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Effects of Cyclic Mechanical Stretch on Extracellular Matrix Synthesis by Human Scleral Fibroblasts

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Abstract

In order to understand the effect of mechanical strain on scleral extracellular matrix remodeling, human scleral fibroblasts were subjected to equibiaxial stretch in vitro and the expression of proteoglycans, metalloproteinases (MMPs) and tissue inhibitor of metalloproteinase-2 (TIMP-2) were evaluated.

Isolated human scleral fibroblasts were seeded onto flexible bottom culture plates, and subjected to a cyclic stretch regimen of 15% equibiaxial stretch for 45 seconds followed by 15 seconds of rest for 6 – 48 hours in the presence of $^{35}\text{SO}_4$. Newly synthesized proteoglycans were measured in the medium by CPC precipitation of radiolabelled glycosaminoglycans. MMP-2 activity and expression levels were measured in the medium by, Western blot, gel zymography and real-time PCR. Steady state levels of TIMP-2 mRNA and membrane-type MMP, MT1-MMP (MMP-14) mRNA were measured in the cell layer using real-time PCR.

The predominant gelatinolytic enzyme secreted by scleral fibroblasts was the pro-enzyme form of MMP-2 (*ProMMP-2*). Mechanical stretch resulted in a significant increase of *ProMMP-2* after 12 and 48 hours (+76.28%, $p < 0.05$; +19.56%, $p < 0.01$, respectively). Mechanical stretch significantly increased the production of the active form of MMP-2 (*ActiveMMP-2*) after 48 hours (+59.72%, $p < 0.05$) and decreased levels of TIMP-2 mRNA (–22%, $p < 0.05$). The rate of scleral proteoglycan synthesis and the steady state levels of MMP-2 and MMP-14 mRNA were not significantly affected by mechanical stretch.

These results suggest that mechanical strain stimulates the activation of MMP-2 by scleral fibroblasts, possibly through increased levels of *ProMMP-2* and reduced levels of TIMP-2. Increased levels of *ActiveMMP-2* in the sclera would be expected to contribute to scleral extracellular matrix degradation, scleral thinning and possible ocular ectasia.

Keywords

mechanical stretch; MMP-2; sclera; extracellular matrix

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Introduction

The human sclera is a dense connective tissue characterized by a collagenous extracellular matrix interspersed with relatively few scleral fibroblasts (Rada et al., 2006). The sclera extends from the cornea back to the optic nerve and functions to support the retina, provides a site for insertion of the extraocular muscles and determines the overall size and shape of the eye. Animal models have shown that the sclera is not simply a static container of the eye, but rather is a dynamic tissue, capable of altering its extracellular matrix composition and biomechanical properties in response to changes in the visual environment (McBrien and Gentle, 2003; Rada et al., 2000a, 2000b; Siegwart and Norton, 1999; Rada et al., 1992). The activity of the matrix-degrading metalloproteinase, MMP-2, has been shown to increase in the sclera of tree shrews and chicks when eyes are rapidly elongating (during the development of experimental myopia) and decrease when eyes are slowing their rates of elongation during the recovery from myopia (compensation for myopic defocus) or during the compensation for positive lenses (Guggenheim and McBrien, 1996; Rada et al., 1999; Siegwart and Norton, 2001). It has been speculated that increased MMP-2 activity in the posterior sclera leads to degradation of the scleral extracellular matrix resulting in scleral thinning and increased scleral distensibility (Rada et al., 1999, Siegwart and Norton, 1999).

Matrix metalloproteinases (MMPs) are a family of proteinases that initiate the degradation of collagen and other extracellular matrix components. MMP-2 (Gelatinase A, 72-kDa gelatinase, Type IV collagenase) is a protein that is initially secreted as a pro-enzyme (*ProMMP-2*) and when cleaved becomes the active enzyme form (*ActiveMMP-2*). This mechanism is not fully understood, but involves membrane-type MMPs (MT-MMPs) such as MT1-MMP (MMP-14), the most potent activator of MMP-2 by cleaving the N-terminal domain of *ProMMP-2* (Sato et al., 1994; Cao et al., 1995). The endogenous inhibitor of MMP-2, tissue inhibitor of metalloproteinases -2 (TIMP-2) has been shown to inhibit both the activation of MMP-2 and activity of MMP-2 by blocking further cleavage of the proform active site cleft and blocking the catalytic domain of both active MMP-2 and MMP-14 (Will et al., 1996; Corcoran et al., 1996; Murphy et al., 1999). Moreover, steady state levels of TIMP-2 mRNA have been shown to decrease in the fibrous sclera of chick eyes which are actively elongating, and increase in the sclera of chick eyes which are slowing their rate of axial elongation, suggesting that TIMP-2 plays a role in the regulation of scleral extracellular matrix remodeling in chick sclera associated with changes in ocular elongation rates (Rada et al., 1999).

