Alarmins from conjunctival fibroblasts up-regulate matrix metalloproteinases in corneal fibroblasts

Lin Chen¹, Ye Liu², Xiao-Shuo Zheng¹, Hui Zheng¹, Ping-Ping Liu¹, Xiu-Xia Yang¹, Yang Liu¹

¹Department of Ophthalmology, the Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai 519000, Guangdong Province, China
²Department of Pathology, the Fifth Affiliated Hospital, Sun Yat-sen University, 52 Road Meihuadong, Zhuhai 519000, Guangdong Province, China

Correspondence to: Yang Liu. Department of Ophthalmology, the Fifth Affiliated Hospital, Sun Yat-sen University, 52 Road Meihuadong, Zhuhai 519000, Guangdong Province, China. yangliu_manu@163.com

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Abstract

- **AIM:** To explore the effects of alarmins produced by necrotic human conjunctival fibroblasts on the release of matrix metalloproteinases (MMPs) by human corneal fibroblasts (HCFs).

- **METHODS:** A necrotic cell supernatant (NHCS) was prepared by subjecting human conjunctival fibroblasts to three cycles of freezing and thawing. The amounts of interleukin (IL)-1β and tumor necrosis factor (TNF)-α in NHCS were determined by enzyme-linked immunosorbent assays. HCFs exposed to NHCS or other agents in culture were assayed for the release of MMPs as well as for intracellular signaling by immunoblot analysis. The abundance of MMP mRNAs in HCFs was examined by reverse transcription and real-time polymerase chain reaction analysis.

- **RESULTS:** NHCS increased the release of MMP-1 and MMP-3 by HCFs as well as the amounts of the corresponding mRNAs in the cells. NHCS also induced activation of mitogen-activated protein kinase (MAPK) signaling pathways mediated by extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK) as well as elicited that of the nuclear factor (NF)-κB signaling pathway by promoting phosphorylation of the endogenous NF-κB inhibitor IκB-α. Inhibitors of MAPK and NF-κB signaling as well as IL-1 and TNF-α receptor antagonists attenuated the NHCS-induced release of MMP-1 and MMP-3 by HCFs. Furthermore, IL-1β and TNF-α were both detected in NHCS, and treatment of HCFs with these cytokines induced the release of MMP-1 and MMP-3 in a concentration-dependent manner.

- **CONCLUSION:** Alarmins, including IL-1β and TNF-α, produced by necrotic human conjunctival fibroblasts triggered MMP release in HCFs through activation of MAPK and NF-κB signaling. IL-1β and TNF-α are therefore potential therapeutic targets for the amelioration of corneal stromal degradation in severe ocular burns.

- **KEYWORDS:** alarmin; ocular burn; matrix metalloproteinase; corneal fibroblast; conjunctival fibroblast; inflammation

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INTRODUCTION

Ocular burns, including alkali, acid, and heat burns, are common and serious ocular emergencies, accounting for 8%-18% of ocular trauma[1]. A recent study reported that the incidence of chemical eye burns was comparatively high[2]. Severe ocular burns, even if treated in a timely fashion, often give rise to persistent corneal stromal ulcers, corneal perforation, and corneal neovascularization, eventually leading to loss of vision. Various medical and surgical treatments have been proposed and confirmed to improve the prognosis of ocular burns: steroids, tetracycline, or ascorbic acid; tendinoplasty; amniotic membrane patch[3-4]. However, the effectiveness of these therapies in treating persistent corneal stromal melting remains unsatisfactory. More effective treatments for ocular burns are needed.

