanning densitometer (model GS-700 Imaging Densitometer; Bio-Rad, Hercules, CA). Transilluminating image scans of five parallel mid-sagittal-stained sections from each eye were collected at a resolution of 1200 dpi (50 μm). Data analysis was performed using Molecular Analyst software (Bio-Rad).

FIGURE 6. (A) MMP-1 immunoreactivity in trabecular meshwork, surrounding Schlemm's canal (SC), and surrounding aqueous collector channels (CC). (B) Control section similarly processed except for omission of primary antibody. Magnification, ×70.
RESULTS

Western Blot Analysis

Western blot analysis yielded two sharp bands with apparent molecular weights of 49 kDa and 62 kDa (Fig. 1). The 49-kDa band corresponds to a 49-kDa protein from retinal pigment epithelial cells identified as MMP-1. The 62-kDa band corresponds to a 62-kDa protein secreted by ciliary smooth muscle cells in vitro and identified as the proenzyme form of MMP-1. These results support the specific recognition of MMP-1 by this antibody.

Immunohistochemical Analysis

Examination of immunoperoxidase-stained sections at low-power magnification revealed immunoreactivity in the ciliary muscle, iris, trabecular meshwork, and sclera (Fig. 2). At a higher magnification, MMP-1 immunoreactivity was seen to be localized into narrow strips separated by minimally stained strips of ECM (Figs. 3A, 3B). These immunoreactive strips contained bundles of parallel ciliary smooth muscle cell fibers (Figs. 3C, 3D). Similar staining was observed in all five eyes (Table 1). Ciliary muscle sections also were double-stained using antibodies to MMP-1 and to calponin, an actin-binding protein found in smooth muscle cell cytoplasm. Examination of these sections by confocal scanning laser microscopy revealed that the image of the smooth muscle cells stained with calponin (Fig. 3D) coincided with the distribution of MMP-1 immunoreactivity (Fig. 3F). This suggested that most of the MMP-1 immunoreactivity in the ciliary muscle was inside the smooth muscle cells.

MMP-1 immunoreactivity in the iris was observed at the anterior border layer, adjacent to pigmented cell clusters within the iris stroma, and adjacent to the pigment epithelium (Fig. 4). At the anterior border layer, immunoreactivity surrounded the clusters of pigmented melanocyes. Melanocyte-free regions of iris stroma contained less MMP-1 immunoreactivity than adjacent regions with melanocytes (Fig. 4A, asterisk). MMP-1 immunoreactivity also was observed in the iris sphincter muscle (not shown) and in the iris root (Figs. 4C, 4D). The iris root staining was more easily seen than staining in the central region of the iris because there were less pigment-containing cells in the iris root.

Long strands of MMP-1 immunoreactive material corresponding to collagen bundles were regularly observed in the sclera (Figs. 1A and 5A). MMP-1 immunoreactivity usually was less in the corneal stroma than in the sclera (compare Figs. 5C and 5A, respectively). In two of the eyes examined, there was moderate staining within corneal epithelial cells (Table 1; Figs. 5C, 5D). There was light staining of the corneal epithelium in the remaining eyes. In contrast, moderate-to-intense staining of the corneal endothelium was observed in all eyes examined (Figs. 5E, 5F).

Light-to-moderate staining was observed in the beams of the trabecular meshwork (Fig. 6), consistent with prior reports of MMP-1 production by trabecular meshwork cells. Immunoreactivity also was observed surrounding Schlemm's canal and surrounding nearby aqueous collector channels.

Within the ciliary pigmented epithelium, moderate-to-intense MMP-1 immunoreactivity was observed in all examined eyes (Figs. 7A, 7B). Immunoreactivity also was present in ciliary pigment epithelial cells. Light-to-moderate immunostaining was observed in blood vessel endothelial cells and in smooth muscle surrounding arterioles (Figs. 7C, 7D).

Quantitative Analysis

Densitometry was used to compare the intensity of MMP-1 immunoreactivity in the ciliary muscle, iris root, sclera, corneal stroma, and trabecular meshwork. The iris root was better for densitometric analysis than the main iris stroma because typically it contained less endogenous pigment (Fig. 4). To allow...
compensation for endogenous optical density (OD) present within all tissues, measurements were collected from the sections processed with and without primary antibody.

Optical density along two line segments overlying each anterior segment tissue, near its widest portion, was scored as illustrated in Figure 8. The mean OD scores were calculated by dividing the area score of OD (OD X millimeters) by the length of the line segments (in millimeters). Correction for the OD arising from the glass slide and mounting medium was achieved by collecting an OD measurement along a line segment adjacent to but not overlying the tissue and subtracting this mean OD score from the raw scores. Pigmented epithelia of the ciliary body and iris pigment epithelia readings were excluded from consideration. The mean OD score for each eye was calculated and compared with corresponding scores from control sections. The paired Student’s t-test was used to compare OD scores from immunostained sections with control sections of the same tissue. The net OD score for each tissue type was calculated by subtracting the OD scores from the sections processed with primary antibody from the OD scores from the control sections. Finally, the net OD scores for each tissue type were compared by ANOVA and the Student-Newman-Keuls test.

As shown in Table 2, the ciliary muscle, iris root, and sclera OD scores from the sections processed with primary antibody were 5- to 10-fold greater than the corresponding control scores. These differences all were statistically significant. In contrast, the differences between the immunostained and control corneal stroma and trabecular meshwork scores were not significant. The scores for the corneal stroma were quite low, indicating that the light staining observed in the corneal stroma is nonspecific. In the case of the trabecular meshwork, the defined nature of the observed staining suggested that it is specific. However, high variability among these scores from the different eyes may explain the absence of a statistically significant result. The Student-Newman-Keuls test showed that the differences among the net OD scores for the ciliary muscle, iris root, and sclera were insignificant (Table 3). Likewise, the differences between the net scores for the corneal stroma and the trabecular meshwork were insignificant. In contrast, the mean scores from the ciliary muscle, iris root, and sclera each were significantly greater than the mean OD scores for the corneal stroma or the trabecular meshwork.

