Form-Deprivation Myopia Induces Activation of Scleral Matrix Metalloproteinase-2 in Tree Shrew

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Purpose. To investigate whether structural changes to the sclera during form-deprivation myopia are caused by active tissue remodeling, the gelatinase activity of tree shrew scleras was studied in normal animals, form-vision deprived animals, and animals recovering from myopia.

Methods. Infant tree shrews were monocularly deprived (MD) of form vision with translucent occluders for 5 days. Recovery animals were allowed 3 days of binocularly unoccluded vision after the period of form deprivation. Eyes were removed and dissected to provide scleral samples corresponding to equatorial and posterior regions. Gelatinase activity was assessed by quantitative sodium dodecyl sulfate-polyacrylamide gel electrophoresis or SDS-PAGE, gelatin, zymography of scleral matrix metalloproteinase (MMP) extracts.

Results. The major gelatinolytic species present in tree shrew sclera was found to be MMP-2 (gelatinase A). In normal (nondeprived) animals, most of the MMP-2 was found to be in the latent form (the ratio of active-to-latent MMP-2 was 0.23 ± 0.05 and 0.34 ± 0.06 in equatorial and posterior samples, respectively; n = 10 eyes from five animals). After 5 days of MD, there was a threefold increase in the amount of active scleral MMP-2 in myopic eyes compared to contralateral control eyes, whereas latent MMP-2 activity levels were not altered significantly. This increase in active MMP-2 was seen in both the equatorial and posterior sclera of myopic eyes (active-to-latent MMP-2 ratios were 0.53 ± 0.10 and 0.81 ± 0.09 in equatorial and posterior regions, respectively; n = 6 animals). Contralateral control eyes had levels of both active and latent MMP-2 not significantly different from normal eyes. After only 3 days of unoccluded vision, previously deprived eyes that were now recovering from myopia had a fivefold lower level of active MMP-2 than that seen in deprived eyes after 5 days of MD. In fact, active (and latent) MMP-2 levels were reduced in recovering eyes even below the levels found in their contralateral control eyes. Active-to-latent ratios for recovering eyes were 0.11 ± 0.03 and 0.15 ± 0.03 in equatorial and posterior sclera, respectively (n = 5 animals).

Conclusions. These results demonstrate that form-deprivation myopia and recovery from myopia alter scleral catabolism and provide further support for the theory that changes in eye size during mammalian refractive development are the result of active tissue remodeling rather than passive scleral stretching alone. Invest Ophthalmol Vis Sci. 1996;37:1380-1395.

The prevalence of myopia in the adult populations of western Europe and the United States is approximately 25% to 30%. In a significant minority of persons with myopia, the degree of myopia is sufficiently high to result in visual loss from ocular pathology associated with the excessive elongation of the eye. Pathologic consequences of high myopia, such as retinal detachment and Fuch's spot, mean that myopia is the fourth or fifth leading cause of blindness in developed countries. High myopia in humans is associated with enlargement of the globe in all meridians, but principally in the axial dimension. Commonly associated with this axial elongation of the myopic human eye is a marked thinning of the posterior sclera and a reduction in scleral collagen fibril diameter. Based on these findings, it has been proposed that the ocular enlargement found in high myopia could be the result of scleral stretching. Further support for this essentially "passive scleral stretch" hypothesis of myopic...
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progression comes from observations of embryonic and neonatal eye growth under extremes of intraocular pressure. If the intraocular pressure of the embryonic chick eye is reduced artificially, normal eye enlargement does not occur. Conversely, in human neonates suffering from buphthalmos, elevated intraocular pressure typically leads to ocular enlargement.

In a small minority of human subjects, high myopia may be found as part of a syndrome that results from a known single-gene defect. In the defects reported to date, the disease locus has been mapped to a gene encoding a structural component of the extracellular matrix (ECM), such as collagen or elastin, or to an enzyme involved in the biosynthesis of such structural components. These defects may predispose affected persons to the development of myopia by changing the biomechanical properties of the sclera, choroid, or vitreous. Perhaps the simplest theory to explain the development of myopia in such conditions is that each gene defect produces a weakened sclera, which then stretches excessively under physiological intraocular pressure. This theory is supported by evidence that collagen gene defects can affect profoundly the biomechanical properties of the tissues in which they are expressed and that the biomechanical properties of the sclera are affected in human high myopia.

The excessive axial elongation, posterior scleral thinning, and reduction in collagen fibril diameter noted in human high myopia are also evident in mammalian models of axial myopia (monkey and tree shrew). However, recent findings from animal models of myopia question a passive scleral stretch hypothesis and give support to the idea of active scleral tissue remodeling. It has been found that the thinning of the posterior sclera in high myopic tree shrew eyes is not simply caused by a passive stretching of the sclera to cover the enlarged eye because there is a significant loss of scleral tissue (dry weight) in myopic eyes, particularly at the posterior pole. In the chick model of myopia, increases in the synthesis of scleral protein, proteoglycan, and DNA have been reported in eyes developing myopia. In both chick and tree shrew, it has been found that eyes are able to recover from induced axial myopia when form deprivation ceases. These eyes are able to slow or stop their rate of elongation, relative to contralateral control eyes, during the recovery period. This recovery effect cannot be accounted for solely by changes in choroidal thickness and does not appear to be related to changes in intraocular pressure. In addition, in chick and tree shrew, when myopia is induced by producing a defocused retinal image with negative lenses, once the eye has compensated for the defocus (by reaching a functional emmetropia), it arrests its increased rate of elongation to maintain a clear image. Findings from recovery experiments and lens-induced defocus experiments demonstrate that eye enlargement in these animal models of axial myopia can be modulated. This argues against a model in which passive scleral stretch is the sole causative mechanism of myopia development because passive scleral stretch would predict gradual and continuous axial elongation that is incapable of such fine control.

