Induction of MMP-1 and -3 by cyclical mechanical stretch is mediated by IL-6 in cultured fibroblasts of keratoconus

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Received December 9, 2016; Accepted March 16, 2017

DOI: 10.3892/mmr.2017.6433

Abstract. In order to understand the effect of mechanical stretch on corneal extracellular matrix remodeling, human keratoconus fibroblasts (HKCFBs) were subjected to cyclic stretch *in vitro* and the expression of matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinases (TIMPs), and inflammatory cytokines were evaluated. HKCFBs were seeded into a flexible membrane base and subjected to a cyclic stretch regimen of 10% equibiaxial stretch at a stretching frequency of 1 Hz for 6 h using a Flexcell tension unit. An antibody directed against interleukin-6 (IL-6 Ab) was used to investigate the roles of IL-6 on mechanical stretch mediated regulation of MMP in HKCFBs. Culture supernatants were assayed using an enzyme-linked immunosorbent assay for MMP-1 and -3, TIMP-1 and -2, and IL-6. Total RNA from the cells was extracted, and quantitative polymerase chain reaction was used to determine mRNA for MMP-1 and -3, TIMP-1 and -2, and IL-6. In stretched cells, levels of MMP-1 and -3 demonstrated an increase compared with unstretched cells, but levels of TIMP-1, and -2 revealed a decrease. Mechanical stretch significantly increased the mRNA expression and protein synthesis of IL-6 compared with unstretched cells. IL-6 induced MMP-1 and -3 expression, whereas no significant effects were observed in levels of TIMP-1 and -2 compared with the untreated control groups. Additionally, the IL-6 Ab markedly inhibited the stretch-induced increase in MMP-1 and -3 in culture supernatants in a dose-dependent manner. No significant differences in TIMP-1 and -2 protein levels were detected between stretched cells treated with IL-6 Ab and stretched cells without IL-6 Ab treatment. These results indicate that cyclical mechanical stretch augments IL-6 production and MMP expression, and reduces levels of TIMP in HKCFBs. Thus, it is suggested that IL-6 mediates the stretch-induced MMP expression.

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Key words: keratoconus, mechanical stretch, IL-6, MMPs, TIMPs

Introduction

Keratoconus is a progressive disease characterized by thinning and protrusion of the cornea, resulting in an irregular, conical shape (1). The characteristics of keratoconus have been known for at least 200 years, but causes of keratoconus have not been clearly established. Case reports showed associations between keratoconus and atopy (2), allergy (3), family history (4), other systemic genetic disorders (4), contact lens wear (5), exposure to ultraviolet radiation (6), eye-rubbing (7), disorders of enzyme function (6), and epithelial trauma and the production of inflammatory mediators (8). However, in a multivariate analysis of keratoconus risk factors, eye-rubbing was the most significant predictor for the development and/or progression of this disease (9).

Two large studies of keratoconus indicated that approximately 50% of subjects reported frequent, chronic, vigorous or abnormal rubbing of at least one eye (10,11). Vigorous eye-rubbing in keratoconus has been shown to be up to 10 times more forceful than normal eye-rubbing (12,13). Previous studies have found that the values of corneal biomechanical measurements are significantly lower in keratoconic eyes than in normal eyes (14,15). A study of the application of experimental digital forces to human eyes revealed that, for an open eye with normal intraocular pressure (IOP) of 15 mm Hg, light and firm digital forces increased IOP by 100 and 300%, respectively (16). The IOP in keratoconus responses may be greater when more force is used and rubbing episodes are longer. In addition, some studies have reported that the human cornea is a viscoelastic tissue (17) and depending on the nature of mechanical forces to which the human cornea is subjected, there may be an (almost) instantaneous deformation, which is the elastic part of the viscoelasticity (18,19). We therefore hypothesized that human keratoconus fibroblasts (HKCFBs) within the intraocular environment may be lengthened in response to the dynamic forces applied to the cornea caused by constant changes in the levels of IOP.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that are responsible for the degradation of extracellular matrix (ECM) and connective tissue protein (20). Elevated levels of MMPs are found in keratoconus

corneas, and considerable degradation of the extracellular matrix occurs, indicating that MMPs may be involved in the pathogenesis of keratoconus (21-25). Tissue inhibitors of metalloproteinases (TIMPs) inhibit the activity of MMPs by binding to them (20). The balance between the MMPs and TIMPs determines the extent of proteolysis linked with tissue remodeling or degradation of ECM components including collagen and elastin (20).

Several studies have suggested that many diseases have been associated with an imbalance of ECM synthesis and degradation and mechanical factors have been found involved in the pathogenesis of these diseases (26,27). Mechanical stimulation is involved in the regulation of MMPs in some ocular tissues, such as sclera (28), trabecular meshwork (29) and lamina cribrosa cells (30). The cornea is a dynamic tissue, capable of altering its ECM composition and biomechanical properties in response to changes in the visual environment (31,32). In addition, corneal fibroblasts have been demonstrated to respond actively to local tension changes in the ECM (33), and fibroblasts are a major type of mechanoresponsive cell (34). However, to date, there have been no reports regarding the effect of mechanical stretch on expression of MMP and TIMP in HKCFBs.

