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Involvement of Mechanical Stretch in the Gelatinolytic Activity of the Fibrous Sclera of Chicks, In Vitro

Hajime Fujikura*, Yuko Seko*,
Takashi Tokoro*, Manabu Mochizuki* and Hitoyata Shimokawa†

*Department of *Ophthalmology and Visual Science, Tokyo Medical and Dental University Graduate School, Tokyo, Japan; †Department of Dental Pharmacology, Tokyo Medical and Dental University Graduate School, Tokyo, Japan*

Purpose: To investigate the role of mechanical stretch in the regulation of matrix metalloproteinase-2 (MMP-2) in scleral fibroblasts of chick embryos.

Methods: Scleral fibroblasts derived from chick embryos were seeded onto flexible bottom culture plates, and subjected to a pulsatile stretch when the cells became subconfluent. After stretching, the cells and the conditioned medium were harvested. One portion of the conditioned medium was activated by reduction, alkylation, and 4-aminophenylmercuric acetate (APMA) treatment. The conditioned medium, with and without the treatment, was subjected to gelatin zymography and quantitative assays. Total cytoplasmic RNA was extracted from the cells, and the expression of MMP-2 and the tissue inhibitor of metalloproteinase-2 (TIMP-2) mRNA was examined by Northern blot analysis.

Results: The predominant gelatinolytic enzyme secreted by scleral fibroblasts was MMP-2. The mechanical stretch increased the gelatinolytic activities significantly in the conditioned medium with reduction, alkylation, and APMA treatment. Mechanical stretch also enhanced the expression of MMP-2 and TIMP-2 mRNA in scleral fibroblasts significantly.

Conclusions: These results suggest that mechanical stretch may be involved in the regulation of the extracellular matrix in the fibrous sclera of chicks in vivo. **Jpn J Ophthalmol 2002; 46:24–30**

Key Words: Extracellular matrix, matrix metalloproteinase-2, mechanical stretch, myopia, sclera.

Introduction

In pathological myopia, excessive axial elongation induces severe disorders in the retina and choroid of human eyes. Therefore, it is important to study the mechanism of axial elongation in myopic eyes. An experimental model of myopia, form deprivation myopia in chicks,[1] was the basis for this study. In this model, axial elongation is induced by visual deprivation in neonatal chicks. Experimental evidence suggests that the scleral changes are strongly associated with the axial elongation in form deprivation myopia.[2]

The chick sclera consists of an inner cartilaginous layer and an outer fibrous layer.[2] In form-deprived eyes of chicks, the inner cartilaginous layer is thicker and the outer fibrous layer is thinner than the corresponding layers in normal chick eyes.[2] The morphological changes in the fibrous layer of the chicks is similar to that of highly myopic human[3] and monkey eyes.[4] Recently, it is hypothesized that the fibrous sclera in form deprivation myopia regulates the remodeling of the extracellular matrix in the cartilaginous layer of the sclera.[5]

Previous studies have suggested that matrix metalloproteinase-2 (MMP-2) might play a role in the scleral remodeling process during the development of form deprivation myopia.[6] MMP-2 belongs to the gelatinase subgroup of the MMP family. Although

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Correspondence and reprint requests to: Hajime FUJIKURA, MD, Department of Ophthalmology and Visual Science, Tokyo Medical and Dental University Graduate School, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

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the mechanisms by which the activities of MMPs are regulated in the myopic sclera remain to be determined, MMPs have been reported to be modulated by mechanical stretch in glomerular mesangial cells[7,8] and cardiac fibroblasts.[9]

In the human eye, experimental[10] and epidemiological[11] data suggest the involvement of accommodation in axial elongation. As for the mechanism by which the sclera is mechanically stretched by accommodation, it has been suggested that the tonus of the ciliary muscle plays a role in the mechanism by determining the tension in the choroid, thereby regulating the net-pressure on the sclera.[12]

Based on these data, we hypothesized that the mechanical stretch induced by accommodation might modulate the activity of MMPs in the fibrous sclera. The balance between MMPs and their intrinsic inhibitors, tissue inhibitors of metalloproteinases (TIMPs), is essential for regulating extracellular matrix. To investigate the role of mechanical stretch in the regulation of MMP and TIMP in the fibrous sclera, we measured the gelatinolytic activity and mRNA expression of MMP-2 and TIMP-2 in vitro. An apparatus that automatically applies mechanical stretch to cultured cells was used.

