Original Research

Expression of MMP-2, MT1-MMP, and TIMP-2 by cultured rabbit corneal fibroblasts under mechanical stretch

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Abstract

Refractive surgery not only leads to tissue injury but also evokes mechanical stress increase of the cornea. How the mechanical stress affects the corneal matrix remodeling, specifically, matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases; TIMPs) is not well understood. In this study, cultured rabbit corneal fibroblasts *in vitro* were subjected to regimen of 5%, 10%, or 15% equibiaxial stretch at 0.1 Hz for 3 or 24 h. MMP-2 protein level was measured by gelatin zymography and Western blotting. MMP-2, membrane type 1 MMP (MT1-MMP), and TIMP-2 mRNA levels were quantified by real-time quantitative PCR. Extracellular regulated protein kinase (ERK) phosphorylation protein levels were assessed by Western blotting. Our results showed that a 15% stretch resulted in increases in MMP-2 protein, MMP-2 mRNA, and MT1-MMP mRNA levels, but a decrease in TIMP-2 mRNA level. However, a 5% stretch caused decreases in MMP-2 protein and mRNA level, but an increase in TIMP-2 mRNA level, and no change in MT1-MMP mRNA level. A 15% stretch also caused a significant increase in ERK1/2 phosphorylation. Inhibition of the mitogenactivated protein kinase (MEK) pathway with PD98059 attenuated stretch-induced increase in MMP-2 production and ERK activity. These results suggest that small-magnitude stretching may promote corneal matrix synthetic events, whereas large-magnitude stretching promotes corneal matrix degradation by changing the balance between MMPs and TIMPs in corneal fibroblasts. Large-magnitude stretch-induced increase in pro-MMP-2 production was in an ERK-dependent manner.

Keywords: Cyclic stretch, corneal fibroblasts, matrix metalloproteinases, TIMPs, ERK

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Introduction

The method of refractive surgery is a procedure to correct ametropia by ablating the central area of the human cornea. The aim of refractive surgery is to manipulate the curvature of the anterior cornea to produce a better quality of vision. The removal of the stroma induces a flattening of the central cornea.[1] The tissue weakens and the membrane stress increases. On the other hand, the daily intraocular pressure (IOP) fluctuates physiologically, and the deformation of the corneal shape due to variation in IOP is observed in the human eye.[2] The role of biomechanics is therefore important to consider in routine refractive surgery and in special cases where the biomechanical status of the cornea is abnormal (such as keratoconus).[3] However, little is known about the effects of mechanical stress on the ECM remodeling in the corneal repair process and anomalies that lead to complications.

Matrix metalloproteinases (MMPs) are a family of enzymes capable of degrading various components of the extracellular matrix. Tissue inhibitor of metalloproteinases (TIMPs) plays an essential role in regulating the activity of MMPs by binding to them. Changes in MMPs and TIMPs expression are thought to be responsible for the corneal wound healing.[4–6] An imbalance in MMPs/TIMPs plays an important role in the development of keratoconus corneas.[7,8] Membrane type 1 (MT1)-MMP or MMP-14 has been reported to regulate matrix turnover either directly through collagenolytic activity against collagen types 1, II, and III, or by activating downstream MMPs such as MMP-2.[9] Expression of MT1-MMP in the epithelium and stroma is also significantly elevated in keratoconus.[10]

It is well established that mechanical stimuli play a key role in regulating growth and function in a variety of cell types. Some studies have demonstrated that mechanical stretch can modulate MMP-2 expression and activity in

trabecular meshwork cells and scleral fibroblasts.[11–13] However, how corneal fibroblasts respond to mechanical stress is not well understood. Petroll *et al*.[14] investigated the response of isolated corneal fibroblasts to local mechanical stimulation using a 3-D collagen matrix model. Their results suggested that corneal fibroblasts actively responded to increases or decreases in local matrix stress in an attempt to maintain tensional homeostasis (constant tension). In addition, tensile stress also influenced the migratory activity of the corneal epithelia of embryos in culture.[15]

Based on these studies, we speculate that the application of mechanical stretch on the corneal fibroblasts may regulate the process of the corneal extracellular matrix remodeling. This study evaluated the effects of cyclic stretch on the regulation of MMPs and TIMPs in cultured rabbit corneal fibroblasts *in vitro*. Our results suggested that stretching magnitude determined whether the cornea underwent matrix synthesis or degradation by changing the balance between MMPs and TIMPs in corneal fibroblasts. We also demonstrated that a 15% stretch stimulated MMP-2 protein production in an ERK-dependent manner. Thus, mechanical stretch would be an important factor to modulate the homeostasis of extracellular matrix in the cornea.

