Molecular mechanism of the inhibition effect of Lipoxin A4 on corneal dissolving pathology process

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Abstract

AIM: Excessive dissolve of corneal tissue induced by MMPs which were activated by cytokins and chemokines will lead to corneal ulcer. The molecular mechanism of Lipoxin A4 (LXA4) on corneal collagen degradation in three dimensions was investigated.

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 solidified 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 MMP-1, –3 and TMMP-1,–2 was performed. MMP –2, –9 was detected by the method of Gelatin zymography. Cytotoxicity assay was measured.

RESULTS: LXA4 inhibited corneal collagen degradation in a dose and time manner. LXA4 inhibited the IL–1β induced increases in the pro–MMP–1, –2, –3, –9 and active MMP–1, –2, –3, –9 in a concentration dependent manner. LXA4 also inhibited the IL–1β induced increases in TIMP–1, –2.

CONCLUSION: As a potent anti–inflammation reagent, LXA4 can inhibit corneal collagen degradation induced by IL–1β in corneal fibroblasts thus inhibiting corneal dissolving pathology process.

KEYWORDS: LXA4; IL–1β; cornea; collagen; dissolution

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INTRODUCTION

Extracellular matrix proteins concerned with matrix metalloproteinase (MMPs) controlling ECM balance in vitro [1–3]. MMPs cleave cellular, extracellular and extracellular matrix substrates regulating tissue structure [4]. Excessive dissolve of corneal stroma will lead to corneal ulcer. IL–1β is a major proinflammatory factor [5]. Lipoxin A4 (LXA4), as the predominant endogenously generated lipoxin, was a high potency of anti-inflammatory lipid mediator. Its molecular formula was C₂₀H₃₂O₅ [6]. LXA4 and its receptor (FPR2/ALX) was used as marker for resolving inflammation and tissue injury [7,8]. LXA4 inhibited LPS-induced production of NO, IL–1β and TNF-α in a concentration-dependent manner via NF-κB, ERK, p38 MAPK and AP-1 signaling pathways in LPS-activated microglia [8]. LXA4 blocked IL–1β and TNF-α-mediated upregulation of E-selecting and intercellular cell adhesion molecule-1 (ICAM-1) and consequent binding of lymphocytes by activating its receptor [9]. LXA4-mediated reduction of MMP–1, –2, –3, –9 in different cell lines and are attributed to increased TIMP-1 expression [11–13].

Three dimensional culture of corneal fibroblasts in collagen gel mimics the in vivo situation [14]. Previously we showed that pseudomonas aeruginosa elastase and IL–1β can stimulate corneal collagen degradation by corneal fibroblasts [9]. We also showed that the female sex hormone inhibited IL–1β–induced collagen degradation by corneal fibroblasts [10]. In this paper, we examined whether LXA4 inhibited collagen degradation by rabbit corneal fibroblasts in response to IL–1β. As LXA4 was a strong mediator of inflammation. We want to clarify the mechanism and effect of LXA4 on corneal collagen degradation by a three dimensional culture system. We will manifest whether LXA4 can be a new potent option in the treatment of corneal desquamese pathology process.
LXA4 inhibition on corneal dissolving process

MATERIALS AND METHODS

Materials Dulbecco's phosphate-buffered saline (DPBS), Minimum Essential Medium Eagle (MEM) and trypsin-EDTA were obtained from Weibo Chem company. Type I collagen (acid solubilized), 5×Dulbecco's modified Eagle's medium (DMEM) were from Nitta Gelatin Co, LTD. Fetal bovine serum (FBS) was from Shanghai Yantuo Biotecnology (Shanghai, China). Bovine plasminogen, protease inhibitor cocktail and LXA4 were from Chemicalbook (Shanghai, China). Recombinant human IL-1β was obtained from R&D Systems. Mouse monoclonal antibodies to rabbit MMP-1 and MMP-3 were obtained from antibodies-online (German). An enhanced chemiluminescence (ECL) kit was from GE Healthcare (Qfbio, Shanghai, China). Coomassie brilliant blue and gelatin were obtained from Bio-Rad (Seajet scintic Inc, Beijing, China). A cytotoxicity assay (CytoTox 96Non-Radioactive) was from Promega (Beijing, China).

Methods

Cell isolation Rabbit corneal fibroblasts were isolated and maintained as described previously [16-19]. 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°C. After mechanical removal of the epithelial sheet, the remaining tissue was treated with collagenase (2mg/mL, in MEM) at 37°C until a single-cell suspension of corneal fibroblasts was obtained. The isolated corneal fibroblasts were cultured under a humidified atmosphere of 5% CO₂ at 37°C in 60-mm culture dishes 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 [16-19]. In brief, corneal fibroblasts were harvested by exposure to Trypsin followed by centrifugation at 15 000g for 5 minutes, and they were then suspended in serum-free MEM. Acid-solubilized collagen type I (3mg/mL), 5×DMEM, collagen reconstitution buffer, and corneal fibroblast suspension (2.2×10⁶/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 5% CO₂ at 37°C, 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 [16-19]. 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°C, and the amount of hydroxyproline in the hydrolysate was determined by measurement of absorbance at 558nm with a spectrophotometer.