In our experiment, HKCFBs were subjected to cyclical mechanical stretch *in vitro* and the expression of MMP-1, MMP-3, TIMP-1, and TIMP-2 were evaluated. The present study appears to be the first experimental evidence to show significantly induced levels of MMP and reduced levels of TIMP in HKCFBs after mechanical stretch, thereby providing support for the possible association between the thinning and ectasia of a keratoconus cornea and mechanical stretch. In addition, we used IL-6 antibody (IL-6 Ab) to examine the role of IL-6 on stretch mediated regulation of MMP in HKCFBs. Results indicated that IL-6 may be a potential mediator of stretch-induced effects on expression of MMP and IL-6 Ab might provide a new modality to prevent further progression of some forms of keratoconus.

Materials and methods

Isolation and cell culture. Keratoconus cornea was collected from patient with keratoconus (a 16 year old donor) who was undergoing corneal transplantation within 6-12 h following surgery from Shanxi Eye Hospital (Tai yuan, Shanxi, China). The study was undertaken with the approval of the local ethics committee and after obtaining written informed consent from patient. The epithelium and endothelium of cornea were mechanically removed. The corneal stroma was cut into several pieces with diameter of 1.0 mm. Subsequently, the small tissue pieces were digested into single cells with DMEM/F12 medium containing 2 mg/ml of type II collagenase. Cells were cultured in DMEM/F12 containing fetal bovine serum and in an air -5% CO_2 incubator at 37˚C. Experiments were performed using HKCFBs from passages 3-5.

Mechanical stretch application. For experiment, HKCFBs were seeded at 6-well Bioflex® plates (Flexcell Int. Corp., Hillsborough, NC, USA) with an initial density of $5x10^5$/well. After the cells reached subconfluency, the cells were serum starved using DMEM/F12 with 0.1% FBS for 24 h. After 24 h, the media was replaced with FBS-free media (DMEM/F12). HKCFBs were then subjected to cyclical stretch (1 Hz) at 10% maximum elongation for 6 h using a Flexcell® Tension Plus™ FX-4000™ system (Flexcell Int. Corp., Hillsborough, NC, USA) at 37˚C in a humidified incubator with an atmosphere containing 5% CO_2. Cells plated on Bioflex® plates but not subjected to stretch served as controls. At the end of the experiment, the cells and supernatants from cell cultures were collected for gene or protein detection, respectively.

Treatment with IL-6. The relationship between IL-6 and expression of MMP and TIMP was investigated. HKCFBs were seeded at 6-well Bioflex® plates (Flexcell Int. Corp., Hillsborough, NC, USA) with an initial density of $5x10^5$/well. When the cultured cells became confluent, the cells were serum starved using DMEM/F12 with 0.1% FBS for 24 h. After 24 h, the media was replaced with FBS-free media (DMEM/F12) and treated with IL-6 (R&D Systems, Minneapolis, MN, USA) at a final concentration of 0, 12.5, 25 or 50 ng/ml. The cells were cultured for 6 h. No significant cytotoxic effect on cell proliferation (data not shown). At the end of the experiment, the cells and supernatants from cell cultures were collected for gene or protein detection, respectively.

Effect of IL-6 Ab. IL-6 Ab (Peprotech, Rocky Hill, NJ, USA) was used to determine if mechanical stretch-induced expression of MMP by HKCFBs was mediated by IL-6. The stretch experiments were repeated in the presence and absence of a IL-6 Ab (15, 30 or 60 ng/ml). No significant cytotoxic effect on cell proliferation (data not shown). At the end of the experiment, the supernatants from cell cultures were collected for protein detection.

Real-time polymerase chain reaction (RT-PCR). The procedure used for RT-PCR was similar to that described elsewhere. Briefly, total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer-recommended procedures. 1 μg of RNA was converted to cDNA with the PrimeScript™ RT reagent Kit (TaKaRa Biotechnology CO., Dalian, China) by gradient PCR device (Eppendorf, Germany). Reverse transcription was performed for 2 min at 42˚C, 15 min at 37˚C, and 5 sec at 85˚C, followed by cooling to 4˚C. Then, 2 μl of 30-fold-diluted cDNA products were amplified with SYBR® Premix Ex Taq™ II (TaKaRa Biotechnology CO) by the StepOnePlus™ RT-PCR System (Applied Biosystems, USA) using the gene specific primers (Table I) designed by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). For quantification, all target mRNA expression were normalized to the expressed housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RT-PCR conditions were as follows: 95˚C for 30 sec and then 40 cycles at 95˚C for 5 sec, 60˚C for 30 sec, and 72˚C for 1 min, followed by 72˚C for 10 min. The relative quantity of mRNA was calculated using the $2^{-\Delta\Delta C_T}$ method, in which C_T is the threshold cycle. The resulting data were expressed as a ratio to the control value denoted as one.