Similar to other connective tissues such as ligament and cartilage, scleral extracellular matrix remodeling is likely to be influenced by tensile and/or compressive forces applied to the tissue. Developmentally, intraocular pressure (IOP) has been demonstrated to be necessary for the normal development of the chick eye (Coulombre, 1961). Reduction of IOP by daily intubation of the developing chick eye results in a decreased eye size as compared to their contralateral controls suggesting that IOP is essential for normal growth of the eye (Coulombre, 1956). Additionally, children with congenital glaucoma demonstrate significant ocular enlargement, known as buphthalmus, mainly due to elevated IOP and elasticity of their growing eyes (Toker et al., 2003). However, little is known about the mechanisms by which intraocular pressure affects changes in the biomechanical properties of the sclera which ultimately lead to changes in eye size and refraction.

Using in vitro stress/strain systems, tension and compression have been shown to significantly affect extracellular synthesis and degradation. Several studies have revealed stretch induced changes of MMPs in other systems, including bovine (Okada et al., 1998; WuDunn, 2001) and porcine TM cells (Bradley, 2001), human ligament fibroblasts (Zhou et al., 2005) and chick scleral fibroblasts (Fujikura, 2002). Embryonic chick scleral fibroblasts, subjected to pulsatile mechanical stretch demonstrate increased levels of MMP-2 activity and TIMP-2 expression

(Fujikura et al., 2002). Mechanically stretched trabecular meshwork (TM) cells have reported increases in MMP-2 protein and gene expression (WuDunn., 2001; Bradley et al., 2003; Vittal, 2005), increased MMP-14 protein levels (Bradley et al., 2003), and either no change or a decrease in TIMP-2 (Okada et al., 1998; Bradley et al., 2001, 2003). However, there is conflicting evidence whether changes in response to stretch occur at the level of transcription or post transcriptionally (Bradley et al., 2003). Additionally, the synthesis of the cartilage proteoglycan, aggrecan, has been observed to be significantly increased in mechanically compressed tendon fibrocartilage (Koob et al., 1992; Evanko and Vogel, 1993; Vogel, 2004).

Based on these studies, we speculate that the application of tension on the sclera induces changes in extracellular matrix remodeling and altered biomechanical properties of the sclera, ultimately facilitating vitreous chamber elongation. The present study evaluates the effect of equibiaxial mechanical stretch on the regulation of proteoglycan synthesis, MMP-2, MMP-14 and TIMP-2 in cultures of human scleral fibroblasts. Results of these studies suggest that scleral fibroblasts rapidly respond to tensile forces by increasing the synthesis and activation of *ProMMP-2*. The increased MMP-2 activity would be expected to participate in the remodeling of the scleral matrix and facilitate compliance to intraocular pressure, ultimately leading to ocular elongation.

2. Methods

2.1. Primary Culture of Human Scleral Fibroblasts

Human scleral fibroblasts from a 29 year old donor (passages 3–5) were plated in 100mm plates containing Dulbecco's modified Eagle's medium (DMEM) with 1X antibiotic/antimycotic (Penicillin-Streptomycin/Amphotericin B Solution; Invitrogen Corp., Carlsbad, CA) and 15% fetal bovine serum (FBS) then incubated at 37°C (5% CO₂). The cells were trypsinized in 1X Trypsin-EDTA (Invitrogen, Carlsbad, CA) after reaching confluency and pelleted using a Sorvall® RT 6000D centrifuge at 1100 x g for 10 minutes in an equal volume of DMEM + 15% FBS. The supernatant was removed, the cell pellet was reconstituted with DMEM + 15% FBS, and then transferred into 35mm-6 well BioFlex collagen type I-coated stretch plates (Flexcell Int. Corp., Hillsborough, NC) and incubated at 37°C. After the cells reached subconfluency, the cells were serum starved using DMEM with 1X antibiotic/antimycotic and 0.1% FBS for 48 hours. After 48 hours, the media was replaced with serum free media (DMEM + 1X antibiotic/antimycotic; 2 ml/well) containing ³⁵SO₄ (100 uCi/ml; PerkinElmer Inc., Wellesley, MA).

