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Comments
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Differential Effect of Proinflammatory Cytokines on Corneal and Conjunctival Epithelial Cell Mucins and Glycocalyx

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Purpose: Ocular surface mucins and glycocalyx are critical for providing ocular hydration as well lubrication and repelling pathogens or allergens. Elevated levels of tear proinflammatory cytokines in dry eye may have detrimental effect on mucins and glycocalyx. The present study tested the effect of proinflammatory cytokines IL-6, TNF-α, and IFN-γ on membrane-tethered mucins expression, glycocalyx, and viability of ocular surface epithelial cells.

Methods: Stratified cultures of human corneal and conjunctival epithelial cells were exposed to different concentrations of IL-6, TNF-α, and IFN-γ for 24 hours. The mucins gene and protein expressions were quantified by real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). The glycocalyx was imaged using confocal microscopy after staining with Alexa 488-conjugated wheat germ agglutinin lectin. Apoptotic and necrotic cell death was quantified using flow cytometry.

Results: IL-6, TNF-α, and IFN-γ treatment resulted in a significant increase in mucins (MUC)1 and MUC4 gene and protein expression in human corneal epithelial cells but caused no significant changes in the levels of these mucins in conjunctival epithelial cells. Moreover, these cytokines decreased MUC16 expression in both corneal and conjunctival epithelial cells. Moreover, no notable change in glycocalyx or apoptotic cell death in corneal and conjunctival epithelial cells was noted with any of the tested cytokines, but IL-6 and TNF-α exposure increased necrotic cell death in corneal and conjunctival epithelial cells, respectively.

Conclusions: Our results demonstrate that proinflammatory cytokines have differential effects on human corneal and conjunctival epithelial cell mucins expression, but do not cause any damage to ocular surface epithelial cell glycocalyx.

Introduction

The apical surface of human corneal and conjunctival epithelial cells is covered with a layer of glycocalyx that forms a boundary between these cells and the tear film.1-3 Glycocalyx is a network made up of membrane-tethered mucins, their side chains, and galectin-3.1-7 Glycocalyx and mucins are critical for ocular health as they serve many functions, including repelling pathogens, keeping the ocular surface hydrated, protecting the cells against mechanical and chemical damage, as well as reducing friction during blinking.1-7

Dry eye or keratoconjunctivitis sicca is a common ocular condition with a high global prevalence. The disease is characterized by loss of tear film homeostasis, tear hyperosmolarity, ocular surface inflammation, and neurosensory abnormalities.8,9 Although multiple factors contribute to the pathogenesis of dry eye, the immune-mediated inflammatory response plays a central role in its onset and progression.10-14 Hyperosmolar stress has been shown to trigger the release of proinflammatory cytokines IL-1, IL-6, and TNF-α from ocular surface epithelial cells.15,16 Hyperosmolar stress due to increased tear osmolarity in dry eye disease and the associated release of cytokines can contribute to the early stage of ocular surface
inflammation. Levels of IL-6 and TNF-α have been reported to be elevated in the tears of patients suffering from dry eye.17 These proinflammatory cytokines along with chemokines are also responsible for activation, maturation, and migration of antigen-presenting cells in the early stages of dry eye disease.10–14 Subsequently, activated antigen-presenting cells recruit T cells, which have been shown to secrete IL-17 and IFN-γ.10–14 T cell-derived cytokines contribute to the amplification stage of inflammation in dry eye disease.10–14

The promoter region of genes encoding for mucins have response elements for signaling pathways activated by proinflammatory cytokines including IL-6, TNF-α, and IFN-γ suggesting that these cytokines can modulate the gene expression of mucins.18–21 Additionally, these cytokines have been shown to induce the expression of enzymes, such as heparanase and hyaluronidase that may have a detrimental effect on the glycocalyx.22,23 Immunological and conjunctival impression cytology studies have shown that the levels of mucins or the degree of their glycosylation is notably altered in both the cornea as well as conjunctiva of patients suffering from non-Sjogren and Sjogren syndrome-related dry eye disease.1,2,24 Therefore, the present study is designed to investigate the effect of two early-stage (IL-6 and TNF-α) and one amplification stage (IFN-γ) proinflammatory cytokines on membrane-tethered mucins (MUC) MUC1, MUC4, MUC16, ocular surface glycocalyx, and cell viability of human stratified corneal and human stratified conjunctival epithelial cells. Because patients suffering from dry eye have elevated tear levels of these cytokines in picogram range, the present study tested the effect of these cytokines on ocular surface mucins and glycocalyx at the pathologically relevant picogram concentrations.

