Effect of proinflammatory cytokines on the human MUC5AC promoter activity in vitro and in vivo

I-Jong Wang[1]
Chun-Yin Wu[1]
Fung-Rong Hu[2]

[1]Far Eastern Memory Hospital, Taipei County, Taiwan; [2]Department of Ophthalmology, National Taiwan University Hospital, Taipei, Taiwan

Purpose: To investigate the effect of inflammatory cytokines on the activity of the human MUC5AC promoter in vitro and in vivo.

Methods: Conjunctival epithelial cells transfected with MUC5AC-luciferase plasmids challenged with different cytokines (tumor necrosis factor-α [TNF-α], interleukin [IL]-1β, IL-2, IL-6, and IL-8) at various concentration (1, 10, 20, 50, 100, 200, 500, 1000, and 2000 pg/mL) for 24 hours. A Helio Gene Gun system (Bio-Rad Laboratories, Hercules, CA, USA) was used to deliver MUC5AC-luciferase plasmids into rabbit conjunctivas, which were also challenged with these cytokines (1000 pg/mL) at the frequency of every six hours for 48 hours. The activity of MUC5AC-luciferase was then evaluated using the luciferase assay.

Results: Results of the studies demonstrated that IL-1β and TNF-α upregulated the activity of MUC5AC-luciferase in cultured conjunctival cells, while IL-2, IL-6, and IL-8 had no effect. In rabbit conjunctival tissues, TNF-α, IL-1β, IL-2, IL-8, and IL-6 significantly upregulated MUC5AC gene expression.

Conclusions: This suggests that MUC5AC mucin gene expression is regulated by proinflammatory cytokines, which could have implications in ocular surface disorders.

Keywords: cytokines; MUC5AC; mucins

Introduction

The ocular surface composed of conjunctiva and cornea is considered as an interface between the external environment and the host. The ocular surface is covered by a mucus layer, the function of which predominantly is determined by mucin glycoproteins. The mucus layer acts as lubricants of the corneal and conjunctival epithelial surfaces during the eyelid blink, as stabilizers of the preocular tears film to prevent desiccation of the underlying epithelium, and as an important barrier to pathogen penetration (Gipson and Inatomi 1998).

The human mucin (MUC) gene family currently comprises at least 17 genes (MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6–9, and MUC 11–17) and their expression is highly cell- and tissue-specific (Gipson 2004). A major portion of each gene consists of tandem repeat domains which vary in number, length, and sequence. All the repeat domains characterized to date contain a high percentage of threonine, proline, and/or serine and form a large part of the coding region of mucins. Of the known mucins, six have been detected on the ocular surface: MUC1, MUC2, MUC4, MUC5AC, MUC7, and MUC16 (Gipson 2004). The nongoblet conjunctival epithelial cells produce the membrane-spanning mucins MUC1, MUC4, and MUC16. Conjunctival goblet cells are thought to be the major source of the gel-forming mucin MUC5AC (Gipson 2004).

To respond to the various stresses from the external environment and to the internal requirements of the ocular surface epithelial cells, it is necessary to control the volume, composition, and structure of the tear film. Deficiencies in any of the tear film

Correspondence: I-Jong Wang
Department of Ophthalmology, National Taiwan University Hospital, 7, Chung-Shan South Road, Taipei, TAIWAN 100
Tel +886 2 23970800 ext 5729
Fax +886 2 23412875
Email ijong@ha.mc.ntu.edu.tw

components can result in symptoms of dry eye diseases. The function of the mucus layer is predominantly determined by ocular mucin glycoproteins. Secreted mucin mixes with the aqueous tears and is estimated to occupy up to 70% to 90% of the tear film thickness (Argueso and Gipson 2001). Environmental, physiological, and pathological factors can alter mucin gene expression. Previous reports by Tei and colleagues (2000) have demonstrated in rat eyes that rMuc4 and rMuc5AC are down-regulated by vitamin A deficiency. Some studies have shown that inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), and IL-9 can stimulate mucin synthesis in respiratory epithelial cells (Levine et al 1995; Dabbagh et al 1999; Longphre et al 1999). Enss and colleagues (2000) have demonstrated that proinflammatory cytokines such as TNF-α, IL-1, and IL-6 could upregulate MUC gene expression in an intestinal cancer cell line. However, no known studies examining a single cytokine have provided evidence that inflammatory mediators also stimulate mucin synthesis in the ocular surface epithelium. Therefore, this study sought to examine the effects of inflammatory mediators on MUC5AC promoter activity in vivo and in vitro.

