TNF- α Stimulates MMP-2 and MMP-9 Activities in Human Corneal Epithelial Cells via the Activation of FAK/ERK Signaling

Yan-Ning Yang^a Fang Wang^a Wei Zhou^b Zhi-Qing Wu^a Yi-Qiao Xing^a

^aDepartment of Ophthalmology, and ^bThe Institute of Gastroenterology and Hepatology, Renmin Hospital of Wuhan University, Wuhan, China

Key Words

Herpes simplex keratitis • Herpes simplex virus type 1 • Tumor necrosis factor- α • Focal adhesion kinase • Extracellular regulated protein kinase

Abstract

Aims: Herpes simplex virus type-1-induced herpes simplex keratitis (HSK) is a common immunological cornea disease. While previous studies have addressed the role of tumor necrosis factor (TNF)- α and matrix metalloproteinases (MMPs) in HSK, the mechanistic link between TNF- α and MMPs in the pathogenesis of HSK remains elusive. **Methods:** We first established a HSK mice model and measured the levels of TNF- α , MMP-2 and MMP-9 in the corneas at different time points by ELISA. Next, we employed cultured human corneal epithelial (HCE) cells as an in vitro model and performed gelatin zymography analysis. **Results:** We observed that the change in the TNF- α level shared a similar pattern to that of MMP-2 and MMP-9 in the HSK mice model. Furthermore, TNF- α stimulated MMP-2 and MMP-9 activities in a dose-dependent manner, but either knockdown of focal adhesion kinase (FAK) by short interference RNA or inhibition of extracellular regulated protein kinase (ERK) by chemical inhibitor could block TNF- α -stimulated MMP-2 and MMP-9 activities in vitro.

Taken together, our results provide in vivo evidence that the TNF- α level is positively correlated with MMP-2 and MMP-9 levels in a HSK model and in vitro evidence that TNF- α stimulates MMP-2 and MMP-9 activities via the activation of FAK/ERK signaling in HCE cells. **Conclusions:** Our findings shed new light on the pathogenesis of HSK and open up new possibility of modulating the TNF- α -FAK-ERK signaling cascade to pursue therapeutic measures for HSK.

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Introduction

Tumor necrosis factor- α (TNF- α) is a proinflammatory mediator that plays an important role in a variety of corneal diseases [1]. Massingale et al. [2] compared the tears from 7 patients with dry eye disease and 7 healthy volunteers and found that the concentrations of cytokines including TNF- α were significantly increased in the tears of dry eye patients as compared to normal controls, suggesting that TNF- α may play an important role in dry eye disease. Furthermore, Kimura [3] reported that TNF- α disrupted the barrier function of human corneal epithelial (HCE) cells and contributed to ocular inflammation.

Herpes simplex virus type 1 (HSV-1)-induced herpes simplex keratitis (HSK) is a common immunopathological cornea disease characterized by recurrent destructive inflammation and progressive scarring in the corneal stroma [4]. The production of a large amount of TNF- α has been reported in HSV-infected murine corneas [5]. Importantly, a family of extracellular endoproteinases called 'matrix metalloproteinases' (MMPs) has been implicated in HSK [6]. The two most widely studied members of this family, MMP-2 and MMP-9 (gelatinase A and gelatinase B), are known as the primary extracellular matrix remodeling enzymes that participate in pathologic conditions of the cornea such as infection, dry eye and neovascularization [7–9]. However, few studies have addressed the potential link/interaction between TNF- α and MMPs in HSK.

Interestingly, it was recently shown that TNF- α stimulated MMP-9 production in cholangiocarcinoma and this was mediated by the activation of focal adhesion kinase (FAK) [10]. FAK is a non-receptor tyrosine kinase that plays an important role in the survival, proliferation and migration of normal and tumor cells [11, 12]. The formation of the FAK-Src-p130Cas-Dock180 complex activated Rac and JNK, leading to increased expression and activities of MMP-2 and MMP-9 in fibroblasts [13]. However, it remained unclear whether TNF- α could stimulate the expression of MMP-2 and MMP-9 in HCE cells and whether FAK signaling is involved in this process.