Active remodeling of connective tissues is a complex process. Because extracellular matrices provide structural strength and form to organs, remodeling requires a delicate balance between degradation and synthesis. The matrix metalloproteinases (MMPs) have been implicated in the degradation of many connective tissues during development and pathology. MMPs are zinc-containing, Ca++-dependent proteolytic enzymes capable of degrading most ECM components. There are at least 10 known members of the MMP family, each with a separate, though usually overlapping, substrate specificity. All MMPs are secreted as inactive precursors (zymogens, pro-enzymes) activated by cleavage of an inhibitory domain, with an accompanying reduction in molecular weight.

To search for evidence that eye enlargement during the development of form-deprivation myopia results from active tissue remodeling, the current study monitored the levels of scleral gelatinases in the eyes of normal, monocularly deprived (MD), and recovering tree shrews.

MATERIALS AND METHODS

Animals

Tree shrews (Tupaia belangeri) were reared in our breeding colony on a 15-hour light–9-hour dark cycle. Food and water were available ad libitum. Animals were assigned randomly to one of three groups: normal, MD, or recovery. On the 15th day after eye opening, all animals were removed from the maternal cage and housed in individual cages. Monocularly deprived and recovery group tree shrews were given anesthesia and were fitted with a head-mounted goggle containing a translucent occluder, essentially as described. The eye to be occluded was assigned on a random basis.

Optical and Structural Measures

On the 20th day after eye opening, animals from all groups were anesthetized, and optical and structural measures were taken as previously described (Cottriall CL, McBrien NA, p. 1368 this issue). Briefly, anterior
corneal curvature was measured by a modified keratometer in the horizontal and vertical meridians. In 5-day MD and normal animals, atropine cycloplegia was not performed before ocular measures to avoid long-term effects on recovery. Therefore, in recovery animals, pupil dilation was achieved by instillation of two 15-μl drops of phenylephrine 2%. Axial ocular dimensions were measured by A-scan ultrasonography (10-MHz focused transducer; Lecroy [Geneva, Switzerland] digital storage oscilloscope). On completion of the optical measures, MD and normal animals were deeply anesthetized (80 mg/kg sodium pentobarbital), and the eyes were enucleated. Recovery animals were permitted 3 days of binocularly unoccluded visual experience, optical measures were repeated (phenylephrine mydriasis), and eyes were enucleated.

Enculcated eyes were trimmed of extraneous orbital tissue and weighed, and equatorial dimensions were measured with digital calipers. The cornea and an accompanying 1.5-mm rim of anterior sclera were removed, as was the lens. A posterior pole button was excised with a 7-mm diameter trephine. This button and the residual equatorial ring of sclera were cleaned of vitreous and of retinal and choroidal tissue and then frozen in liquid nitrogen. Tissue was stored at −80°C until use. Zymographic analysis was performed “blind,” without knowledge of whether tissue was derived from treated or control eyes. All animals were treated in accordance with the ARVO Statement for the use of Animals in Ophthalmic and Vision Research.

Choice of Extraction Procedure

We found the choice of MMP extraction procedure to be crucial to the success of this investigation. In preliminary experiments, with an extraction technique involving repeated rounds of freezing and thawing in the presence of a nonionic detergent, we found a considerable variation in the scleral gelatinase activity between individual, and even between the two eyes of, normal tree shrews. A subsequent comparison with other extraction techniques demonstrated that the freeze-thaw method was highly inefficient for scleral tissue. We chose an improved extraction procedure that used sodium deoxycholate (SDS) in conjunction with a calcium chelating agent (EGTA). The rationale for the choice of this extraction method was that SDS would partially denature matrix proteins and dissociate MMP–tissue inhibitor of metalloproteinases (TIMP) complexes. Chelating agents were used to sequester extracellular calcium, which may have altered the conformation of the calcium-dependent MMPs or prevented any calcium-dependent attachment to their substrate. Although both EDTA and EGTA were found to be equally effective extraction agents (appearing to act synergistically with SDS, at the concentrations tested; Fig. 1A), EGTA was chosen because it chelates zinc ions to a lesser extent than EDTA (by approximately four orders of magnitude). Further advantages to the use of SDS and EGTA were that they both inhibit conversion of latent MMPs to their active forms (Fig. 1B). This ensured that the levels of latent and active MMPs measured byzymography represented, as closely as possible, those present in the tissue.

Extraction of Metalloproteinases

Scleral samples were freeze-dried, and their dry weights were recorded in triplicate (dry weights of both posterior and equatorial samples approximately 2 mg each; weighing accuracy ± 10 μg). Each sample was rehydrated for 3 minutes at room temperature by submersion in a 50-μl droplet of water (18 Ω conductivity). The tissue was then snap frozen in liquid nitrogen and powdered in a freezer mill (Mikrodisembrator; B. Braun, Melsungen GmbH, Germany). The powder was recovered as a suspension in a total volume of 950 μl water (preliminary experiments demonstrated that the recovery of tissue was >95%). An aliquot of extraction solution (2% wt/vol SDS; 200 mM EGTA; 100 mM Tris–HCl, pH 7.5) was added to give a final concentration of 0.1% SDS and 10 mM EGTA. The solution was vortexed, and then the extraction was allowed to proceed for 20 minutes at 37°C. Insoluble material was pelleted by centrifugation (13,000 rpm × 5 minutes, at room temperature) and the supernatant, containing the extracted MMPs, was removed and freeze-dried.

