·Basic Research·

Molecular mechanism of the inhibition effect of Celecoxib on corneal collagen degradation in three dimensions

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Abstract

• AIM: To clarify the molecular mechanism of Celecoxib on corneal collagen degradation and corneal ulcer.

• METHODS: Rabbit corneal fibroblasts were harvested and suspended in serum-free MEM. Type I collagen, DMEM, collagen reconstitution buffer and corneal fibroblast suspension were mixed on ice. The resultant mixture solidify in an incubator, after which test reagents and plasminogen was overlaid and the cultures were returned to the incubator. The supernatants from collagen gel incubations were collected and the amount of hydroxyproline in the hydrolysate was measured. Immunoblot analysis of MMP1, 3 and TIMP1, 2 was performed. MMP2, 9 was detected by the method of Gelatin zymography. Cytotoxicity Assay was measured.

• RESULTS: Celecoxib inhibited corneal collagen degradation in a dose and time manner; Celecoxib inhibited the IL-1β induced increases in proMMP1, 2, 3, 9 and active MMP1, 2, 3, 9 in a concentration-depended manner. Celecoxib can also inhibit the IL-1β induced increases in the TIMP1, 2.

• CONCLUSION: Celecoxib can inhibit corneal collagen degradation induced by IL-1β , this effect is the consequence of the reduction of MMP1, 2, 3, 9 and TIMP1, 2. The results of the present study provide new insight into Celecoxib in corneal ulcer treatment.

• KEYWORDS: Celecoxib; corneal ulcer; IL-1β ; collagen degradation
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INTRODUCTION

Homeostasis of corneal stroma depend on the balance of matrix remodeling proteins and matrix disrupting proteins [1]. Structural extracellular matrix proteins such as fibronectin and collagen concerned with matrix metalloproteinase (MMPs) regulating ECM degradation[2-4]. Members of the MMPs families cleave cellular, extracellular and extracellular matrix substrates modulating tissue structure[5]. Conversion of matrix plasminogen to plasmin by proteases of the plasminogen activator (PA) system promote proMMPs to their active form. MMP production can be induced by varied cytokines, such as the proinflammatory cytokines TNF-a, IL-1,IL-6 [6]. Cytokines and chemokines attract PMN to the cornea thus inducing corneal ulcer.IL-1β is critical to regulation of the corneal bacterial inflammatory process[7].

We previously showed that Pseudomonas aeruginosa elastase and IL-1β stimulated corneal collagen degradation by corneal fibroblasts induced by IL-1β [8]. We also manifested female sex hormone inhibitrf corneal collagen degradation induced by IL-1β [9,10]. As a selective cyclooxygenase-2(COX-2) inhibitor, Celecoxib is a specific nonsteroidal antiinflammatory drugs (NSAID). Celecoxib down-regulates the inflammatory mediators such as IL-1β, IL-2, IL-4, IFNγ, TNF-α, iNOS, COX-2, MMP1, 2, 3, and PGE(2)to a level that inhibits inflammation[11-14].

MMP1, 3 lead to a marked increase in COX-2 expression, PGE2 secretion, and MMP9 expression. Celecoxib can block Proteinase-induced MMP9 expression [15,16]. Inhibition

of COX-2 reduces the collagen fibrillogenesis associated with tumor cell infiltration [17].Celecoxib, has been reported to have COX-2-independent immunomodulatory effects[18]. Previously we showed that the female sex hormone inhibited IL-1β-induced collagen degradation by corneal fibroblasts [9,10]. In this study, we examined whether Celecoxib inhibited collagen degradation by rabbit corneal fibroblasts in response to IL-1β. We want to clarify the mechanism and potency of COX-2 inhibitor Celecoxib on the treatment of corneal dissolvability disease and corneal ulcer.