The cornea is made up mostly of stroma. The extracellular matrix (ECM) and keratocytes are two major elements of corneal stroma[5]. Keratocytes are generally quiescent cells; however, under pathological conditions (e.g., infection, injury), keratocytes are activated to become corneal fibroblasts or myofibroblasts[6]. In response to injury and/or stress, activated keratocytes release matrix-degrading enzymes, such as metalloproteinases (MMPs). Experiments performed in an animal model of alkali-burn injury revealed increased levels of collagenase (MMP-1) and stromelysin (MMP-3) in the cornea[7].
The expression of MMP-1 and MMP-3 has been shown to be significantly associated with collagen degradation. The investigation of mechanisms underlying MMP homeostasis may offer new insight into the treatment of ocular burns. Damage-related molecular patterns (DAMPs), also known as alarmins, are endogenous molecules released by damaged cells into the extracellular environment in response to non-infectious stress or injury. The signals sent by alarmins to surrounding tissues recruit inflammatory cells to injured tissue and trigger a sterile inflammatory response. Severe ocular burns can induce necrosis of ocular surface tissue and thereby give rise to sterile inflammation, which plays a crucial role in the pathogenesis of subsequent corneal stromal ulceration. Given that the conjunctiva occupies a larger area of the eye in the pathogenesis of subsequent corneal stromal ulceration, alarmins up-regulate MMPs in corneal fibroblasts as alarmins, are endogenous molecules released by damaged cells into the extracellular environment in response to non-infectious stress or injury. Alarmins released by necrotic corneal epithelial cells can induce intact corneal epithelial cells to release inflammatory factors, effectively destroying the barrier function of corneal epithelial cells. However, little is known about the effects of alarmins from human conjunctival fibroblasts on initiation and amplification of the non-infectious inflammatory response in corneal stroma.

In current study, we investigated the effects of alarmins produced by necrotic human conjunctival fibroblasts on the release of MMPs by human corneal fibroblasts (HCFs) and sought to identify the underlying mechanisms.

**MATERIALS AND METHODS**

**Materials** Phosphate-buffered saline (PBS), HEPEs, 0.25% trypsin-EDTA, 10% fetal bovine serum (FBS) and Eagle’s minimum essential medium (MEM) were purchased from Invitrogen-Gibco company (Rockville, MD, USA). Cell culture dishes and flasks were purchased from Corning company (Corning, NY, USA). Monoclonal antibodies to extracellular signal-regulated kinase (ERK), phospho-ERK, p38, phospho-p38, c-Jun NH2-terminal kinase (JNK), phospho-JNK, IκB-α, and phospho-IκB-α were obtained from Cell Signaling Technology company (Beverly, MA, USA). Goat polyclonal antibodies to human MMP-1 and MMP-3 as well as an interleukin-1 receptor (IL-1R) antagonist was obtained from R&D Systems (Minneapolis, MN, USA). A TNF-α receptor antagonist (R7050) was purchased from MCE (Billerica, CA, USA). ERK inhibitor (PD98059), JNK inhibitor II, p38 inhibitor (SB203580), and nuclear factor-κB (NF-κB) endogenous inhibitor [IκB kinase (IKK)-2 inhibitor] were purchased from Merck Millipore (Temecula, CA, USA). Mouse monoclonal antibodies to β-actin, penicillin and streptomycin were purchased from Boster (Wuhan, China). Cell counting kit-8 (CCK8) reagents was obtained from Dojindo (Kumamoto, Japan). All reagents and media used for cell culture in the present study were endotoxin minimized.

**Cell Culture** Human conjunctival fibroblasts and HCFs were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). Cells were passaged in 10% FBS-MEM [Eagle’s MEM supplemented with 10% FBS, streptomycin (100 μg/mL), and penicillin (100 U/mL)]. HCFs were cultured at 37°C under normoxic conditions (21% oxygen, 5% CO₂, 74% nitrogen), stock cultured in 75- or 175-cm² flasks, and passaged every 10-12d. HCFs were then isolated by treatment with trypsin-EDTA. Cells at passage 4-6 were used for subsequent experiments.

**Preparation of Necrotic Cell Supernatant** Necrotic human conjunctival fibroblasts were induced by repeated freezing and thawing, as described previously. Briefly, confluent human conjunctival fibroblasts were washed twice with PBS, then isolated by treatment with trypsin-EDTA at 37°C, and collected in 10% FBS-MEM. After two washes with MEM, cells were resuspended in MEM at a density of 1×10⁶ cells/mL. Cells were subject to three rounds of rapid freezing at -196°C for 3min in liquid nitrogen, then thawed at 37°C for an additional 3min in a 37°C water bath. After cell lysates were centrifuged at 15 000 rpm for 10min at 4°C, necrotic human conjunctival fibroblast supernatants (NHCS) was collected.