MMP-2 Standards

Primary cultures of tree shrew skin fibroblasts were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum until confluent. The cells were washed in serum-free medium and then incubated for 24 hours with serum-free medium containing 100 ng/ml phorbol 12-myristate 13-acetate to stimulate MMP expression. The conditioned medium was collected, concentrated 50-fold over a 10 kDa-cut ultrafiltration membrane, divided into aliquots, and stored at −80°C. Zymographic analysis showed that MMP-2 accounted for >95% of the gelatinolytic activity of the conditioned medium; the activity of the active and latent forms was approximately equal (as judged by scanning densitometry). A 1:128,000 dilution of the conditioned medium standard was the minimum amount that could be detected with our zymography tech-
FIGURE 1. Characterization of scleral gelatinases. (A) Sodium dodecyl sulfate (SDS) and EGTA act synergistically in the extraction of matrix metalloproteinase (MMP-2) from sclera. Duplicate samples were extracted with 0.1% SDS in the presence (+ EGTA) or absence (- EGTA) of 10 mM EGTA and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) gelatin zymography. EGTA enhanced extraction approximately twofold. (B) Time-course of SDS-EGTA extraction of MMP-2 from sclera. MMPs were extracted for periods of 0, 5, 10, 20, 30, 60, or 300 minutes, centrifuged for 5 minutes, and analyzed by zymography. There was no conversion of latent MMP-2 to the active form during the course of the extraction. (C) The effect of reduction, alkylation, and APMA activation on tree shrew scleral gelatinase activity. Samples were reduced and alkylated (+ R/A), and/or APMA activated (+ APMA) before MMP extraction and zymographic analysis. APMA activation converted the latent (Mr 64 kDa) gelatinase to active forms (61, 59, and 45 kDa; lanes 2 and 4). Reduction and alkylation resulted in the appearance of a 72-kDa band (lanes 3 and 4). (D) The 64/59 kDa gelatinase doublet from tree shrew sclera is immunologically related to human MMP-2. Reduced and alkylated scleral MMP extracts were subjected to immunoprecipitation with a sheep polyclonal antibody to MMP-2-TIMP-2 (10 µg IgG, lane 1; 20 µg IgG, lane 2), a control antibody (20 µg preimmune sheep IgG, lane 4), or in the absence of antibody (lane 3), and immunoprecipitates were analyzed by zymography. Gelatinases were immunoprecipitated only in the presence of the specific anti-MMP-2-TIMP-2 antibody.
concentration between samples would have produced bands of variable width on the final gel.

Zymography was carried out as described previously, with slight modifications. Ten-microliter samples of scleral extract were run on 0.5-mm thick 10% SDS–PAGE mini-gels containing 1 mg/ml gelatin (Sigma G-9382). Conditioned medium MMP-2 standards were included on every gel. After electrophoresis, gels were rinsed at room temperature for 15 minutes in 0.03% SDS and then for 45 minutes in 2.5% Triton X-100. These steps served to remove residual EGTA from the gel and to re-renature SDS-denatured MMPs. Gelatinase activity was initiated by incubation in reaction buffer (50 mM Tris–HCl, pH 7.5; 500 mM NaCl, 50 mM CaCl2) and continued for 18 hours at 37°C. To visualize regions of gelatin degradation, gels were stained with Coomassie blue R, destained, immersed with glycerol, and dried between sheets of cellophane.

Quantification by Scanning Densitometry

Dried gels were scanned at high resolution (64 μm × 64 μm) on a Howtek (Hudson, NH) Scanmaster 3 scanner. Images were analyzed with the Quantity One software package from PDI (Huntington Station, NY) running on a Sun Sparc computer (Sun Microsystems, Mountain View, CA). Zymogram images were “contrast-inverted” (to give images with dark bands on a light background), and the background was subtracted by taking the average pixel density over a 30% area of the gel containing no bands. The pixel density of individual bands was integrated over a constant area of the gel containing no bands. The pixel density of individual bands was integrated over a constant area of the gel containing no bands. The pixel density of individual bands was integrated over a constant area of the gel containing no bands. The pixel density of individual bands was integrated over a constant area of the gel containing no bands.

Characterization of Gelatinases

Normal tree shrew sclera (dry wt ≈ 2 mg) was powdered in a freezer mill, as described above in Extraction of Metalloproteinases, and collected as a suspension in 2 ml of water. Five hundred-microliter aliquots of this suspension were distributed to microcentrifuge spin-filter columns (500 μl capacity, 0.22 μm cellulose acetate membrane; Costar, Cambridge, MA) to facilitate a series of efficient solution changes. Each sample was resuspended in 400 μl of 50 mM Tris–HCl (pH 7.5), 2 mM CaCl2, and 0.5 mM phenylmethylsulfonyl fluoride, and transferred to a 37°C water bath. Two samples were reduced and alkylated (5 mM dithiothreitol, 30 minutes; 20 mM iodoacetamide, 30 minutes). One of these samples and one of the untreated samples were then activated with p-aminophenyl mercuric acetate (APMA; 1 mM, 60 minutes). Metalloproteinases were eluted from the powdered tissue by resuspension in (20 μl/mg initial dry weight of EGTA-containing zymography sample buffer, and 10-μl aliquots were analyzed by gelatin zymography.

To test whether the 64/59 kDa tree shrew gelatinase doublet was immunologically related to human MMP-2, we performed a zymographic analysis of scleral MMPs immunoprecipitated by a polyclonal antibody to human MMP-2–TIMP-2 (generously donated by Dr. G. Murphy, Strangeways Research Laboratory, Cambridge, UK). This antibody was raised (in sheep) against purified MMP-2 and crossreacts with both MMP-2 and TIMP-2. To prevent immunoprecipitation of scleral gelatinase-TIMP complexes by TIMP immunoreactivity, we reduced and alkylated samples (to remove TIMPs) before immunoprecipitation. Briefly, powdered tree shrew sclera was reduced and alkylated (as described above) and rinsed twice with water. MMPs were then extracted from the tissue with 0.1% SDS + 10 mM EGTA, diluted fourfold in buffer (1% bovine serum albumin, 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM EGTA, 0.2% Triton X-100), and incubated with protein G-sepharose beads that had been primed with 20 μg of either sheep anti-MMP-2–TIMP-2 immunoglobulin G (IgG) or control (preimmune) sheep IgG. After a 16-hour incubation, beads were washed extensively, and immunoprecipitated proteins were eluted with EGTA-containing zymography sample buffer and analyzed by zymography, as described above.