MATERIALS AND METHODS

Materials Minimum essential medium eagle (MEM), Dulbecco's phosphate-buffered saline (DPBS), antibiotic-antimycotic mixture, and trypsin-EDTA were obtained from Weibo Chem Company; native porcine type 1 collagen (acid solubilized),5×Dulbecco's modified Eagle's medium (DMEM), and collagen reconstitution buffer were from Nitta Gelatin (Osaka, Japan). Fetal bovine serum (FBS) was from Shanghai Yantuo Biotecnology (Shanghai, China). Bovine plasminogen, protease inhibitor cocktail, and Celecoxib were from Sigma-Aldrich (Beijing, China). Recombinant human IL-1β was obtained from R&D Systems China (Shanghai, China). Mouse monoclonal antibodies to rabbit MMP1, 3 and TIMP1, 2 were obtained from antibodies-online (German). An enhanced chemiluminescence (ECL) kit as well as horseradish peroxidase-conjugated goat polyclonal antibodies to rabbit or mouse immunoglobulin G were from GE Healthcare (Piscataway, NJ). Coomassie brilliant blue and gelatin were obtained from Bio-Rad (Hercules, CA). A cytotoxicity assay (CytoTox 96Non-Radioactive) was from Promega (Beijing, China). All media and reagents used for cell culture were endotoxin minimized.

Methods

Cell isolation Rabbit corneal fibroblasts were isolated and maintained as described previously [9,10,19,20]. In brief, the enucleated eye was washed with DPBS containing antibiotic-antimycotic mixture, the endothelial layer of the excised cornea was removed mechanically, and the remaining corneal tissue was incubated with dispase (2mg/mL, in MEM) for 1 hour at 37℃. After mechanical removal of epithelial sheet, the remaining tissue was treated with collagenase (2mg/mL, in MEM) at 37℃ until a single-cell suspension of corneal fibroblasts was obtained. The isolated corneal fibroblasts were cultured under a humidified atmosphere of 5% CO_2 at 37℃ in 60mm culture dishes containing MEM supplemented with 10% FBS. Proliferating cells were harvested for experiments at the subconfluent stage after four to seven passages in monolayer culture.

Three-Dimensional culture system Culture collagen gels were prepared as described [9,10,19,20]. In brief, corneal fibroblasts were harvested by exposure to trypsin-EDTA followed by centrifugation at 15 000×g for 5 minutes, and they were then suspended in serum-free MEM. Acid-solubilized collagen type I (3mg/mL), 5 × DMEM, collagen reconstitution buffer [0.05mol/L NaOH, 0.26mol/L Na_2CO_3, 0.2mol/L HEPES (pH 7.3)], and corneal fibroblast suspension (2.2×10^6/mL in MEM) were mixed on ice at a volume ratio of 7:2:1:1. The resultant mixture (0.5mL) was added to each well of a 24-well culture plate and allowed to solidify in an incubator containing 50mol/L CO_2 at 37℃, after which 0.5mL of serum-free MEM containing test reagents and plasminogen (60μg/mL) was overlaid and the cultures were returned to the incubator for 48 hours.

Assay of collagenolytic activity Collagen degradation was measured as previously described. In brief, the supernatants from collagen gel incubations were collected, and native collagen fibrils with a molecular size of >100kDa were removed by ultrafiltration. The filtrate was subjected to hydrolysis with 6mol/L HCl for 24 hours at 110℃, and the amount of hydroxyproline in the hydrolysate was determined by measurement of absorbance at 558nm with a spectrophotometer.

Immunoblot analysis Immunoblot analysis of MMP1 and MMP3 was performed as described previously. In brief, culture supernatants from collagen gel incubations were subjected to SDS polyacrylamide gel electrophoresis on a 10% gel, and the separated proteins were transferred electrophoretically to a nitrocellulose membrane. Nonspecific sites of the membrane were blocked, and it was then incubated with antibodies to MMP1 or to MMP3. Immune complexes were detected with the use of horseradish peroxidase-conjugated secondary antibodies and ECL reagents.