Methods

Stratified Human Corneal and Conjunctival Epithelial Cell Culture

Telomerase-transformed human corneal and conjunctival epithelial cell lines were used for this study. Human corneal epithelial cells were propagated in keratinocyte growth medium supplemented with bovine pituitary extract (0.004 mL/mL), human epidermal growth factor (0.125 ng/mL), human insulin (5 μg/mL), hydrocortisone (0.33 μg/mL), epinephrine (0.39 μg/mL), transferrin (10 μg/mL), and calcium chloride (0.15 mM; PromoCell GmbH, Heidelberg, Germany). To induce stratification, the cells were plated on a collagen-coated 3.0 μm PTFE transwell membrane inserts (Corning Inc., Corning, NY, USA) and the media was switched to supplemented keratinocyte growth medium containing a higher concentration of CaCl₂ (1.15 mM). This media was added both on the inserts as well as the bottom well until the cells reached 100% confluence. Once the cells reached 100% confluence, the media was added to the bottom of the well only, leaving the membrane inserts containing the cells exposed to air for 1 week to achieve stratification into a multilayered epithelium.25

The human conjunctival epithelial cells were propagated in keratinocyte serum-free medium supplemented with 25 μg/mL bovine pituitary extract, 0.2 ng/mL epidermal growth factor, and 0.4 mM CaCl₂ (Gibco-Invitrogen Corp., Rockville, MD, USA). Once the cells reached 50% confluence, the media was switched to a 1:1 mixture of supplemented keratinocyte serum-free medium and low-calcium DMEM without HEPES/F12 media (Gibco-Invitrogen Corp., Rockville, MD, USA). When the cells reached 100% confluence, the media was replaced with DMEM/F12 media supplemented with 1 mM CaCl₂, 10% Calf serum, and 10 ng/mL EGF for about 5 to 7 days to induce stratification.26,27

Cytokine Treatments

The cytokines were purchased from PeproTech (Cranbury, NJ, USA). To test the effects of proinflammatory cytokines on mucin expression, glycocalyx, and epithelial viability, the stratified cultures of human corneal and conjunctival epithelial cells were separately exposed to IL-6, TNF-α, and IFN-γ at five different concentrations (30, 60, 125, 250, 500 pg/mL) for 24 hours. Each experiment was conducted in quadruplicate.

Isolation of mRNA and Preparation of cDNA

The total RNA from the control and cytokine exposed stratified corneal and conjunctival epithelial cells was isolated using a commercially available kit (Qiagen’s RNeasy Mini Kit, Valencia, CA, USA) as per the manufacturer’s instructions. The RNA was immediately reverse transcribed to cDNA using a commercially available kit (SuperScript III First- Stand; Invitrogen, Carlsbad, CA, USA).

Mucin Gene Expression Quantification

The gene expression of MUC1, MUC4, and MUC16 was quantified using real-time polymerase chain reaction (PCR). A 20 μL reaction mixture containing 2 μL of cDNA and 18 μL of SYBR Green
Master Mix was run at a universal cycle (95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 55°C for 60 seconds) using real-time thermocycler (Bio-Rad CFX Thermal Cycler; Bio-Rad Laboratories, Hercules, CA, USA). β-actin was used as the housekeeping gene. The relative change in gene expression was calculated using the ΔΔCT method.

Protein Extraction and Mucin Enzyme-Linked Immunosorbent Assays

To prepare protein extracts, control, and cytokine exposed stratified corneal and conjunctival epithelial cells were incubated for 30 minutes on ice in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor (Thermo Fisher Scientific, Rockford, IL, USA) on a shaker followed by the removal of any adherent cells using a cell scraper. The supernatant was collected as total cell lysate, and protein concentration was determined by the BCA method using a commercially available kit (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific). Protein levels of MUC1 (Thermo Fisher Scientific), MUC4, and MUC 16 were quantified in the cell lysate using commercially available enzyme-linked immunosorbent assay (ELISA) kits (LifeSpan Biosciences, Inc., Seattle, WA, USA). For MUC1 and MUC4, 100 μL of undiluted cell lysate was used. For MUC16, corneal and conjunctival cell lysates were diluted 4 times and 200 times, respectively.