Materials and Methods

Cell Culture

The sclera was separated from the enucleated eyeballs of 14-day-old chick embryos and divided into the cartilaginous layer and the fibrous layer. Scleral fibroblasts were harvested from the fibrous layer according to procedures described previously.[13,14] The fibroblasts derived from primary cultures were seeded onto BioFlex collagen I culture plates coated with type-I collagen (Flexcell International, McKeesport, PA, USA) at a concentration of 2.0×10^5 cells per dish. A single plate consists of 6 dishes (diameter: 35 mm), and the bottom of each plate is made of a silicone elastomer. The seeded cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 2 days until they were subconfluent. Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Bioserum, Victoria, Australia) was used as growth medium.

Application of Mechanical Stretch

The subconfluent cells on the silicone elastomer bottom culture plates were washed with calcium- and magnesium-free phosphate-buffered saline (Gibco, Grand Island, NY, USA), and the media were replaced with 1.5 mL of DMEM containing 1% FBS. The culture plates were placed on a computerized Flexercell Strain Unit gasketed baseplate in the incubator (Flexcell). The apparatus, a modification of one described by Banes et a1,[15] consists of a computer-controlled vacuum unit. The computer system controls the frequency of deformation and the ratio of elongation of the silicone elastomer bottom. In our study, the subconfluent cells were subjected to mechanical stretch in a pulsatile pattern of load at 0.2 Hz for 30 seconds, followed by a 30-second rest period. The 60-second cycle was repeated for 48 hours (Figure 1).[16] Cells cultured in the same elastomer bottom plates in the same incubator, but not subjected to mechanical stretch, were used as controls.

Activation of MMPs and Inactivation of TIMPs

All MMPs are synthesized as inactive proenzymes with propeptides. The latency is retained through the coordination of the zinc (Zn) atom at the active site and the cystein (Cys) residue in the propeptide.[17,18] Pro-MMPs are activated in vitro by 4-aminophenylmercuric acetate (APMA).[19,20] APMA breaks the Cys-Zn coordination in the latent MMPs, resulting in activation.[21,22] In this study, we attempted to measure the gelatinolytic activities of MMPs excluding the inhibitory effects of TIMPs and propeptides in pro-MMPs. For this purpose, the conditioned media were incubated in the presence of 1 mM APMA at 37°C for 60 minutes to activate latent MMPs. TIMPs were inhibited by a reduction and alkylation treatment because TIMPs contain disulfide-bonds in the molecules.[6] To inactivate TIMPs, the conditioned media were reduced with 2 mM dithiothreitol (Sigma) for 30 minutes at 37°C and alkylated with 5 mM iodoacetamide (Sigma) for 30 minutes at 37°C. These media were designated as APMA(+) media. The media without the treatment were designated as APMA(−).

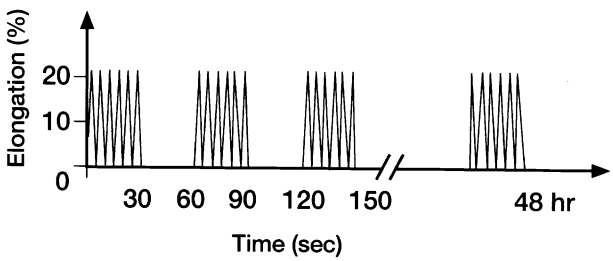

Figure 1. Diagrammatic representation of pattern of stretching. Mechanical load at 0.2 Hz is applied for 30 seconds followed by 30-second rest period. This 60-second cycle is continued for 48 hours.

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Gelatin Zymography

Gelatin zymography was performed using a Gelatin Zymography Kit (Yagai, Yamagata) according to the manufacturer's instructions with some modification. The conditioned media were harvested from each dish immediately after mechanical stretch. To examine whether gelatinolytic activity was derived from MMPs, zymography was performed after selective inactivation of MMPs. Instead of using the reaction buffer supplied in the kit, 50 mM Tris-HCl (pH 8.0; Sigma) and 10 mM ethylenediaminetetraacetic acid (EDTA; Sigma) were used.[23] Because zinc and calcium ions are required for the activities of MMPs,[24] the gelatinolytic activities of MMPs are masked under these conditions. Trypsin was used as a positive control because it does not belong to the MMP family but has gelatinolytic activity. APMA(+) media were used in zymography to characterize the MMPs derived from the conditioned media.