Gelatin zymography Gelatin zymography was performed as described previously. In brief, culture supernatants from collagen gel incubations were mixed with SDS sample buffer by the ratio of 2:1, and 5μL of the resulting mixture were subjected to SDS polyacrylamide gel electrophoresis in the dark at 4℃ on a 10% gel containing 0.1% gelatin. The gel was then washed with 2.5% Triton X-100 for 1 hour before incubation for 18 hours at 37℃ in a reaction mixture containing 50mmol/L Tris-HCl (pH 7.5), 5mmol/L $CaCl_2$, and 1% Triton X-100. The gel was finally stained with Coomassie brilliant blue.

Cytotoxicity assay LDH release was measured by Non-Radioactive Cytotoxicity assay kit. In brief, 2×10^4 cells were cultured in 10% FBS in 96-well plates for 24 hours. After washing, the cells were secreted by the compounds in serum-free for extra 24 hours. 1g/L triton was taken as positive control. Supernatants and substrate were mixed in assay buffer in a new plate (1:1 v:v, 30 minutes, RT). Stop

Solution was added and absorbance was recorded on spectrophotometer at 400nm.

Statistical Analysis Data are presented as means±SEM and were analyzed with Dunnett's multiple comparison test. *P* value <0.0001 was considered statistically significant.

RESULTS

Inhibition effect of Celecoxib on IL −1β induced collagen degradation by corneal fibroblasts We manifested that proinflammatary factor IL-1β markedly increased the extent of collagen degradation by cultured corneal fibroblasts [9,10,19,20]. To investigate and analyze the inhibition effect of Celecoxib on collagen degradation resulting from IL-1β stimulation in three dimensional cultures of rabbit corneal fibroblasts, the cells incubated for 48 hours with Celecoxib (1-100μmol/L) resulted in a concentration-depended inhibition of collagen degradation in the presence of IL-1β (0.1ng/mL, Figure 1).

Except the results above, we carried out the time course of collagen degradation by corneal fibroblasts in the absence or presence of IL-1β (0.1ng/mL) or 10μmol/L Celecoxib. In different time points, the amount of degraded collagen increased gradualy. Compared to the amount of collagen degradation by plasminogen, IL-1β increased the amount of degraded collagen dramatically at 36 and 48 hours. This effect was inhibited by 10μmol/L Celecoxib at 36 and 48 hours (Figure 2).

Effects of Celecoxib on the expression of MMP1, 3 MMP1, 3 expression were detected using the methods of immunoblot analysis. Corneal fibroblasts were cultured in collagen gels for 48 hours in the absence or presence of IL-1β and in the presence of Celecoxib (1μmol/L-100μmol/L). Coincident with our previous result [9,10,19,20], immunoblot analysis with antibodies to human biotinylated MMP-1 revealed that the culture supernatants of cells incubated without IL-1β contained relatively small amounts of 61 and 57-kDa immunoreactive proteins corresponding to pro MMP1 as well as of 49kDa-45kDa immunoreactive proteins corresponding to active MMP1. In contrast, large amount of these bands were detected in the culture supernatants of cells incubated with IL-1β. Celecoxib inhibited the IL-1β induced increases in proMMP1 and active MMP1 in a dose dependent manner (Figure 3).

Immunoblot analysis with antibodies to MMP3 detected small amount of proMMP3 in culture supernatants of cells incubated in the absence of IL-1β. In contrast, 57kDa-45kDa immunoreactive proteins corresponding to proMMP3 and active MMP3, respectively, were apparent in the culture supernatants of cells incubated with IL-1β. Celecoxib inhibited the IL-1β induced increases in proMMP3 and active MMP3 in a concentration-depended manner (Figure 4).