### DISCUSSION

This study demonstrates the presence of substantial MMP-1 immunoreactivity in the ciliary muscle, iris, sclera, corneal endothelium, and ciliary epithelium in normal human eyes. Less immunoreactivity was found in the corneal epithelium, blood vessels, trabecular meshwork, and associated collector channels. The results of western blot analysis are consistent with specific recognition of human MMP-1 by the polyclonal anti-MMP-1 antibody.

The distribution of MMP-1 is well positioned to play an important role in regulating uveoscleral outflow. Tracer studies with fluoresced dextrans indicate that the uveoscleral outflow pathway includes the iris, the ciliary muscle, the adjacent sclera, and, to a smaller degree, the posterior sclera. More recent tracer studies indicate that uveoscleral flow in the ciliary muscle passes through the interstitial ECM regions among ciliary smooth muscle fiber bundles. Such flow is supported by the observation of a pressure gradient between the anterior chamber and suprachoroidal space in vivo. Increased MMPs may reduce the amount of ECM in the ciliary muscle portion of the uveoscleral outflow pathway and thereby decrease hydraulic resistance to flow. In recent studies that support this possibility, induction of ciliary muscle MMPs was observed after topical prostaglandin treatment of cynomolgus monkey eyes (authors’ unpublished data). Moreover, there is a simultaneous reduction in the specific content of collagen types I, III, and IV in ciliary muscle (authors’ unpublished data). In view of previous evidence indicating FP receptors in corneal endothelium, iris, and ciliary processes, the present observation that these tissues contain MMP-1 suggests that they might be involved in the ocular response to prostaglandins as well. Moreover, endogenous prostaglandin release by human trabecular meshwork cells also may influence adjacent ciliary muscle cells and therefore might affect their release of MMPs.

It is not known whether MMP-1 immunoreactivity within the sclera adjacent to the ciliary body has any physiological or pathologic significance. Previous studies have shown that when fluoresceinated dextran is injected into the anterior chamber it becomes distributed within the scleral stroma adjacent to the ciliary muscle and suggest that there is bulk fluid movement across the sclera. This view is supported by

<table>
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<tr>
<th>Site</th>
<th>OD&lt;sub&gt;c&lt;/sub&gt; ± SD</th>
<th>OD&lt;sub&gt;c&lt;/sub&gt; ± SD</th>
<th>P</th>
<th>OD&lt;sub&gt;net&lt;/sub&gt; ± SD&lt;sub&gt;net&lt;/sub&gt;</th>
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<tr>
<td>Ciliary muscle</td>
<td>0.096 ± 0.013</td>
<td>0.011 ± 0.011</td>
<td>&lt;0.001</td>
<td>0.085 ± 0.024</td>
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<tr>
<td>Iris root</td>
<td>0.098 ± 0.014</td>
<td>0.021 ± 0.015</td>
<td>&lt;0.001</td>
<td>0.077 ± 0.029</td>
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<tr>
<td>Sclera</td>
<td>0.073 ± 0.017</td>
<td>0.010 ± 0.009</td>
<td>&lt;0.001</td>
<td>0.063 ± 0.026</td>
</tr>
<tr>
<td>Corneal stroma</td>
<td>0.003 ± 0.003</td>
<td>0.002 ± 0.001</td>
<td>0.31</td>
<td>0.001 ± 0.004</td>
</tr>
<tr>
<td>Trabecular meshwork</td>
<td>0.018 ± 0.018</td>
<td>0.011 ± 0.007</td>
<td>0.37</td>
<td>0.007 ± 0.025</td>
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TABLE 3. Comparison of OD<sub>net</sub> by Student-Newman-Keuls Test

<table>
<thead>
<tr>
<th>Site</th>
<th>Iris</th>
<th>Sclera</th>
<th>Corneal Stroma</th>
<th>Trabecular Meshwork</th>
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<tbody>
<tr>
<td>Ciliary muscle</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Iris root</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sclera</td>
<td>+</td>
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<tr>
<td>Corneal stroma</td>
<td>-</td>
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+ P < 0.05; - P > 0.05.
the observation of sodium pertechnetate-labeled saline movement across scleral plugs. Finally, potential specializations that could facilitate such movements are suggested by the demonstration of transscleral passages extending from the suprachoroidal space deep into the sclera of human eyes. As previously suggested by Toris et al., it is possible that the sclera adjacent to the ciliary body is part of the uveoscleral pathway.

The significance of MMP-1 immunoreactivity within the iris also is not known. Movement of aqueous protein between the ciliary muscle and the anterior chamber aqueous humor has been supported by recent tracer studies. In particular, these studies have shown that a significant fraction of protein normally present in the aqueous humor originates in the ciliary and iridal processes and that it diffuses anteriorly through the iris stroma to the anterior chamber. On the other hand, aqueous humor proteins may gain access to the uveoscleral outflow pathway via the iris root. Also, it is possible that MMP-1 may be biosynthesized in the iris root tissue. Further studies will be required to evaluate these possibilities.

MMP-1 activity is one of many factors that can regulate ECM deposition in situ. This activity may reflect changes in biosynthesis and changes in the production of agents that regulate the activity of these enzymes as tissue inhibitors of MMP. In addition, changes in ciliary muscle ECM may reflect the net effect of changes in the biosynthesis and assembly of the matrix components themselves.

Acknowledgment

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References