2.2. Equibiaxial Mechanical Stretch

Subconfluent cells in radiolabelled (³⁵SO₄) serum free media were subjected to a cyclic stretch regimen of 15% equibiaxial stretch (45 seconds flex/15 seconds relax) using the FlexCell® FX-4000 vacuum-driven system together with the Bioflex Loading Stations baseplate (Flexcell Int. Corp., Hillsborough, NC) for 0, 12, 24 and 48 hour intervals at 37°C (95% air/5% CO₂) to deliver a highly controlled regimen of equibiaxial tension to human scleral fibroblasts. Cells plated on Bioflex plates but not subjected to stretch served as controls for each time point.

2.3. Cetyl Pyrimidium Chloride (CPC) Precipitation of radiolabelled Glycosaminoglycans (GAGs)

Following mechanical stretch, the cells and the culture medium were harvested separately. A portion of the radiolabelled culture medium from control and stretched culture wells was digested with 0.05% (w/v) Proteinase K (Type XXVIII Protease, Sigma) in 10mM EDTA, 0.1 M sodium phosphate (pH 6.5) overnight at 60°C. An aliquot of each Proteinase K digest was used to measure newly synthesized proteoglycans released into the medium of control and stretched cultures using cetyl pyrimidium chloride (CPC) as previously described (Rada and

Matthews, 1994). Briefly, unlabelled chondroitin sulfate (Sigma, 1mg/ml dH₂O) was added to each sample digest and all glycosaminoglycans (GAGs) were precipitated by the addition of 0.5% CPC in 0.002M NaSO₄ (1ml/tube; 37°C for 30 minutes). Precipitated GAGs were collected on Whatman GF/F filters (Fisher Scientific) using a 12-port vacuum manifold (Millipore Corp., Bedford, Massachusetts) followed by extensive washing with CPC (0.1% in 0.05M NaCl), followed by distilled water to remove unincorporated ³⁵SO₄. Radioactivity was measured on each filter by scintillation counting.

2.4. Measurement of MMP-2 Activity in Culture Medium

MMP-2 (Gelatinase-A) activity was assayed on aliquots of culture medium by gelatin zymography using pre-cast 10% tris-glycine acrylamide gels containing 0.1% gelatin (NOVEX gels, Invitrogen Corp., Carlsbad, CA) according to manufacturer's protocol. Gel images were captured using a bioimaging system (Chemigenius, Syngene USA, Frederick, MD) and bands were quantified on digitized images using NIH Image v. 1.63.

2.5. RNA, DNA and Protein Isolation

Following removal of the culture supernatants, total RNA, DNA and protein were isolated from the human sclera fibroblast cell layers of stretched and control plates using TRIZOL Reagent following the standard protocol (Invitrogen Corp., Carlsbad, CA). RNA concentration and purity was determined spectrophotometrically at an optical density ratio of 260/280 (OD₂₆₀/OD₂₈₀). DNA in the 48 hour control and stretch samples was quantified at 260nm using PicoGreen® dsDNA Quantitation Reagent (Molecular Probes; Eugene, Oregon) and a TBS-380 Mini-Fluorometer (Turner BioSystems, Sunnyvale, CA). Total protein concentrations in all 6, 12, 24, 48 hour control and stretch samples were assayed using the Bradford method (Bio-Rad Protein Assay, Bio-Rad USA, Hercules, CA) and absorbance measured at 595nm and compared to a standard curve of bovine serum albumin (0.025 – 2 µg/µl).