Enzyme-linked immunosorbent assay (ELISA). Human MMP-1, MMP-3, TIMP-1, TIMP-2, and IL-6 ELISA kits (BioSource International, Camarillo, CA, USA) were used to

Table I. Primer sequences for RT-PCR.

Gene	5'-3' sequences (forward; reverse)
MMP-1	For: GGGAGATCATCGGGACAACTC
	Rev: GGGCCTGGTTGAAAAGCAT
MMP-3	For: TGGCATTCAGTCCCTCTATGG
	Rev: AGGACAAAGCAGGATCACAGTT
TIMP-1	For: TTGTTGCTGGGCTGATAGC
	Rev: CAGGATTCAGGCTATCTGGG
TIMP-2	For: GCACATCACCCTCTGTGACTT
	Rev: AGCGCGTGAT CTT GCACT
IL-6	For: CCTGAACCTTCCAAAGATGGC
	Rev: CTTGGGGTTCTTGCTGATGT
GAPDH	For: AAGGTCGGAGTGAACGGATTTG
	Rev: TTCACCAGGCAAGTCTCCTCA

MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinases; IL-6, interleukin-6; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

measure levels of MMP-1, MMP-3, TIMP-1, TIMP-2, and IL-6 in the cell supernatants according to the manufacturer's recommendations. The optical density was measured at 450 nm in a microplate reader (Multiskan Go, Thermo Scientific, USA).

Statistical analysis. All results were presented as the mean ± standard deviation of three independent experiments and statistically performed by using SPSS v.19.0 software and one-way analysis of variance (ANOVA) analysis. P<0.05 was considered to denote a statistically significant difference.

Results

Cyclical stretch up-regulates MMP-1 and -3 and down-regulates TIMP-1 and -2. The results indicated that the mRNA expression of MMP-1 and -3 were up-regulated by the cyclic stretch, while in contrast the mRNA expression of TIMP-1 and -2 were down-regulated by the cyclic stretch. The ratios of stretch to nonstretched control values of MMP-1, MMP-3, TIMP-1, and TIMP-2 were 3.07±0.31, 2.83±0.22, 0.44±0.04, and 0.61±0.04, respectively (P<0.05; Fig. 1A).

The concentrations of MMP-1 and -3 in cell culture supernatants showed an increase in stretched cells relative to unstretched cells, while in contrast the concentrations of TIMP-1 and -2 showed a decrease in stretched cells compared to unstretched cells: MMP-1 in unstretched cells, 419.27±33.58 ng/ml, and stretched cells, 486.48±26.32 ng/ml (P<0.05); MMP-3 in unstretched cells, 217.44±12.08 ng/ml, and stretched cells, 270.34±10.15 ng/ml (P<0.05); TIMP-1 in unstretched cells, 204.94±10.67 ng/ml, and stretched cells, 176.06±8.58 ng/ml (P<0.05); and TIMP-2 in unstretched cells, 31.65±2.45 ng/ml, and stretched cells, 19.51±1.55 ng/ml (P<0.05; Fig. 1B).

Cyclical stretch increases concentration ratios of MMP/TIMP. The MMP/TIMP ratios in cell culture supernatants were increased in stretched cells relative to unstretched cells: MMP-1/TIMP-1 ratio in unstretched cells was 2.05±0.24 and 2.77±0.23 in stretched cells (P<0.05; Fig. 2A); MMP-1/TIMP-2 ratio in unstretched cells was 13.28±1.05 compared to 25.14±2.13 in stretched cells (P<0.05; Fig. 2A); MMP-3/TIMP-1 ratio in unstretched cells was 1.06±0.07 as opposed to 1.55±0.09 in stretched cells (P<0.05; Fig. 2B); MMP-3/TIMP-2 ratio in unstretched cells was 6.89±0.56 compared with 12.96±1.32 in stretched cells (P<0.05; Fig. 2B).

Cyclical stretch causes secretion of IL-6. Cyclical stretch augmented IL-6 mRNA expression and protein synthesis. The ratio of stretch to nonstretched control mRNA value of IL-6 was 3.21±0.24 (P<0.05; Fig. 3A). It was found that the mean concentration of IL-6 in cell culture supernatants rose from 7.12±0.58 ng/ml before stretch to 9.77±0.85 ng/ml after stretch (P<0.05; Fig. 3B).

IL-6 induces MMP-1 and -3 expression. Fig. 4A showed that MMP-1 and -3 mRNA expression were enhanced in the presence of IL-6 with a dose-dependent manner. The ratios of mRNA for MMP-1 and -3 to IL-6-untreated control were 2.35±0.21 and 1.78±0.13 at a concentration of 12.5 ng/ml, 6.64±0.58 and 4.65±0.41 at 25 ng/ml, and 7.87±0.74 and 6.23±0.49 at 50 ng/ml, respectively (P<0.05).

As shown in Fig. 4B, IL-6 induced MMP-1 and -3 protein synthesis in a dose-dependent manner. Similarly, the ratios of protein for MMP-1 and -3 to IL-6-untreated control were 1.27±0.09 and 1.25±0.08 at a concentration of 12.5 ng/ml, 1.58±0.11 and 1.66±0.12 at 25 ng/ml, and 1.98±0.13 and 2.22±0.17 at 50 ng/ml, respectively (P<0.05).