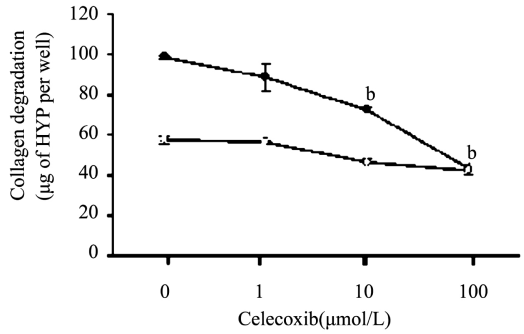

Figure 1 Dose −dependent inhibition effect by Celecoxib of IL−1β−induced collagen degradation by corneal fibroblasts Rabbit fibroblasts were cultured in collagen gels in the presence of plasminogen, in the absence (open symbols)) or presence (closed symbols) of IL-1β (0.1ng/mL) and in the presence of the indicated concentrations of Celecoxib. After incubation of the cells for 48 hours, the amount of degraded collagen in the culture supernatants was determined. Data are expressed as micrograms of hydroxyproline (HYP) per well and are means ±SEM of values from an experiment that was repeated a total of three times with similar results. $^{b}P<0.001$ (Dunnett's test) *vs* the value for cells cultured with IL-1β in the absence of Celecoxib.

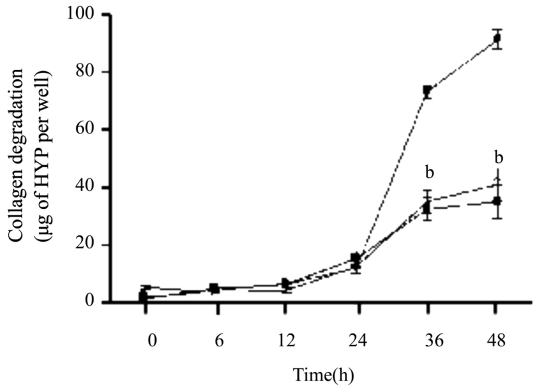

Figure 2 Time −dependent inhibition effect of Celecoxib on IL−1β induced collagen degradation by corneal fibroblasts Cells were cultured in collagen gels for the indicated times in the presence of plasminogen 60μg/mL and in the absence (open symbols) or presence of 0.1ng/mL IL-1β (close symbols), in the absence (circles) or presence (squares) of 10μmol/L Celecoxib, after which the amount of degraded collagen was determined. Data are mean ±SEM of values from three experiments. $^{b}P<0.001$ (Dunnett test) *vs* the corresponding value for cells cultured with IL-1β and plasminogen.

Effects of Celecoxib on the expression of MMP2, 9 The expressions of MMP2, 9 were detected by gelatin zymography. Culture supernatants of corneal fibroblasts incubated without IL-1β for 48 hours revealed two major bands of 65 and 57kDa corresponding to pro- MMP2 and active MMP2 and a faint band of 77kDa band corresponding to active MMP9. Cells cultured in the presence of IL-1β (0.1ng/mL) resulted in an increase in the intensity of the band corresponding to active MMP2 and the appearance of bands at 92 and 77kDa corresponding to proMMP9 and

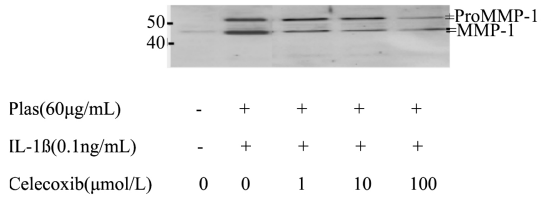

Figure 3 Effects of Celecoxib on the expression of proMMP1 and MMP1 by corneal fibroblasts Cells were cultured in collagen gels for 48 hours in the presence of plasminogen, in the absence or presence of IL-1β (0.1ng/mL), and in the presence of the indicated concentrations of Celecoxib. The culture supernatants were then subjected either to immunoblot analysis with antibodies to MMP1. Data are representative of three independent experiments. The positions of bands corresponding to the pro MMP1 and MMP1 are indicated on the right, and those of molecular size are shown on the left.