2.6. Real-Time Polymerase Chain Reaction (Real-Time PCR)

Real-time PCR was used to compare the levels of steady state mRNA for several genes in stretched and non-stretched cultures of human scleral fibroblasts. cDNA was generated from total RNA by reverse transcription using MuLV reverse transcriptase, together with random hexamers, dNTPs in the presence of PCR buffer, 25 mM MgCl₂, and RNase inhibitor at 42°C for 15 minutes, 99°C for 5 minutes, and 4°C for 5 minutes using a kit (GeneAmp; Applied Biosystems, Foster City, CA). Real-time PCR was performed on dilutions, in triplicate, of each cDNA sample using gene-specific primers together with SYBR Green (Molecular Probes, Eugene, Oregon) in a 96 well plate format, using an i-Cycler iQTM Multi-Color Real Time PCR Detection System (Bio-Rad, Hercules, CA). All primers were designed, selected and ordered using BLAST, Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and Sigma-Genosys (St. Louis, MO), respectively, and diluted to 15µM in RNase-free water. Primers in this study include matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-14 (MMP-14), tissue inhibitor of metalloproteinase-2 (TIMP-2) and the housekeeping gene to normalize for starting cDNA concentration, hypoxanthine guanine phosphoribosyl transferase (HPRT). GenBank accession numbers, forward and reverse sequences, product size and optimal melting temperature (T_m) are shown in Table 1. Denaturation was performed for 45 seconds at 95.0°C, primer annealing for 45 seconds at the indicated temperatures (T_m) presented in Table 1, and extension for 60 seconds at 72.0°C.

The mean cycle threshold (cT) value was calculated automatically using MyiQ™ Optical System Software version 1.0 (Biorad Labs., Hercules, CA) and represents the PCR cycle at which the fluorescent signal crosses a threshold line in the exponential phase of the amplification curve. Temperature gradients were run for all primers to determine the optimal

melting temperature (T_m). Primer efficiencies were 88% (TIMP-2), 90% (HPRT), 90% (MMP-2) and 98% (MMP-14), which were calculated using serial dilutions of scleral fibroblast cDNA (1:1 – 1:1000) using the equation $E = e^{-1/\text{slope}}$ (Pfaffl, 2001) where s = the slope of the line generated by plotting C_T values for each serial dilution of cDNA. To compare relative levels of gene expression between control and recovering eyes, the mean normalized expression (MNE) values (Perikles, 2003) were determined for each group according to the equation:

$$\text{MNE} = (E_{\text{reference}})^{CT_{\text{reference, mean}}} \div (E_{\text{target}})^{CT_{\text{target, mean}}}$$

where $E_{\text{reference}}$ and $CT_{\text{reference}}$ are the efficiency and mean threshold cycles of the PCR reaction of the reference gene, HPRT, and E_{target} and CT_{target} are the efficiency and mean threshold cycles for the genes of interest. All samples were run in triplicate with melt curve analysis to ensure the presence of one PCR product. DNA agarose-gel electrophoresis was performed to confirm product size and ensure the absence of primer dimer formation.

2.7. Western blot analysis

Aliquots of culture media (50 μ l) from each control or stretched culture well were concentrated by speed-vacuum, reconstituted in dH₂O and applied to 10% Bis-Tris Gel NuPAGE™ SDS-PAGE gels (Invitrogen Corp., Carlsbad, CA). Gel samples were electrophoresed under reducing conditions and electroblotted onto a nitrocellulose membrane using an electro-transfer unit (XCELL Sureback™ Electrophoresis Cell, Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Blots were probed with Rabbit anti-MMP-2 (Gelatinase A) antibody (Chemicon International; 100 μ g, 1mg/mL) at a 1:1000 dilution in blocking buffer [PBS containing 0.1% Tween-20 and 0.2% I-Block (Tropix, Bedford, MA)] for three hours at room temperature, followed by incubation with goat-anti-rabbit IgG (whole-molecule) conjugated to alkaline phosphatase (Sigma Chemical Co, St. Louis, MO) at a dilution of 1:3000 for one hour at room temperature. Between incubations the blot was washed three times for ten minutes per wash with 1X PBS containing 0.05% Tween20. CDP-Star® Ready-to-Use with Nitro-BlockII™ (Tropix, Bedford, MA) was added to the blot for 5 minutes and then the blot was exposed for one hour on film and visualized on a Chemigenius imager (Syngene USA, Frederick, MD).

