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Role of MMP-9 in the breakdown of barrier integrity of the corneal endothelium in response to TNF-α

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ABSTRACT

TNF-α induces loss of barrier integrity of the corneal endothelium through mechanisms involving the activation of p38 MAP kinase. This study has investigated the role of matrix metalloproteinase-9 (MMP-9), known to be activated by mechanisms downstream of p38 MAP kinase, on the breakdown of the barrier integrity. Experiments were performed with primary cultures of bovine corneal endothelium. Changes in the trans-endothelial electrical resistance (TER), a measure of barrier integrity, were measured by electric cell-substrate impedance sensing. The integrity of the apical junctional assembly was imaged by immunolocalization of ZO-1. MMP-9 activity in the conditioned medium of cells treated with TNF-α was visualized by gelatin zymography. Transcriptional activation of MMP-9 was assessed by real-time RT-PCR. Exposure to TNF-α led to significant disruption of ZO-1 and also caused a continuous decline in TER for more than 20 h. These effects were opposed by cycloheximide (protein synthesis inhibitor), GM-6001 (broad spectrum inhibitor of MMPs), minocycline (MMP-2 and MMP-9 inhibitor), and MMP-9 inhibitor I (selective MMP-9 inhibitor). Cycloheximide, GM-6001, and MMP-9 inhibitor I also attenuated the increase in permeability to FITC-dextran (10 kDa). In addition, TNF-α led to an increased MMP-9 activity in the conditioned medium as well as a nearly 20-fold increase in mRNA for MMP-9 but not for MMP-2. The functional activity and increase in mRNA levels of MMP-9 were blocked by SB-203580 (selective p38 MAP kinase inhibitor) and cycloheximide. In conclusion, transcriptional and translational activation of MMP-9, downstream of p38 MAP kinase signaling, is involved in the (TNF-α)-induced loss of corneal endothelial barrier integrity.

1. Introduction

The corneal endothelial dysfunction and/or loss of its barrier integrity are implicated in a number of conditions including aging, genetic disorders such as Fuchs' dystrophy, uveitis, and iatrogenic effects (Edelhauser, 2006; Srinivas, 2010, 2012). Currently, there are no pharmacological strategies for treating the endothelial dysfunction, and the only alternative is corneal transplantation. Allogeneic corneal transplantation often triggers immune responses of the host, leading to elevated levels of pro-inflammatory cytokines (Niederkorn 2001; George and Larkin, 2004; Niederkorn, 2007). TNF-α is one such cytokine whose levels are shown to be elevated in the aqueous humor during allograft rejection as well as uveitis (Rayner et al., 2000; Maier et al., 2010, 2011; Valentincic et al., 2011; Borkenstein et al., 2013).

As a pro-inflammatory cytokine, TNF-α is known to elicit dysfunctions in the epithelial and endothelial monolayers by pleiotropic mechanisms that induce apoptosis, upregulation of intercellular adhesion molecule, oxidative stress, microtubule disassembly, and/or barrier disruption (Goldblum et al., 1993; Nusrat et al., 2000; Walsh et al., 2000; Nwariaku et al., 2002; Bruewer et al., 2003; Petrache et al., 2003; Ma et al., 2005; Turner, 2006; Ye et al., 2006; McKenzie and Ridley, 2007). Loss of the barrier integrity is also known to occur in the corneal endothelium (Yin and Watsky, 2005; Shivanna and Srinivas, 2009, Shivanna et al. 2010a,b; Srinivas, 2010, Srinivas, 2012; Hu et al., 2013). However, the underlying molecular mechanisms are not

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yet completely understood. Recently, we have shown that the (TNF-α)-induced loss of barrier integrity involves microtubule disassembly (Shivanna and Srinivas, 2009, Shivanna et al. 2010a,b). We have also demonstrated that inhibition of p38 MAP kinase opposes the (TNF-α)-induced barrier dysfunction (Shivanna et al. 2010a,b). Additional mechanisms are implicated in the (TNF-α)-induced barrier dysfunction in a variety of epithelial and endothelial cell types (Goldblum et al., 1993; Walsh et al., 2000; Shaw et al., 2001; Nwariaku et al., 2002; Bruewer et al., 2003; Petrache et al., 2003; Ma et al., 2005; Turner, 2006; Ye et al., 2006; McKenzie and Ridley, 2007).