Data Analysis

Latent and active MMP-2 activity data were transferred to a spreadsheet (Excel; Microsoft) for paired sample statistical analyses (t-test). Analysis of variance (Mini-
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TABLE 1. Optical and Structural Measures

<table>
<thead>
<tr>
<th></th>
<th>Refractive Status (D)</th>
<th>Anterior Segment Depth (mm)</th>
<th>Lens Thickness (mm)</th>
<th>Vitreous Chamber Depth (mm)</th>
<th>Axial length (mm)</th>
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<tr>
<td>Normals</td>
<td></td>
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<td>Age-matched to 5 day MDs</td>
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<tr>
<td>Right eye</td>
<td>+8.3 ± 0.5</td>
<td>1.05 ± 0.02</td>
<td>3.19 ± 0.03</td>
<td>2.83 ± 0.04</td>
<td>7.07 ± 0.05</td>
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<td>Left eye</td>
<td>+8.2 ± 0.6</td>
<td>1.05 ± 0.02</td>
<td>3.18 ± 0.01</td>
<td>2.81 ± 0.04</td>
<td>7.04 ± 0.05</td>
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<tr>
<td>Difference (right − left)</td>
<td>+0.1 ± 0.2</td>
<td>0.00 ± 0.01</td>
<td>0.01 ± 0.02</td>
<td>0.02 ± 0.02</td>
<td>0.03 ± 0.02</td>
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<tr>
<td>MDs</td>
<td></td>
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<tr>
<td>After 5 days MD</td>
<td></td>
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<tr>
<td>Deprived eye</td>
<td>+1.3 ± 0.7</td>
<td>1.09 ± 0.02</td>
<td>3.20 ± 0.01</td>
<td>2.94 ± 0.03</td>
<td>7.23 ± 0.06</td>
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<td>Control eye</td>
<td>+8.7 ± 0.1</td>
<td>1.06 ± 0.02</td>
<td>3.21 ± 0.02</td>
<td>2.79 ± 0.02</td>
<td>7.06 ± 0.05</td>
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<tr>
<td>Difference (deprived − control)</td>
<td>−7.4 ± 0.7†</td>
<td>0.03 ± 0.01</td>
<td>−0.01 ± 0.01</td>
<td>0.15 ± 0.02†</td>
<td>0.17 ± 0.02†</td>
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<td>Recoveries</td>
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<tr>
<td>After 5 days MD</td>
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<tr>
<td>Deprived eye</td>
<td>+0.8 ± 0.8</td>
<td>1.09 ± 0.01</td>
<td>3.19 ± 0.02</td>
<td>2.91 ± 0.02</td>
<td>7.20 ± 0.05</td>
</tr>
<tr>
<td>Control eye</td>
<td>+8.5 ± 0.1</td>
<td>1.07 ± 0.01</td>
<td>3.22 ± 0.02</td>
<td>2.76 ± 0.02</td>
<td>7.05 ± 0.03</td>
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<tr>
<td>Difference (deprived − control)</td>
<td>−7.7 ± 0.8‡</td>
<td>0.03 ± 0.02</td>
<td>−0.03 ± 0.01</td>
<td>0.15 ± 0.02‡</td>
<td>0.15 ± 0.02‡</td>
</tr>
<tr>
<td>5 days MD + 3 days recovery</td>
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<tr>
<td>Recovering eye</td>
<td>+5.6 ± 0.7</td>
<td>1.08 ± 0.02</td>
<td>3.25 ± 0.03</td>
<td>2.84 ± 0.02</td>
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</tr>
<tr>
<td>Control eye</td>
<td>+8.8 ± 0.2</td>
<td>1.08 ± 0.01</td>
<td>3.24 ± 0.03</td>
<td>2.77 ± 0.01</td>
<td>7.09 ± 0.04</td>
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<tr>
<td>Difference (recovering − control)</td>
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<td>0.01 ± 0.02</td>
<td>−0.01 ± 0.01</td>
<td>0.07 ± 0.02*</td>
<td>0.07 ± 0.03</td>
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</tbody>
</table>

Values are mean ± SEM; n = 5 for normal and recovery groups; n = 6 for MD group. Significant differences on paired t-test indicated as *P < 0.05, †P < 0.01, and ‡P < 0.001.

RESULTS

Refractive Effects of Form Deprivation and Recovery

Five days of monocular deprivation induced −7.4 ± 0.7 D of myopia in occluded eyes compared to contralateral control eyes (Table 1). This difference in refraction was accounted for by an elongation of the vitreous chamber in occluded eyes relative to contralateral control eyes. There were no significant differences in corneal curvature, anterior segment depth (corneal thickness + anterior chamber depth) or lens thickness in any of the three groups.

Three days of binocular visual experience (recovery) after 5 days of MD reduced the amount of myopia in previously occluded eyes, compared to their contralateral controls, by 60% (Table 1). This demonstrated that the 3-day recovery period was sufficient to allow a significant emmetropization response to occur in recovering eyes. This reduction in myopia was associated with a significant reduction (P < 0.002) in the relative difference in vitreous chamber depth between the two eyes. An interesting finding was that the reduction in vitreous chamber difference was achieved predominantly by the myopic eye actually becoming smaller (see Table 1), an effect that did not appear to be caused by choroidal thickening (unpublished observations, 1995). Recently, a similar finding was reported, and a more detailed investigation of this effect will be presented in a separate communication.