**Immunoblot Analysis** Immunoblot analysis was performed to measure levels of MMP-1, MMP-3, ERK, JNK, p38, and IκB-α, as described previously in detail. Immunoblot analysis was performed to measure levels of MMP-1, MMP-3, ERK, JNK, p38, and IκB-α, as described previously in detail. HCFs were first cultured in 60-mm dishes (5×10⁵ cells per dish) for 24h in MEM supplemented with 10% FBS. After an additional 24h in serum-free medium, HCFs were incubated for 24h in serum-free medium supplemented with one of various concentrations of NHCS (0, 0.1, 0.3, 1, or 3×10⁵ cells/mL), or incubated in serum-free medium supplemented with 3×10⁵ cells/mL NHCS for various lengths of time. Samples of culture supernatant (30 μL) or cell lysate (10 μg protein) were fractionated by SDS-polyacrylamide gel electrophoresis in a 10% gel. Segregated proteins were moved to a polyvinylidene difluoride membrane, which was then exposed to blocking solution [20 mmol/L Tris-HCl (pH 7.4), 5% skim milk, 0.1% Tween-20] for 1h at room temperature. Membranes were then incubated overnight at 4°C with primary antibody in blocking solution at a dilution of 1:1000. Membranes were washed 4 times (10min each) in washing buffer. Membranes were washed again after incubation with horseradish peroxidase-conjugated secondary antibody for 1h at room temperature. Immune complex levels
Assay of IL-1α, IL-1β, and TNF-α
NHCS was induced by repeated freezing and thawing of human conjunctival fibroblasts. After centrifuged at 15 000 rpm for 10 min at 4℃, TNF-α, IL-1α and IL-1β release in the NHCS was examined with ELISA kits according to the instruction.

Quantitative Reverse Transcription-Polymerase Chain Reaction Analysis
Serum-deprived HCFs were incubated with NHCS (3×10^5 cells/mL) for 24h. Total RNA was isolated from cells with the use of the Trizol reagent and was subjected to reverse transcription (RT). The resulting cDNA was subjected to quantitative RT-polymerase chain reaction (RT-PCR) analysis essentially as previously described with the use of a LightCycler instrument (Roche Molecular Biochemicals, Indianapolis, IN, USA). The sequences of the PCR primers were as follows: MMP-1 sense, 5’-CGACTCTAGAACAAGAGGCAAGA-3’; MMP-1 antisense, 5’-AAGGTTAGTTACTGTCACACGC TT-3’; MMP-3 sense, 5’-GCCACAATATGGGCACTTTA-3’; MMP-3 antisense, 5’-CCGGCAAGATACAGATTCAC-3’; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5’-GCCAAAAGGGTCATCATCTC-3’; and GAPDH antisense, 5’-ACCACCTGGTGCTCAGTGTA-3’. The PCR protocol comprised denaturation at 94℃ for 15s, annealing at 58℃ for 20s, and elongation at 72℃ for 13s for the amplification. Real-time PCR data were analyzed with LightCycler ver.3.1 software. The abundance of MMP-1 and MMP-3 mRNAs was normalized by that of GAPDH mRNA.

Assay of Cell Viability
HCFs (5×10^3 cells/well) were seeded into a 96-well plate. After incubation for 24h in serum-free medium, HCFs were treated with NHCS (0, 0.1, 0.3, 1, or 3×10^5 cells/mL) for 24h. CCK8 reagent (10 μL) was added to cells, which were then incubated at 37℃ for an additional 2h. Cell viability was measured with a microplate reader (BIO-RAD, USA). Human conjunctival fibroblasts were counted with a cellometer (Nexcelom Bioscience, MA, USA).

