Direct Matrix Metalloproteinase Enhancement of Transscleral Permeability

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PURPOSE. Previous studies have shown that increased transscleral permeability after exposure to certain prostaglandins is associated with increased intrascleral matrix metalloproteinases (MMPs). The present study was undertaken to determine whether these MMPs could directly alter transscleral permeability.

METHODS. Freshly enucleated mouse eyes were incubated with human MMP-1, -2, and -14 for 4 hours at 37°C. The eyes then were incubated with 10 or 70 kDa dextran-tetramethylrhodamine-lysine for 16 to 32 minutes at 37°C. Two methods of analysis were used. In the first, quickly isolated retinas were homogenized and centrifuged. Fluorescence in the supernatants was determined by microspectrofluorimetry. In the second, the eyes were fixed in 4% paraformaldehyde, and frozen sections were prepared. After the identity of the sections was masked, the intensity of fluorescence in anterior, middle, and posterior regions of the outer retina and inner retina was scored with a 7-point grading scheme.

RESULTS. The concentration of 10-kDa fluorescent dextran was 5.14 ± 1.61 μg/mL (mean $\pm$ SD, $n = 33$) in the control retinal supernatants, and 6.37 ± 2.67 μg/mL ($n = 40$) in the retinal supernatants from the MMP-treated eyes. This increase was statistically significant ($P < 0.02$, t-test). The structural organization of the retina and other ocular tissues was maintained in all experimental conditions. Histologic scoring of fluorescence found significantly increased dextran in the outer retina of eyes treated with MMPs for 32 minutes (the score of control eyes was 2.5 ± 0.4 and of MMP-treated eyes was 3.5 ± 0.1, mean $\pm$ SD; $P = 0.02$, $n = 3$). Analysis by region found greater scores in the third of the retina nearest to the optic nerve head.

CONCLUSIONS. These results show that MMP-1, -2, and -14 can directly increase transscleral permeability and support the view that the increased MMP-1 and -2 observed after topical PG treatment could contribute to increased uveoscleral outflow. (*Invest Ophthalmol Vis Sci.* 2007;48:752–755) DOI:10.1167/iovs.06-0334

Transscleral permeability of scleral organ cultures to macromolecular tracers can be enhanced by exposure to prostaglandins (PGs) and PG analogues that can lower intraocular pressure.[1,2] This permeability increase is accompanied by in-creased scleral gene transcription,[3] biosynthesis,[4] and release[1] of certain matrix metalloproteinases (MMPs), neutral proteases that can specifically initiate cleavage of extracellular matrix components.[5] Although this evidence raise the possibility that these increased MMPs mediate the increased transscleral permeability, direct evidence of this capability is lacking. We have found that subconjunctivally injected fluorescent 70-kDa dextran reproducibly permeates the mouse eye sclera.[6] To test the hypothesis that increased scleral MMPs could directly mediate increased transscleral permeability, the present study was undertaken to assess whether exposure of isolated mouse eyes to MMPs would increase transscleral delivery of high-molecular-weight dextrans to the retina.

METHODS

MMP Incubation

The procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult Swiss white mice weighing 28 to 35 g were anesthetized by intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (9 mg/kg), and the eyes were quickly harvested. Control eyes were incubated in Hanks'-buffered saline with calcium, magnesium, and glucose (Invitrogen-Gibco, San Diego, CA) and supplemented with 10 μM zinc chloride. These incubations were run for 4 hours at 37°C in a shaking water bath with gentle agitation. The incubation medium for the experimental eyes also contained 1 μg/mL each of purified human proMMP-1 and activated MMP-2 and -14 catalytic domain (EMB/Calbiochem, San Diego, CA). After these incubations were complete, the eyes were transferred to Hanks'-buffered saline containing 2.5 μg/mL 10- or 70-kDa dextran conjugated to tetramethylrhodamine (TMRD) and to lysine (Invitrogen-Molecular Probes, Eugene, OR) and then incubated at 37°C for 4 to 64 minutes. Transscleral permeability of these dextrans has been studied previously.[1,6] Pilot studies found the greatest sensitivity after incubations lasting 16 and 32 minutes.