Assay of Gelatinolytic Activities

To examine quantitatively whether mechanical stretch was involved in gelatinolytic activities of scleral fibroblasts, a Type IV Collagenase Assay Kit (Yagai, Yamagata) was used according to the manufacturer's instructions. The zymography results indicated that the gelatinolytic activity of the scleral fibroblasts was derived predominantly from MMP-2, which belongs to type IV collagenase. Using this kit, gelatinolytic activity was determined by measuring the digestion of fluorescein-labeled type IV collagen. APMA(+) and APMA(−) media were used in this gelatinolytic assay. In one experiment, the media from six dishes were examined separately and a statistical analysis was performed. Two independent experiments were repeated in the same manner.

Northern Blot Analysis

Northern blot analyses were performed to examine whether mechanical stretch affected the expression of MMP-2 and TIMP-2 mRNA as well as the gelatinolytic activities. TIMP-2 preferentially forms complexes with pro-MMP-2 to prevent activation of pro-MMP-2.[25–27] Total cytoplasmic RNA was prepared using Isogen (Nippon Gene, Toyama) according to the manufacturer's instructions. In each lane, 10 μg of total cytoplasmic RNA extracted from six dishes of fibroblasts was electrophoresed in a formaldehyde-agarose (Sigma) gel and transferred to a nylon membrane (Boehringer Mannheim Biochemica, Mannheim, FRG).

To synthesize the probes for hybridization, the combinations of 5′ and 3′ primers described below were used. These primers were derived from chick sequences.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH):

5′-GACCACTGTCCATGCCATCAC-3′ (5′ sense)
5′-TCCACAACACGGTTGCTGTAT-3′ (3′ antisense)
probe size: 453 bp

MMP-2:

5′-AATGGTGATGGACAGCCCTG-3′ (5′ sense)
5′-CGGAAGTTCTTGGTGTAGGT-3′ (3′ antisense)
probe size: 443 bp

TIMP-2:

5′-ACCCGCAGCAGGCCTTCTGCAAC-3′ (5′ sense)
5′-ATGTCGAGAAACTCCTGCTT-3′ (3′ antisense)
probe size: 553 bp

Polymerase chain reaction (PCR) was performed using these primers, and the sequences of the PCR products were confirmed. The probes were labeled with [α-^{32}P]-dCTP (ICN Biomedicals, Costa Mesa, CA, USA) by Klenow enzyme using Ready To Go DNA labeling kit (-dCTP; Amersham Pharmacia Biotech, Bucks, UK). Hybridization was performed according to the methods described previously.[28] The hybridization of MMP-2 and GAPDH was performed at the same time, and the TIMP-2 blot represented a strip of their probe and reprobe experiment using the same membrane. The density of the detected bands was quantified by a fluoroimage analyzer (FLA-2000, Fuji Film, Tokyo). The values for MMP-2 and TIMP-2 were normalized by the value of GAPDH. The values derived from the stretched cells were divided by the values derived from control cells. The divided values were designated as ratios (stretch/control).

Statistical Analyses

Data are reported as mean ± SD. The Mann-Whitney *U*-test or the Student *t*-test was used to compare results between the stretched and the control cells. $P < .05$ was accepted as statistically significant.

Results

Gelatin Zymography

To examine whether the gelatinolytic activities are derived from MMPs, reaction buffer including a

