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Interleukin-32 induced thymic stromal lymphopoietin plays a critical role in the inflammatory response in human corneal epithelium

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ABSTRACT

Interleukin (IL)-32, a novel cytokine, participates in a variety of inflammatory disorders. Thymic stromal lymphopoietin (TSLP) plays important roles in mucosal epithelial cells, especially in allergy-induced inflammation, through the TSLP-TSLPR (thymic stromal lymphopoietin receptor) signalling pathway. However, the association of IL-32 with TSLP on the ocular surface remains unclear. The present work aimed to assess the functional association of IL-32 with TSLP in the control of pro-inflammatory cytokine levels in the corneal epithelium. Human corneal tissue specimens and human corneal epithelial cells (HCECs) were administered different concentrations of IL-32 in the presence or absence of various inhibitors to assess TSLP levels and localization, as well as the molecular pathways that control pro-inflammatory cytokine production. TSLP mRNA levels were determined by real time RT- PCR, while protein levels were quantitated by ELISA and immunohistochemical staining. TSLP protein expression was examined in donor corneal epithelium samples. IL-32 significantly upregulated TSLP and pro-inflammatory cytokines (TNFα and IL-6) in HCECs at the gene and protein levels. The production of pro-inflammatory molecules by IL-32 was increased by recombinant TSLP. Interestingly, both NF-κB (quinazoline) and caspase-1 (VX-765) inhibitors suppressed the IL-32-related upregulation of pro-inflammatory cytokines (TNFα and IL-6). These findings demonstrate that IL-32 and IL-32-induced-TSLP are critical cytokines that participate in inflammatory responses through the caspase-1 and NF-κB signalling pathways in the corneal epithelium, suggesting new molecular targets for inflammatory diseases of the ocular surface. The effects of IL-32 on cell proliferation and apoptosis were investigated by MTT assays and RT-PCR,respectively. The results demonstrated that IL-32 inhibits cells apoptosis in HCECs.

1. Introduction

Interleukin (IL)-32, a newly discovered cytokine, induces crucial inflammatory cytokines and is upregulated in inflammatory autoimmune disorders, cancer, and viral infections [1–3]. The IL-32 gene was first found in activated T cells [4]. However, IL-32 has also been detected in other immune and nonimmune cells [5,6]. Studies reported that IL-32 is produced by natural killer cells, mast cells, keratinocytes, eosinophils, monocytes, and epithelial cells [7–11]. Our recent study demonstrated that IL-32 is expressed in human corneal epithelial cells [12]. In addition, the stimulation of IL-32 expression by *M. tuberculosis* depends on endogenous interferon-γ (IFN-γ) production [13]. IL-32 is defined as a pro-inflammatory cytokine because it can induce interleukin 1β (IL-1β), tumour necrosis factor-α (TNFα), IL-6, and IL-8 and activate the nuclear factor-kB (NF-κB), p38 mitogen-activated protein kinase (MAPK), and caspase-1 pathways [8]. IL-32 also participates in the modulation of signalling pathways controlled by Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD) ligands [8].

Thymic stromal lymphopoietin (TSLP), a newly described cytokine that induces Th2 cytokines, such as thymus- and activation-regulated chemokine (TARC), takes part in allergic responses, e.g., IgE expression [14–16]. TSLP is associated with allergic conjunctivitis, allergic rhinitis, and atopic dermatitis [17,18]. Moreover, TSLP is found primarily in keratinocytes (KCs) and mucosal epithelial cells and is constitutively produced by thymic and intestinal epithelial cells [19–22]. Pro-inflammatory cytokines, Th2-related factors, and IgE are associated with TSLP production, indicating an amplification cycle for Th2 responses [23]. TSLP acts through tight binding to the heterodimeric receptor comprising IL-7 receptor alpha (IL-7Rα) and TSLP receptor (TSLPR), transmitting signals through STAT5 induction [24,25]. The TSLP-TSLPR interaction is critical for triggering immune responses to intestinal parasitic pathogens [18]. In addition, TNF-α and IL-1β can

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induce TSLP in human airway smooth muscle cells through the MAPK, p38 and extracellular-signal-regulated kinase (ERK) pathways [26]. Li et al. recently detected TSLP in primary corneal epithelial cells treated with a water extract of fat-free SRW (short ragweed) pollen [27].

Jeong et al. revealed that IL-32 strongly induces TSLP production in the THP-1 and in human blood monocytes via the activation of caspase-1 and nuclear factor-κB [28]. Our research team reported that IL-32 is produced by human corneal epithelial cells [12]. However,the functions and associated mechanisms of IL-32 and TSLP in corneal epithelial cells are largely undefined. The present work is the first to demonstrate that IL-32-induced TSLP has a critical function in the inflammatory response of the human corneal epithelium.

2. Materials and methods

2.1. Materials and reagents

Dulbecco's modified Eagle's medium (DMEM), amphotericin B, Ham F-12, gentamicin, and 0.25% trypsin containing 0.03% EDTA were obtained from Thermo Fisher Scientific (Carlsbad, CA). Foetal bovine serum (FBS) was obtained from HyClone (Logan, UT). Secondary antibodies were obtained from Molecular Probes (Eugene, OR). Recombinant human IL-32 and TSLP were obtained from R&D Systems (Minneapolis, MN). The anti-TSLP antibody was obtained from ProSci Incorporated (Poway, CA). The rabbit anti-p65 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enzyme-linked immunosorbent assay (ELISA) DuoSet kits detecting human TSLP, TNFα, and IL-6 were obtained from R&D Systems. TaqMan gene expression assays and the real-time PCR master mix were both obtained from Applied Biosystems (Foster City, CA).