Characterization of Scleral Gelatinases

Zymographic analysis of MMP extracts from tree shrew sclera demonstrated the presence of two major gelatinases that migrated as a doublet of 64 and 59 kDa (Fig. 1). This doublet accounted for >95% of scleral gelatinase activity. Highly concentrated scleral extract samples consistently demonstrated the presence of other minor gelatinases (a ≈90 kDa form, which may have been MMP-9, and a ≈28 kDa form, which may have been the gelatinolytically active cleavage product of a larger MMP). All gelatinase activity could be inhibited by replacing the Ca ++ in the zymography reaction buffer with EDTA (not shown), but activity was not inhibited by the irreversible serine protease inhibitor phenylmethylsulfonyl fluoride (Fig. 1C).

Activation of scleral samples with APMA converted some of the 64 kDa gelatinase to the 59-kDa form, suggesting that the 64-kDa and 59-kDa bands represented the latent and active forms, respectively, of a
single metalloproteinase (Fig. 1C, lanes 2, 4). This activation process was accompanied by the appearance of a 61-kDa band of gelatinase activity, suggestive of an intermediary product formed as part of the 64/59 kDa conversion reaction.

Several lines of evidence suggested that the 64/59 kDa gelatinase doublet represented the latent and active forms of tree shrew MMP-2 (gelatinase A, 72-kDa type IV collagenase). Most important, the molecular weights corresponded to those expected for latent MMP-2 and its activated form when run under nonreducing SDS-PAGE conditions. The appearance of an intermediary gelatinase product after APMA-activation is also characteristic of MMP-2. When several gelatinases were reduced and alkylated before zymography, a new band of gelatinase activity with an M, of 72 kDa appeared (Fig. 1C, lanes 3, 4), again consistent with results expected for MMP-2 (this 72-kDa band corresponds to a reducing agent-induced, conformationally distinct form of latent MMP-2). The 64/59 kDa scleral gelatinase doublet comigrated with the gelatinase doublet secreted by tree shrew skin fibroblasts when stimulated by the tumor promotor, phorbol 12-myristate 13-acetate (phorbol esters have been shown to induce the expression of MMPs, including MMP-2, in many fibroblast cell lines and also to induce the activation of a considerable proportion of the secreted zymogens). The similar electrophoretic migration patterns of the fibroblast-secreted gelatinase doublet and the scleral gelatinase doublet suggest that these proteins are closely related. Finally, the scleral gelatinase doublet could be immunoprecipitated by an antibody to human MMP-2-TIMP-2, but not by a control (sheep IgG) antibody, used at the same concentration (Fig. 1D). In these experiments, immunoprecipitation was conducted on scleral extracts that had undergone reduction and alklyation to remove TIMPs to prevent immunoprecipitation of gelatinase-TIMP complexes by TIMP immunoreactivity. This result demonstrates that the scleral gelatinase doublet is immunologically related to human MMP-2. In conclusion, we propose that the 64/59 kDa scleral gelatinase doublet represents the latent and active forms of tree shrew MMP-2.

Active and Latent MMP-2 Are Present in Developing Tree Shrew Sclera

Zymograms of the gelatinase activity of normal tree shrew sclera demonstrated the presence of active and latent MMP-2 (Fig. 2). The absolute amounts of latent and active MMP-2 found in the right and left eyes of normal animals (n = 5) are shown in Table 2 and Figure 3A. There were no significant differences in latent or active MMP-2 levels between right and left eyes. Although there was no difference in the absolute amount of latent MMP-2 between equatorial and posterior sclera, there was significantly more active MMP-2 in posterior, compared to equatorial, sclera (n = 10 eyes from five animals; P < 0.01).

In the normal animals, most of the scleral MMP-2 was in the latent form (Fig. 3D). The ratio of active-to-latent MMP-2 was 0.23 ± 0.05 and 0.34 ± 0.06 (n = 10 eyes from five animals) in the equatorial and posterior sclera, respectively. This equatorial-posterior difference in active-to-latent ratios was significant (P < 0.01), caused by the higher level of active MMP-2 in posterior sclera noted above. These results suggest that the posterior sclera undergoes more intensive remodeling than the equatorial region at this stage of normal ocular development (20 days after eye opening).

Form-Deprived Myopic Eyes Show Increased Levels of Active MMP-2

There were substantial differences in the appearance of zymograms from animals that had undergone 5 days of form deprivation, compared to normals (Fig. 4). There appeared to be much greater amounts of active MMP-2 in equatorial and posterior sclera of form-deprived myopic eyes compared to their contralateral controls. These differences were confirmed by quantitative densitometric analysis (Table 2, Fig. 3B).

There was a significant increase in the absolute amount of active MMP-2 in form-deprived myopic eyes compared to their contralateral control eyes (P < 0.001 and P < 0.02 in equatorial and posterior sclera, respectively). There were no corresponding differences in latent MMP-2 levels between control and myopic eyes in either the equatorial or the posterior sclera (P = 0.12 and P = 0.78, respectively). When data from the two eyes of MD animals were pooled (i.e., activity in the equatorial region of both eyes versus activity in the posterior region of both eyes), the equatorial sclera had significantly higher latent MMP-2 levels than posterior sclera (161.8 ± 15.7 versus 119.6 ± 9 U, respectively; P < 0.005). This difference is unlikely to represent a methodological effect because the equatorial and posterior samples from individual animals were processed simultaneously.

