• Basic Research •

Expressions of matrix metalloproteinases 1 and 3 and their tissue inhibitors in the conjunctival tissue and fibroblasts cultured from conjunctivochalasis

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Abstract

• AIM: To investigate the expression of matrix metalloproteinases 1 and 3 (MMP-1 and MMP-3) and their tissue inhibitors of metalloproteinases 1 and 3 (TIMP-1 and TIMP-3) in the conjunctiva of eyes with conjunctivochalasis (CCh).

• METHODS: The conjunctival tissue was obtained from the CCh patients and controls, the MMPs/TIMPs expression concentration was determined by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence staining. The expression levels of MMPs/TIMPs in the CCh fibroblasts were determined by analyzing its concentration in the cellular supernatant that was abstracted from the *in vitro* cultured CCh fibroblasts.

• RESULTS: MMP-1 and MMP-3 levels determined by ELISA were both significantly higher in the CCh group than that in the control group (*P*=0.042, 0.022, respectively), so was the levels of TIMP-1 (*P*=0.010). No significant difference in the expression of TIMP-3 in conjunctiva was found between the two groups (*P*=0.298). The expression of MMP-1 and MMP-3 were both up-regulated significantly in the CCh group (*P*=0.040, 0.001, respectively) on immunofluorescence staining. MMP-1 and MMP-3 expression in the fibroblasts were both significantly higher in the CCh group than that in the control group (*P*=0.027, 0.001, respectively), while neither the TIMP-1 nor TIMP-3 expression was significantly different between the two groups (*P*=0.421, 0.237, respectively).

• CONCLUSION: The overexpression of MMP-1 and MMP-3 in conjunctival tissue and fibroblasts may play an important role in the pathogenesis and development of CCh.

• KEYWORDS: conjunctivochalasis; relaxed conjunctiva; fibroblast; matrix metalloproteinase; tissue inhibitor of matrix metalloproteinase

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INTRODUCTION

Conjunctivochalasis (CCh) is defined as redundant, nonedematous conjunctiva typically located between the eyeball and the lower eyelid. It commonly occurs in the elderly and can cause intermittent epiphora, ocular irritation, and subconjunctival hemorrhage[1-2]. The mechanism behind CCh has yet to be elucidated, as the age-related and inflammatory changes and mechanical friction from blinking eyelids have been suggested as risk factors that could incur a change in the bulbar conjunctiva, tear, and eyelid. Inflammation is believed to alter the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in the conjunctival fibroblasts[3], which is unpinned by the finding that MMP-9 is significantly reduced after CCh surgery[4]. PTX3 and TSG-6 mediate the transcription and activation of MMP-1 and MMP-3 in CCh[5-6]. So MMPs and TIMPs might be the most important enzyme in the pathogenesis of CCh. This study is designed to detect the expression of MMPs and TIMPs in both the conjunctiva and *in vitro* cultured conjunctival fibroblasts and to further assess the role of MMPs/TIMPs in the pathogenesis of CCh.

SUBJECTS AND METHODS

Subjects Based on the standard described in a previous paper[7], 46 subjects (18 males, 28 females; mean age: 72.77±7.42y) with CCh of more than grade II that underwent conjunctival resection surgery were recruited as the CCh group in this study. Twenty-seven eyes of 27 healthy cataract patients without CCh (10 males, 17 females; mean age: 72.25±7.83y) who received phacoemulsification were included as the control group. This

study adhered to the Declaration of Helsinki and was approved by the institutional review board. All the included subjects were fully informed of the risks and benefits and the consents were signed before proceeding with the examinations and treatments. The subjects who wore contact lenses or who had lid congruity disorders, meibomian gland disease, blepharitis, a history of recent ocular surgery, systemic or topical drug use, or other systemic or eye diseases having an ocular surface presentation were excluded from the study.