In addition, no significant differences or changes were observed in mRNA and protein levels of TIMP-1 and -2 between cells treated with and without IL-6 (P>0.05; Fig. 4).

IL-6 Ab inhibits stretch-induced MMP-1 and -3 expression. As depicted in Fig. 5A, the protein expression of MMP-1 and -3 induced by mechanical stretch were significantly reduced in a dose-dependent manner by the specific IL-6 Ab treatment. IL-6 Ab markedly decreased mechanical stretch-induced MMP-1 and -3 protein production by 16±1.2 and 18±1.4% at a concentration of 15 ng/ml, 35±2.9 and 54±4.9% at 30 ng/ml, and 51±4.5 and 68±6.1% at 60 ng/ml, respectively (P<0.05). Additionally, TIMP-1 and -2 concentrations reduced by mechanical stretch were not affected by the addition of IL-6 Ab (P>0.05; Fig. 5B).

IL-6 Ab down-regulates concentration ratios of MMP/TIMP. The fact that decreases in mechanical stretch-induced MMP-1 and -3 secretion after IL-6 Ab treatment were not accompanied by changes in the amount of secreted TIMP-1 and -2, resulted in shifting the MMP/TIMP ratios. For example, concentration ratios of MMP-1/TIMP-1 and MMP-1/TIMP-2 showed decrease in cells stretched in the presence of IL-6 Ab relative to the IL-6 Ab-untreated stretched cells by 13±0.9 and 17±1.3% at a concentration of 15 ng/ml, 27±2.2 and 42±3.6% at 30 ng/ml, and 61±5.5 and 56±4.3% at 60 ng/ml, respectively (P<0.05; Fig. 5C). Similarly, a significant reduction was demonstrated for the MMP-3/TIMP-1 and MMP-3/TIMP-2 ratios in stretched cells treated with IL-6 Ab, compared with the IL-6 Ab-untreated stretched cells by 14±1.0 and 21±1.6% at