Statistical Analysis
SPSS version 19.0 (IBM Corp.) was used for data analysis. Quantitative data are presented as mean±standard deviation (SD). Differences among groups were analyzed by one-way ANOVA, followed by LSD test. P<0.05 was considered statistically significant.

RESULTS
Effects of NHCS on the Release of MMP-1 and MMP-3 by HCFs
HCFs were obtained from serum, then exposed to various concentrations of NHCS (0, 0.1, 0.3, 1, or 3×10^5 cells/mL) for the same time (24h). Immunoblot analysis showed that treatment with NHCS increased the production of MMP-1 and MMP-3 in a concentration-dependent manner (Figure 1). HCFs also showed time-dependent increases in the production of MMP-1 and MMP-3 after exposure to 3×10^5 cells/mL NHCS for various durations of time (Figure 2). The effect of NHCS on the abundance of MMP-1 and MMP-3 mRNA in the cells was examined by quantitative RT-PCR analysis. Incubation of HCFs with NHCS (3×10^5 cells/mL) for 24h resulted in significant increase of the amount of MMP-1 and MMP-3 mRNA (Figure 3).

Effects of MAPK and NF-κB Signaling Inhibitors on NHCS-Induced Release of MMP-1 and MMP-3 by HCFs
To identify possible roles of NF-κB and MAPK signaling molecules in the expression of MMP-1 and MMP-3 by HCFs,
serum-deprived HCFs were first incubated with MAPK or IKK-2 inhibitor (10 μmol/L) for 2h, then incubated with 3×10^5 cells/mL NHCS for another 24h. Immunoblot analysis indicated that the NHCS-induced production of MMP-1 and MMP-3 by HCFs was inhibited by treatment with an inhibitor of ERK, p38, JNK II, or IKK-2 (Figure 5).

Effects of IL-1 Receptor and TNF-α Receptor Antagonists on NHCS-Induced Release of MMP-1 and MMP-3 by HCFs

Serum-deprived HCFs were first incubated with IL-1 receptor antagonist (IL-1RA, 100 ng/mL) or TNF-α receptor antagonist (R7050, 1000 ng/mL) for 2h, then incubated with 3×10^5 cells/mL NHCS for another 24h. We found that exposure of HCFs to an IL-1 receptor antagonist or TNF-α receptor antagonist resulted in a marked decrease in the release of MMP-1 and MMP-3 (Figure 6).

Presence of IL-1β and TNF-α in NHCS and Effects on MMP-1 and MMP-3 Released by HCFs

To examine the possible alarmins released by necrotic conjunctival fibroblasts, we measured the TNF-α, IL-1α and IL-1β expression in the NHCS with ELISA assay. The release of TNF-α and IL-1β was found in the NHCS (Figure 7A), but that of IL-1α was not detected. To check the effect of these cytokines on MMPs expression, serum-deprived HCFs was incubated with TNF-α or IL-1β (0-100 pg/mL) for 24h. Immunoblot analysis indicated that IL-1β and TNF-α induced the expression of MMP-1 and MMP-3 by HCFs in a dose-dependent manner (Figure 7B).

Effect of NHCS on HCF Viability and Proliferation

The results of CCK8 and cell counting assays showed that exposure to different concentrations of NHCS [(0-3)×10^5 cells/mL] for 24h had no significantly effect on the proliferation or viability of HCFs (Figure 8).

**DISCUSSION**

In the present study, we show that NHCS stimulated the release of MMP-1 and MMP-3 by HCFs at protein and mRNA level. NHCS activated the MAPK pathway via ERK, p38, and JNK and activated the NF-κB pathway via IκB-α. The production of MMP-1 and MMP-3 by HCFs was decreased by inhibition of TNF-α or IL-1β (0-100 pg/mL) for 24h. Immunoblot analysis indicated that IL-1β and TNF-α induced the expression of MMP-1 and MMP-3 by HCFs in a dose-dependent manner (Figure 7B).
Figure 5 Effects of inhibitors of MAPK and NF-κB signaling on NHCS-induced MMP-1 and MMP-3 production by HCFs. A: HCFs were treated with or without PD98059, SB203580, JNK inhibitor II, or IKK2 inhibitor (each at 10 µmol/L) for 2h and then in the additional absence or presence of NHCS (3×10⁵ cells/mL) for 24h. The release of MMP-1 and MMP-3 in the cell supernatants were examined by immunoblot analysis; B: Immunoblots subjected to densitometric analysis in order to determine band intensity. Error bars represent SD. *P<0.05 compared with the corresponding value of cells incubated without treatment; †P<0.05 compared with the corresponding value for cells incubated with NHCS only.