Materials and methods

Cell isolation and culture

Male rabbits (body weight 2.0–2.5 kg) were obtained from Shanxi Medical University, Shanxi province, China. Rabbit corneal fibroblasts were isolated and maintained as described previously.[16] In brief, the epithelial sheet and endothelial layer of the cornea were removed mechanically and the remaining tissue was treated with collagenase (2 mg/mL, in DMEM/F12) (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C until a single-cell suspension was obtained. Isolated corneal fibroblasts were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells from the third to the fifth passage were employed in this study.

Equibiaxial mechanical stretch

For cyclic stretch studies, corneal fibroblasts were seeded onto 6-well Bioflex® plates (Flexcell Int. Corp., Hillsborough, NC, USA) at 3×10^5 cells/well. Bioflex® plates contain a collagen I coated silicon membrane bottom which enables precise deformation of cultured cells by computer-controlled vacuum. When cells reached subconfluency, media was removed and replaced with 1% FBS media and the cells were exposed to cyclic stretch. A Flexercell® Tension Plus™ FX-4000T™ system (Flexcell Int. Corp., Hillsborough, NC, USA) was used to apply cyclic equibiaxial stretch (0–5%, 0–10%, and 0–15% elongation, 0.1 Hz, 3 h or 24 h, sine waveform). Unstretched cells were treated as controls.

For inhibitor studies, cells were serum starved using DMEM/F12 with 2% FBS for 16 h, and pretreated with the mitogenactivated protein kinase (MEK) inhibitor PD98059 (30 μM) (Sigma, St Louis, MO, USA) for 1 h prior to stretch.

Conditioned media and cells were harvested after the stretching. Protein concentration was measured using a BCA kit with BSA as a standard. Cellular injury was measured by a lactate dehydrogenase (LDH) detection kit. No increase in LDH release was observed, indicating that strain was not inducing any cellular injury.

Real-time quantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted from corneal fibroblasts using RNAiso Plus according to the manufacturer's instructions. RNA concentration and purity was determined spectrophotometrically at an optical density ratio of 260/280 (OD260/OD280) (Multiskan GO, Thermo Fisher Scientific Inc, Waltham, MA, USA). All polymerase chain reaction (PCR) reagents were purchased from Takara (Dalian, China).

Real-time PCR was used to compare the levels of steady state mRNA for MMP-2, TIMP-2, and MT1-MMP genes of corneal fibroblasts. Complementary DNAs (cDNA) were generated from total RNA by reverse transcription using the PrimeScript™ RT reagent Kit. All primers were designed, selected, and ordered using Primer 5.0 according to the corresponding sequences at Genbank (Table 1) and diluted to 10 mM in RNase-free water. The housekeeping gene GADPH was used as the internal control. The GenBank accession numbers, forward and reverse sequences, and product size are shown in Table 1. PCR amplification was done in a 20 μL total volume containing appropriate diluted cDNA, 0.2 μM of each primer, and 10 μL 2 × Q-PCR Mix (containing SYBR Green I). Negative controls missing the cDNA template were also included in this experiment. Real-time quantitative PCR was performed using a thermocycler (StepOne™, Applied Biosystems, Foster City, CA, USA) as follow: 30 s pre-denaturation at 95°C, 1 cycle; 5 s denaturation at 95°C and 30 s annealing and collection fluorescence at 60°C, 40 cycles. All samples were run in triplicate with a melt curve analysis to ensure the presence of one PCR product. DNA agarose-gel electrophoresis was performed to confirm product size and to ensure the absence of primer dimer formation. Quantification of the gene expression was done using the comparative $2^{-\Delta\Delta CT}$ method.

Gelatin zymography

Gelatin zymography was used to analyze the relative amounts of MMP-2 secreted into the cell culture media. Conditioned media (10 μg) were separated through 8% SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) gel containing 0.1% gelatin. Gels were washed in 2.5% Triton-X-100 (2.5% Triton-X-100, 5 mM $CaCl_2$, 50 mM Tris pH7.5) for 45 min × 2 and incubated in renaturing buffer (10 mM $CaCl_2$, 50 mM Tris pH7.5, 10 mM NaCl, 0.02% NaN_3) at 37°C for 24 h. Gels were stained with 0.05% Coomassie blue (0.05% Coomassie blue, 60% distilled water, 30% methanol, 10% acetic acid) for about 2 h and de-stained (60% distilled water, 30% methanol, 10% acetic acid) until bands were

clearly visible. Gels were then scanned, and band intensities were quantitated using Image Pro Plus 5.1 software (Media Cybernetics Inc, Bethesda, MD, USA). Bands were identified based on molecular weight and co-migration with known standard marker.