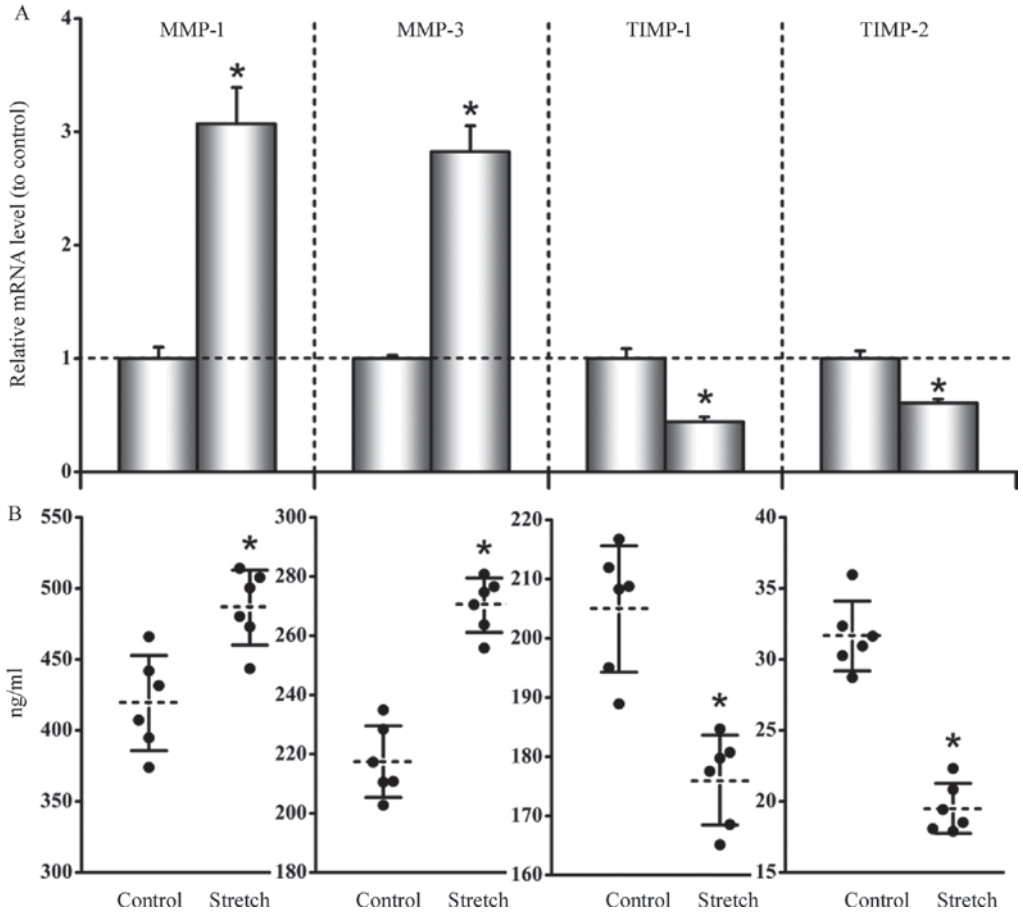

Figure 1. Effect of cyclical mechanical stretch on MMP and TIMP levels in HKCFBs. Cells were subjected to cyclical stretch for 6 h. Total RNA and cell-culture supernatants were then collected. mRNA levels determined by RT-PCR from total RNA and protein levels measured using human ELISA kits from cell-culture supernatants. (A) The MMP-1 and -3 mRNA expression were increased in stretched cells relative to unstretched cells. But in stretched cells, TIMP-1 and -2 mRNA expression showed a decrease compared to unstretched cells. (B) The concentrations of MMP-1 and -3 in culture supernatants showed an increase in stretched cells as opposed to unstretched cells, while in contrast the concentrations of TIMP-1 and -2 decreased in stretched cells compared to unstretched cells. Data are represented as the mean ± standard deviation (n=6 per group). *P<0.05, stretched vs. nonstretched control. MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinases; HKCFBs, human keratoconus fibroblasts.

a concentration of 15 ng/ml, 47±4.1 and 59±5.5% at 30 ng/ml, and 67±5.8 and 69±6.2% at 60 ng/ml, respectively (P<0.05; Fig. 5D).

Discussion

It might be thought that the eye-rubbing is coincidental and not causal, but the large proportion of keratoconus giving a history of chronic habits of abnormal eye-rubbing appeared leaves little doubt that chronic and abnormal eye-rubbing may cause the cornea to give way and is also possibly responsible for the progression of some forms of keratoconus (7). Based on the above hypothesis, we speculate that corneal stromal fibroblasts may be exposed to mechanical stress caused by abnormal chronic eye-rubbing in some patients with keratoconus. *In vitro* cell stretch systems have rapidly become standard models for studying the effects of mechanical forces on a variety of cell types, including scleral fibroblasts (28), trabecular meshwork cells (29) and lamina cribrosa cells (30). These studies have attempted to model intraocular forces by introducing mechanical distortion (4-15% amplitude strain) to ocular cells for static or dynamic (0.3 Hz or 1 Hz) loading for 30 min -72 h (28-30). Because the forces exerted at the cornea represent transient changes in force caused by constant changes in the levels of IOP, we selected a stretch protocol for HKCFBs *in vitro* employing a regimen of cyclical dynamic stretch. In our study, flexible-bottom culture dishes were subjected to distension, using the Flexcell® Tension Plus™ FX-4000™ system to deliver a highly controlled regimen of sinusoidal stretch with a strain amplitude of 10% at a frequency of 1 Hz (60 cycles per min) for 6 h.

In the human eye, studies of MMPs and TIMPs have been performed in the aqueous humor (35), vitreous (36), retina (37), trabecular meshwork (38), keratoconus corneas (39), and corneas during wound healing (40). Large studies have revealed a relationship between mechanical stretch and MMPs and TIMPs in many kinds of eye cells. For example, Shelton *et al* (28) reported that mechanical strain increased levels of MMP-2 and reduced levels of TIMP-2 by human scleral fibroblasts. Kirwan *et al* (30) reported that cyclical stretch induced MMP-2 expression in human lamina cribrosa cells. Mechanically stretched trabecular meshwork cells have reported increases in MMP-2 protein and gene expression (29,41), increased MMP-14 protein levels (29), and either no change or a decrease in TIMP-2 (29,42). In these cultured cells, mechanical stretch shifted the MMP-TIMP balance and

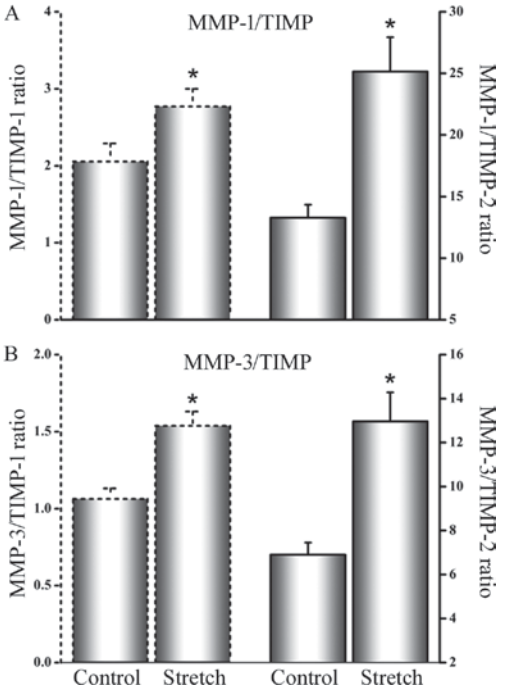

Figure 2. Effect of cyclical mechanical stretch on MMP/TIMP ratios in culture supernatants of HKCFBs. Concentration ratios of (A) MMP-1/TIMP-1, MMP-1/TIMP-2, (B) MMP-3/TIMP-1, and MMP-3/TIMP-2 were higher in stretched cells than in unstretched cells. Data are represented as the mean ± standard deviation (n=6 per group). *P<0.05, stretched vs. nonstretched control. MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinases; HKCFBs, human keratoconus fibroblasts.