Materials and methods
Cell culture

Conjunctival specimens (0.8 cm × 0.4 cm) were obtained by biopsy from the superior bulbar conjunctiva from six patients with no history of ocular surface disease undergoing cataract surgery in the Department of Ophthalmology of the National Taiwan University Hospital, Taipei (Taiwan). The use of human tissue was in strict accordance with the principle set forth in the Declaration of Helsinki. Informed consent was obtained from each patient. Fresh explants were immediately placed in storage medium (keratinocyte defined serum-free medium +5% fetal calf serum) and stored at 4 °C until further procedures. The conjunctival sheets were then rinsed in sterile phosphate-buffered saline (PBS), minced into 2–3 mm pieces with a sterile scalpel, and placed into 35-mm dishes with dispase II solution (1.2 U/mL in PBS) for 1 hour at 37 °C, in 5% $CO2$. The loosened epithelial cells were dispersed by gentle pipetting, rinsed twice with keratinocyte serum-free medium (KSFM), and then put in culture. From each biopsy, approximately $3–5 \times 10^6$ cells were obtained. Primary cells were seeded into 75 mm^2 tissue culture flasks at a density of approximately $1–3 \times 10^6$ cells per flask in 15 mL of medium. Cells were permitted to grow until 60% confluence, and each flask contained approximately 10^7 cells. Only first or second passages of conjunctival epithelial cells were studied and were treated with cytokines at least 48 hours after seeding. Each experiment was conducted at least three times.

Animals

All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. New Zealand white rabbit (male, weighing 1.5 kg) were purchased from the Laboratory Animal Center, National Taiwan University, College of Medicine (Taipei, Taiwan). The animals were housed in cages that were kept in pathogen-free, environmentally controlled, laminar flow hoods, and had free access to autoclaved chow and water.

Transfection of plasmids into conjunctival epithelial cells

A 3.7 kb segment of the 5' flanking region of the human MUC5AC gene (Li et al 1998) was cloned into pGL3-Basic luciferase vector (Promega Corp., Madison, WI, USA). The cytomegalovirus-beta-galactosidase, was used as a control plasmids and to measure transfection efficiency. Conjunctival epithelial cells were seeded in 24-well tissue culture plates (5×10^4 cells/well) and left for 18 hours in complete growth medium. Cell transfection was performed using TransFast™ (Promega) in accordance with the instructions of the manufacturer. For each well, 0.5 µg DNA and 1.5 µL TransFast™ Reagent were mixed in 0.2 mL D-MEN serum-free medium and incubated for 15 minutes at room temperature. The culture medium was replaced with the prepared TransFast™ reagent/DNA mixture. After 1 hour, cells were overlaid with complete growth medium and were allowed to recover overnight. The transfected conjunctival cells were challenged with different cytokines (TNF-α, IL-1β, IL-2, IL-6, or IL-8) in various concentration (1, 10, 20, 50, 100, 200, 500, 1000, and 2000 pg/mL) for 24 hours.

Gene gun delivery of plasmids

New Zealand white rabbits were anesthetized with intramuscular injection of ketamine hydrochloride and 2-(2.6-xylidino)-5.6-dihydro-4H-1.3-thiazine-hydrochloride. The MUC5AC-pGL3 basic luciferase vector was transferred to the rabbit conjunctivae using particle bombardment-mediated DNA transfer with the Helios Gene Gun System (Bio-Rad Laboratories, Hercules, CA, USA). DNA delivery to the rabbit conjunctivae using Helios Gene Gun was performed in accordance with the instructions of the manufacturer, with the following conditions for DNA transfer: Helium pressure: 300 psi; Microcarrier loading quantity (MLQ): 0.5 mg/cartridge;

Microcarrier: 1.0 µg gold; DNA loading ratio (DLR): 5 µg DNA/mg gold). After 36 hours, different proinflammatory cytokines (TNF-α, IL-1β, IL-2 , IL-6, and IL-8, all at 1000 pg/mL) were administered to the transfected rabbit conjunctival tissue via eye drops every 6 hours for 48 hours. Rabbits were killed immediately after exposure by an intracardiac injection of pentobarbital sodium (50 mg/kg of Pentothal; Abbott Australasia, Sydney, Australia). The entire conjunctival tissue was then collected and stored in one 1.5-mL tube.