Experiment Material The apparatus such as CO_2 incubator (SANYO Company, Japan), clean bench (Antai Company, China), inverted microscope (Wilovert Company, Germany), high-speed low-temperature centrifuge (Eppendorf Company, Germany), enzyme-labeled detector (Bio-Rad Company, USA), Dulbecco's Modified Eagle's medium (DMEM), mycillin solution, 0.25% trypsin solution (Shanghai Bogoo Biotechnology Company, China); and fibroblast growth supplement (Beijing Yuhengfeng Biotechnology Company, China) were used. The agents like MMP-1, MMP-3, TIMP-1, TIMP-3, and enzyme-linked immunosorbent assay (ELISA) kit (Mingrui Biotech Company, China). MMP-1 antibody and MMP-3 antibody (Abcam, England) were also used.

Conjuctival Tissue Sample Collection The samples of relaxed conjunctiva were collected from the CCh patients undergoing the CCh surgery. Conjunctival resections for CCh were all performed by the same surgeon. The collection of normal control conjunctiva was made in the cataract patients receiving phacoemulsification, the protocol of which was established and described in a previous study[8]. The control samples during cataract surgery were also obtained from the inferior conjunctiva as 3×3-mm samples so that the tissues could be fairly compared.

Experiment Methods

Enzyme-linked immunosorbent assays of conjunctiva Conjunctival specimens were put into sterile test tubes immediately, and buffered salt or saline solution was added to them. Ultrasound breaking at 400 Hz was conducted instantly for 99 times with a time span of 3s. The supernatant was obtained by centrifugation at 12 000 rpm, and then refrigerated at -80℃ for detection.

MMP-1, MMP-3, TIMP-1, and TIMP-3 were prepared as 10 000, 5000, 2500, 1250, 625, 312, and 156 pg/mL standard substances. Each standard substance (0.1 mL) was added into seven wells in order and a sample dilution into one well as zero-well to obtain the standard curve. Samples and biotin-labeled antibodies were added into a 96-well ELISA plate in order for reaction, and washed with tris-buffered saline (TBS). Attenuated avidin-peroxidase compound was added for coloration with substrate 3,3',5,5'-tetramethylbenzidine (TMB) after being completely washed with TBS. TMB turned blue under peroxidase catalyst and finally to yellow under the action of acid. This color shade is positively related to MMP-1, MMP-3, TIMP-1, and TIMP-3 in the samples. Concentration of all samples can be obtained according to absorbance values and standard curve.

Immunofluorescence staining of conjunctival specimens The excised conjunctival samples were placed in formalin and fixed for 48h. They were then washed and dehydrated to make it transparent, and later waxed, embedded, then excised for detection.

After dewaxing, the samples were hydrated as follows: dewaxed sections were infiltrated with 100% alcohol, 95% alcohol, 75% alcohol, and double-distilled water, successively, for 3min. Antigen retrieval was conducted by placing the sample in 0.01 mol/L sodium citrate-buffered solution for high-pressure repair for 20-30min. During microwave, the sample should be cooled naturally to avoid boiling the solution. The sample was washed with 0.02 mol/L PBS for 5min thrice. Primary antibodies (MMP-1 and MMP-3) diluted in appropriate proportion were dripped as required and incubated under 4℃ overnight. The sample was then rinsed with 0.02 mol/L PBS for 5min thrice with frequent vibration (to remove unnecessary nomadic antibodies). Fluorescent second antibodies diluted in appropriate proportion were dripped and incubated under room temperature for 1h. X-ray was taken with fluorescence microscope after mounting.

Human fibroblasts cultures The excised bulbar conjunctival tissue was placed in prepared sterile disposable mixing bowl and rinsed with 9 g/L normal saline three times. Then, the tissue was cut with a microscopic ophthalmic scissor into blocks of size 0.5-1 mm^2 and laid into a six-well plate, which was then cultured in a CO_2 incubator with 5% volume fraction under 37℃ with upside down. When the blocks in the six-well plate were almost dry, the plate was turned bottom down, and 3-mL DMEM nutrient solution with fetal calf serum and mycillin in 10% volume and 0.1% FGS was added slowly. Adherence and culture condition of inoculated tissue blocks were observed under inverted phase contrast microscope. The positive cell area was calculated to represent the relative value of fluorescence staining intensity.