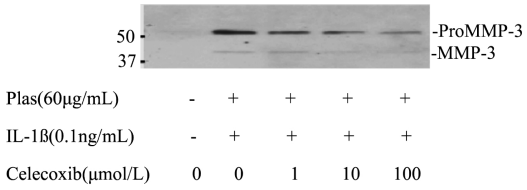

Figure 4 Effects of Celecoxib on the expression of proMMP3 and MMP3 by corneal fibroblasts Cells were cultured in collagen gels for 48 hours in the presence of plasminogen, in the absence or presence of IL-1β (0.1ng/mL), and in the presence of the indicated concentrations of Celecoxib.The culture supernatants were then subjected either to immunoblot analysis with antibodies to MMP3. Data are representative of three independent experiments. The positions of bands corresponding to the pro MMP3 and MMP3 are indicated on the right, and those of molecular size are shown on the left.

active MMP9 respectively. Celecoxib inhibited the IL-1β induced increases in the amounts of the proMMP9, active MMP9 and active MMP2 in a concentration-dependent manner (Figure 5).

Effects of Celecoxib on the expression of TIMP1,2 Further analysis emphasized the effects of Celecoxib on the expressions of TIMPs secreted by corneal fibroblasts. Immunoblot analysis with antibodies to TIMP1 revealed that the culture supernatant of cells maintained in collagen gels for 48 hours in the presence or absence of plsminogen with or without Celecoxib contained a 28kDa immunoreactive protein corresponding to TIMP1 (Figure 6). TIMP1 protein levels increased in the presence of IL-1β (0.1ng/mL) and plasminogen. The increased secretion of TIMP1 was inhibited by Celecoxib in a dose-dependent manner. Celecoxib also inhibited the expression of TIMP2 in a dose dependent manner, which corresponding to 21kDa immunoreactive protein (Figure 7).

LDH detection Measurement of LDH release revealed that Celecoxib at 1, 10, 100μmol/L had no cytotoxic effect on corneal fibroblasts (Figure 8).

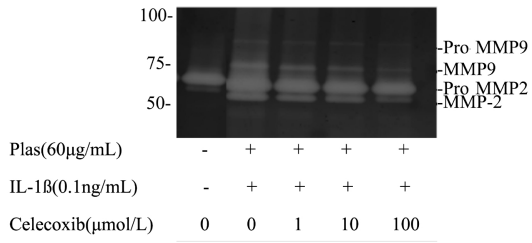

Figure 5 Effects of Celecoxib on the expression MMP2, 9 by corneal fibroblasts Cells were cultured in collagen gels for 48 hours in the presence of plasminogen and in the absence or presence of IL-1β (0.1ng/mL) and the indicated concentrations of Celecoxib. Culture supernatants were then subjected to gelatin zymography. The positions of bands corresponding to proMMP2 and proMMP9 and active MMP2, 9 are indicated on the right, and those of molecular size are shown on the left. Data are representative of three independent experiments.

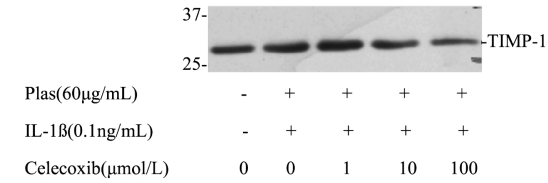

Figure 6 Effects of Celecoxib on the expression of TIMP1 by corneal fibroblasts Cells were cultured in collagen gels for 48 hours in the presence of plasminogen, in the absence or presence of IL-1β (0.1ng/mL), and in the presence of the indicated concentrations of Celecoxib. The culture supernatants were then subjected to immunoblot analysis with antibodies to TIMP1. Data are representative of three independent experiments. The positions of bands corresponding to the TIMP1 are indicated on the right, and those of molecular size are shown on the left.

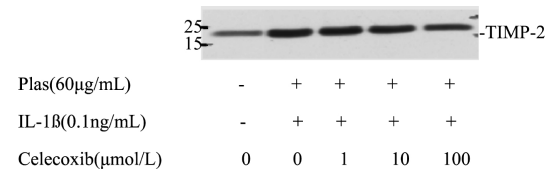

Figure 7 Effects of Celecoxib on the expression of TIMP2 by corneal fibroblasts Cells were cultured in collagen gels for 48 hours in the presence of plasminogen, in the absence or presence of IL-1β (0.1ng/mL), and in the presence of the indicated concentrations of Celecoxib. The culture supernatants were then subjected to immunoblot analysis with antibodies to TIMP2. Data are representative of three independent experiments. The positions of bands corresponding to the TIMP2 are indicated on the right, and those of molecular size are shown on the left.