Western blotting analysis

Cell lysates (30 μg) or concentrated conditioned media from the cell culture (13 μg) were separated through 8% SDS-PAGE gel. Then the gel was transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) using a wet transfer apparatus (JunYi, Beijing, China). The membrane was blocked with TBST (Tris-buffered saline containing 0.2% Tween-20) containing 5% non-fat milk and then probed overnight at 4°C with primary antibodies. Primary antibodies were diluted in blocking buffer:anti-MMP-2 antibody (1:2000); anti-phospho ERK1/2 antibody (1:10,000); anti-ERK1/2 antibody (1:4000); and anti-GAPDH antibody (1:10,000) (Abcam, Cambridge, UK). Membranes were then washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Abcam) at a dilution of 1:6000. Phospho ERK1/2 was normalized with total ERK, and GAPDH was used for controls of equal protein loading. The band intensities were quantitated by Image Pro Plus 5.1 software.

Statistical analysis

All data from three independent experiments were expressed as mean ± standard deviation. Statistical analysis was performed using one-way ANOVA analysis followed by *post hoc* Tukey testing, or using a Student's *t*-test to compare experiments consisting of only two groups. The level of significance was considered when $P < 0.05$.

Results

Effects of cyclic stretch on MMP-2 protein production

First, we examined the effects of cyclic stretch on the production of MMP-2 *in vitro*. Only 72 kDa MMP-2 (pro-form) was observed in this study (Figure 1a). There was no detectable active-MMP-2 in corneal fibroblasts in both unstretched and stretched conditions. At 3 h, compared to the controls, no significant differences were found in the 5% and 10% stretch groups, while a 15% stretch significantly increased pro-MMP-2 production by 52% ($P < 0.05$) At 24 h, pro-MMP-2 production of the 5% stretch group decreased by 20% ($P < 0.05$), a 10% stretch resulted in a 9% decrease of the pro-MMP-2 production, while pro-MMP-2 increased by 25% in response to a 15% stretch ($P < 0.05$) (Figure 1b).

The gelatin zymogramy results were also confirmed by Western blotting analysis (Figure 2a). Compared to the control, a 5% stretch for 24 h decreased pro-MMP-2 production by 56% ($P < 0.05$), a 10% stretch had no significant effect on pro-MMP-2 production with only a 6% decrease, while a 15% stretch increased pro-MMP-2 production by 98% ($P < 0.05$) (Figure 2b). Although the exact changes (x-fold) were seldom identical when the gelatin zymography and Western blotting were compared, similar responses to stretching were indicated by both methods.

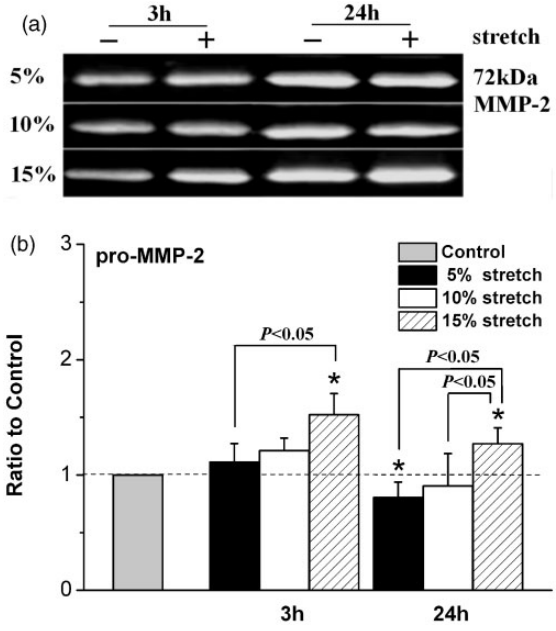

Figure 1 Effects of cyclic stretch on 72 kDa pro-MMP-2 production in conditioned media. (a) Representative picture of gelatin zymogramy in conditioned media following 3 and 24 h of unstretched culture or cyclic equibiaxial stretch. (b) The indicated quantitative data refer to ratio to control. The data are means of three different experiments ($n = 3$). *$P < 0.05$ versus control