Immunoblot analysis Immunoblot analysis of MMP-1, -3 was performed as described previously [16-19]. 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 electrophotorectively to a nitrocellulose membrane. Nonspecific sites of the membrane were blocked, and it was then incubated with antibodies to MMP-1, -3. 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 [16-19]. In brief, culture supernatants from collagen gel incubations were mixed with SDS sample buffer by the ratio of 2:1, and the resulting mixture were subjected to SDS-polyacrylamide gel electrophoresis in the dark at 4°C 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°C in a reaction mixture containing 50mmol/L Tris-HCl (pH 7.5), 5mmol/L CaCl₂, 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, 2×10⁶ 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. 0.1% Triton was taken as positive control. Supernatants and Substrate were mixed in assay buffer in a new plate (1:1 vol, 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 LXA4 on IL-1β induced collagen degradation by corneal fibroblasts IL-1β can markedly increases the extent of collagen degradation by cultured corneal fibroblasts as before [16-19]. To investigate and analysis the inhibition effect of LXA4 on collagen degradation resulting from IL-1β stimulation in three dimensional cultures of rabbit corneal fibroblasts, the cells incubated for 48 hours with LXA4 (1μmol/L-100μmol/L) resulted in a concentration dependent 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 10nmol/L LXA4. In different time points, the amount of degraded collagen increased

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gradually. 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 10 nmol/L LXA4 at 36 and 48 hours (Figure 2).

**Effects of LXA4 on the expressions of MMP-1, -3**

MMP-1, -3 expressions were detected using the methods of immunoblot analysis. Corneal fibroblasts were cultured in collagen gels for 48 hours in the presence of IL-1β and in the presence of LXA4 (1 nmol/L-100 nmol/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 with IL-1β contained large amounts of 61-kDa and 57-kDa immunoreactive proteins corresponding to pro-MMP-1. 49-kDa and 45-kDa immunoreactive proteins were in correspondence to active MMP-1. LXA4 can inhibit the IL-1β induced increases in pro-MMP-1 and active MMP-1 in a dose dependent manner (Figure 3).

Immunoblot analysis with antibodies to MMP-3 detects 57-kDa and 45-kDa immunoreactive proteins corresponding to pro-MMP-3 and active MMP-3 in the culture supernatants of cells incubated with IL-1β. LXA4 inhibited the IL-1β-induced increases in the pro-MMP-3 and active MMP-3 in a concentration-dependent manner (Figure 4).

**Effects of LXA4 on the expressions of TIMP-1, -2**

Further analyses emphasized the effects of LXA4 on the expressions of TIMPs secreted by corneal fibroblasts. Immunoblot analysis with antibodies to TIMP-1 revealed that TIMP-1 protein levels increased in the presence of IL-1β (0.1 ng/mL) and plasminogen. The increased secretion of TIMP-1 protein levels in the presence of IL-1β (0.1 ng/mL) and plasminogen was inhibited by LXA4. The increased secretion of TIMP-2 protein levels in the presence of IL-1β (0.1 ng/mL) and plasminogen was also inhibited by LXA4.

**Effects of LXA4 on the expression of TIMP-1, -2**

Further analyses emphasized the effects of LXA4 on the expressions of TIMPs secreted by corneal fibroblasts. Immunoblot analysis with antibodies to TIMP-1 revealed that TIMP-1 protein levels increased in the presence of IL-1β (0.1 ng/mL) and plasminogen. The increased secretion of TIMP-2 protein levels in the presence of IL-1β (0.1 ng/mL) and plasminogen was inhibited by LXA4.
TIMP-1 can be inhibited by LXA4 in a dose-dependent manner (Figure 5). LXA4 can also inhibit the expression of TIMP-2 by corneal fibroblasts (Figure 6). TIMP-1 and TIMP-2 are produced in relation to urokinase (uPA) and MMP-1, -9. TIMP-1, -2, -3, -9 in corneal fibroblasts exposed to IL-1β in a dose-dependent manner.

Fibroblast cells in affected areas trigger cytokine and chemokine production, which recruit leukocytes and activate MMPs, thus destroying the inflammation tissue [20]. The anti-MMP treatment may provide a therapeutic tool for the pathological destruction of ECM and chronic inflammatory disease [21].

IL-1β is an important marker of inflammation in many diseases [22-24]. We proved that IL-1β can induce collagen degradation by corneal fibroblasts and activate MMPs expression as before. LXA4 can inhibit MMPs expression induced by IL-1β, thus inhibiting the corneal collagen degradation.

As an endogenous, anti-inflammatory mediator, LXA4 was involved in the resolution of inflammation. LXA4 can inhibit the expressions of MMPs and stimulate the expressions of TIMPs in many pathological processes. LXA4 can be a negative feedback loop opposing inflammatory cytokine induced inflammation [11-13,25-26].

We found that LXA4 can inhibit the expressions of TIMP inhibitors of MMPs (TIMPs) not as anticipated. TIMP-1, -2, -3, -9 are produced in relation to urokinase (uPA) and MMP-1, -9 in corneal 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 [27]. LXA4 can inhibit corneal collagen degradation induced by IL-1β, this effect was the consequence of the reduction of MMP-1, -2, -3, -9 and TIMP-1, -2. LXA4 showed no evident cytotoxicity on corneal fibroblasts. Therefore, LXA4 may have therapeutic potential for corneal dissolved collagen degradation induced by cytokine thus can be a potent drug for corneal inflammation disease.

REFERENCES

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Figure 5 Effects of LXA4 on the expressions of MMP-2, -9 by corneal fibroblasts

Figure 6 Effects of LXA4 on the expression of TIMP-1 by corneal fibroblasts

Figure 7 Effects of LXA4 on the expression of TIMP-2 by corneal fibroblasts

Figure 8 Lack of a cytotoxic effect of LXA4 on corneal fibroblasts


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