Spectrophotometric Analysis of Permeability

After incubation in 2.5 mg/mL TMRD conjugated to 10-kDa dextran (10 kDa-TMRD) for 30 minutes, the retinas were isolated and homogenized on ice with 60 μL of PBS using a ground-glass homogenizer. The homogenate was then centrifuged at 14,000g for 10 minutes. Fluorescence in 2-μL samples of supernatant was measured directly using a microspectrofluorimeter (ND-3300; Nanodrop Technologies, Wilmington, DE). Calibration of the measurements was achieved by measuring fluorescence in serial dilutions of 10 kDa-TMRD standards.

Histologic Assessment of Permeability

The isolated eyes were incubated in 2.5 mg/mL TMRD conjugated to 70-kDa dextran (70 kDa-TMRD) for 4, 8, 16, 32, or 64 minutes, quickly rinsed in plain Hanks'-buffered saline, and then transferred to 4% formaldehyde (freshly prepared from paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) at 4°C for 4 hours. This step chemically cross-linked the lysine groups on the fluorescent 70 kDa-TMRD to lysine residues within the tissue proteins and thus prevented any further diffusion of the dextran during subsequent tissue processing. The eyes were placed sequentially in 10% sucrose in phosphate-buffered saline

TABLE 1. MMPs Increase Dextran in Retinal Supernatants

Experimental Group	Dextran Concentration*	Eyes (N)	t-Test (P)
Control	5.14 ± 1.61	33	
MMP-treated	6.37 ± 2.67	40	0.0194
Percent change	+24%	—	—

* Data are in micrograms per milliliter, mean ± SD.

(PBS), 20% sucrose in PBS, and 30% sucrose in PBS, to protect against ice crystal formation during freezing. The eyes were then placed in plastic molds that were half filled with tissue-freezing medium (TFM; Triangle Biomedical Sciences, Durham, NC) and covered with more TFM. The molds were snap frozen by immersion in a 2-methyl butane and dry ice mixture, and the frozen tissue was sectioned axially at 12 μm on a cryostat. The sections were sequentially mounted on positively charged slides (Positive-charged Microscope Slides; BioGenex, San Ramon, CA) and dried overnight. Coverslips were applied with a nonfluorescent mounting medium (Fluoromount-G; Southern Biotechnology Associates, Birmingham, AL). The identity of the slides was masked before analysis.

Four to six sections from each eye were examined with fluorescence microscopy and scored. The average of these determinations was considered the final score for each eye, to minimize the potential influence of a frozen section that might have been thicker than average (which could produce higher fluorescence scores). For each experiment containing 8 to 10 control and experimental eyes, the scoring was performed in one session at the microscope without changing any of the illumination settings between slides. For the purpose of analysis, the span of the retina on each side of the optic nerve was divided into thirds including the anteriormost third, adjacent to the ciliary body, the middle region extending from the equator to halfway back to the optic nerve head, and the posterior region adjacent to the optic nerve head. Fluorescence intensity within each region's inner and outer retina was graded separately for the brightness of the fluorescence by a subjective 7-point grading scale as follows: absent, 1; uniformly very dim, 2; generally very dim with moderately dim areas, 3; moderately dim, 4; moderately dim with moderately bright areas, 5; moderately bright, 6; and moderately bright with highly bright areas, 7. Scores from the corresponding regions on each side of the optic nerve head were combined.

The final scores from the outer retina were considered separately from the inner retina as dextran concentration in the inner retina might be reduced as it passed from the outer retina to the inner retina. Statistical analysis considered all regions together as well as separately. In the former case, the mean of the final scores from each corresponding region was obtained before statistical comparison using the unpaired Student's t-test. The retinal regions were considered separately to assess the possibility that certain regions might show more change in response to the MMP treatment than other regions. Results were deemed significant when $P < 0.05$.

