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Infliximab counteracts tumor necrosis factor-α-enhanced induction of matrix metalloproteinases that degrade claudin and occludin in non-pigmented ciliary epithelium

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ABSTRACT

Infliximab, a monoclonal antibody directed against human tumor necrosis factor-alpha (TNF-α), effectively treats anterior uveitis, which can accompany Behçet's disease. Here, we investigated the underlying mechanism of this action. We examined human, non-pigmented ciliary epithelial cells (HNPCECs), which make up the blood–aqueous barrier (BAB) in the uvea. We measured the expression levels of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs in the presence or absence of TNF-α using quantitative, real-time polymerase chain reaction and enzyme-linked immunosorbent assays. The expression of MMP-1, MMP-3, and MMP-9 increased in the presence of TNF-α, and the addition of infliximab reversed the increase. The TNF-α effects were more attenuated when infliximab was added before than when it was added after TNF-α exposure. Gelatin zymography demonstrated that the protease activity of these MMPs was also increased in the presence of TNF-α and attenuated with infliximab. Immunostaining showed that MMP-1, MMP-3, and MMP-9 degraded claudin-1 and occludin in HNPCECs and in non-pigmented ciliary epithelial cells of the swine ciliary body. In a monolayer of HNPCECs, we found that permeability was significantly increased with MMP treatment. Thus, TNF-α increased levels of MMPs in cells that form the BAB, and MMPs degraded components of the tight junctions in the BAB, which increased permeability through the cellular barrier. Furthermore, infliximab effectively attenuated the TNF-α-induced increases in MMP expression in cells that make up the BAB. These findings might suggest a basis for the clinical prevention of anterior uveitis.

1. Introduction

Uveitis, which can accompany Behçet's disease, is associated with increased tumor necrosis factor-alpha (TNF-α) in the serum and aqueous humor [1–5]. TNF-α is a monocyte-derived cytokine, a signaling molecule with a wide variety of functions. It has cytotoxic effects under certain conditions, which can alter and disturb the cells of the blood–ocular barrier consisting of a blood–aqueous barrier (BAB) and blood–retinal barrier (BRB). Disruptions of these barriers are clinically recognized as anterior and posterior uveitis, respectively.The BAB comprises the tight junctions of the ciliary process non-pigmented epithelium, the inner wall endothelium of Schlemm's canal, and the iris vasculature, and the outward-directed active transport systems of the ciliary processes [6]. Secretion of aqueous humor is attributed to the non-pigmented ciliary epithelial cells. The endothelium of intraretinal blood vessels is considered as the main component of the inner BRB [7]. The outer BRB is composed of three structural entities: the fenestrated endothelium of the choriocapillaries, Bruch's membrane, and the retinal pigment epithelial cells (RPEs). The BRB resembles the blood–brain barrier (BBB) because it prevents elements in the blood from entering the neural parenchyma.

In a previous study, we focused on human retinal microvascular endothelial cells and human RPEs, major components of the BRB. In

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that work, we showed that the presence of TNF-α caused increased expression of matrix metalloproteinases (MMPs) and decreased expression of tissue inhibitors of MMPs (TIMPs) [8]. These altered expression levels were reversed by the addition of infliximab (Remicade®), a monoclonal antibody directed against human TNF-α, which is used as an effective treatment for uveitis in Behçet's disease [9–17]. We reasoned that the pathological alterations in the BRB associated with TNF-α exposure and the attenuations associated with infliximab could simulate the occurrence of posterior uveitis and the treatment process, respectively.

TNF-α is a pro-inflammatory cytokine that plays a key role in anterior uveitis [18], and TNF-α inhibition suppresses experimental autoimmune anterior uveitis [18] and has the same effect in humans [19]. In the present study, we investigated the mechanism underlying anterior uveitis induced by TNF-α in human non-pigmented ciliary epithelial cells (HNPCECs), an essential constituent of the posterior BAB. In addition, we assessed whether infliximab could attenuate the effects of TNF-α in this context. First, with quantitative, real-time polymerase chain reaction (qPCR) assays and enzyme-linked immunosorbent assays (ELISA), we analyzed the expression levels of MMP and TIMP in HNPCECs in the presence of TNF-α and investigated the effects of infliximab on these altered expression levels. Next, we used gelatin zymography to analyze the changes in protease activity caused by enhanced MMP activity and the effect of adding infliximab. With immunohistochemistry techniques, we examined the tight junctions of non-pigmented ciliary epithelial cells, the primary constituents of the posterior BAB, and evaluated the effects of increased MMPs on claudin-1 and occludin, the two most important components in these junctions [20,21]. Finally, we analyzed the permeability of HNPCECs before and after MMP digestion to evaluate the pathological consequences. Our results showed that infliximab could effectively attenuate the TNF-α-induced increases in MMP expression in cells that make up the posterior BAB. This finding could represent a pathway to target for preventing anterior uveitis.