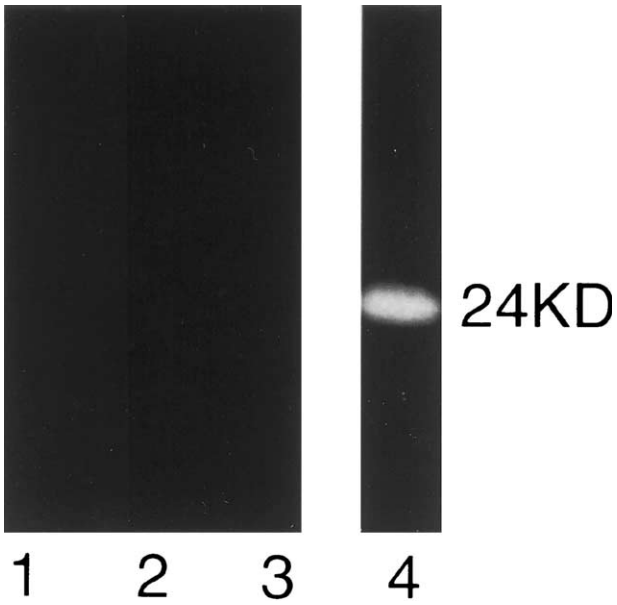

Figure 2. Gelatin zymogram of 4-aminophenylmercuric acetate (APMA)(+) conditioned media in the presence of 10 mM ethylenediaminetetraacetic acid. Lane 1: unstretched control; lane 2: stretched for 48 hours; lane 3: Dulbecco's modified Eagle's medium containing 1% fetal bovine serum; lane 4: 5 ng trypsin. Trypsin was used as positive control because it does not belong to matrix metalloproteinase family but has gelatinolytic activity. Gelatinolytic activity was not detected in either stretched or unstretched APMA(+) media in presence of chelating agent. However, gelatinolytic activity was detected in positive control, trypsin, at 24 kD.

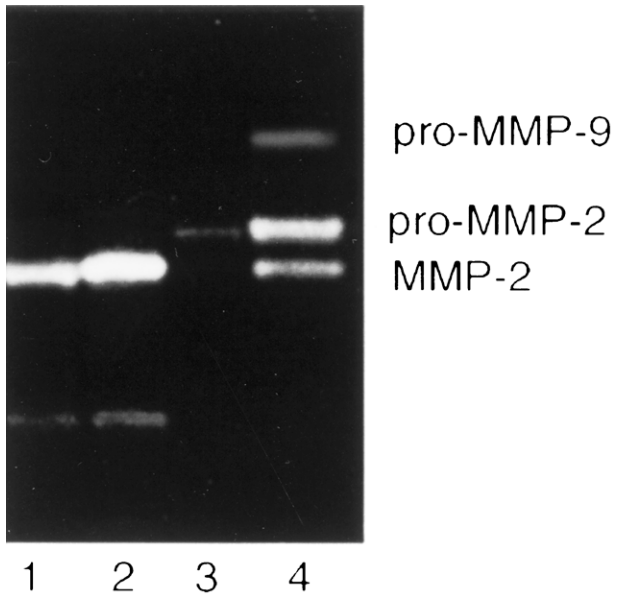

Figure 3. Gelatin zymogram of conditioned media. Lane 1: 4-aminophenylmercuric acetate (APMA)(+) of unstretched control; lane 2: APMA(+) stretched for 48 hours; lane 3: Dulbecco's modified Eagle's medium containing 1% fetal bovine serum; lane 4: mixture of promatrix metalloproteinase (MMP)-9, pro MMP-2, and MMP-2. In lanes 1 and 2, two bands were detected. Higher molecular weight band represents active MMP-2. Lower molecular weight band may also represent active form of

chelating agent was used. The gelatinolytic activities were not detected in either stretched or unstretched APMA(+) media in the presence of a chelating agent (Figure 2). However, gelatinolytic activity was detected in a positive control, trypsin, at 24 kD. These results indicate that the gelatinolytic activities in the conditioned media were derived from MMPs.

To characterize the MMPs derived from the conditioned media, APMA(+) media were examined by gelatin zymography (Figure 3). A positive control used in this experiment, which was a constituent of the commercial kit, contained pro-MMP-9 (92 kD), pro-MMP-2 (72 kD), and active MMP-2 (67 kD) (lane 4). In lanes 1 and 2, two bands were detected. The higher molecular weight band represents active MMP-2. The lower molecular weight band may also represent the active form of MMP-2 because it has been reported that MMP-2, lacking both N and C terminal portions, migrates at 41 kD.[29] These results suggest that the gelatinolytic activities in the media collected from the cultured scleral fibroblasts were derived from MMP-2.

Assay of Gelatinolytic Activities

The gelatinolytic activities were examined in APMA(+) and APMA(−) media. The gelatinolytic activities were increased by the mechanical stretch (Table 1). In APMA(−) media, the gelatinolytic activities from the stretched cells were higher than those from unstretched controls, and the differences were statistically significant in one of two experiments. In APMA(+) media, the gelatinolytic activities from the stretched cells were higher than those from unstretched controls, and the differences were statistically significant in two experiments.