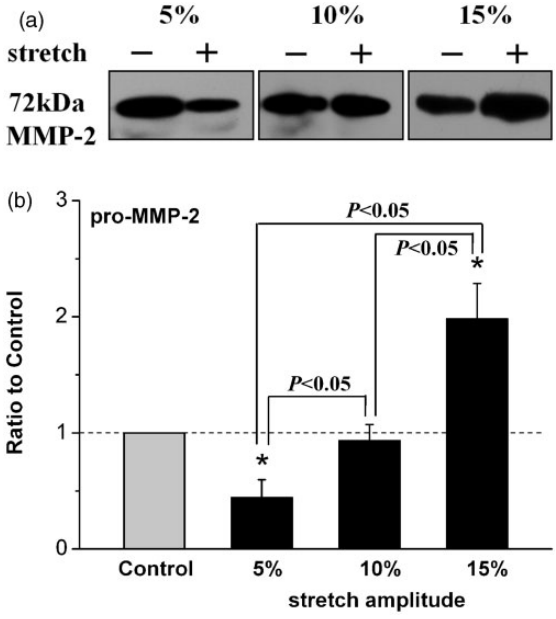

Figure 2 Effects of cyclic stretch on 72 kDa ProMMP-2 production in conditioned media. (a) Representative picture of Western blotting in conditioned media following 24 h of unstretched culture or cyclic equibiaxial stretch. (b) The indicated quantitative data refer to ratio to control. The data are mean of three different experiments ($n = 3$). *$P < 0.05$ versus control

Effects of cyclic stretch on the mRNA expression of MMP-2 MT1-MMP, and TIMP-2

We next investigated the mRNA expression of MMP-2, MT1-MMP, and TIMP-2 of corneal fibroblasts in the presence of cyclic stretch. Compared to the controls,

MMP-2 mRNA levels did not significantly change in response to cyclic stretch for 3 h in all groups. However, a 5% stretch resulted in a decrease by 41% in MMP-2 mRNA levels after 24 h ($P < 0.05$); a 10% stretch did not affect MMP-2 expression levels significantly; whereas a 15% stretch increased the mRNA levels of MMP-2 significantly by 81% ($P < 0.05$) (Figure 3a). Only the 15% stretch for 24 h increased MT1-MMP mRNA levels by 36% ($P < 0.05$) (Figure 3b).

At 3 h, compared to the controls, 5% and 10% stretches increased TIMP-2 mRNA levels by 69% and 73%, respectively ($P < 0.05$), and a 15% stretch decreased TIMP-2 mRNA levels by 63% ($P < 0.05$). At 24 h, though the 5% stretch showed a slight increase in TIMP-2 mRNA levels, there was no significant difference between the stretched and the control. The TIMP-2 mRNA level began to decrease in response to 10% and 15% stretches by 21% and 20%, respectively ($P < 0.05$) (Figure 3c).

Cyclic stretch-induced increase in pro-MMP-2 is in an ERK-dependent manner

Considering the positive relationship between ERK activation and MMP-2 production,[17,18] we first examined whether ERK phosphorylation was activated in corneal fibroblasts stimulated by stretching. Exposure to a 15% stretch alone for 1 h resulted in a significant increase (1.91 ± 0.17 fold, $P < 0.05$ versus control) in phospho-ERK1/2 activity without affecting total ERK1/2 protein expression, while PD98059 significantly attenuated stretch-induced increases in phospho-ERK1/2 activity (1.07 ± 0.09 fold, $P < 0.05$ versus 15% stretch) (Figure 4(a) and (b)).

We subsequently determined whether the ERK pathway was involved in the MMP-2 production induced by stretching. Corneal fibroblasts were preincubated with PD98059, an MEK inhibitor (30 μM, 1 h), prior to stretching (15%, 24 h). As shown in Figure 5(a) to (c), a 15% stretch significantly increased pro-MMP-2 release ($P < 0.05$ versus control). However, the stretch-induced increase in MMP-2 was attenuated by pretreatment of corneal fibroblasts with PD98059 ($P < 0.05$ versus 15% stretch). These results indicate that large-magnitude stretching may invoke the ERK1/2 pathway to stimulate pro-MMP-2 production.