Therefore, in the present study, we first established a HSK mice model and examined the levels of TNF- α and MMPs in the corneas in order to investigate the correlation between TNF- α and MMP levels during HSK. Next, we employed cultured HCE cells as a model to further explore the mechanistic link between TNF- α and MMPs during HSK. We found that TNF- α stimulated MMP-2 and MMP-9 activity by cultured HCE cells in a dose-dependent manner and this could be inhibited by knock-down of FAK or chemical inhibitor of extracellular regulated protein kinase (ERK), suggesting that TNF- α may play a role in HSK by stimulating MMP activity via the FAK/ERK pathway.

Methods

Virus

The HSV-1 (KOS strain) was kindly provided by Dr. Wei Hou (Institute of Virology, Wuhan University Medical School, Wuhan, China). The virus was isolated and expanded on Vero cells and the virus-containing supernatants were collected. The plaque-forming units were determined in a standard plaque assay.

Mice

All animal studies were conducted according to the Chinese National Guidelines for the Care and Use of Laboratory Animals. Female BALB/c mice aged 6–8 weeks were anesthetized intraperitoneally, and the right cornea was scratched eight times. Then, 10^5 plaque-forming units of HSV-1 was applied to the cornea, as described elsewhere [14].

TNF- α Level in the Cornea

The cornea was taken from the mice and homogenized at days 2, 7, 14 and 28 after infection. Then, the TNF- α level was measured by TNF- α ELISA kit (BD Pharmingen) according to the manufacturer's protocol. The TNF- α level was determined based on photometric measurement of optical density at 450 nm by comparison with standards.

MMP-2 and MMP-9 Levels in the Cornea

The cornea was taken from the mice and homogenized at days 2, 7, 14 and 28 after infection. Then, MMP-2 and MMP-9 levels were measured by MMP-2 and MMP-9 ELISA kits (Ray-Biotech) at the same time point, according to the manufacturer's protocol.

Cell Culture

An SV-40 transformed HCE cell line was kindly provided by Dr. Lixin Xie (Shandong Eye Institute, China). The cells were cultured in supplemented hormone epithelial medium which consisted of DMEM/F-12 medium supplemented with 10% fetal bovine serum, 5% DMSO, 5 ng/ml epidermal growth factor and 0.005 mg/ml insulin.

Short Interference RNA Transfection

HCE cells were cultured in 6-well plates and transfected with 12 μ l FAK short interference RNA (siRNA; Santa Cruz Biotech) or scramble control siRNA (Santa Cruz Biotech) using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. After culture for 7 h at 37°C in a CO₂ incubator, 1 ml DMEM/F-12 medium supplemented with 20% fetal bovine serum was added into every well and the cells were incubated for an additional 24 h before the following experiments. Transfection efficiency was examined by Western blotting.

TNF- α Treatment

HCE cells were incubated in serum-free medium for 24 h. Then, the cells were randomly divided into 4 groups and treated with different concentrations of TNF- α (0, 1, 10 and 100 ng/ml).

Zymography

The conditioned media of each group of cells were collected and centrifuged at 4,000 rpm for 10 min at 4°C. The supernatant was collected and the protein concentration was quantitated by the BSA method. 5 μ g protein was mixed with SDS-PAGE sample buffer without boiling or reduction, then loaded onto 8% polyacrylamide gels containing gelatin (1 mg/ml) for electrophoresis at 100 V for 90 min at 4°C. The gels were soaked in 0.25% Triton X-100 for 30 min at room temperature to remove SDS and incubated in digestion buffer containing 5 mM PMSF at 37°C overnight to allow proteinase digestion of its substrate. Gels were rinsed again in distilled water, stained with 0.25% Coomassie brilliant blue R-250 in 40% isopropanol for 2 h and destained with

Fig. 1. TNF- α , MMP-2 and MMP-9 levels are correlated in the cornea of HSV-1-infected mice. The corneas were taken from HSV-1-infected mice, and TNF- α , MMP-2 and MMP-9 levels in the homogenized corneas were detected by ELISA. Data were expressed as the mean $\pm$ SD from three independent experiments.

7% acetic acid. Gelatinolytic activity appeared as clear bands of digested gelatin against a dark blue background of stained gelatin.

Immunoblotting

Proteins were extracted from each group of cells using cell lysis buffer and quantitated by BSA method. 10 μ g protein was separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk for 1 h at room temperature, then incubated with first antibodies (Santa Cruz) overnight at 4°C. The membrane was washed three times in Tris-buffered saline with Tween-20, pH 8.0, and then incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce) for 1 h at room temperature. The membrane was washed three times in Tris-buffered saline with Tween-20 and developed using ECL kit (Pierce) and exposed to X-ray film.