Figure 6 Effects of IL-1 receptor and TNF-α receptor antagonists on NHCS-induced MMP-1 and MMP-3 release by HCFs. A: HCFs were incubated with or without IL-1RA (100 ng/mL) or the TNF-α receptor antagonist R7050 (1000 ng/mL) for 2h and then in the additional absence or presence of NHCS (3×10⁵ cells/mL) for 24h. The release of MMP-1 and MMP-3 in the cell supernatants were examined by immunoblot analysis; B: Immunoblots subjected to densitometric analysis in order to determine band intensity. Error bars represent SD. *P<0.05 compared with the corresponding value of cells incubated without treatment; †P<0.05 compared with the corresponding value for cells incubated with NHCS only.

Figure 7 Presence of IL-1β and TNF-α in NHCS and effects of these cytokines on MMP release by HCFs. A: The concentrations of IL-1β and TNF-α in NHCS (3×10⁵ cells/mL) were measured with ELISA. Error bars represent SD; *P<0.05 versus corresponding value for serum-free MEM (SF-MEM). B: Serum-deprived HCFs were incubated in the presence of the indicated concentrations of IL-1β or TNF-α for 24h. The release of MMP-1 and MMP-3 in the cell supernatants were examined by immunoblot analysis. Data are representative of three independent experiments.
the MAPK and NF-κB pathway, as well as treatment with an antagonist of IL-1 or TNF-α receptor.

MMPs mediate degradation of the ECM in a broad array of tissues and participate in the occurrence and progression of numerous diseases [16-17]. ECM remodeling and proteolytic degradation requires homeostasis among MMPs, endogenous MMP inhibitors, and tissue inhibitors of metalloproteinases (TIMPs) [17]. The overexpression of MMPs can destroy the homeostasis of the corneal stroma, leading to persistent corneal epithelial defects, stromal ulcer, and poor wound healing [18]. In intact cornea, MMPs are expressed at barely detectable levels. However, the transcription and immunoreactivity of MMPs are significantly elevated after chemical burn and during corneal wound healing [17]. Up-regulated expression of MMP-1 and MMP-3 was found in a corneal stromal wound rabbit model [19]. In the present study, we found that NHCS enhanced the secretion of MMP-1 and MMP-3 by HCFs in a time- and dose-dependent pattern. Our findings suggest that the necrosis of conjunctival fibroblasts may contribute to degradation of the corneal stroma by inducing corneal fibroblasts to overexpress MMP-1 and MMP-3. An imbalance between the activities of TIMPs and MMPs has been involved in the pathogenesis of corneal ulceration [19]. Besides MMP-1 and MMP-3, it is reported that other MMPs such as MMP-2, MMP-9, MMP-8 or MMP-13 [21-22], as well as that of TIMPs are abnormally expressed during the process of corneal ulceration. MMP-9 or MMP-8 were significantly up-regulated in the tear fluid of patients with peripheral noninfectious corneal ulcers, whereas TIMP-1 and TIMP-2 concentrations did not change [22]. Both active MMP-2 and MMP-9 were detected in tear fluid in patients with corneal ulcer or ocular burn [24]. The effect of NHCS on the production of these TIMPs and MMPs remain to be investigated, however.