Discussion

MMPs play a critical role in extracellular matrix synthesis and turnover in ocular tissues under both healthy and disease conditions.[19] MMPs are also associated with corneal wound healing[4–6] and keratoconus.[7,8] Mechanical stretching has been shown to modulate the activity of MMPs and TIMPs in several ocular cells types, such as sclera fibroblasts and trabecular meshwork cells.[11–13] The cornea is exposed to cyclic stretching under IOP in physiological state, and refractive surgery leads to mechanical stress increase of the cornea.[2] Therefore, it is important to determine how corneal fibroblasts respond to mechanical load in order to better understand corneal fibroblasts

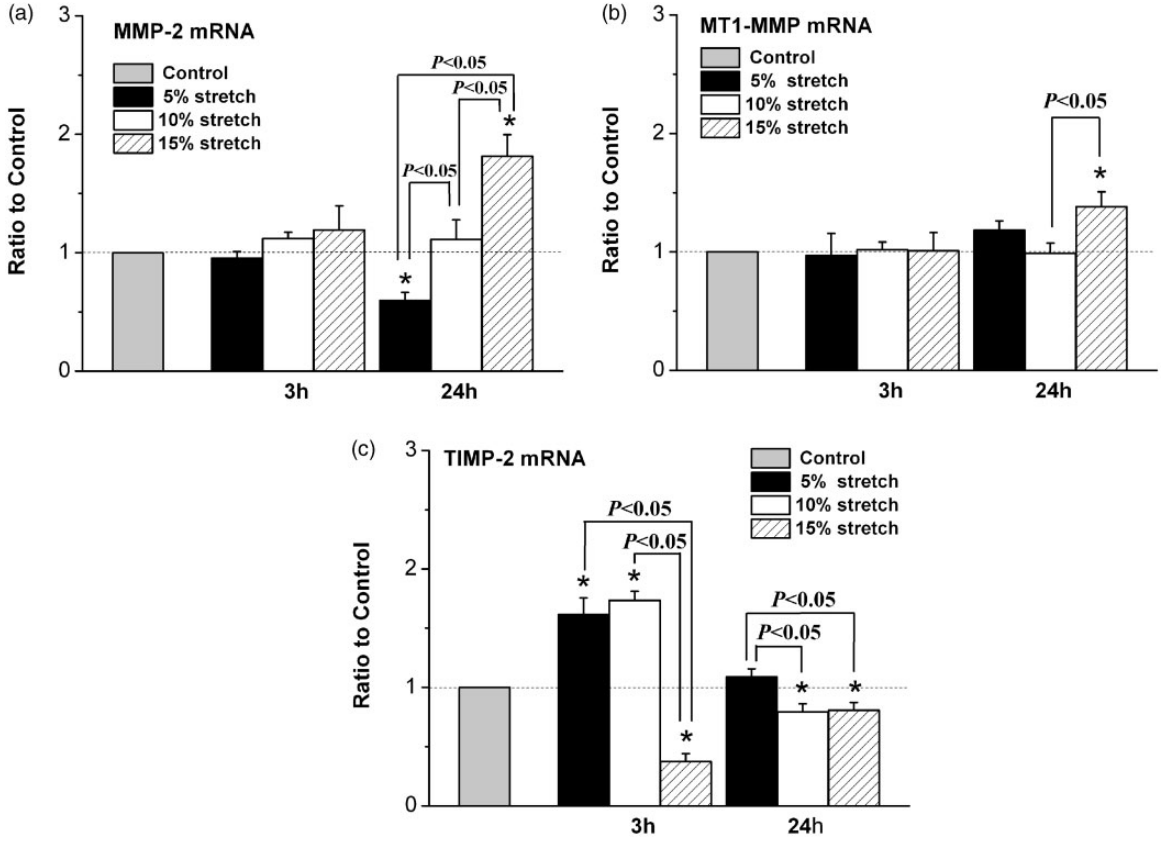

Figure 3 The mRNA expression of MMP-2, MT1-MMP, and TIMP-2 in response to cyclic equibiaxial stretch. (a) MMP-2, (b) MT1-MMP, and (c) TIMP-2. The indicated quantitative data refer to ratio to control. The data are mean of three different experiments ($n = 3$). *$P < 0.05$ versus control