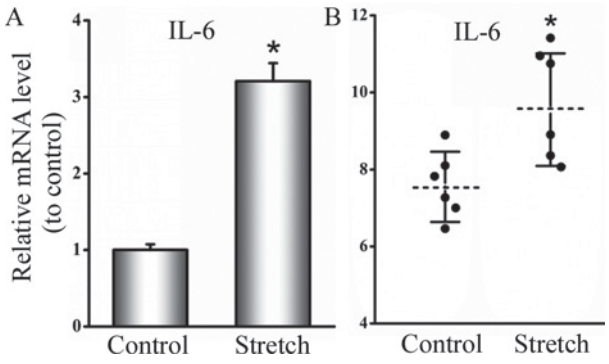

Figure 3. Effect of cyclical mechanical stretch on IL-6 mRNA expression and protein synthesis in HKCFBs. Cells were subjected to cyclical stretch for 6 h. Total RNA and cell-culture supernatants were then collected. mRNA levels assayed by RT-PCR from total RNA and protein levels measured using a human IL-6 ELISA kit from cell-culture supernatants. (A) The mRNA expression of IL-6 were induced by the cyclical stretch. (B) IL-6 production increased by cells during mechanical cyclical stretch, compared with the nonstretched cells. Data are represented as the mean ± standard deviation (n=6 per group). *P<0.05, stretched vs. nonstretched control. IL-6, interleukin-6; HKCFBs, human keratoconus fibroblasts.

these results suggest mechanical stretch may be involved in the pathogenesis of these diseases (29,30).

MMP-1, -2, -3, -7, -9 and -13 have been found to be elevated in the tear film of patients with keratoconus (22-25,43,44) and decreased levels of TIMP-1 have been found in keratoconus corneas and their cell cultures (6,45), indicating that MMPs and TIMPs may be involved in the pathogenesis of keratoconus. Our studies have focused primarily on MMP-1, MMP-3, TIMP-1, and TIMP-2. To the best of our knowledge, this was the first investigation to determine the effect of cyclical mechanical stretch on MMP-1, MMP-3, TIMP-1 and TIMP-2 mRNA expression and protein synthesis in HKCFBs *in vitro*.

Our data showed that mechanical stretch increased MMP-1 and -3 production and decreased TIMP-1 and -2 production, resulting in an increased MMP-1/TIMP-1 ratio, MMP-1/TIMP-2 ratio, MMP-3/TIMP-1 ratio, and MMP-3/TIMP-2 ratio. MMP-1, an interstitial collagenase, can degrade native fibrillar collagen types I, II, III, IX, and XI (20). MMP-3, or stromelysin-1, has a broad substrate specificity that includes casein, proteoglycans, fibronectin, elastin, and laminin, as well as collagen types III, IV, V, IX, and IX (20). Type I and type III collagen accounts for about 85% of corneal ECM (46). Keratoconus corneal stroma has decreased levels of type I, III, V, XII and VI collagens (47). Therefore, it is conceivable that the increase in the ratios of MMP/TIMP by stretched HKCFBs may facilitate the degradation of the corneal ECM. A balance between ECM synthesis and degradation is a prerequisite for maintaining the structural and functional integrity of the cornea (27). Moreover, previous studies have revealed that the thinning and ectasia of a keratoconus cornea has been mainly attributed to the increased degradation of ECM (21,25,48). Since keratoconus is characterized by the thinning of the corneal stroma, taken together, the up-regulation of MMP/TIMP ratios caused by mechanical stretch which may represent one of the main causes of the damage of corneal tissue, thereby contribute to the development and/or progression of some forms of keratoconus.

Keratoconus is defined as a non-inflammatory disease of the cornea, however, in recent years, several groups have demonstrated that the tear film in keratoconus showed increased levels of inflammatory molecules (IL-4, -5, -6, -8, TNF-α, -β) compared with normal controls (22,23,25,49). The extent of the increase was found to be associated with the severity of keratoconus (22,23,49). This suggested that the pathogenesis of keratoconus may involve chronic inflammatory events.

IL-6 is a pleiotropic cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation and oncogenesis and IL-6 could play an important role in the generation of inflammation within the cornea (50). A recent study has indicated that IL-6 may rank as important factors in the pathogenesis of keratoconus (25). Previous studies have documented the effect of mechanical stretch on IL-6 synthesis in a variety of cell types (51). Additionally, IL-6 can modulate the expression of MMPs and TIMPs by human corneal epithelial cells (52). However, to date, there have been no reports regarding the correlation between mechanical stretch and IL-6 expression and the effect of IL-6 on the production of MMPs and TIMPs in HKCFBs.