In the sclera of normal tree shrews, most of the MMP-2 was in the latent form (see previous section). This was also the case in the control eye of MD animals, but in deprived eyes in which myopia was developing, there was a considerable increase in the active-to-latent ratio so that, in some cases, there was almost as much active MMP-2 as latent MMP-2. Differences in the active-to-latent MMP-2 ratio between deprived and control eyes were significant (P < 0.01 for both equatorial and posterior sclera). Additionally, the higher active-to-latent ratio of the posterior sclera,
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Standards

Equatorial Left eye

Active (59 kDa)
Latent (64 kDa)

Active (59 kDa)
Latent (64 kDa)

Posterior

1 2 3 4 5

FIGURE 2. SDS–PAGE gelatin zymography of normal group tree shrew sclera. MMP were extracted from sclera and analyzed by zymography as described in Materials and Methods. (upper and lower gel panels) Equatorial and posterior scleral extracts, respectively (from a single animal). Lanes 1 to 5, MMP-2 standards (256 U to 1 U per lane). Lanes 6 to 8, scleral extracts from right eye (fourfold serial dilution). Lanes 9 to 11, scleral extracts from left eye (fourfold serial dilution). Comparisons between the two eyes were possible directly from the gel because sample loadings were normalized on the basis of tissue dry weight.

compared to the equatorial sclera, of deprived eyes was significant ($P < 0.01$), as was found for normal animals. In fact, the active-to-latent ratio for the posterior sclera of deprived eyes was significantly higher than the active-to-latent ratio of all other groups ($P < 0.01$, Tukey's).

Recovering Eyes Show Reduced Levels of MMP-2

The levels of scleral MMP-2 in animals recovering from form-deprivation myopia are shown in Table 2 and Figure 3C. A typical zymogram from a recovery group animal is shown in Figure 5. After only 3 days of recovery, there was a fivefold reduction in the level of active MMP-2 in both the equatorial and posterior sclera of eyes recovering from myopia, when compared to the deprived eyes of MD animals (equatorial $P < 0.05$; posterior $P < 0.01$; Tukey's). No significant differences in latent MMP-2 levels were found between eyes recovering from myopia and the deprived eyes of MD animals. These findings resulted in significant reductions in the active-to-latent MMP-2 ratio of both the equatorial and posterior regions of recovering eyes, compared to the deprived eyes of MD animals (equatorial $P < 0.05$; posterior $P < 0.01$; Tukey's).

The amount of active MMP-2 was reduced slightly in recovering eyes, compared to their contralateral controls in both equatorial and posterior sclera. How-

<table>
<thead>
<tr>
<th>Equatorial Sclera</th>
<th>Normals</th>
<th>MDs</th>
<th>Recoveries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
<td>Deprived</td>
</tr>
<tr>
<td>Active</td>
<td>35.0 ± 15.9</td>
<td>39.6 ± 13.9</td>
<td>86.7 ± 13.8</td>
</tr>
<tr>
<td>Latent</td>
<td>146.3 ± 18.8</td>
<td>155.1 ± 16.9</td>
<td>172.6 ± 19.1</td>
</tr>
<tr>
<td>Total</td>
<td>181.3 ± 33.4</td>
<td>194.6 ± 23.4</td>
<td>259.3 ± 23.1</td>
</tr>
<tr>
<td>Active/Latent Ratio</td>
<td>0.21 ± 0.07</td>
<td>0.26 ± 0.08</td>
<td>0.55 ± 0.10</td>
</tr>
<tr>
<td>Posterior Sclera</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td>65.1 ± 20.9</td>
<td>67.6 ± 27.9</td>
<td>99.3 ± 16.3</td>
</tr>
<tr>
<td>Latent</td>
<td>172.3 ± 21.0</td>
<td>183.3 ± 28.8</td>
<td>121.2 ± 15.4</td>
</tr>
<tr>
<td>Total</td>
<td>237.4 ± 38.5</td>
<td>250.9 ± 54.8</td>
<td>220.5 ± 39.8</td>
</tr>
<tr>
<td>Active/Latent Ratio</td>
<td>0.36 ± 0.09</td>
<td>0.33 ± 0.09</td>
<td>0.81 ± 0.09</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; $n = 5$ for normal and recovery groups, $n = 6$ for MD group. Activity measured in arbitrary units.
FIGURE 3. MMP-2 activity of tree shrew sclera. MMP activity was quantified from zymograms as described in Materials and Methods. (A,B,C) Absolute activity of latent and active MMP-2 in equatorial and posterior sclera. (D,E,F) Ratio of active-to-latent MMP-2 activity in equatorial and posterior sclera. Normal animals (n = 5; age-matched to 5-day monocularly deprived animals). (A,D) Five-day monocularly deprived animals (n = 6). (B,E) Three-day recovery after 5-day monocular deprivation (n = 5). (C,F) Error bars = 1 SEM. Significant differences between fellow eyes indicated as *P < 0.05 and **P < 0.01; paired t-test).
ever, this difference only reached statistical significance when the equatorial and posterior enzyme activities of each eye were pooled ($P < 0.05$, $n = 10$ pairs of measurements). Interestingly, for posterior sclera, the level of latent MMP-2 was also reduced in recovering eyes compared to contralateral control eyes ($P < 0.05$). Monocularly deprived animals showed no such difference in latent MMP-2 levels (see previous section). The reduction in both active and latent MMP-2 levels in the posterior sclera of eyes recovering from axial myopia resulted in an active-to-latent ratio that was not significantly different to that of contralateral control eyes ($P = 0.10$). Conversely, for the equatorial sclera, there was a significant reduction in the active-to-latent ratio of recovering eyes compared to control eyes ($P < 0.02$) because, equatorially, latent MMP-2 levels were similar in both recovering and control eyes. There were no significant differences in either active or latent MMP-2 levels, nor in active-to-latent ratios, between the contralateral control eyes of recovery group animals and the eyes of normals (it should be noted, however, that normals were age matched to 5-day MD animals and not to recovery group animals).

**DISCUSSION**

We hypothesized that if ocular enlargement during the development of form-deprivation myopia in tree shrews was the result of active tissue remodeling rather than just passive scleral stretching, we would find evidence of increased ECM degradative activity in the scleras of myopic eyes. As potential markers of ECM degradative activity, we chose to study scleral gelatinases. Results demonstrated that scleral gelatinase activity is increased markedly during axial myopia development and is reduced during the recovery from myopia.