Luciferase assay

Luciferase activity was measured according to the manufacturer's recommendations (Promega). Briefly, 50 µL of Promega "lysis buffer" (500 µL for rabbit conjunctival tissues) was added to each well (or tube), and plates were agitated for 1 hour at room temperature. Wells were then scraped with a rubber cell scraper, and the contents of the wells were transferred into 1.5-mL tubes and briefly centrifuged to remove cell debris. The supernatant (20 µL) was added to 100 µL of luciferase assay reagent, and light units were measured for 0.6 second on an automated Packard Model TopCount microplate scintillation and luminescence counter (Packard Instrument Company, Meriden, CT, USA). All transfections were carried out in triplicate. Luciferase activity was normalized with respect to relative light units emitted from serum-free, medium-treated controls.

A univariate analysis of variance (U-ANOVA) was performed to compare the quantitative variables for all groups and a Bonferroni post-hoc test was used to evaluate the difference between each group. All clinical and microscopical determinations performed during the study were included as variables. In acknowledgement of the multiple U-ANOVA tests, $P < 0.01$ was considered as statistically significant.

Results
Effect of proinflammatory cytokines on MUC5AC gene expression in vitro

The effect of proinflammatory cytokines on MUC5AC gene expression was assessed by measuring the luciferase activity in cultured human conjunctival epithelial cells. TNF-α and IL-1β (at the concentration of 1000 pg/mL) respectively induced a 1.9- and 6.5-fold increase in MUC5AC-luciferase activity (Figure 1A and 1B, Bonferroni post-hoc test of U-ANOVA tests, $P = 0.0013$ and $P = 0.0001$). However, IL-2, IL-6, and IL-8 incubation did not increase MUC5AC gene expression (Figure 1C, 1D, and 1E).

Effect of proinflammatory cytokines on MUC5AC gene expression in rabbit conjunctiva in Vivo

Next, we investigated the effect of proinflammatory cytokines on MUC5AC gene expression in rabbit conjunctiva in vivo using the luciferase assay system. TNF-α and IL-1β significantly upregulated MUC5AC gene expression (approximately 3- and 6.3-fold increase compared with controls, Bonferroni post-hoc test of U-ANOVA tests, $P = 0.0011$ and $P = 0.0001$) in the rabbit conjunctival tissue in vivo. IL-2, IL-6, and IL-8 also upregulated MUC5AC to a lesser extent (2.6-, 2.5-, and 2.1-fold respectively) (Bonferroni post hoc-test of U-ANOVA tests, $P = 0.012$, $P = 0.021$, and $P = 0.0023$, respectively).

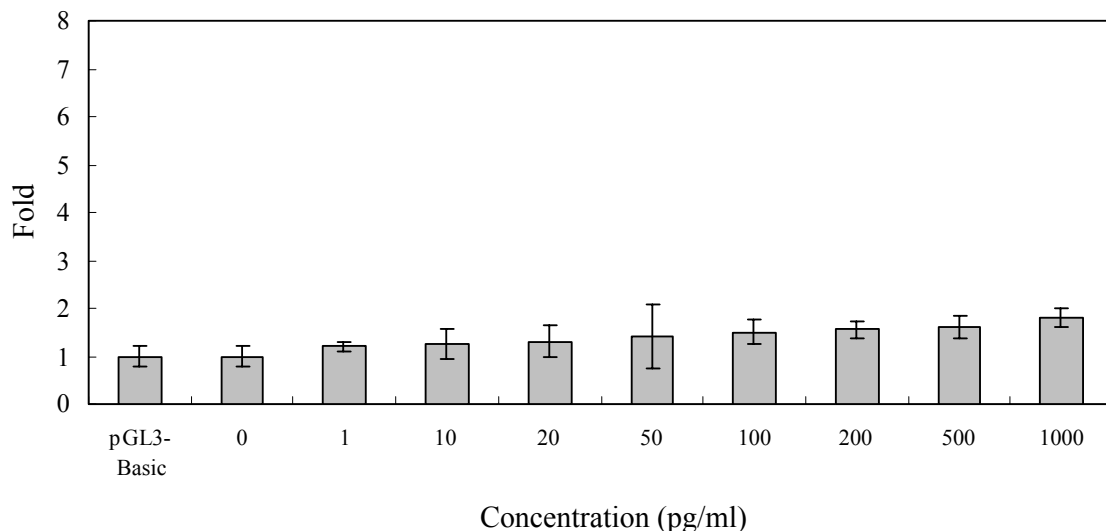**Figure 1A** Effect of TNF-α on the MUC5 AC-luciferase activity