2.2. Ex vivo model of human corneal epithelial tissue for TSLP induction

We used a total of 6 corneoscleral tissue samples. Each corneoscleral tissue sample was divided into 4 parts, which were placed into the wells of eight-chamber slides with the epithelial side facing up in serum-free SHEM and incubated in the presence or absence of 10 ng/ml IL-32 for 24 h at 37 °C. After treatment, the specimens were snap frozen in liquid nitrogen and cut into sections for immunohistochemical staining of TSLP.

2.3. TSLP and inflammatory cytokine induction in the primary human corneal epithelium

Freshly collected human corneoscleral tissue samples were obtained from donors at the Affiliated Hospital of Qingdao University after study approval was obtained from the institutional ethics committee [29]. All donors died of non-inflammatory diseases and their corneoscleral tissue was normal. The human experiments conformed to the Declaration of Helsinki. Human corneal epithelial cell (HCEC) culture was carried out as previously reported [30,31], in supplemented hormonal epidermal medium (SHEM) with 5% FBS, according to our previously reported method [32]. Close attention was paid to HCEC growth, and only epithelial cultures that were not contaminated with visible fibroblasts were used. Confluent HCECs were transferred to serum-free SHEM and administered recombinant IL-32 at various concentrations (0, 2, 10 and 50 ng/ml) for 1–24 h. After cell treatment and lysis, total RNA was extracted for mRNA level determination. The experiments were repeated three times. Supernatant aliquots collected after 24–48 h of treatment were stored at −80 °C for immunoassays.

2.4. Caspase-1 enzymatic activity assessment

Caspase-1 activity was tested with a specific kit (BioVision, CA), as directed by the manufacturer. The Caspase-1/ICE Colorimetric Protease Assay Kit provides a simple and convenient means ofassaying the activity of caspases that recognize the sequence YVAD. The assay is based on the spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labelled substrate YVAD-pNA. pNA light emission can be quantified using a spectrophotometer or a microtiter plate reader at 400 or 405 nm. Comparison of the absorbance of pNA from a treated sample with that of an untreated control allows the determination of the fold increase in caspase-1 activity.

2.5. NF-κB signalling evaluation

HCECs were first administered recombinant human TSLP (10 ng/ml) or the NF-κB inhibitor quinazoline (10 μM) for 1 h, followed by treatment with IL-32 for 4, 6, 24 and 48 h [30]. Total RNA was extracted from cells cultured in 12-well plates, and TSLP and inflammatory cytokine (TNFα and IL-6) expression levels were measured using quantitative RT-PCR (qRT-PCR). After 24–48 h of treatment, the cells were subjected to lysis in RIPA buffer for ELISA.

2.6. Quantitative RT-PCR

Total RNA extraction from HCECs was performed with a Qiagen RNeasy® Mini kit; RNA quantitation was performed on a NanoDrop® ND-1000 Spectrophotometer. Reverse transcription was carried out with 1 μg of total RNA with Ready-To-Go You-Prime First-Strand Beads. Then, qRT-PCR was carried out on a Mx3005P™ system (Stratagene) in 20 μl reactions containing 5 μl of cDNA, 1 μl of TaqMan® Gene Expression Assays specific for TSLP, TNFα, IL-1β, IL-6, IL-8,caspase-3,caspase-8, caspase-9 and GAPDH, and 10 μl of Master Mix (Life Technologies, CA). Amplification was performed at 50 °C (2 min), 95 °C (10 min), and 40 cycles of 95 °C (15 s) and 60 °C (1 min). GAPDH was used as an internal control. The following primers (F, forward; R, reverse) were used: TSLP, 5′-TCCCCCGCGCCACATT-3′ (F) and 5′-ACAG CCGAGAATTACTGCCA-3′ (R); TNFα, 5′-TGCTTGTTCCTCAGCCTCTT -3′ (F) and 5′-CAGAGGGCTGATTAGAGA GAGGT-3′ (R); IL1β, 5′-GCT GATGGCCCTAAAC AGATGAA-3′ (F) and 5′-TCCATGGCCAC AACAAC TGAC-3′(R); IL6, 5′-AA GCCAG AGCTGTGCAGATGAGTA-3′(F) and 5′-CCATCTTTGGAAGG TTCAGGTTG-3′ (R); IL8, 5′-TCTTGGCAGCCTT CCTGATT-3′ (F) and 5′-AACTTCTCCACA ACCCTCT G-3′ (R); caspase9, 5′-CTGCGTGGTGGTCATTCT-3′ (F) and 5′-ACAGGGCATCCATCTGTG-3′ (R); GADPH, 5′-TGGCACCCAGCACAATGAA-3′(F) and 5′-CTAAGTCAT AGTCCGCCTAG AAGCA-3′ (R).

2.7. Elisa

ELISA was performed to detect the protein concentrations of human TSLP, TNFα, and IL-6, as instructed by the manufacturer, in culture supernatants or cell lysates from different treatments. Absorbance was measured at 450 nm on a VERSAmax microtiter plate reader (Molecular Devices, Sunnyvale, CA).

2.8. Immunoblot

After cell lysis in RIPA buffer (1 h), the samples were centrifuged to remove cellular debris, and protein concentrations were assessed. Then, SDS sample buffer was added, and the mixtures were boiled. Total protein was separated by 10% PAGE-SDS, followed by transfer onto PVDF membranes. After blocking with 5% BSA, the samples were incubated overnight with primary antibodies targeting different human proteins at 4 °C, followed by the addition of the appropriate peroxidase-conjugated secondary antibodies at 37 °C for 1 h. Enhanced chemiluminescence (ECL; Thermo Scientific) was employed for development.

2.9. Immunohistochemical staining

Indirect immunostaining of TSLP was applied based on our previously reported methods [3]. Briefly, frozen human cornea sections