In normal tree shrew sclera, MMP-2 was found to be by far the most prominent gelatinase (a ≈90-kDa enzyme presumed to be latent MMP-9 accounted for less than 5% of all activity on gelatin zymograms). Most of the scleral MMP-2 was found to be in the latent form. This suggests that in vivo the enzyme is prevented from autocatalytic activation, probably by an association with the inhibitor TIMP-2. The discovery of a proportion of MMP-2 in the active form indicates that at this stage of normal scleral development, some active tissue remodeling is occurring because activated MMP-2 is catalytically active, even in the presence of TIMP-2.49,50

In myopic eyes that underwent 5 days of MD, the level of active MMP-2 was increased approximately threefold over that in contralateral control eyes and twofold over that in age-matched normal eyes. Latent MMP-2 levels were not significantly different between the deprived and control eyes of MD animals. The increase in active MMP-2 content shifted the balance of active-to-latent MMP-2 in the sclera of deprived, myopic eyes so that, in posterior sclera, there was almost as much active MMP-2 as latent MMP-2 (see Figs. 3B, 4). Although the zymography technique does not
standards recover eye control eye

**FIGURE 5.** SDS-PAGE gelatin zymography of recovery group tree shrew sclera. (upper and lower gel panels) Equatorial and posterior scleral extracts, respectively (from a single animal). Lanes 1 to 5, MMP-2 standards (256 U to 1 U per lane panels). Lanes 6 to 8, scleral extracts from recovering eye (fourfold serial dilution). Lanes 9 to 11, scleral extracts from control eye (fourfold serial dilution). Comparisons between the two eyes are possible directly from the gel.

measure the in situ level of gelatinase activity, because it is independent of scleral TIMP/TIMP-2 concentrations, the observed increase in the level of active MMP-2 indicates that scleral gelatinolytic activity is increased markedly in form-deprived myopic eyes. Two other studies, both in chick, have found differences in scleral gelatinase activity during the development of form-deprivation myopia. Using zymography, Jones et al. found increased latent and active gelatinase activity in chick sclera from myopic form-deprived eyes compared to controls, coupled with an increased prevalence of low molecular weight cleavage products. The gelatinase activity was attributed to an enzyme of appropriate molecular weight to be MMP-2. A more detailed study of chick scleral gelatinase activity has been reported recently by Rada and Brenza. Using a [3H]gelatin degradation assay rather than the zymography technique, this group reported an increase in latent, but not active, gelatinase activity in the posterior sclera of eyes developing form-deprivation myopia compared to their contralateral control eyes. This contrasts with our results on tree shrew sclera showing that increased active gelatinase levels were apparent in deprived eyes. As in the current study, however, the metalloproteinase responsible for gelatinolytic activity was demonstrated to be MMP-2. In chick sclera, major differences in MMP-2 activity between anterior and posterior regions were apparent in deprived and contralateral control eyes, but there were no significant differences in equatorial scleral gelatinase activity between the two eyes. This again contrasts with our results from tree shrew sclera showing similar levels of gelatinase activity in equatorial and posterior sclera and demonstrating that the effects of form-deprivation on MMP-2 levels were quantitatively similar in the two regions. A surprising result from the study of Rada and Brenza was that in the chick, the only difference found in gelatinase, collagenase, or proteoglycanase activity between deprived and control eyes was for the latent (inactive) form of gelatinase in posterior sclera. Because marked differences in ECM turnover are apparent in chick sclera during form deprivation, this result suggests either that the turnover of ECM components is regulated primarily by synthesis rates alone (not by a combination of synthesis and degradation rates) or that at the time tested (11 days of MD), scleral ECM degradatory activity had returned to basal levels. An argument in support of the latter explanation is that if MMP-2 synthesis and activation were regulated independently, MMP-2 activation in chick sclera may have been downregulated while enzyme synthesis was still in an upregulated state. This would have led to an increased abundance of MMP-2 in the latent form, consistent with the findings of Rada and Brenza. However, the above hypothesis infers that by 11 days of deprivation, the rate of chick scleral ECM turnover in deprived eyes has slowed to levels comparable with control eyes—a suggestion that seems inconsistent with reports of turnover rates of, for example, chick scleral proteoglycans. Therefore, it seems likely that differences in scleral MMP-2 activity between chick and tree shrew eyes undergoing myopia development are mainly caused by species differences. Unlike...
mammalian sclera, which is composed of predominantly type I collagen, chick sclera is composed of both a cartilaginous layer and a thinner fibrous layer (containing predominantly types II and I collagen, respectively). The response of chick sclera to form deprivation is to increase the rate of ECM component synthesis in the cartilaginous sclera, leading to a thickening of the tissue. Conversely, in tree shrew, there is a thinning of the sclera and a reduction in proteoglycan synthesis in response to form deprivation (chick fibrous sclera has been reported to become thinner and does not show increased synthesis of proteoglycans). The opposing responses of chick and tree shrew sclera to form deprivation, in terms of thickness changes and proteoglycan synthesis, suggest that active tissue remodeling in these two species may be operating in different directions (i.e., greater synthesis than degradation in [cartilaginous] chick sclera, and greater degradation than synthesis in tree shrew sclera).

Because Rada and Brenza measured scleral gelatinase activity with \([^{3}H]\)gelatin degradation assays rather than by zymography, they were able to assess the role of TIMPs in determining the final degree of scleral gelatinase activity. The zymography technique used in the current study measures the activity of gelatinases when they have been separated from TIMP inhibitors. Because TIMP and MMP-2 expression may be regulated independently, determining TIMP levels in tree shrew sclera will be an informative next step.