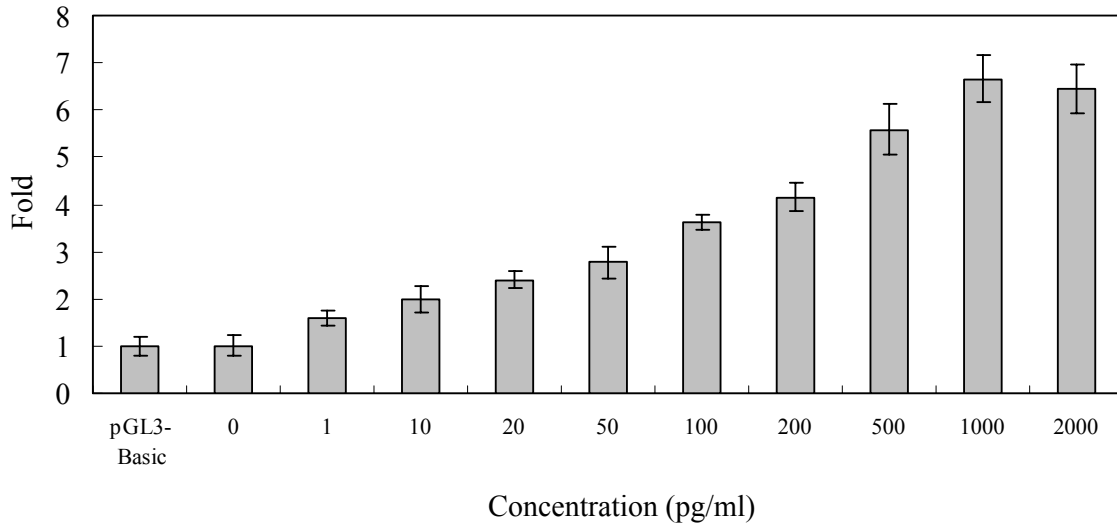**Figure 1B** Effect of IL-β on the MUC5 AC-luciferase activity

Concentration (pg/ml)

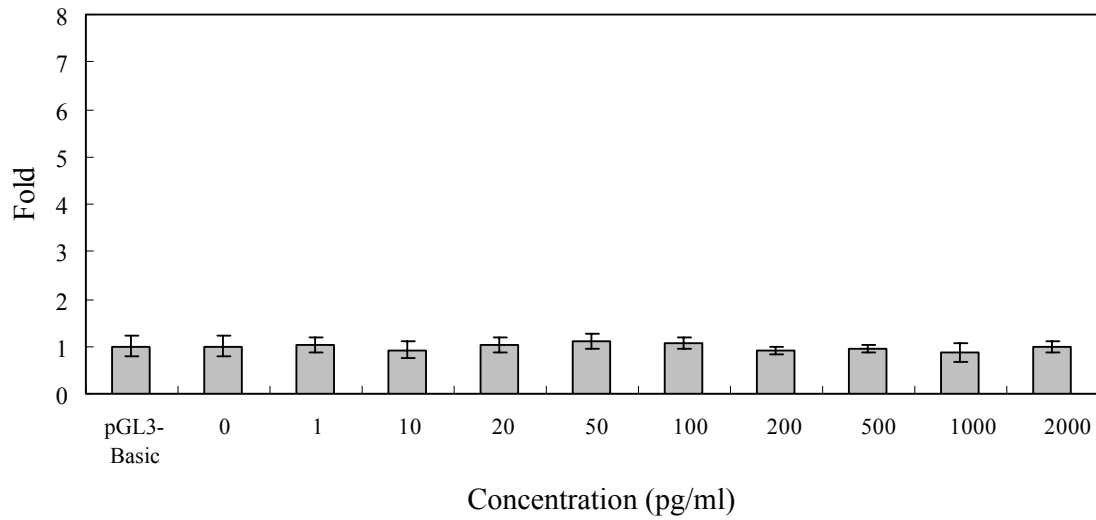**Figure 1C** The Effect of IL-2 on the MUC5 AC-luciferase activity