Glycocalyx Staining

The stratified cultures of control and cytokine-exposed human corneal and conjunctival epithelial cells were incubated in the dark at room temperature for 10 minutes in a solution containing 1:2000 propidium iodide and 1:300 FITC-conjugated annexin V (Cayman Chemical, Ann Harbor, MI, USA). The PI positive and FITC negative cells were quantified as necrotic. To calculate apoptotic cells, PI positive FITC negative cells were subtracted from PI positive + FITC negative cell population. The populations of stained cells were analyzed using BD FACSverse flow cytometer (BD Sciences, San Jose, CA, USA). The data were analyzed using FlowJo software.

Statistics

The data are presented as mean ± standard error of mean. Statistical analysis was performed using GraphPad Prism software (GraphPad Prism, version 8; GraphPad, San Diego, CA, USA). The data were analyzed using 1-way ANOVA, followed by Dunnett's post hoc test. A P value of <0.05 was considered statistically significant.

Results

Effect of IL-6, TNF-α, and IFN-γ on Membrane-Tethered Mucins Gene and Protein Expression in Stratified Human Corneal and Conjunctival Epithelial Cells

To test the effect of IL-6, TNF-α, and IFN-γ on ocular surface mucins gene expression, the stratified human corneal and conjunctival epithelial cells were exposed to five concentrations of these cytokines. IL-6 treatment caused a significant increase in the gene expression of MUC1 and MUC4 but caused a notable decrease in the gene expression of MUC16 in the human corneal epithelial cells (Fig. 1A). For both MUC1 and MUC4, IL-6 showed a bell-shaped dose response curve with a less pronounced effect at 30 pg/mL followed by a dose-dependent significant (P < 0.05 compared to control cells not exposed to IL-6) increase in gene expression at 60, 125, and 250 pg/mL then a subsequent decrease in effect at 500 pg/mL. In congruence with the gene expression data, IL-6 treatment also caused a significant increase (P < 0.05 compared to control cells not exposed to IL-6) in protein levels of MUC1 and MUC4 and a
Figure 1. Effect of IL-6 on MUC1, MUC4, MUC16 gene, and protein expression in stratified cultures of human corneal (A, B) and conjunctival (C, D) epithelial cells. The cells were exposed to IL-6 for 24 hours. *P < 0.05 compared to control cells (C) that were not exposed to IL-6.

Figure 2. Effect of TNF-α on MUC1, MUC4, MUC16 gene, and protein expression in stratified cultures of human corneal (A, B) and conjunctival (C, D) epithelial cells. The cells were exposed to TNF-α for 24 hours. *P < 0.05 compared to control cells (C) that were not exposed to TNF-α.
significant decrease ($P < 0.05$ compared to control cells not exposed to IL-6) in MUC16 in corneal epithelial cells (Fig. 1B). Compared to corneal epithelial cells, very low levels of MUC1 and MUC4 proteins were detected in human conjunctival epithelial cells. Furthermore, IL-6, in contrast to the corneal epithelial cells, did not significantly change MUC1, MUC4 gene and protein expression, and MUC16 gene expression in stratified human conjunctival epithelial cells but it caused a significant decrease in MUC16 protein levels (Figs. 1C, 1D).

TNF-α treatment also caused significant ($P < 0.05$ compared to control cells not exposed to TNF-α) increase in the gene expression of MUC1 and MUC4 in stratified human corneal epithelial cells but did not cause any notable change in MUC16 (Fig. 2A). It is worth noting that the various tested concentrations of TNF-α caused a similar increase in the gene expression of MUC1 and MUC4 suggesting that the effect of TNF-α may not be dose-dependent but a likely response of all or no pattern. Consistent with the changes in gene expression, 250 pg/mL dose of TNF-α caused a significant ($P < 0.05$ compared to control cells not exposed to TNF-α) increase in MUC1 and MUC4 protein levels but this dose also caused a significant decrease in MUC16 protein levels (Fig. 2B). On the other hand, TNF-α did not cause any notable change in MUC1, MUC4 gene and protein expression, and MUC16 gene expression in the stratified human conjunctival epithelial cells, However, similar to the effect observed in corneal epithelial cells, TNF-α caused a significant decrease in MUC16 protein expression (Fig. 2D).