Northern Blot Analysis

Northern blot analyses were performed in three experiments and a representative result is shown in Figure 4. The expression of MMP-2 and TIMP-2 mRNA in stretched fibroblasts appeared to be greater than in the control. The mRNA expression was quantitatively measured using a fluoroimage analyzer. The values for either MMP-2 or TIMP-2 mRNA were normalized with the values for GAPDH mRNA. The values of stretched cells were divided by those of control cells and analyzed statistically. These re-

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Table 1. Gelatinolytic Activity in Conditioned Media Following Application of Mechanical Stretch

Experiment	Sample*	Gelatinolytic Activity† (unit/mL)	
		Control	Stretch
1	APMA (−)	0.24 ± 0.05 (n = 6)	0.31 ± 0.05 (n = 6)‡
	APMA (+)	1.80 ± 0.20 (n = 6)	2.12 ± 0.19 (n = 6)‡
2	APMA (−)	0.18 ± 0.02 (n = 6)	0.20 ± 0.04 (n = 6)
	APMA (+)	2.28 ± 0.11 (n = 6)	2.48 ± 0.10 (n = 6)‡

*APMA: 4-aminophenylmercuric acetate.
†Data expressed as mean ± SD.
‡$P < .05$ stretch vs control (Mann-Whitney *U*-test).

sults are shown in Table 2. The mRNA expression was increased significantly by the application of mechanical stretch in MMP-2 as well as TIMP-2.

Discussion

In this study, we have examined the influence of mechanical stretch on the turnover of extracellular matrix in the fibrous sclera of chicks. Our data demonstrated that mechanical stretch altered the gelatinolytic activities in scleral fibroblasts in vitro, and that the gelatinolytic activities were derived predominantly from MMP-2. The data also suggest that mechanical stretch increased the expression of MMP-2 and TIMP-2 mRNA in scleral fibroblasts.

Gelatin zymography was performed to examine qualitatively the enzymatic source of the gelatinolytic activity. Figure 2 reveals no gelatinolytic activities in the conditioned media, whereas the trypsin control produced a clear band. This suggests that the gelatinolytic activities in media conditioned by scleral fibroblasts were derived from MMPs. Among MMPs, MMP-2 was found to be the major type, and gelatinolytic activity derived from other enzymes such as MMP-9 was not detected in the conditioned media (Figure 3). Because the gelatin zymography experiments showed that the gelatinolytic activities were derived predominantly from MMP-2, a quantitative analysis of gelatinolytic activities was performed by measuring the digestion of fluorescein-labeled type IV collagen (Table 1).

Experimental mechanical stretch significantly increased the gelatinolytic activities treatment in scleral fibroblasts. An earlier study showed that MMP-2 degrades collagen type I,[30] which is the major component in the fibrous sclera of chicks.[31] In the chick model of form deprivation myopia, the fibrous sclera is thinner than in the control eye.[2] A previous study demonstrated that the human sclera, which consists predominately of type I collagen, is thinner in myopic eyes than in normal eyes.[3] In addition, MMP-2 mRNA expression has been shown to increase in the fibrous sclera of form deprivation myopic eyes.[32] Taken together, these results suggest that mechanical stretch influences the metabolism of type I collagen by altering MMP-2 activities, and that these effects are associated with accommodation not only in chicks, but also in humans, resulting in morphological changes in the sclera.

The results shown in Figure 4 and Table 2 indicate that mechanical stretch enhances MMP-2 and TIMP-2 mRNA expression in scleral fibroblasts. This is compatible with the quantitative results of the gelatinolytic activities, ie, the gelatinolytic activities in APMA(+) were increased and the ones in APMA(−) were not increased by mechanical stretch. A recent study, however, demonstrated that MMP-2 mRNA expression increased and TIMP-2 mRNA expression decreased in the fibrous sclera of form deprivation myopic eyes of chicks.[32] The discrepancy in TIMP-2 mRNA expression between our study and the previous study may be due to the differences in the experimental system, ie, in vitro vs in vivo. For instance, we used cultured fibroblasts in a single layer which is different from the thicker structure of the in vivo fibrous sclera interacting with other tissue such as the cartilaginous sclera.[5] In addition, factors other than mechanical stretch may be involved in the regulation of the balance between MMP-2 and TIMP-2 in the fibrous sclera of myopic eyes.