DISCUSSION

The present study examined whether Celecoxib can inhibit the degradation of the extracellular matrices during corneal inflammation. We observed that Celecoxib inhibited the amount of collagen degradation of rabbit corneal fibroblasts induced by IL-1β in concentration and time-depended manner. Celecoxib can suppress the synthesis and activation of MMP1, 2, 3, 9 in corneal fibroblasts exposed to IL-1β in

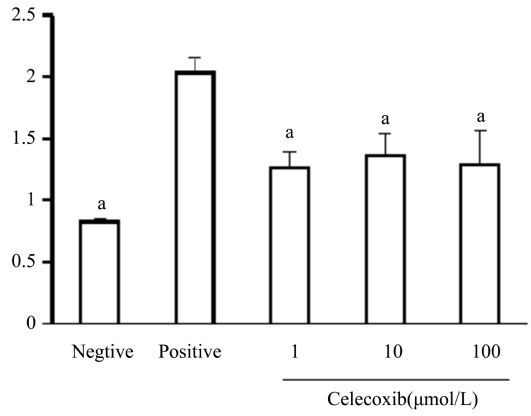

Figure 8 Lack of a cytotoxic effect of Celecoxib on corneal fibroblasts Cells were incubated for 24 hours in MEM and in the absence (negative control) or presence of 10μmol/L or 100μmol/L Celecoxib, after which the culture supernatants were assayed for LDH activity with a colorimetric assay. The amount of LDH released from cells by 0.1% Triton was determined as a positive control. Data are means±SEM from three independent experiments. [a] $P< 0.05$ *vs* 0.1% Triton (Dunnett's test).

a concentration-depended manner. MMPs play an important role in degradation of the matrix which allows the inflammatory cell such as leucocytes and mononuclear assembling into the infected tissue site [21]. IL-1β can alter MMPs expression involving in leukocyte influx which has a close relationship to the pathogen and resident cells in the inflammation process[22]. Medication of anti IL-1β treatment to bacterial corneal infection mice resulted in a significant reduction in expression of MMP2, 9, TIMP1, 2 [23]. We proved that Celecoxib inhibited MMPs expression induced by IL-1β thus inhibiting the corneal collagen degradation.

Celecoxib at 100nmol/L reduced the IL-1β induced of MMP1, 3, iNOS and NO in human articular chondrocytes[24]. Celecoxib could inhibit PGE2 production and MMP-2 secretion of Tca8113 cell [25]. Selective cyclooxygenase-2 inhibitor can inhibit gingival tissue MMP8 expression [26]. COX-1 is constitutively expressed in many tissues, whereas COX-2 is induced by inflammatory mediators.COX-2 was expressed in the epithelium, endothelium, and stromal cells of the inflamed cornea [27]. COX-2 can be induced by proinflammatory factors such as IL-1β and TNF. PGE produced in inflammatory tissues by COX-2, play a main role in ocular inflammation[28,29].

We found that Celexicob inhibited the expressions of TIMP1,2 not as anticipated. Tissue inhibitors of MMPs (TIMPs) and plasminogen activator inhibitor (PAI-1) are produced in relation to urokinase (uPA) and MMP1, 9 in cornea injury. There is an imbalance between the expression of this proteolytic enzyme and its inhibitors, which may contribute to changes in the wound healing process and ultimately lead to corneal ulcer development[30].

Celecoxib reduced type II collagen and MMP1,3 induced by IL-1β in human osteoarticular cartilage [31]. Celecoxib can inhibit corneal collagen degradation induced by IL-1β, this effect was the consequence of the reduction of MMP1, 2, 3, 9 and TIMP1, 2. Celecoxib showed no evident cytotoxicity on corneal fibroblasts. The results of the present study provide new insight into Celecoxib in corneal ulcer treatment.

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