2. Materials and methods

2.1. Cell culture

HNPCECs were purchased from ScienCell® Research Laboratories (Carlsbad, CA, USA) and maintained in tissue culture flasks (Biocoat® Poly-D-Lysine Cellware; Becton, Dickinson and Company Labware, Bedford, MA, USA) at 37 °C under an atmosphere of 5% CO_2. For immunohistochemistry, the cells were maintained in tissue culture plates (poly-L-lysine–coated, 24-well Cell Disk Plate LF1®; Sumitomo Bakelite Co. LTD., Tokyo, Japan) that were precoated with BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Bedford, MA, USA) diluted at 1:100. Cells were cultured in Epithelial Cell Medium (EpiCM; ScienCell® Research Laboratories) supplemented with 2% fetal bovine serum (FBS), epithelial cell growth supplement (EpiCGS, Cat. No. 4152; ScienCell® Research Laboratories), and penicillin/streptomycin solution (P/S, Cat. No. 0503; ScienCell® Research Laboratories). The cells were allowed to reach confluence and were then subcultured in a 6-well plate (Biocoat® Poly-D-Lysine Cellware; Becton, Dickinson and Company Labware) at 6.0×10^5 cells/well until confluent. For immunostaining analysis, the cells were cultured at 1.5×10^5 cells/well until confluent. Our pilot study showed that HNPCECs required 2% FBS and EpiCGS to maintain cell viability for more than 24 h.

2.2. Cell treatment

For the following experiments, we used recombinant human TNF-α (10 μg; R&D Systems, Inc., Minneapolis, MN, USA) and infliximab in the form of Remicade® (100 mg; Centocor/Mitsubishi Tanabe Pharma, Osaka, Japan). HNPCECs incubated in EpiCM were divided into four groups: group 1 was treated with human TNF-α (0, 0.4, 2.0, 10, or 50 ng/mL) for 24 h; group 2 was treated with infliximab (0.04, 0.2, 1.0, or 5.0 μg/mL) for 24 h; group 3 was initially treated with infliximab (0.04, 0.2, 1.0, or 5.0 μg/mL), then 2 h later, 10 ng/mL TNF-α was added; and group 4 was initially treated with 10 ng/mL TNF-α, followed by addition of infliximab (0.04, 0.2, 1.0, or 5.0 μg/mL) 2 h later. The cells in groups 3 and 4 were continuously cultured for 22 h.

2.3. RNA isolation and cDNA synthesis

Total cellular RNA was isolated with the SV Total RNA Isolation System® (Promega, Madison, WI, USA) according to the manufacturer's instructions. Total RNA was used as a template for cDNA synthesis with random primers and the Super Script VILO cDNA Synthesis Kit® (Invitrogen–Gibco, Grand Island, NY, USA) according to the manufacturer's protocol.

2.4. qPCR

cDNA was synthesized from total RNA (50 ng) for each treatment group. The cDNA served as the template for the qPCR assays. The primer sequences were human MMP-1 forward, 5′-AGCTAGCT-CAGGATGACATTGATG-3′, and reverse, 5′-CTCCCCGAATCGTAGTTA-TAGCAT-3′; human MMP-2 forward, 5′-CGCAGATGCCTGGAATGC-3′, and reverse, 5′-TCAGGTAATAGGCACCCTTGAAG-3′; human MMP-3 forward, 5′-TGGCATTCAGTCCCTCTATGG-3′, and reverse, 5′-AGGACAAAGCAGGATCACAGTT-3′; human MMP-9 forward, 5′-CCCTGGAGACCTGAGAACCA-3′, and reverse, 5′-CCACCCGAGTG-TAACCATAGC-3′; human TIMP-1 forward, 5′-CACCCACA-GACGGCCTTCT-3′, and reverse, 5′-TCTGGTGTCCCCACGAACTT-3′; and human TIMP-2 forward, 5′-CAACAGGCGTTTTGCAATG-3′, and reverse, 5′-TATCCTTCTCAGGCCCTTTG-3′. The qPCR gene expression results were normalized to the expression of the endogenous control gene, glyceraldehyde-3-phosphate dehydrogenase (forward, 5′-TGAACGGGAAGCTCACTGG-3′, and reverse, 5′-TCCAC-CACCCTGTTGCTGTA-3′), as described previously [8]. The qPCR reaction was carried out with the Takara Thermal Cycler Dice Real Time System TP800® (Takara Bio, Inc., Shiga, Japan).

2.5. ELISAs

Conditioned media were analyzed with ELISAs. These assays were performed with the following ELISA kits: ELISpot® Human Total MMP-1, Quantikine® Human Total MMP-3, and Quantikine® Human Total MMP-9 (R&D Systems, Inc.). All procedures were performed according to the manufacturer's instructions.

2.6. Gelatin zymography

HNPCECs were maintained in 24-well plates. The four treatment groups described in section 2.2 were incubated for 24 h; then, the cell media were collected and concentrated up to 10 fold with the Amicon® Ultra-0.5, Ultracel-10 Membrane 10 kDa (EMD Millipore Corporation, Billerica, MA, USA). Aliquots with equal amounts of protein (20 μg) were assayed with zymography, performed with Novex®10% Zymogram Gelatin Gels and a Novex® Colloidal Blue Staining Kit (Life Technologies, Catalog Number LC6025, Carlsbad, CA, USA), according to the manufacturer's instructions. Gelatinolytic band densities were quantified with Image J software (US National Institutes of Health, http://www.rsb.info.nih.gov/ij/). As a positive control, 0.1 μg of MMP-1, 0.1 μg of MMP-3, and 0.01 μg of MMP-9 were loaded on each gel. The gels were developed for 24 h to evaluate MMP-1 and

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