The alarmins released by damaged cells include high mobility group box (HMGB)-1, heat shock proteins, S100 proteins, IL-1α, calreticulin, and fibrinogen degradation products [25]. These molecules can trigger non-infectious inflammation to promote wound healing and restore homeostasis [26]. The IL-1 cytokine is a typical DAMPs. This molecule mediates a variety of inflammatory reactions. The IL-1 receptor antagonist is an inhibitor that attenuates the biological activity of IL-1 by binding to the IL-1 receptor [25,27]. TNF-α is an inflammatory cytokines known to be involved in corneal inflammation. One previous study conducted in a mouse model of alkali burn showed that TNF-α is highly upregulated within 24h, ultimately mediating corneal perforation and/or scarring [28]. In animal and clinical studies, anti-TNF-α antibodies have been shown to be effective in protecting the cornea and promoting wound healing [29]. In our study, we found that TNF-α and IL-1β was released in the NHCS and treatment of HCFs with TNF-α and IL-1β induced the release of MMP-3 and MMP-1 in a concentration-dependent manner. In addition, an IL-1 receptor antagonist or a TNF-α receptor antagonist inhibited the NHCS-induced expression of MMP-3 and MMP-1 by HCFs. This finding suggests that IL-1β and TNF-α signaling mediate necrosis-triggered MMP expression in HCFs. IL-1β and TNF-α are dangerous signals released by necrotic human conjunctival fibroblasts that mediate the sterile inflammatory response.

Several signaling pathways, including those mediated by MAPK and NF-κB, are considered to underlie the pathogenesis of corneal ulceration, which is characterized by excessive degradation of the corneal stroma. The MAPK pathway comprises a family of highly conserved serine/threonine protein kinases. This pathway is thought to regulate various physiological or pathological processes, including cell proliferation and differentiation, inflammation, and apoptosis [30-32]. Three major MAPK family members are ERK, p38, and JNK [33-34]. The NF-κB pathway mediates inflammation. NF-κB usually binds to its inhibitory protein (IκB) in the inactive state. NF-κB signaling is activated after stimulation by various pathological factors and after the phosphorylation and degradation of IκB-α [35]. Various MMP genes are thought to be targets of the transactivation activity of NF-κB [36]. Both MAPK and NF-κB signaling can be activated by IL-1 or TNF-α. These cytokines may regulate the production of MMP-3 and MMP-1 through MAPK or NF-κB signaling in different cells differentially.
induces activation of the MMP-1 gene promoter in corneal fibroblasts and this effect is mediated by the transcription factors NF-kB[33]. ERK, but not NF-kB pathways and JNK, is particularly important for IL-1β regulating MMP-1 expression in chondrocytes[10]. TNF-α stimulates MMP-3 production through PGE2 signalling and via p38 MAPK and NF-κB pathway in a murine cementoblast cell line[39]. However, TNF-α induced MMP-3 or MMP-1 release to participate in the regulation of inflammation-associated cancer metastasis through C/EBPβ signal and activation of p38 MAPK, but not other MAPKs or NF-kB[39]. Our results demonstrated that NHCS increases the release of MMP-1 and MMP-3 by HCFs and activated of MAPKs and NF-κB signaling. The NHCS-induced release of MMP-1 and MMP-3 in HCFs was attenuated by treatment with MAPK ERK, p38, JNK or IKK-2 inhibitors. These results thus indicate that the NHCS-induced production of MMP-1 and MMP-3 by HCFs is mediated by three MAPKs and NF-kB signaling.

In summary, these results suggest that the IL-1β and TNF-α released by necrotic conjunctival fibroblasts trigger the secretion of MMPs from corneal fibroblasts via MAPK and NF-kB signaling. IL-1β and TNF-α represent potential therapeutic targets for the treatment of corneal stromal degradation in patients with severe ocular burns.

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REFERENCES


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20 Riley GP, Harrall RL, Watson PG, Cawston TE, Hazleman BL. Collagenase (MMP-1) and TIMP-1 in destructive corneal disease associated with rheumatoid arthritis. Eye (Lond) 1995;9( Pt 6):703-718.


25 Martin SJ. Cell death and inflammation: the case for IL-1 family cytokines as the canonical DAMPs of the immune system. FERS J 2016;28(14):2599-2615.