A prominent mechanism downstream of p38 MAP kinase is the activation of matrix metalloproteinases (MMPs) (Peifer et al., 2006; Cuenda and Rousseau, 2007; Schindler et al., 2007; Zhang et al., 2007). These are zinc-dependent endopeptidases, which breakdown the extracellular matrix (ECM) (Nagase and Woessner, 1999). Additionally, MMPs are known to act on a variety of substrates including cytokines, chemokines, growth factor binding proteins, cell adhesion molecules, and other proteinases (Fini et al., 1998). Thus far, about 23 different human MMPs have been identified (Visse and Nagase, 2003). Synthesized as pro-enzymes, MMPs are secreted before conversion to their active form. MMPs are modulated at several levels including transcription, pro-enzyme activation, or by their endogenous inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) (Visse and Nagase, 2003; Khokha et al., 2013). An increased expression of MMPs is induced upon inflammation. Upregulation of MMPs is associated with failure of cellular barriers (Lohmann et al., 2004). Specifically, the (TNF-α)-induced loss of blood-cerebrospinal fluid barrier is disrupted by enhanced activity of MMPs. In mice, targeted deletion of MMPs prevents barrier dysfunction upon inflammation (Manicone and McGuire, 2008). MMP-9 (gelatinase B) and MMP-2 cleave several components of the extracellular matrix. A consequent loss of cell-matrix adhesion can have a profound impact on the actin cytoskeleton, which has structural and biochemical associations with the focal adhesion complex (Huveneers and Danen, 2009; Wehrle-Haller, 2012; Kuo, 2013). Consequently, a loss of the cell-matrix adhesion can dismantle the actin cytoskeleton, including the pool of actin cytoskleleton that forms the peri-junctional actomyosin ring (PAMR) in the corneal endothelium. As demonstrated in a number of our previous studies, disruption of PAMR breaks down the barrier integrity of the corneal endothelium concomitant with the dislocation of ZO-1 (Srinivas, 2010, 2012). As an intracellular adapter molecule at the apical junctional complex, (Walsh et al., 2000; Shaw et al., 2001; Bruewer et al., 2003) ZO-1 is contiguous at the cell borders. Thus, dislocation of ZO-1 at the cell border, induced by agents/drugs that disrupt the PAMR, results in loss of barrier integrity (Satpathy et al., 2004; Jalimarada et al., 2009; Shivanna and Srinivas, 2009, Ramachandran and Srinivas, 2010; Srinivas, 2010, 2012). In our earlier studies with TNF-α, we also observed that p38 MAP kinase inhibition not only prevented the disruption of the PAMR but also suppressed the loss of barrier integrity (Shivanna and Srinivas, 2009; Shivanna et al., 2010a,b). Therefore, here we have examined whether the loss in barrier integrity in response to TNF-α involves the activation of MMP-9 and MMP-2.

2. Materials and methods

2.1. Materials

TNF-α (biological activity of 2×10^7 U/mg; free of endotoxin), cycloheximide, GM-6001 (a broad spectrum inhibitor of MMPs), and FITC dextran (10 kDa) were purchased from Sigma Aldrich (St. Louis, MO). SB-203580, minocycline, and MMP-9 inhibitor I were obtained from EMD Biosciences (Cat. No. 444278; La Jolla, CA). Goat anti-mouse Alexa-488 and anti-fade agent were purchased from Molecular Probes (Eugene, OR). ZO-1 antibody was from Zymed (Long Island, NY). Gold electrodes (8W10E+) for trans-endothelial electrical resistance (TER) measurements were from Applied Biophysics, Inc. (Troy, NY). Cell culture supplies were from Invitrogen (Long Island, NY). The porous tissue culture inserts for measurement of FITC-dextran permeability were obtained from Fisher Scientific, (Pittsburgh, PA).

2.2. Cell culture

Primary cultures of bovine corneal endothelial cells (BCEC) from fresh eyes were established as previously described (Satpathy et al., 2004; Srinivas et al., 2004; Jalimarada et al., 2009; Ramachandran and Srinivas, 2010; Shivanna et al., 2010a,b). The growth medium contained Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum and an antibiotic-antimycotic mixture (Penicillin 100 U/mL, Streptomycin 100 μg/mL, and amphotericin-B 0.25 μg/mL). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was replaced every 2–3 days. Cells of the first and second passages were harvested, seeded onto glass coverslips, gold electrodes, and porous filters, and allowed to reach confluence before use.

2.3. Immunocytochemistry

As a cellular marker of disruption of the PAMR and the barrier integrity, the intracellular ZO-1 was stained in cellular monolayers using protocols as described previously (Shivanna and Srinivas, 2009, Ramachandran and Srinivas, 2010; Shivanna et al., 2010a,b). Following a typical experiment, monolayers on coverslips were washed with PBS, and then fixed with 3.7% paraformaldehyde. Cells were stained for ZO-1 after permeabilization using 0.01% saponin. This was followed by exposure to blocking buffer for 45 min at RT and incubation with the antibody for ZO-1 (1:25) in a mixture of 0.01% saponin in PBS-goat serum (1:1) overnight at 4 °C. The excess antibody was washed with 0.01% saponin and then incubated with secondary antibody (goat anti-mouse IgG Alexa Fluor 488 at 1:1000) for 1 h at RT. Stained cells were mounted with anti-fade medium and imaged using an epi-fluorescence microscope equipped with a 60× oil immersion objective (Nikon, Tokyo, Japan).

2.4. Trans-endothelial electrical resistance

TER was measured by the principle of electric cell-substrate impedance sensing (ECIS; 1600R, Applied Biophysics, Inc., Troy, NY) as described previously (Jalimarada et al., 2009; Shivanna and Srinivas, 2009, Ramachandran and Srinivas, 2010; Shivanna et al., 2010a,b). Briefly, electrodes were stabilized with an aqueous solution of L-cysteine (10 mM) for 15 min at 37 °C prior to seeding 5×10^5 cells/mL on the gold electrodes. The approach to confluence was monitored overnight until steady state electrical resistance was attained. Then, the monolayer on the electrodes was washed gently with serum-free medium twice and used for experiments after 1 h. Following treatment with desired drugs/agents, TER normalized to its value at time zero was taken as a measure of barrier integrity (Satpathy et al., 2004; Srinivas et al., 2004; Jalimarada et al., 2009; Shivanna and Srinivas, 2009, Ramachandran and Srinivas, 2010; Shivanna et al., 2010a,b; Srinivas, 2010, Srinivas, 2012).

2.5. Measurement of permeability to FITC dextran

Cells were grown to confluence on 0.2 μm pore-size tissue culture inserts. The inserts were coated with collagen IV (1 mg/mL)