Concentration (pg/ml)

Figure 1d The Effect of IL-6 on the MUC5 AC-luciferase activity
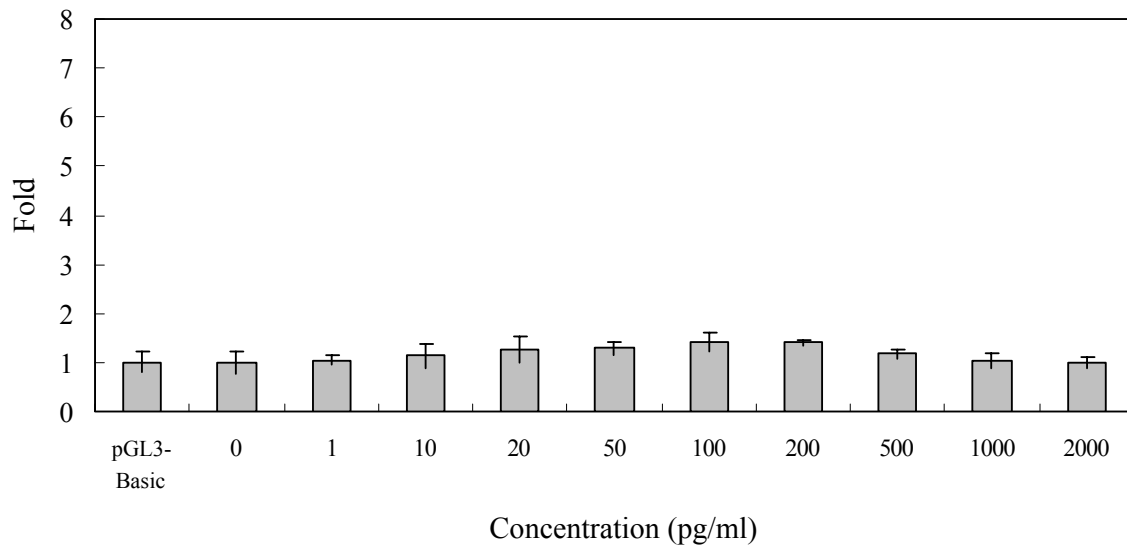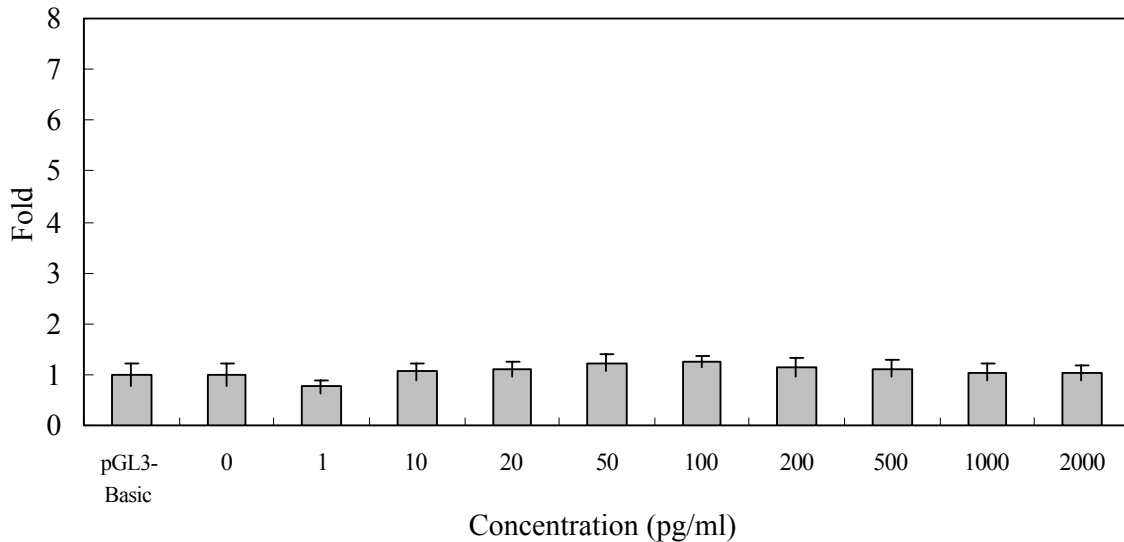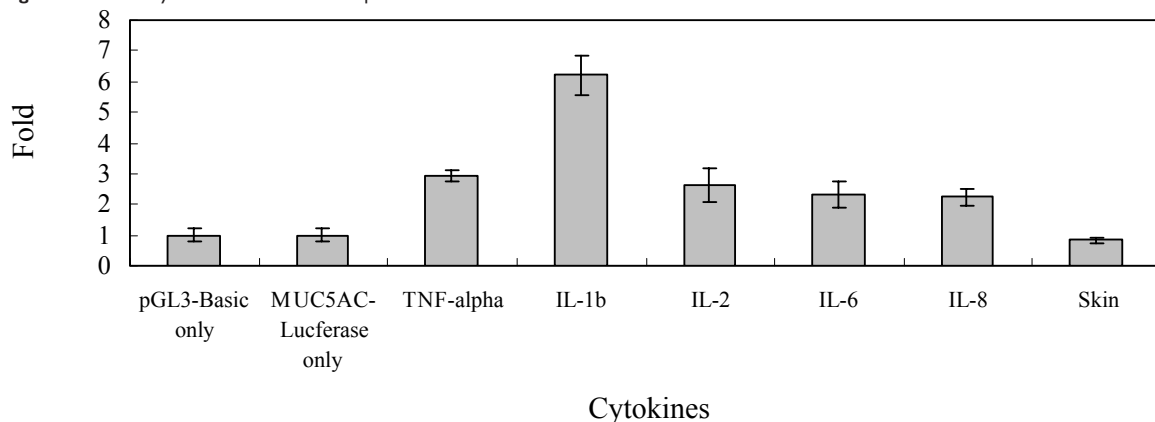