Enzyme-linked immunosorbent assays of cultured cellular supernatant Cells of three to six generations in logarithmic growth were selected. After dissociating the cells growing to 90%, DMEM nutrient solution with 10% fetal calf serum was used to prepare cell suspension for inoculation in a 24-well plate at a concentration of 2.5×10^5 cells/mL with 1 mL per well. After culturing for 24h, the original nutrient solution was abandoned, and 1 mL DMEM nutrient solution without serum was added per well and three complex wells per group. Then, the wells were cultured in the CO_2 incubator with 5% volume fraction for 48h under 37℃. The cultured supernatant fluid was collected from each well and centrifuged at a high

Table 1 ELISA assay of MMPs/TIMPs concentration

Parameters	*n*	$\bar{x} \pm s$ (ng/mL)	*U*	*W*	*Z*	*P* (both sides)	*P* (singles side)
MMP-1			72.00	108.00	-1.73	0.08	[a]0.042
CCh	30	489.10±164.49					
Control	8	304.17±218.75					
MMP-3			51.00	117.00	-2.01	0.04	[a]0.022
CCh	17	1236.96±1419.53					
Control	11	213.79±7.45					
TIMP-1			96.00	162.00	-2.33	0.02	[a]0.010
CCh	33	1529.92±2147.49					
Control	11	93.51±51.20					
TIMP-3			162.00	228.00	-0.53	0.60	0.298
CCh	33	604.60±107.77					
Control	11	548.00±154.10					

[a]*P*<0.05.

speed of 1500 rev/rain for 20min. The supernatant was placed in a sterile Eppendorf tube and cryopreserved under -20℃ after split charging. The concentrations of MMP-1, MMP-3, TIMP-1, and TIMP-3 were detected using ELISA, and the concentrations of various samples were obtained according to absorbance values and standard curves.

Statistical Analysis The SPSS16.0 software was used for statistical analysis. The average concentration was presented as mean±SD according to the individual concentration calculated from the standard curve and absorbance values. Difference was analyzed using one-way analysis of variance (LSD *t*-test) and independent-sample *t*-test if the data were normally distributed while Wilcoxon rank-sum test was used if not. *P*<0.05 was considered statistically significant.

RESULTS

Enzyme-linked Immunosorbent Assay for Measurement of Matrix Metalloproteinases/Tissue Inhibitors of Metalloproteinases Levels in Conjunctiva MMP-1 was 489.10±164.49 ng/mL in concentration in the CCh group and 304.17±218.75 ng/mL in the control group with a statistical significance (*P*=0.042). MMP-3 was 1236.96±1419.53 ng/mL in concentration in the CCh group and 213.79±7.45 ng/mL in the control group with a statistical significance (*P*=0.022).

TIMP-1 was 1529.92±2147.49 ng/mL in concentration in the CCh group and 93.51±51.20 ng/mL in the control group with a statistical significance (*P*=0.010). TIMP-3 was 604.60±107.77 ng/mL in concentration in the CCh group and 548.00±154.10 ng/mL in the control group with no statistical significance (*P*=0.298) (Table 1).

Immunofluorescence for Measurement of Matrix Metalloproteinases Expression in Conjunctiva The positive cells area of MMP-1 was 716.78±39.81 μm^2 in the CCh group and 661.50±39.11 μm^2 in the control group with a statistical significance (*t*=2.322, *P*=0.040). The positive cells area of MMP-3 was 756.56±55.84 μm^2 in the CCh group and 620.25±19.82 μm^2 in the control group with a great statistical significance (*t*=4.654, *P*=0.001) (Table 2; Figure 1).

Table 2 Immunofluorescence assay of MMPs μm^2

Groups	MMP-1	MMP-3
CCh	716.78±39.81	756.56±55.84
Control	661.50±39.11	620.25±19.82
t	2.322	4.654
P	0.040	0.001

Human Fibroblasts Cultures The tissue blocks were closely attached to the 6-well plate after 2-5d of primary culture using the tissue block adherence method. Under an inverted microscope, tissue blocks displayed poor transparence with a dark black area and spilled cells around them. Cell proliferation was observed after 10-15d. The cell rings of the tissue blocks were spread around, in which mosaic-like cells with inhomogeneous morphologies and unclear boundaries were observed. After 20d, the cells grew to above 80% and were used for passage. Pure fibroblast cells could be obtained after two passages (Figure 2). All cells are fiber-like and radially arranged. They have homogenous sizes, long-spindles or biapiculate shapes, an oval nucleus in the plasma, and cell processes with different lengths for cross-linking. And the cells were identified by keratin and vimentin immunofluorescence staining, vimentin and keratin flow cytometry, which were confirmed to be fibroblast cells[9].