Consistent with the effects observed with IL-6 and TNF-α, IFN-γ exposure also caused a significant ($P < 0.05$ compared to control cells not exposed to IFN-γ) increase in the both the gene and protein expression of MUC1 and MUC4 in the stratified human corneal epithelial cells but did not cause any change in MUC16 gene and protein expression (Figs. 3A, 3B). Various doses of IFN-γ also caused a similar increase in the gene expression of MUC1 and MUC4 suggesting that the effect of IFN-γ is not dose-dependent but follows all or no response. Akin to the other two cytokines, IFN-γ also did not cause any significant change in the gene expression of MUC1, MUC4, and MUC 16 (Fig. 3C) in stratified human conjunctival epithelial cells but it caused a significant decrease in MUC16 protein levels (Fig. 3D).
Effect of IL-6, TNF-α, and IFN-γ on Stratified Human Corneal and Conjunctival Epithelial Cell Glycocalyx

The integrity of glycocalyx is important for the barrier, lubrication, and hydration functions of ocular surface epithelial cells. Glycocalyx, along with mucins, contains side-chain structural and chemical components to form a network. Proinflammatory cytokines have been shown to increase enzymes that can digest these components and damage glycocalyx. Therefore, we next tested the effect of these proinflammatory cytokines on human corneal and conjunctival epithelial cell glycocalyx. Wheat germ agglutinin lectin binds to the sialic acid residues and has been previously used to stain the glycocalyx. Figure 4 shows the top projection of 3-D confocal images of glycocalyx (stained green with wheat germ agglutinin) in stratified human conjunctival epithelial cells. As shown in Figure 4, treatment of corneal epithelial cells with IL-6, TNF-α, and IFN-γ (250 pg/mL) did not cause any detrimental effect on corneal epithelial cell glycocalyx. Digital quantification of randomly captured images revealed an average stained area of 92 ± 6% in control corneal epithelial cells, 93 ± 8% in IL-6 treated corneal epithelial cells, 90 ± 10% in TNF-α treated corneal epithelial cells, and 92 ± 9% in IFN-γ treated stratified human corneal epithelial cells.

Effect of IL-6, TNF-α, and IFN-γ on Stratified Human Corneal and Conjunctival Epithelial Cell Necrosis, Apoptosis, and Morphology

Loss of epithelial cell viability can compromise ocular surface health and perpetuate inflammation. Therefore, we next tested the effect of IL-6, TNF-α, and IFN-γ on human corneal and conjunctival epithelial cell necrosis and apoptosis by flowcytometry (scatter plot, Supplementary Fig.). None of the cytokines caused any significant increase in apoptotic cell death of human corneal epithelial cells (Fig. 6A). Interestingly, IL-6 treatment (250 pg/mL) caused an increase in necrotic cell death (see Fig. 6A). Like the effect observed in human corneal epithelial cells, none of the tested cytokines caused any increase in apoptotic cell death of human conjunctival epithelial cells (Fig. 6B). In contrast to human corneal epithelial cells, TNF-α instead of IL-6 caused an increase in

Figure 4. Representative confocal Z-stack images showing glycocalyx staining in stratified cultures of human corneal epithelial cells exposed to IL-6, TNF-α, and IFN-γ (250 pg/mL) for 24 hours. Nuclei are stained blue with DAPI and glycocalyx is stained green with Alexa-488 conjugated wheat germ agglutinin lectin. Scale bar = 100 μm.
Figure 5. Representative confocal Z-stack images showing glycocalyx staining in stratified cultures of human conjunctival epithelial cells exposed to IL-6, TNF-α, and IFN-γ (250 pg/mL) for 24 hours. Nuclei are stained blue with DAPI and glycocalyx is stained green with Alexa-488 conjugated wheat germ agglutinin lectin. Scale bar = 100 μm.