2.9. Data Analysis

Statistical comparisons between two groups were conducted by use of Students two tailed t-tests for samples with unequal variances and comparisons of multiple groups were made using one-way ANOVAs with Bonferroni adjustments using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA).

3. Results

3.1. Effects of Mechanical Stretch on Proteoglycan Synthesis in Human Scleral Fibroblasts

The rate of proteoglycan synthesis was evaluated in cultures of human scleral fibroblasts following 48 hours of equibiaxial stretch (Figure 1). No significant differences were detected in the levels of newly synthesized proteoglycans in stretched cells as compared to unstretched controls (Figure 1A; $p = 0.564$). Similarly, no significant differences were detected in total protein concentration in the culture supernatants of scleral fibroblasts (Figure 1B; $p = 0.686$). Additionally, no significant differences in DNA concentration were noted among 48 hour non-stretched and stretched human scleral fibroblasts ($p = 0.923$) (data not shown).

3.2. Effects of Mechanical Stretch on MMP-2 Synthesis in Human Scleral Fibroblasts

MMP-2 (Gelatinase A) activity was assessed in cultures of human scleral fibroblasts by gelatin zymography of the culture medium (2ng of total protein) following 6 – 48 hours of cyclic stretch on BioFlex culture dishes and compared to their unstretched controls (Figure 2). Due to variability in staining intensities between zymogram gels, the densities of the 72-kDa proenzyme form of MMP-2 (*ProMMP-2*) and the 62-kDa active form of MMP-2 (*ActiveMMP-2*) zymogram bands were normalized to that of the average of the control group *ProMMP-2* and *ActiveMMP-2* zymogram bands for each experiment, respectively. A significant increase in secretion of *ProMMP-2* into the medium of human scleral fibroblasts was apparent following 12 and 48 hours of mechanical stretch (+76.28%, $p < 0.05$; +19.56%, $p < 0.01$, respectively) (Figure 2A, D). After 48 hours, levels of *ActiveMMP-2* were significantly elevated in human scleral fibroblasts undergoing cyclic stretch (+59.72%, $p < 0.05$) as compared to the unstretched control cultures (Figure 2B, D). Levels of *ActiveMMP-2* were not detectible on gels following 6 and 12 hours of stretch. Levels of total protein increased significantly in both control and stretched fibroblast cultures over the initial 24 hour culture period (+51.5% in 24 hour control cultures as compared with 6 hour control cultures, $p < 0.01$, ANOVA; and +33.1% in 24 hour stretched cultures compared with 6 hour stretched cultures $p < 0.01$, ANOVA). Additionally, total protein in the culture medium was significantly elevated following 6 hours of cyclic stretch when compared with 6 hour controls (+16.9%, $p < 0.05$) (Figure 2C), however no significant differences in total protein were observed between control and stretched cells following longer periods of culture and mechanical stretch. Western blot analysis confirmed the presence of the 72-kDa MMP-2 proenzyme in stretched and control cultures following 24 hours of culture (Figure 3). *ActiveMMP-2* was not recognized by the anti-MMP-2 antibody used in this experiment. MMP-14 protein levels were undetectable using Western blot analysis (data not shown).

3.3. Gene Expression in Response to Mechanical Stretch

The expression of MMP-2, as well as two known regulators of MMP-2 activation, TIMP-2 and MMP-14, was quantified using real-time PCR and normalized to the expression levels of the housekeeping gene, HPRT (Figure 4). No significant changes in MMP-2 or MMP-14 mRNA expression were detected in response to 48 hours of equibiaxial cyclic stretch, ($p = 0.348$ and $p = 0.799$, respectively, ANOVA). TIMP-2 mRNA expression was significantly decreased in stretched human scleral fibroblasts when compared to their non-stretched controls (–22%, $p < 0.05$, ANOVA) (Figure 4A). Ethidium bromide gels of PCR products were used to verify the correct size and amplification of HPRT, MMP-2, MMP-14 and TIMP-2 following 40 cycles of PCR (Figure 4B).