After analysis, the slides were photographed using a cooled digital camera (Spot Digital Camera System; Diagnostic Instruments, Sterling Heights, MI). For each experiment, photography was performed in one session at the microscope without changing any of the illumination settings between slides.

RESULTS

Spectrophotometric Analysis of Permeability

The mean concentration of 10-kDa fluorescent dextran was 5.1 ± 1.6 μg/mL (SD) in retinal homogenate supernatants from the control eyes and 6.4 ± 2.7 μg/mL in retinal homogenates of the eyes that had been incubated with MMPs (summarized in Table 1). This 24% increase was statistically significant ($P < 0.02$) by the two-tailed Student's t-test.

Preservation of Retinal Architecture

The structural organization of the retina and other ocular tissues was maintained in all experimental conditions. The inner and outer nuclear layers appeared as compact cell layers, with minimal spacing between the cells. The inner and outer plexiform layers appeared as uniformly dense neuropil. There was no difference in the appearance of sections from the control and the sections from the MMP-treated eyes (Fig. 1).

Histologic Assessment of Permeability

When the scores from all regions were considered together, it was observed that there was no statistical difference between fluorescence in the control and MMP-treated inner retina (Table 2). In contrast, fluorescence in the outer retina from the eyes probed with dextran for 32 minutes was significantly greater than in the control retina.

When the scores for each retinal region of eyes probed with dextran for 32 minutes were considered separately, it was observed that the score in the middle region inner retina increased from 2.0 ± 0 to 2.6 ± 0.2 ($P = 0.01$; Table 3). In the middle region outer retina, the score increased from 2.6 ± 0.4 to 3.8 ± 0.1 ($P = 0.01$). In the posterior outer retina, the score increased from 2.6 ± 0.05 to 4.2 ± 0.05 ($P = 0.02$). Within each region, the scores in the inner retina were always less than the scores in the outer retina, though this difference reached significance in the mid and posterior regions of the MMP-treated retinas.
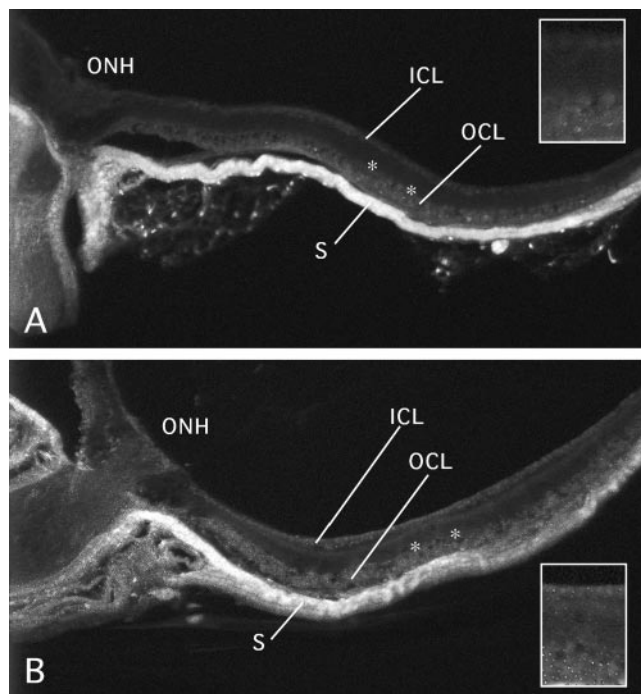

FIGURE 1. Frozen sections of the posterior retina of control (**A**) and MMP-treated (**B**) eyes that were incubated in 70-kDa dextran for 32 minutes. Labeled structures include the sclera (S), retinal inner cell layer (ICL), retinal outer cell layer (OCL), and optic nerve head (ONH). *Insets*: high-magnification views of the retina at similar distances from optic nerve head (between the asterisks [✱] on the lower-magnification images). Note that the fluorescence was brighter in both the inner and outer cell layers of the MMP-treated eye. Magnification: (**A**, **B**) ×50; *inset*: ×100.