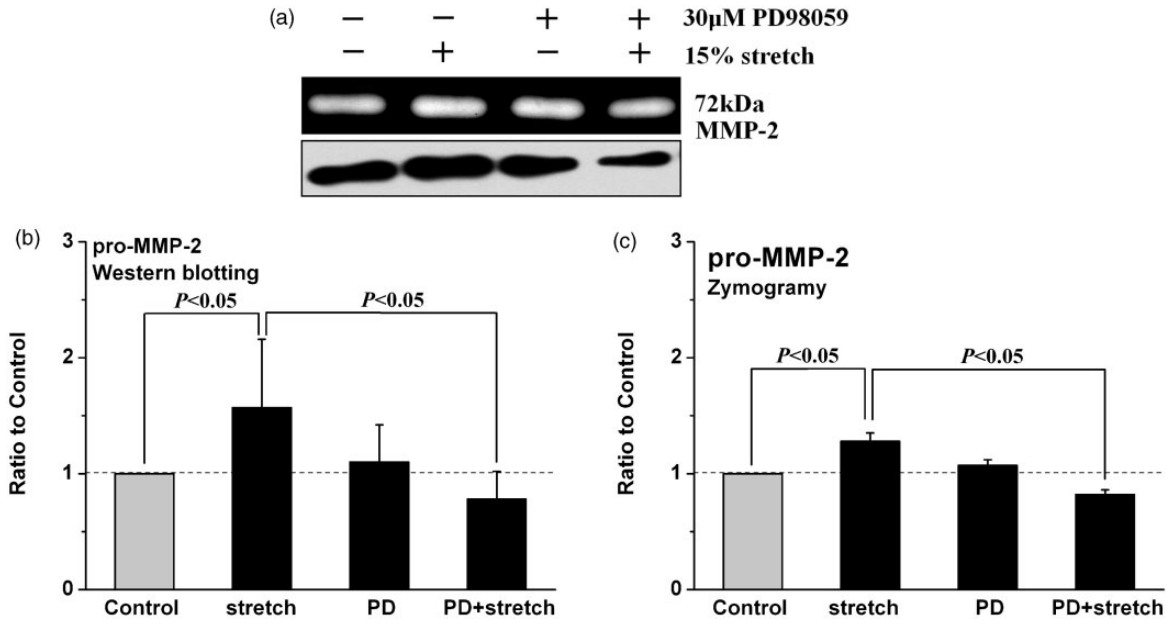

Figure 4 Cyclic stretch enhanced ERK1/2 phosphorylation more than the control. PD98059 inhibited stretch-induced ERK1/2 phosphorylation. Rabbit corneal fibroblasts were pretreated with 30 μM PD98059 (PD) for 1 h prior to stretch (15%, 1 h). (a) Representative picture of Western blotting. (b) The indicated quantitative data refer to ratio to control. The data are mean of three different experiments ($n = 3$)

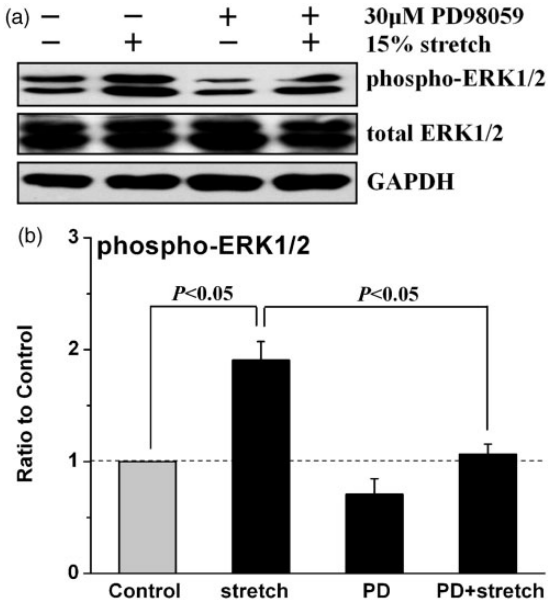

Figure 5 PD98059 significantly attenuated stretch-induced MMP-2 protein production in conditioned media. Rabbit corneal fibroblasts were pretreated with 30 μM PD98059 (PD) for 1 h prior to stretch (15%, 24 h). (a) Representative picture of Zymography (upper panel) and Western blotting (lower panel). The indicated quantitative data refer to ratio to control, (b) Western blotting, and (c) Zymography. The data are mean of three different experiments ($n = 3$)

mechanobiology as well as the wound healing process after refractive surgery.