4. Discussion

In vivo, stresses and strains exerted at the posterior pole of the eye resulting from the sum of a combination of factors including intraocular pressure, muscular tension during accommodation/convergence, weakness near the scleral canal, axial length and scleral thickness may contribute to the development and progression of myopia (Greene, 1991). Supporting this hypothesis is the association between elevated intraocular pressure and myopia development in infants and children (Abdalla et al., 1970; Perkins and Phelps, 1982 and David et al., 1985; Jensen, 1992; Quinn et al., 1995) as well as the finding that scleral changes associated with myopia development are limited to the posterior poles of human and animal eyes, a region where scleral stresses are greatest (Greene, 1991; Rada et al., 1994). Previous studies suggest roles for proteoglycans, MMP-2, MMP-14 and TIMP-2 in scleral extracellular matrix remodeling events associated with eye growth under normal conditions, as well as under conditions leading to the development of myopia (reviewed in Rada et al., 2006; Brown et al., 1994; McBrien et al., 2000; Schippert, 2006). Therefore, we hypothesize that the synthesis

and/or activity of these extracellular matrix molecules are regulated, in part, by biomechanical stresses exerted on scleral fibroblasts.

In vitro biaxial cell stretch systems have rapidly become standard models for studying the effects of mechanical forces on a variety of cell types, including trabecular meshwork cells (Bradley et al., 2001, 2003; WuDunn, 2001), lamina cribrosa cells (Kirwan et al., 2004, 2005) and scleral fibroblasts (Fujikura et al., 2002; Cui et al., 2004). These studies have attempted to model intraocular forces by introducing mechanical distortion (4 – 15% increases in cellular surface area) to ocular cells for durations and frequencies ranging from <1 sec at 1 cycle/second (Kirwan et al., 2004, 2005), 30 seconds at 2 cycles/minute (Fujikura et al., 2002), or constant stretch for 30 minutes – 72 hours (Cui et al., 2004, Bradley et al., 2001). Because the forces exerted at the posterior ocular pole represent a combination of relatively static factors (e.g. scleral thickness, intraocular pressure) superimposed with transient changes in force associated with accommodation/convergence (Greene, 1991), we selected a stretch protocol for scleral fibroblasts in vitro employing a regimen of static stretch for 45 seconds applied cyclically from 6 – 48 hours. In this study, flexible-bottom culture dishes were subjected to distension, using the Flexcell 4000 vacuum-driven system to deliver a highly controlled regimen of 15% equibiaxial strain to human scleral fibroblasts. This stretch apparatus has been used widely by other labs for several years and many results have been published with this system (Brown, 2000).

4.1. Effect of equibiaxial stretch on the rate of proteoglycan synthesis

Previous studies using several animal models of myopia have detailed the close association between myopia development and the rate of proteoglycan synthesis in the posterior sclera (reviewed in Rada et al., 2006). We therefore hypothesize that if changes in scleral proteoglycan synthesis observed during the development of myopia resulted from increased scleral strain, we would expect to observe changes in proteoglycan synthesis by scleral fibroblasts in response to in vitro equibiaxial stretch. Interestingly, we were unable to detect a significant difference in proteoglycan synthesis rates between cells mechanically stretched for 48 hours as compared to their non-stretched controls. However, since proteoglycan synthesis rates were measured in the present study as the rate of $^{35}\text{SO}_4$ incorporation into CPC-precipitable glycosaminoglycans, we can not exclude the possibility that in response to equibiaxial stretch, scleral fibroblasts alter their profile of proteoglycan gene expression, as has been demonstrated for that of fetal bovine deep flexor tendon during cyclic compression (Evanko and Vogel, 1993; Robbins et al., 1997) where synthesis of aggrecan is increased after 72 hours of cyclic compression, but decorin synthesis remains unchanged. In the present study, although the net amount of $^{35}\text{SO}_4$ into newly synthesized glycosaminoglycans is unchanged, the population of proteoglycans may be similarly modified to produce a more compliant extracellular matrix.

4.2. Effect of equibiaxial stretch on MMP-2 activity

The expression and activity of matrix metalloproteinases (MMPs) have been shown to play a critical role in extracellular matrix synthesis and turnover in a variety of tissues and cell types, (Woessner, 1991; Birkedal-Hansen et al., 1993; Corcoran, 1996; Coussens, 2002). Moreover, biomechanical forces such as tension, compression and shear have been shown to modulate the activity of MMP-2 in chondrocytes (Fujisawa et al., 1999), ligaments (Zhou et al., 2005), muscle (O'Callaghan and Williams, 2000; Auluck et al., 2005) and in the cardiovascular system (Milkiewicz et al., 2005; Rastogi et al., 2005).