Discussion

Cytokines play an important role in the initiation and perpetuation of dry eye disease. Exposing ocular surface epithelial cells to hyperosmolar stress causes the release of proinflammatory cytokines. The level of these proinflammatory cytokines is also significantly increased in the tears of patients with dry eye and the conjunctiva of patients with dry eye with Sjogren's syndrome. An inverse correlation has been demonstrated between the levels of these cytokines and the fluorescein staining, and goblet cell density. Further, topical cyclosporine, a potent immunosuppressant that reduces proinflammatory cytokines, has been shown to modulate ocular surface mucins and increase goblet cell density. The apical surface of corneal and conjunctival epithelial cells is covered with glycocalyx. Membrane-tethered mucins are important components of glycocalyx. Multiple studies have shown that proinflammatory cytokines can modulate the expression of membrane-tethered mucins in oral, nasal, and respiratory mucosal epithelium. The results of the present study demonstrate that IL-6, TNF-α, and IFN-γ increase the expression of MUC1 and MUC4 in the corneal epithelial cells at the picogram levels. Interestingly, our data demonstrates that these cytokines do not affect the MUC1 and MUC4 expression in conjunctival epithelial cells. Although both corneal and conjunctival epithelial cells are reported to express the receptors for these cytokines, the differential effect of these cytokines on MUC1 and MUC4 gene expression in corneal and conjunctival epithelial cells may be due to differences in the receptor density, presence of decoy molecules, differential activation of signaling pathways, or different response of mucin promoters in the two epithelial cell types. Multiple studies have demonstrated that MUC1 has anti-inflammatory properties due to the negative regulation of Toll-like receptors. Studies have also demonstrated that MUC1 can act as microbial scavenger to limit Campylobacter jejuni and Helicobacter pylori infection in gastrointestinal tract in mouse model and limit...
Figure 6. Flow cytometry quantification of apoptosis and necrosis in stratified human corneal (A) and conjunctival (B) epithelial cells treatment with IL-6, TNF-α, and IFN-γ (250 pg/mL) for 24 hours. The cells were stained with annexin V/propidium iodide dual staining for flow cytometry quantification. *P < 0.05 as compared to control cells that were not exposed to any cytokine.
Proinflammatory Cytokines and Mucins

Figure 7. Representative phase contrast microscopy images of stratified human corneal (A) and conjunctival (B) epithelial cells exposed to IL-6, TNF-α, and IFN-γ (250 pg/mL) for 24 hours. Scale bar = 100 μm.

*S. pneumoniae* infection in epithelial cells. Therefore, the cytokine-mediated increase in gene expression of corneal epithelial MUC1 and MUC4 could possibly represent a physiological response to enhance the ocular surface defense against microbial stress. Interestingly, these cytokines caused a significant decrease in the expression of MUC16 in both corneal as well as conjunctival epithelial cells. MUC16 is the largest mucin. It has been shown to be linked to actin cytoskeleton thus providing an adhesive function to glycocalyx and a strong barrier function due to its large size and glycosylation. These MUC16 functions are critical for ocular surface health. Therefore, our data raises the possibility that cytokine-mediated decrease in MUC16 may partially contribute to ocular surface damage in conditions associated with ocular surface inflammation and elevated levels of proinflammatory cytokines.

Cytokines have also been shown to induce the expression of enzymes, such as heparanase and hyaluronidase, which can damage the mucin side chains, thus compromising the integrity of glycocalyx. TNF-α has been shown to induce endothelial glycocalyx shedding resulting in increased vascular permeability and aberrant vasodilation. However, results of the present study demonstrate that IL-6, TNF-α, and IFN-γ do not have any detrimental effect on corneal and conjunctival epithelial cell glycocalyx.
Absence of shear stress and blinking effect is a limitation of in vitro stratified epithelia. Thus, it will be worthwhile to further investigate the effect of cytokines on glycocalyx in an in vivo model. Last, none of the cytokines caused an increased in apoptotic cell death, but a small increase in necrotic cell death was noted with TNF-α in conjunctival epithelial cells and with IL-6 treatment in corneal epithelial cells. However, no overt change in morphology or cell density was noted. A difference in receptor density or death signaling pathways activated by TNF-α or IL-6 in the two cell lines may underlie this differential response of corneal and conjunctival epithelial cells to these two cytokines.

In summary, the results of the present study demonstrate that IL-6, TNF-α, and IFN-γ have differential effects on corneal and conjunctival epithelial cells mucins expression and cause an increase in MUC1 and MUC4 gene expression only in corneal epithelial cells but cause a decrease in MUC16. Furthermore, these cytokines did not cause any notable change in the corneal and conjunctival glycocalyx and apoptotic cell death.

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* KS and PM contributed equally to this paper.

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