As our results have shown, when corneal fibroblasts were cultured *in vitro*, they secreted predominantly MMP-2, and MMP-2 was almost constant throughout the experiments. The corneal fibroblasts showed a tendency to produce higher levels of MMP-2 mRNA and pro-MMP-2 over time in the nonstretched condition. Only 72 kDa pro-MMP-2 was observed in this study. Our results also showed that cyclic stretching had a biphasic effect in regulating MMP-2, i.e. at 24 h, a 5% stretch attenuates corneal fibroblasts to produce MMP-2; in contrast, the MMP-2 level was upregulated by a 15% stretch. Other researchers also investigated the effects of stretch magnitude on the inflammatory responses induced by interleukin-1β in tendon fibroblasts. The results suggested that the application of small mechanical stretch (≤4%) to tendons was beneficial in terms of restoring tendon homeostasis. However, a large mechanical stretch (≥4%) may lead to tendon matrix degradation.[20]

Previous studies suggested that MT1-MMP was involved in the activation of pro-MMP-2.[9,10] Our data showed that MT1-MMP mRNA level was elevated in response to a 15% stretch for 24 h, which was accompanied with the increase of MMP-2. Although the active form of MMP-2 was not detected in this study, however, the increase of pro-MMP-2 protein and MT1-MMP mRNA will provide the potential for generating activated MMP-2 under the proper stimulus.

The activity of MMPs is generally regulated by TIMPs. Balance between MMPs and TIMPs may determine net enzymatic activity in the cornea wound healing.[4–6] Therefore, we investigated the effects of cyclic stretching on the expression of TIMP-2, which is known to bind to MMP-2. Our results showed that both at 3 h and 24 h, a 15% stretch decreased the TIMP-2 mRNA level, whereas a 5% stretch increased the TIMP-2 mRNA level at 3 h, but did not change the TIMP-2 mRNA level at 24 h. TIMP-2 protein levels have also been shown to be dramatically reduced, while pro- and active-MMP-2 were elevated in trabecular meshwork cells following 24 h of 10% constant stretching.[21] Moreover, mechanical stretching significantly increased the production of active-MMP-2 at 48 h and decreased the

expression of TIMP-2 mRNA of human scleral fibroblasts.[12] Our results indicate that a small-magnitude stretch may promote corneal matrix synthetic events, whereas large-magnitude stretching may promote corneal matrix degradation by modulating the balance between MMPs and TIMPs.

The mitogen-activated protein kinase (MAPK) family plays important roles in the transduction of mitogenic and differentiation signals from the cytoplasm to the nucleus in response to mechanical strain.[22] Previous studies in a number of cell types showed a positive relationship between ERK1/2 activation and MMP-2 production.[17,18] The present results showed that a 15% stretch not only activated ERK1/2 phosphorylation but also increased MMP-2 secretion, as PD098059, an inhibitor of MEK, inhibited both stretch-induced ERK phosphorylation and MMP-2 secretion. However, inhibition of p38 and JNK using SB203850 and SP600125 did not suppress stretch-induced pro-MMP-2 increase (data not shown), implying that it is not p38 and JNK, but ERK1/2 activation that seems to be required for overload-induced pro-MMP-2 production in rabbit corneal fibroblasts.

However, we have to point out that since our stretch conditions only broadly mimic the changes of mechanical force of the cornea after refractive surgery, it cannot exactly represent the forces as they occur *in vivo*. Our results do, however, demonstrate that corneal fibroblasts are mechanoresponsive, and the balance between MMPs and TIMPs induced by cyclic stretching may determine the direction of the corneal ECM remodeling. Large-magnitude stretching promotes corneal matrix degradation events, which may result in poor mechanical properties of the corneal tissue and lead to post-keratectasia under IOP. This study may explain why excessive ablation in refractive surgery, a thin cornea, or a high IOP is a risky factor for post-keratectasia from the biomechanics viewpoint.

It is well known that the corneal wound healing cascade is complex and involves stromal-epithelial interactions mediated by cytokines. Interleukin-1 (IL-1) appears to be a master modulator of many of the events involved in this cascade. In our current system, we only analyzed the effects of mechanical stretch on MMP-2, MT1-MMP and TIMP-2 in corneal fibroblasts *in vitro*. Further studies will consider the role of IL-1 on the expression of MMPs and TIMPs in corneal fibroblasts under mechanical load.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. CL, PF, XL, JS conducted the experiments, CL wrote the manuscript, XL processed data, and WC and XL designed and guided the experiments.

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