Both the 72-kDa proenzyme form of MMP-2 (*ProMMP-2*) and the 62-kDa active form of MMP-2 (*ActiveMMP-2*) were detectable in the culture medium of human scleral fibroblasts, following 24 hours of culture, although human scleral fibroblasts released very little *ActiveMMP-2*, relative to that of the *ProMMP-2*. In the present study, a trend toward increased

ProMMP-2 levels was observed on gelatin zymograms following 6 hours of equibiaxial stretch, which became statistically significant following 12 hours of equibiaxial stretch, as compared with non-stretched cultures. Due to relatively low amounts of the active 62-kDa form of MMP-2 in culture medium, the *ActiveMMP-2* did not reach detectible levels until 24 hours of culture and significant increases were detected in culture medium following 48 hours of cyclical stretch. This relatively early increase between 6 and 12 hours in *ProMMP-2* levels and subsequent increase in *ActiveMMP-2* levels in response to stretch is suggestive of a direct response by scleral fibroblasts to the applied stress and occurs in a similar time frame as has been reported for human ligament fibroblasts in response to equibiaxial stretch (Zhou et al., 2005) as well as porcine and bovine trabecular meshwork cells following 24 – 72 hours of mechanical stretch or distortion (Bradley, 2001; WuDunn, 2001).

4.3. Quantitation of MMP-2, MMP-14 and TIMP-2 mRNA expression levels

In an effort to determine the mechanism for the observed stretch-induced increases in *ProMMP-2* and *ActiveMMP-2*, steady state mRNA levels were determined for MMP-2, MMP-14 and TIMP-2 following 48 hours of cyclic equibiaxial stretch and compared with non-stretched controls. Both MMP-2 and MMP-14 mRNA expression did not significantly change in response to stretch when compared to their non-stretched controls. These results suggest that the increased synthesis of *ProMMP-2* observed following 12 – 48 hours of applied stretch is not the result of an increased rate of transcription, but rather due to a post-transcriptional mechanism or increased MMP-2 mRNA stability. Similarly, a post-transcriptional mechanism has been suggested for observed increases in MMP-2 and MMP-14 protein levels in trabecular meshwork cells following 24 – 48 hours of mechanical stretch (Bradley et al., 2003).

Significant decreases in TIMP-2 mRNA expression were detected following 48 hours of applied stretch, indicating that TIMP-2 expression is mechanoresponsive. Interestingly, TIMP-2 protein levels have also been shown to be dramatically reduced while *Pro-* and *ActiveMMP-2* are increased in trabecular meshwork cells following 24 hours of 10% constant stretch (Bradley et al., 2001). We speculate that stretch-induced decrease in TIMP-2 mRNA would result in a dis-inhibition of MMP-2 activation, resulting in an increased production of *ActiveMMP-2*. Previous studies on embryonic chick scleral fibroblasts (Fujikura et al., 2002), demonstrated increased levels of MMP-2 and TIMP-2 mRNA in response to stretch in chick scleral fibroblasts. The discrepancy in the results of our study and that of Fujikura et al., are most likely due to the differences between adult human scleral fibroblasts and embryonic chick scleral fibroblasts. Moreover, since the chick sclera contains a distinct cartilaginous layer, the embryonic chick scleral fibroblast cultures would most likely contain both chondrocyte and fibroblast progenitor cells, which would be expected to respond to mechanical stimuli in a manner distinct from that of differentiated human scleral fibroblasts.

Interpretation of the results of the present study should be made with caution, as our in vitro stress/strain system does not model intraocular forces as they occur in vivo. Our results do, however, demonstrate that scleral fibroblasts respond to mechanical stretch/distortion by increasing MMP-2 activity, through increased MMP-2 protein synthesis and decreased TIMP-2 gene expression. Since MMPs are largely involved in ECM turnover, this may suggest one mechanism by which mechanical stresses and strains on posterior ocular globe may alter the compliance of the sclera, and result in an increased axial elongation of the eye. Stretch-induced increases in MMP-2, especially the 62-kDa *ActiveMMP-2*, would be expected to increase the overall MMP activity in the sclera, as well as activate other MMP proteins, thereby hastening scleral extracellular matrix degradation.