Statistical Analysis

Data were statistically analyzed by using ANOVA and a p value <0.05 was considered significant.

Results

TNF- α , MMP-2 and MMP-9 Levels Are Correlated in the Cornea of HSV-1-Infected Mice

Based on the experimental HSK model we established, we detected TNF- α , MMP-2 and MMP-9 levels in the cornea of the HSK mouse by ELISA. As shown in figure 1, MMP-2 and MMP-9 levels were increased in the cor-

Fig. 2. TNF- α activates FAK/ERK signaling in cultured HCE cells. **a** Western blots showing the levels of phosphorylated FAK and ERK1/2 in HCE cells treated with TNF- α (30 min) at the indicated concentration. **b** Western blots showing the levels of phosphorylated FAK and ERK1/2 in HCE cells transfected with control or FAK siRNA and treated with TNF- α (30 min) at the indicated concentration. **c** Evaluation of phosphorylated ERK1/2 expression by immunoblotting. HCE cells were treated with TNF- α (30 min) at the indicated concentration with or without the presence of ERK inhibitor (PB98059, 20 μ M).

neas at day 2 and then reduced at day 7 after infection, but later on, MMP-2 and MMP-9 levels were upregulated at day 14 and 28 after infection. Interestingly, we detected a TNF- α level in the corneas and found that the pattern of the changes in the TNF- α level during the course of HSK was similar to that of MMP-2 and MMP-9 (fig. 1).

Fig. 3. TNF-α enhanced the activity and expression of MMP-2 and MMP-9 by cultured HCE cells via the FAK/ERK pathway. **a** Gelatin zymography of conditioned medium taken from HCE cells treated with TNF-α (24 h) at the indicated concentration. **b** Gelatin zymography of conditioned medium taken from HCE cells transfected with control or FAK siRNA and treated with

TNF-α (24 h) at the indicated concentration. **c** Evaluation of MMP-2 and MMP-9 activity by gelatin zymography. HCE cells were treated with TNF-α (24 h) at the indicated concentration with or without the presence of ERK inhibitor (PB98059, 20 μM). **d** Similar results were observed using immunoblotting analysis. Actin was used as an internal control.

These findings suggest a positive correlation between TNF-α and MMP levels during HSK.

TNF-α Activates FAK/ERK Signaling in Cultured HCE Cells

To further explore the link between TNF-α and MMPs during HSK, we used cultured HCE cells as a model. Because FAK has been shown to play a critical role in TNF-α-stimulated MMP-9 production in cholangiocarcinoma [10], we speculated that TNF-α may promote MMP production in HCE cells via the activation of the FAK/ERK signaling pathway. To test our hypothesis, we treated cul-

tured HCE cells with different concentrations of TNF-α and detected the levels of phosphorylated FAK and ERK1/2 which represent the activation of FAK/ERK signaling. The results showed that TNF-α induced the phosphorylation of FAK and ERK1/2 in HCE cells in a dose-dependent manner (fig. 2a). To confirm that TNF-α activates FAK/ERK signaling in HCE cells, we employed siRNA specific to FAK to knock down FAK in HCE cells and observed that FAK siRNA obviously depleted FAK expression and blocked TNF-α-induced FAK and ERK1/2 phosphorylation (fig. 2b). Furthermore, we treated HCE cells with ERK inhibitor (PB98059) and found that

PB98059 specifically blocked TNF- α -induced ERK1/2 phosphorylation (fig. 3c). Taken together, these results demonstrate that TNF- α activates FAK/ERK signaling in cultured HCE cells.

TNF- α Enhanced the Activity and Expression of MMP-2 and MMP-9 by Cultured HCE Cells via FAK/ERK Pathway

To confirm the role of TNF- α in the regulation of MMP-2 and MMP-9 activity by HCE cells, we performed gelatin zymography to detect the gelatinolytic activity in conditioned media of HCE cells treated by TNF- α . When the cells were incubated with TNF- α for 24 h, the gelatinolytic activity of MMP-2 and MMP-9 was observed to be increased in a dose-dependent manner (fig. 3a). Under these experimental conditions, immunoblot analysis also showed an increased expression of MMP-2 and MMP-9 (fig. 3d). Furthermore, the activity and expression of MMP-2 and MMP-9 in TNF- α -stimulated HCE was significantly attenuated by ERK inhibitor (PB98059) or FAK siRNA (fig. 3b, c). Collectively, these findings suggest that TNF- α enhanced the activity and expression of MMP-2 and MMP-9 by cultured HCE cells via the FAK/ERK pathway.