TABLE 2. Retina Scores after 4-hour MMP Treatment: All Regions Considered Together

	16-Minute Dextran Probe		32-Minute Dextran Probe	
	Inner Retina	Outer Retina	Inner Retina	Outer Retina
Control ($n = 3$)	2.2 ± 0.3	2.4 ± 0.3	2.0 ± 0.1	2.5 ± 0.4
MMPs ($n = 3$)	2.1 ± 0.1	2.8 ± 0.5	2.4 ± 0.2	3.5 ± 0.1
Percent change	-5%	$+17\%$	$+20\%$	$+40\%$
t-Test	NS	NS	NS	$P = 0.02$

* Data are the mean $\pm$ SD.

DISCUSSION

The results of this study indicate that exposure of sclera to MMP-1 and -2, and the catalytic domain of MMP-14 increases the penetration of dextran through the sclera and into the retina. The findings show that MMP-1, -2, and -14 can increase transscleral permeability to macromolecules. Moreover, it supports the view that the increased scleral MMPs observed after topical PG treatment directly contribute to increased uveoscleral outflow.

The composition of the MMP treatment solution was guided by several considerations. MMPs generally are secreted as proenzymes and then activated in the extracellular environment by proteolytic truncation.[5] The proform of MMP-1 is more stable than the active form, because the active form can cleave itself. ProMMP-1 was included because the active form of this MMP efficiently initiates degradation of fibrillar collagen by cutting collagen type I or III (the major components of fibrillar collagen) at a location that allows the collagen molecule to unwind from the fibril. This is an appropriate target, as more than 50% of the dry weight of human sclera is collagen type I.[7] Unwinding of these collagens increases their susceptibility to further degradation by MMP-2, also known as gelatinase A.[5] Active MMP-2 was provided because activation in vivo requires tissue inhibitor of matrix metalloproteinase (TIMP)-2, a 21-kDa protein that also can irreversibly inhibit MMP activation.[5] MMP-14 is unlike most of the MMPs in that it is covalently tethered to the cell membrane. When activated, it efficiently converts proMMP-1 to active MMP-1. The catalytic fragment of MMP-14 retains the specificity and MMP-1-activating capability of activated MMP-14 in vivo, but is soluble. Both MMP-1 and -2 were increased in monkey sclera after topical PG treatments.[4] MMP-14 mRNA is present in fresh human sclera,[3] suggesting that MMP-14 may be available to facilitate MMP-2 activation in vivo. In all cases, MMPs require the presence of calcium or zinc for activation and both were present in the incubation media. The glucose in the Hanks' solution provided for the energy needs of ocular tissues during the incubation. Thus, it was anticipated that the present MMP mixture acted to remove some of the fibrillar collagen elements of the scleral stroma. This action could have enlarged molecular passages through the scleral stroma and thereby could have facilitated transscleral penetration of the dextran tracers.

In the histologic analysis, counterstaining may further help to characterize the distribution of labeled dextran within various portions of the retina. However, it also would be likely to obscure our results by either blocking or quenching fluorescence from the labeled dextrans. Moreover, because standard histologic stains are often fluorescent, counterstaining may have introduced confusing fluorescent signals into the section that could be difficult to distinguish from the dextran-associated fluorescence. Thus, the present study examined noncounterstained sections, using fluorescence and bright field microscopy. Another important consideration is whether there was any separation of the fluorescent tag from the dextran in tissues or biological fluids. Several studies of fluorescent dextrans indicate that such separation is virtually nonexistent,[8,9] although specific experiments to prove this were not performed in the present study.