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Figure 1. Effect of cyclical mechanical stretch on proteoglycan and protein synthesis by human scleral fibroblasts

A. $[^{35}\text{S}]$ Sulfate incorporation into glycosaminoglycans following 48 hours of cyclical stretch did not differ from static controls ($p > 0.05$). **B.** No differences in total protein concentration were observed in the culture medium of stretched and static cultures ($p > 0.05$) following 48 hours of culture. Data are represented as mean $\pm$ SEM ($n = 6$ wells per group).

Figure 2. Effect of cyclical mechanical stretch on MMP-2 activity by human scleral fibroblasts

A. Significant increases in the 72-kDa proenzyme form of MMP-2 (*ProMMP-2*) secretion into the medium of human scleral fibroblast cells after mechanical stretch was observed at 12 and 48 hours as compared to their non-stretched controls. **B.** Levels of the 62-kDa active form of MMP-2 (*ActiveMMP-2*) in the culture medium was observable after 24 hours and significantly increased after 48 hours of cyclical stretch. *ActiveMMP-2* was not detectable prior to 24 hours of culture. **C.** Total protein concentration ($\mu\text{g/ml}$) was significantly increased after 6 hours of cyclical stretch as compared with 6 hour static controls ($p < 0.05$). Significant increases in total protein were observed in control and stretched cultures following 24 hours as compared with 6 hour control and stretched cultures ($p < 0.01$, ANOVA). **D.** Representative gelatin zymograms of culture media following 12 and 48 hours of static culture (control) and cyclical equibiaxial stretch (stretch). Data are represented as mean $\pm$ SEM relative mean density for 6 individually plated wells per plate. * $p \leq 0.05$, $n = 6$; student's two-tailed t-test for unequal variances. Zymogram bands were normalized to that of the average of the control group *ProMMP-2* and *ActiveMMP-2* zymogram bands for each experiment, respectively.

Figure 3. Western blot analyses of ProMMP-2 in culture medium of human scleral fibroblasts following 24 hours of culture

Western blot analysis confirmed the presence of the 72-kDa *ProMMP-2* in both stretched and static control cultures of human scleral fibroblasts. *ActiveMMP-2* was not recognized by anti-MMP-2 used in this experiment. (Lanes 1-3: static control cultures; lanes 4-6: stretched cultures).

Figure 4. Effect of cyclical mechanical stretch on MMP-2, MMP-14 and TIMP-2 mRNA expression in human scleral fibroblasts following 48 hours of culture

A. Average threshold (Ct) values for MMP-2, MMP-14, and TIMP-2 of each individual well (triplicate measurements each of 6 control and 6 stretched cultures) were used to calculate the mean normalized expression (MNE) by normalizing to the housekeeping gene, HPRT (see Methods). No measurable significant differences in either MMP-2 or MMP-14 mRNA expression were detected in control and stretched cultures ($p > 0.05$). TIMP-2 mRNA expression was significantly decreased following 48 hours of cyclical mechanical stretch as compared with static cultures (* $p < 0.05$, student's two-tailed t-test for unequal variances).

B. Representative ethidium bromide gel for PCR products generated during real-time PCR following 40 cycles of PCR in control (lanes 1 – 6) and stretched (lanes 7 – 12) cultures of human scleral fibroblasts.

TABLE 1

Human primers			
Gene	GenBank	Forward Primer	Reverse Primer
HPRT	M31642	5'-GCA GAC TTT GCT TTC CTT GG-3'	5'-AAG CAG ATG GCC ACA GAA CT-3'
MMP-2	NM004530	5'-TGG GGA GTA CTG CAA GTT CC-3'	5'-TAC TTC TTG TCG CGG TCG TA -3'
MMP-14	NM004995	5'-CAT TGG AGG AGA CAC CCA CT-3'	5'-TGG GGT TTT TGG GTT TAT CA-3'
TIMP-2	NM003255	5'-CTG GAC GTT GGA GGA AAG AA-3'	5'-GTC GAG AAA CTC CTG CTT GG-3'
			Product Size (bases)
			321
			300
			314
			345
			Tm (°C)
			59.0
			56.5
			56.5
			59.0