Discussion

HSK is a common kind of corneal disease. Although substantial progress has been achieved in understanding the infection biology of HSV, the pathogenesis of HSK remains incompletely understood [15]. Recent evidence suggests that the development of HSK involves the complex interactions between infiltrating inflammatory cells and corneal resident cells. Consequently, a variety of chemokines and proinflammatory cytokines are produced, leading to a chronic inflammatory reaction that causes damage to the cornea [16]. Among them, TNF- α ranks as an important factor in the pathogenesis of murine recurrent HSK [17]. On the other hand, neovascularization of the cornea is a prominent event following HSV infection, and Zheng et al. [18] suggested that neovascularization of the cornea is a necessary step in the pathogenesis of HSK based on their findings that inhibiting angiogenesis at an early stage could diminish the severity of HSK. Furthermore, they showed that HSV infection upregulated MMP-9, which then promoted the neovascularization process in the corneal stroma in response to HSV infection [19]. While TNF- α has been reported to stimulate the gelatinolytic activity of MMP-9 in cultured conjunc-

tivochalasis fibroblasts [20], few studies have addressed the mechanistic link between TNF- α and MMPs in the pathogenesis of HSK.

In this study, we first investigated the correlation between TNF- α and MMP levels during HSK using a HSK mice model. By ELISA we measured TNF- α , MMP-2 and MMP-9 levels in the corneas at day 2, 7, 14 and 28 after infection. We observed that MMP-2 and MMP-9 levels were upregulated at day 14 and 28 after infection. Moreover, the TNF- α level in the corneas showed a similar pattern of changes to that of MMP-2 and MMP-9. These data indicate a positive correlation between TNF- α and MMP levels during HSK and suggest a potential link between TNF- α and MMPs in the pathogenesis of HSK.

Next, we employed cultured HCE cells as an in vitro model to explore the mechanistic link between TNF- α and MMPs during HSK. By gelatin zymography immunoblotting analysis, we found that TNF- α dose-dependently stimulated MMP-2 and MMP-9 activity and expression by cultured HCE cells, suggesting that TNF- α plays a positive role in the regulation of MMP-2 and MMP-9 expression and activity.

To further elucidate the molecular mechanisms by which TNF- α upregulates MMP-2 and MMP-9 in HCE cells, we focused on FAK/ERK signaling because FAK has been shown to be crucial to TNF- α -stimulated MMP-9 production in cholangiocarcinoma [10]. First, we proved that in HCE cells TNF- α dose-dependently induced the phosphorylation of FAK and ERK1/2, the hallmarks of the activation of FAK/ERK signaling. Second, we demonstrated that siRNA-mediated knockdown of FAK or ERK inhibitor could block TNF- α -induced ERK1/2 phosphorylation. Taken together, these results suggest the existence of a TNF- α -FAK-ERK signaling cascade in HCE cells. We then investigated the role of the TNF- α -FAK-ERK signaling cascade in regulating MMP activity. By gelatin zymography and immunoblotting analysis, we found that siRNA-mediated knockdown of FAK or ERK inhibitor could block TNF- α -stimulated MMP-2 and MMP-9 activities and expressions in conditioned media of HCE cells. Based on these data, we conclude that TNF- α stimulates MMP-2 and MMP-9 activity by cultured HCE cells via the activation of FAK/ERK signaling. We propose that TNF- α -FAK-ERK signaling-mediated upregulation of MMP-2 and MMP-9 activities could promote neovascularization in the corneal stroma and contribute to the development of HSK [18, 19]. Further in vivo experiments are important to test our hypothesis.

In summary, although further studies are necessary to characterize how FAK/ERK signaling acts downstream

of TNF- α to modulate the expression and activities of MMPs, our present results provide in vivo evidence that the TNF- α level is positively correlated with MMP-2 and MMP-9 levels in a HSK model and in vitro evidence that TNF- α stimulates MMP-2 and MMP-9 activities via the activation of FAK/ERK signaling in HCE cells. The mechanistic link between TNF- α and MMPs we revealed in this study sheds new light on the pathogenesis of HSK and opens up new possibilities of modulating the TNF- α -FAK-ERK signaling cascade to pursue therapeutic measures for HSK.

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Disclosure Statement

No potential conflicts of proprietary interest were disclosed.

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