We have attempted to duplicate the conditions of mechanical stretch experimentally by mimicking the physiological conditions of the sclera. However, the physiological stretching conditions have not been established with regard to intensity, polarity, and pattern of stretch, and it is difficult to mimic the physiological conditions in an in vitro experiment using the available apparatus. In this study, we used a pulsatile pattern of stretch (Figure 1). In preliminary experiments, the gelatinolytic activities were significantly

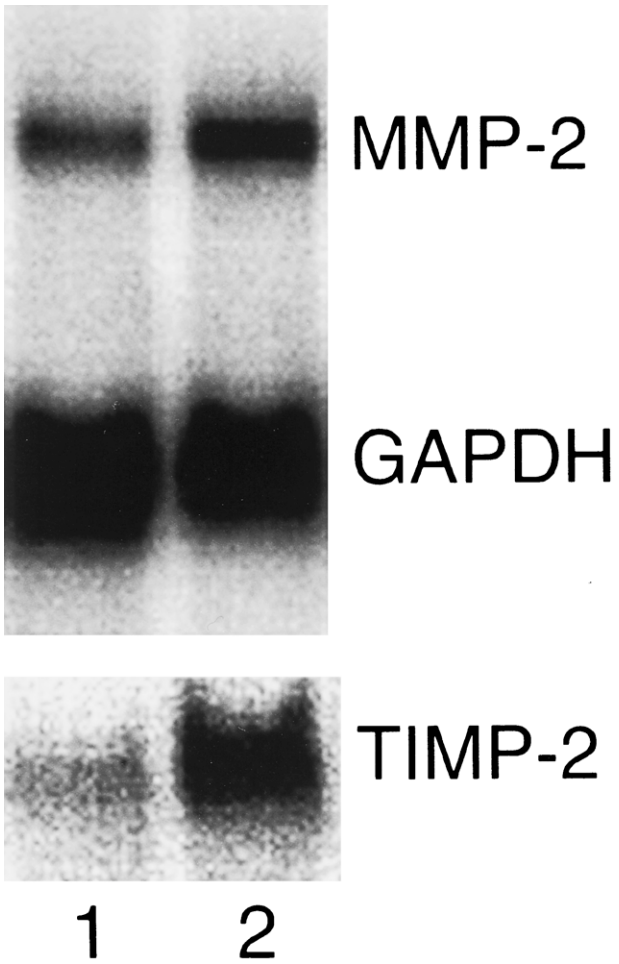

Figure 4. Northern blot analysis of matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-2 (TIMP-2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Lane 1: unstretched control; lane 2, stretched for 48 hours. Expression of MMP-2 and TIMP-2 mRNA in stretched fibroblasts appears to be greater than in control.

different between stretched cells and the controls only after a 20% elongation for 48 hours and not under other conditions, such as a 10% elongation for 48 hours or a 20% elongation for 24 hours (data not shown). Although the pulsatile pattern in this study does not necessarily mimic the physiological condition in accommodation, our results showed that mechanical stretch altered MMP-2 activities in scleral fibroblasts in vitro.

In conclusion, we have used scleral fibroblasts of chicks in vitro to demonstrate that mechanical stretch increases the gelatinolytic activities with APMA treatment and the mRNA expression of MMP-2 and TIMP-2. These results suggest that mechanical stretch is involved in the regulation of the turnover of extracellular matrix in the fibrous sclera in vivo, and that physiological stretch, such as ciliary muscle tone, may be associated with the scleral remodeling of axial elongation in myopia.

Table 2. Quantification by Northern Blot Analysis of Matrix Metalloproteinase-2 (MMP-2) and Tissue Inhibitor of Metalloproteinase-2 (TIMP-2)

Experiment	MMP-2*	TIMP-2*
1	2.51	1.70
2	2.46	2.40
3	2.36	3.03

Values are the ratios of MMP-2 or TIMP-2 mRNA expression normalized by glyceraldehyde-3-phosphate-dehydrogenase in stretched cells to those in controls.

*$P < .05$ stretch vs control (Student t-test).

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