Enzyme-linked Immunosorbent Assay for Measurement of Matrix Metalloproteinases/Tissue Inhibitors of Metalloproteinases Expression in Fibroblasts The MMP-1 levels in fibroblasts were significantly higher in the CCh group (437.62±13.62 ng/mL) than in the control group (404.74±9.68 ng/mL; *t*=3.408, *P*=0.027). The MMP-3 levels were significantly higher in the CCh group (0.29±0.02 ng/mL) than in the control group (0.15±0.016 ng/mL; *t*=8.160, *P*=0.001). There was no significantly difference in the TIMP-1 concentration between the two groups, nor was in the TIMP-3

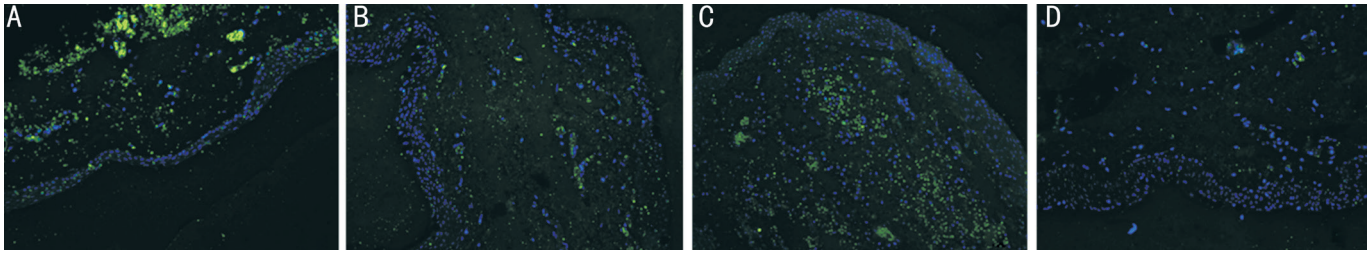

Figure 1 MMPs of conjunctiva detected with immunofluorescence staining (×200) A: MMP-1 in the CCh group; B: MMP-1 in the normal control group; C: MMP-3 in the CCh group; D: MMP-3 in the normal control group. Positive expression is marked as green while cell nuclei as blue.

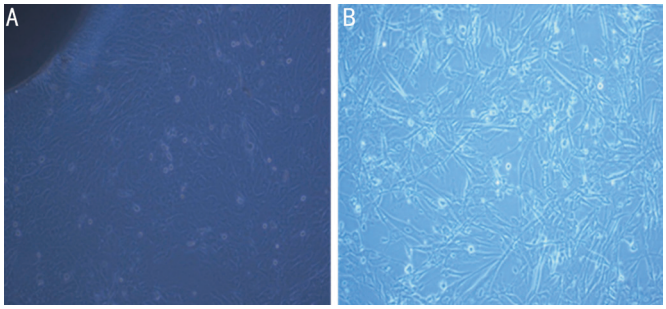

Figure 2 Fibroblast cells from CCh conjunctival tissue of primary culture *in vitro* A: Primary fibroblast cells from conjunctival tissues with CCh (×100); B: Second-passage fibroblast cells from conjunctival tissue with CCh (×100). All cells are fiber-like and radially arranged. They have homogenous sizes, long-spindles or biapiculate shapes, an oval nucleus in the plasma, and cell processes with different lengths for crosslinking.