Our study showed that cyclical stretch increased IL-6 levels, and we also found IL-6 induced MMP-1 and -3 expression in a dose-dependent manner, but did not modify TIMP-1 and TIMP-2 levels, indicating that IL-6 induces a increase of the MMP/TIMP ratios in HKCFBs. The synthesis of larger amounts of IL-6 by epithelial cells could influence a number of immunity and inflammation activities which could lead to corneal damage within the eye (53). We postulate that IL-6 may play a role in the degradation of ECM in

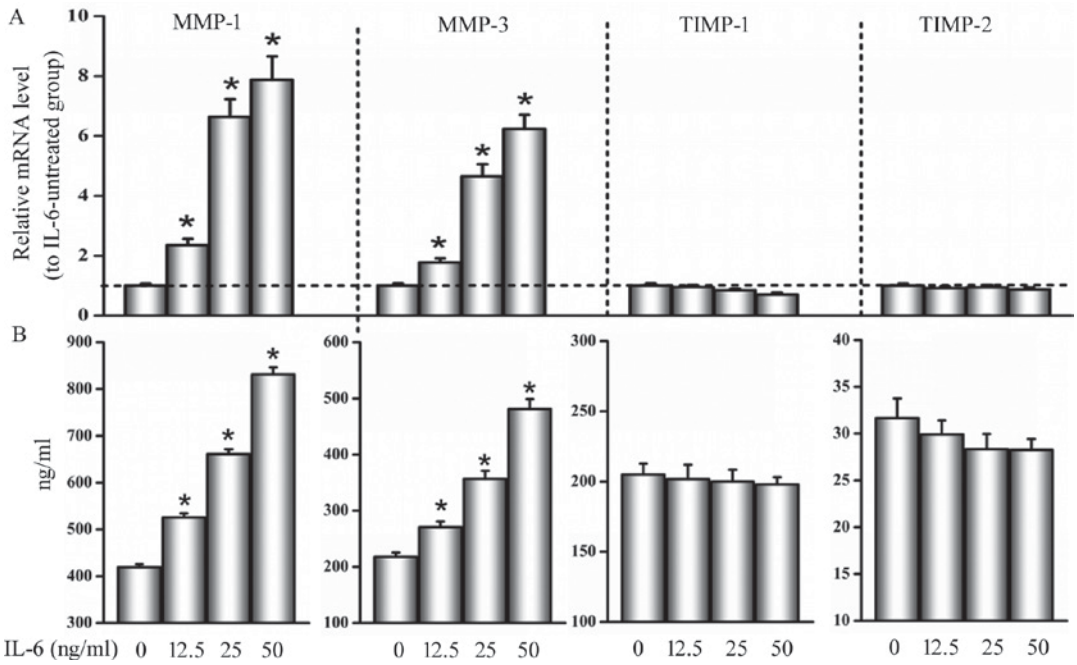

Figure 4. Effect of IL-6 on MMP and TIMP expression in HKCFBs. Cells were treated with IL-6 for 6 h at the indicated concentration. Total RNA and cell-culture supernatants were then collected. mRNA levels determined by RT-PCR from total RNA and protein levels measured using human ELISA kits from cell-culture supernatants. (A) The MMP-1 and -3 mRNA expression were up-regulated in cells treated with IL-6 relative to IL-6-untreated group. No significant differences were observed in mRNA levels of TIMP-1 and -2 between cells treated with and without IL-6. (B) IL-6 enhanced protein synthesis for MMP-1 and -3, whereas no significant effects were observed in protein levels of TIMP-1 and -2. Data are represented as the mean ± standard deviation (n=6 per group). *P<0.05, IL-6-treated vs. IL-6-untreated control. IL-6, interleukin-6; MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinases; HKCFBs, human keratoconus fibroblasts.