A key purpose of the histologic analysis was to assess the possibility that penetration of the tracer depends on the region of the sclera. The fluorescence scores of the anterior outer retina increased by 17%, while the scores of the mid outer retina and posterior outer retina increased 46% and 61%, respectively (Table 3). This pattern may reflect that the anterior mouse sclera is thicker than the mid and posterior sclera and the thicker sclera may have been more resistant to transscleral flow of the dextran tracer. Similarly, the fluorescence scores of the anterior inner retina increased by 10%, whereas the scores of the mid outer retina and posterior outer retina increased 30% and 35%, respectively. Most likely, the reason these increases were smaller than seen in the outer retina is the added resistance associated with dextran transport from the outer retina to the inner retina. It is possible that the lack of statistical significance in the changes presently observed in the anterior and posterior inner retina may reflect lack of sensitivity of the assay. Nevertheless, the observed increase in permeability after exposure to MMPs is consistent with the increased permeability determined in this study by spectrofluorometry. Similar to the mouse, equatorial human sclera is thinner than anterior sclera.[10] Unlike mouse sclera, however, human sclera posterior to the equator increases in thickness. This increase may not limit transscleral delivery of macromolecules to the posterior human retina in vivo, however, because fluorescent dextran placed adjacent to the equator by subconjunctival injection enters the extracellular space of the choroid and then readily redistributes throughout the whole choroid and subepithelial stroma of the ciliary body.[6] Thus, regional differences in scleral permeability are not likely to limit the usefulness MMPs to facilitate transscleral delivery to the posterior retina in vivo.

There is growing acceptance of transscleral delivery as a route for the intraocular delivery of macromolecules because the sclera has a large and accessible surface area, a high degree of hydration rendering it conducive to water-soluble substances, and a hypocellularity with an attendant paucity of

TABLE 3. Scores of Each Retina Region Considered Separately: 32-Minute Dextran Probe*

	Outer Retina			Inner Retina		
	Anterior	Mid	Posterior	Anterior	Mid	Posterior
Control ($n = 3$)	2.3 ± 0.6	2.6 ± 0.4	2.6 ± 0.5	1.9 ± 0.2	2.0 ± 0	2.0 ± 0
MMPs ($n = 3$)	2.7 ± 0.4	3.8 ± 0.1	4.2 ± 0.5	2.1 ± 0.1	2.6 ± 0.2	2.7 ± 0.5
Percent change	$+17\%$	$+46\%$	$+61\%$	$+10\%$	$+30\%$	$+35\%$
t-Test	NS	$P = 0.01$	$P = 0.02$	NS	$P = 0.01$	NS

* Data are expressed as the mean $\pm$ SD.

proteolytic enzymes and protein-binding sites.[10–13] In vitro experiments have demonstrated that the sclera is permeable to large molecules, up to a 150-kDa antibody.[1,13–15] However, these studies, together with the present study, support the view that macromolecules with larger hydrodynamic radius may have limited transscleral permeability. It is well known that macromolecules minimally penetrate the blood–retinal[16] or blood–brain barriers.[17] In addition, several studies have shown that macromolecular penetration may be different in vivo due to the effect of functional blood circulation and lymphatic drainage, suggesting interpretation of ex vivo studies should be made with caution.[18–20] Also, it is possible that differences exist between the permeability properties of human and the much-thinner mouse sclera. Nevertheless, penetration of macromolecules across the sclera into the retina has been demonstrated in vivo.[6,14–17,21] Moreover, because intraocular pressure lowering that occurs with topical prostaglandin analogues is linked with intrascleral MMP production,[2–4] it is possible that MMP-induced reduction of the transscleral pressure gradient (reflecting intraocular pressure) may further facilitate intraocular drug penetration in vivo. In the present study, the normal appearance of the retina and associated structures in the frozen sections suggest that MMP facilitation of transscleral drug delivery is safe. Nevertheless, further studies using electron microscopy and other techniques may be helpful to confirm this point.

There are several ways that the present findings might be adapted to facilitate drug delivery to the posterior retina in patient eyes. First, subconjunctival injections of MMPs could be made either before or with macromolecular therapeutics. Alternatively, molecular vectors might be used to enhance endogenous local production of MMPs. These approaches could accelerate access of macromolecular therapeutic treatments for acute severe damage such as can occur in acute closed-angle glaucoma, central retinal artery occlusion, central retinal vein occlusion, anterior ischemic optic neuropathy, or traumatic optic neuropathy. It also may be useful for enhancing the long-term delivery of macromolecules for the treatment of primary open-angle glaucoma, diabetic retinopathy, or age-related macular degeneration.

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