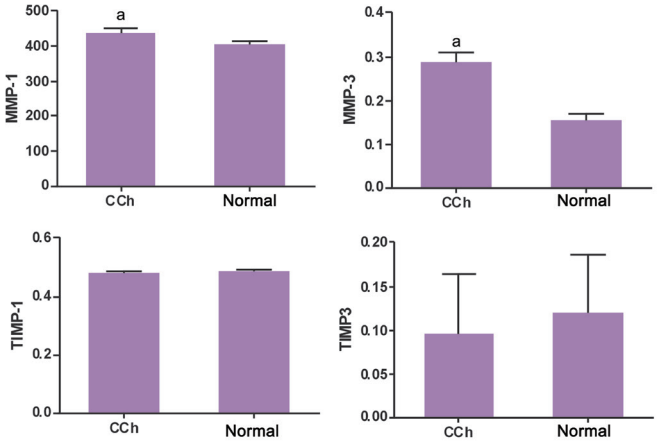

Figure 3 MMPs/TIMPs levels in the CCh fibroblasts *vs* in the normal control using ELISA [a]$P<0.05$ using independent-sample *t*-test.

concentration (0.48±0.00 ng/mL *vs* 0.49±0.01 ng/mL, t=-0.896, P=0.421; 0.17±0.01 ng/mL *vs* 0.15±0.02 ng/mL, t=1.388, P=0.237) (Figure 3).

DISCUSSION

The growth in the number of CCh patients have shown to be increasingly fast with the acceleration of population. The incidence of CCh varies in the archives, as Mimura *et al*[10] have demonstrated that it is 85.24% in the population aged within 1-94y while Zhang *et al*[11] have reported that it is 44.08% in the population aged 60y or above. The conjunctival fold, a common feature of CCh, is commonly viewed as a senile abnormality[12], which may involve the changes in the extracellular components such as degeneration of elastic fibers or increased collagenolytic activity, as was evidenced by several pathological studies in which the reduced elastic fiber and collagen fibril in lamina propria and fascia tissue of bulbar conjunctiva, the melted collagenous fiber, and the degenerated fibroblast were found[13-14]. Meller *et al*[1,3] have suggested that inflammation may initiate the process of collagenous fiber melting, as inflammatory reaction-related proteins have shown to appear in tear[15]. As the fibroblast is the most common cell and it secretes many extracellular matrix components including collagenous fiber and elastic fiber in the connective tissue, the activity level of which, among others, may feed into the onset and development of CCh.

MMPs are something of protein-cleaving enzymes that can hydrolyze extracellular matrix, among which MMP-1, MMP-2, MMP-3, and MMP-9 are commonly seen in human. MMPs can degrade all components of the extracellular matrix and play an important role in many physiological and pathological processes. TIMPs, which are thought to neutralize the activity of MMPs, also assume a role in maintaining the stability of extracellular matrix. The imbalance between the level of MMPs and TIMPs expression is believed to be closely associated with some ophthalmic diseases such as pterygium, ocular injury, cornea lesion, glaucoma, diabetic retinopathy, and proliferative vitreoretinopathy[16-18]. Overexpression of MMP-1 and MMP-3 in cultured bulbar conjunctival fibroblast was also found in CCh[19].

The present study aimed at further understanding the role of MMPs in the pathogenesis of CCh is therefore designed to detect both of the expression of MMPs and TIMPs in CCh using the method of ELISA and immunofluorescence. To strengthen the performance of this study, inclusion and exclusion criteria were strictly respected and the eligible subjects were recruited. The 3rd to 6th generations of generations of cultured fibroblasts were used for study to avert the influence of the epithelia in the primary culture, as is supported by the findings in a previous paper[9].

An up-regulation of MMP-1 and MMP-3 was observed in both of the conjunctival tissue and fibroblasts of CCh subjects.

However, no significant difference was found in the expression levels of TIMPs between the CCh group and control group.

The present study, in which two scientific methods were used, *i.e.* ELISA and immunofluorescence, has validated the early findings of the overexpression of MMP-1 and MMP-3 in loose conjunctival tissue and fibroblasts in CCh. It is generally accepted that MMP-1 could degrade type I, II, and III collagenase, while MMP-3 has a capacity of degrading type IV, V, VII, and X collagenase, elastic protein, and fibronectin. The up-regulation of MMPs means its increased activity of melting the collagen fibers and degrading the elastic fibers with a result of over-degradation of conjunctival matrix and Tenon's capsule, thereby thinning the bulbar conjunctiva, resulting in an accumulation of the conjunctiva in the lower eyelid and between inner and lateral canthus, and forming the conjunctival folds. However, additional studies are required to further understand how the expression of MMPs is exactly regulated in the pathogenesis of CCh.

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