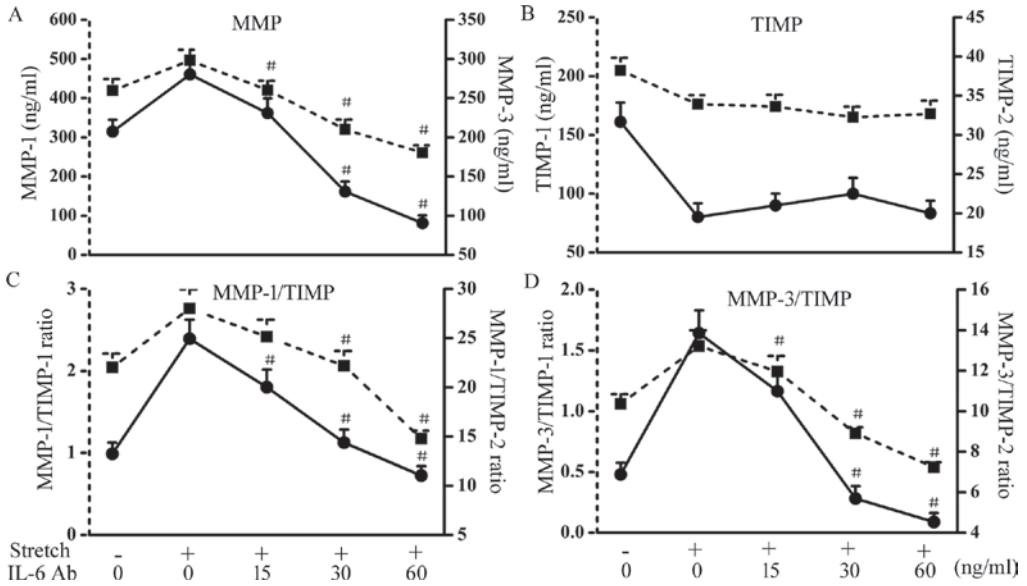

Figure 5. Effect of IL-6 Ab on MMP and TIMP concentrations and the concentration ratios of MMP/TIMP in culture supernatants of stretched HKCFBs. The stretch experiments were repeated in the presence of various IL-6 Ab concentrations. Cell-culture supernatants were then collected and protein levels were detected by human ELISA kits. (A) The increased MMP-1 and -3 protein levels in cell culture supernatants of stretched cells were inhibited during IL-6 Ab treatment. (B) No significant differences in TIMP-1 and -2 concentrations were observed in the culture medium of stretched cultures with the absence or presence of IL-6 Ab. The up-regulated concentration ratios of (C) MMP-1/TIMP-1, MMP-1/TIMP-2, (D) MMP-3/TIMP-1, and MMP-3/TIMP-2 in cell culture supernatants of stretched cells were down-regulated during IL-6 Ab treatment. Data are represented as the mean ± standard deviation (n=6 per group). #P<0.05, stretch in the presence of IL-6 Ab vs. stretched effect without IL-6 Ab. IL-6 Ab, interleukin-6 antibody; MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinases; HKCFBs, human keratoconus fibroblasts.

keratoconus corneas by up-regulating the MMP/TIMP ratios. Balasubramanian *et al* (54) used specific ELISA to measure the amount of inflammatory markers 60 sec after eye-rubbing in normal subjects. They found an increased expression in tear MMP-13, IL-6 and TNF-α in normal subjects. These inflammatory changes after eye-rubbing are thought contribute to the pathogenesis. Put together, our results provide further support for that the pathogenesis of keratoconus may involve chronic

inflammatory events and matrix degradation. Moreover, the mechanical stress caused by eye-rubbing may be a cause of development of inflammatory events and matrix degradation in some types of keratoconus.

Wang *et al* (55) reported that TNF-α mediates the stretch-induced MMP genes expression in human umbilical vein endothelial cells. TNF-α is also widely considered an important cytokine mediator of inflammation and immune regulation that can cause a range of local and systemic biological effects in various cell types (56). Similarly, we speculate that the stretch-induced MMP expression in HKCFBs is dependent on IL-6. In our experiment, IL-6 Ab was used to determine whether mechanical stretched-induced MMP by HKCFBs was mediated by IL-6. Our data showed that the stretch-induced expression of MMP-1 and -3 were markedly inhibited in the presence of IL-6 Ab, whereas no significant changes in TIMP-1 and -2 protein levels were detected in stretched cells with the presence or absence of the IL-6 Ab. Moreover, the IL-6 Ab inhibited significantly the stretch-induced increase in MMP-1 and -3 in culture supernatants in a dose-dependent manner.

Previous work reported that heat shock can increase MMP-1 and -3 and IL-6 at both the mRNA and protein levels in cultured skin fibroblasts and the expression of MMP-1 and -3 increased by heat shock significantly were blocked after treatment with a monoclonal antibody against IL-6, indicating that IL-6 mediates the heat shock-induced MMP-1 and -3 expression (57). Similarly, our study demonstrates that mechanical stretch up-regulated MMP-1 and -3 expression via IL-6 in HKCFBs.

A recent study has found that subsequent treatment of keratoconus patients with cyclosporine A (CyA) reduced tear MMP-9 levels significantly and led to local reduction in corneal curvatures, indicating that CyA might be a novel treatment strategy in keratoconus (58). In addition, it is worth mentioning that several IL-6 blocking agents such as a humanized anti-IL-6 receptor antibody has been developed and successfully applied in clinical trials for the treatment of several diseases (59,60).

Our data showed that MMP/TIMP ratios up-regulated by cyclical stretch were attenuated by IL-6 Ab in a dose-dependent manner. A significant reduction in the MMP/TIMP ratios by IL-6 Ab might provide a new modality to treat patients with keratoconus.

Further studies are required to determine which signaling pathway being involved in the process: Stretch → IL-6 → MMP in keratoconus. It is possible that inhibiting IL-6 may help to dampen degradation of the corneal ECM mediated by mechanical stretch thereby reducing damage to the cornea. This may be realized by inhibiting IL-6 receptors or inhibition of targets downstream of the IL-6 signaling pathway or other pathways targeting IL-6 for treating keratoconus.

In conclusion, our findings indicate that cyclically mechanical stretch induces levels of MMP-1 and -3 and IL-6 and reduces levels of TIMP-1 and -2 in HKCFBs; IL-6 mediates the stretch-induced MMP expression in keratoconus.

Acknowledgements

This study was financially supported by the National Natural Science Foundation of China (grant no. 11402161, 11402162, 11572213, 31300770, 31271005).

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