After only 3 days of unoccluded vision, previously deprived eyes now recovering from myopia showed marked reductions in active MMP-2 levels in both equatorial and posterior sclera. This demonstrates that scleral MMP concentrations can be regulated rapidly (within 5 days of a putative retinal signal, scleral MMP-2 levels had dropped to \(\approx 20\%\) of the level attained in myopic eyes after 5 days of deprivation).

Because MMP-2 levels increase during myopia development but decrease during recovery, there may well be a shift in the balance of degradation versus synthesis during the active scleral remodeling events occurring in mammalian axial myopia development and in the recovery from myopia. The loss of scleral...
FIGURE 7. Total MMP-2 activity of tree shrew sclera. Data from Figures 3B and 3C are replotted to show sum of active plus latent MMP-2 activity. Error bars = 1 SEM. Significant differences between fellow eyes are indicated as *P < 0.05 and **P < 0.01; paired t-test.

dry weight after deprivation and the gain in scleral dry weight after recovery are consistent with this hypothesis.

Although there were no significant differences in scleral gelatinase activity between either MD or recovery contralateral control eyes and the eyes of normal animals (Fig. 6), there was a trend for the posterior sclera of MD control eyes to show a reduced level of latent gelatinase compared to normal eyes. This reduction in latent MMP-2 level in the control eyes of MD animals was matched by a similar reduction in latent MMP-2 level in the posterior sclera of these animals’ deprived eyes. A comparison of latent MMP-2 levels in the posterior sclera for all eyes in the study revealed a significant difference (analysis of variance, P < 0.05) that resulted from the reduction seen in latent MMP-2 levels of eyes from MD animals (n = 12 eyes from six animals) compared to eyes from normal animals (n = 10 eyes from five animals; Tukey’s, P < 0.05). However, this effect was not apparent in equatorial sclera, in which latent MMP-2 levels were similar in deprived, control, recovery, and normal eyes (P = 0.73). If form deprivation does produce changes in MMP-2 levels in contralateral control eyes, a route of nonlocal ocular growth regulation, in addition to local growth control mechanisms, must be operational. Because MMP expression and activation are complex processes that are not fully understood, a number of factors could be envisaged in producing the reduced level of latent MMP-2 seen in the posterior sclera of MD animals.

In addition to demonstrating that form-deprivation myopia induces activation of MMP-2, our results suggest that scleral MMP-2 gene expression is altered during form-deprivation myopia and recovery. Any changes in the total amount of MMP-2 (i.e., latent + active enzyme) in sclera must be the result of changes in either the rate of MMP-2 synthesis or its degradation. A previous study has shown that latent MMP-2 is activated fully during zymography, (that is, the enzyme activity of latent MMP-2 activated during the zymography technique equals the enzyme activity of MMP-2 activated naturally in vivo), although it should be noted that this claim is not universally accepted. In spite of this uncertainty, the total MMP-2 activity measured by zymography provides a credible indication of the total amount of MMP-2 protein present in tissue.

In the deprived eyes of MD animals, total MMP-2 activity was increased in equatorial and posterior sclera, compared to contralateral control eyes (Table 2, Fig. 7; P < 0.01 and P < 0.05 for equatorial and posterior sclera, respectively; n = 6 animals). In recovering eyes, total MMP-2 activity was decreased in equatorial and posterior sclera (although this only reached significance for the posterior sclera; P < 0.05; n = 5 animals). These findings suggest that MMP-2 expression is upregulated during form-deprivation myopia development and downregulated below control levels during recovery from myopia. Alternatively, the rate of MMP-2 protein degradation could be altered during myopia development and recovery.

MMP-2 gene expression is regulated by a range of cytokines and growth factors, some of which already have been implicated in the mechanism of deprevia-
Form-Deprivation Myopia Induces Activation of MMP-2

Among the most likely candidates responsible for induced changes in MMP-2 gene expression during form-deprivation myopia are TGF-β, basic fibroblast growth factor, and the interleukins (for example, IL-1 and IL-10). However, the control of MMP gene expression is likely to be a complex process. Retinoic acid and vasoactive intestinal polypeptide, for example, are other modulators that could play a significant role in determining the degree of MMP expression.

If scleral MMP-2 is secreted as a complex of latent MMP-2 and TIMP-2, as is the case in most connective tissues, then the enzyme would be unable to autoactivate. Therefore, irrespective of changes in MMP-2 expression, the question arises: How does the enzyme become activated? Matrix metalloproteinases often are expressed and activated coordinately, either by each other or by other proteases. The serine protease, plasmin, for example, has been implicated in the activation of several metalloproteinases and may play a major role in the overall cascade of activation of MMPs during active tissue remodeling. At present, the effect of form-deprivation myopia on the plasminogen–plasmin system remains unknown, but it is an area worthy of investigation.

In conclusion, this study provides further evidence that the ocular enlargement that occurs during the development of form-deprivation myopia is the result of active tissue remodeling rather than of passive scleral stretching alone. The involvement of MMP-2 in this response may be particularly relevant to the degradation of scleral collagen because a recent report has demonstrated that MMP-2 is capable of digesting native, fibrillar type I collagen. This finding is in direct contradiction to the conclusions of earlier studies suggesting that only MMP-1 and MMP-8 (interstitial collagenase and neutrophil collagenase, respectively) are capable of degrading fibrillar type I collagen. Because type I collagen constitutes 85% to 90% of tree shrew scleral protein, MMP-2 may be directly responsible for a significant degree of scleral collagen degradation during myopic ocular enlargement. It will now be important to identify the agent(s) responsible for metalloproteinase activation during the development of myopia as a crucial step in elucidating the pathway by which retinal signals modulate eye growth.

Key Words

gelatinase A, matrix metalloproteinase, myopia, sclera, tree shrew

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References