Concentration (pg/ml)

Figure 1E The Effect of IL- 8 on the MUC5 AC-luciferase activity

Figure 1 Proinflammatory cytokines induce MUC5AC gene expression in cultured conjunctival epithelial cells transfected with a pGL3-control luciferase vector and the 3.7-kb MUC5AC luciferase construct and challenged with different kinds of proinflammatory cytokines (A) TNF-α, (b) IL-1β, (c) IL-2, (d) IL-6, (e) IL-8 in different concentration (1, 10, 20, 50, 100, 200, 500,1000, and 2000 pg/ml) for 24 hours. Bars represent means ± SEM (n = 3).
Abbreviations: IL, interleukin; RLU, relative luciferase units; SEM, standard error of mean; TNF-α, tumor necrosis factor-α.

Discussion

Several studies have shown that proinflammatory cytokines can alter the protective mucus layer and result in ocular surface disorders (Jones et al 1998). MUC5AC is the major form of secretory mucins of ocular surface (Inatomi et al 1997; Jumblatt et al 1999). However, studies showing that MUC5AC expression is affected by proinflammatory cytokines were mainly performed in the respiratory tract. For example, mucin hypersecretion is commonly observed in many

inflammatory diseases of the respiratory tract (Richardson and Peatfield 1980). MUC5AC is generally recognized to be a major airway mucin because MUC5AC is also highly expressed in the goblet cells of human airway epithelium (Hovenberg et al 1996). and regulated by various inflammatory cytokines (Adler et al 1994). In these studies, IL-1β and TNF-α vigorously induced MUC5AC gene expression in normal nasal and tracheal epithelial cells (Yoon et al 1999; Smirnova et al 2000; Song et al 2003). IL-1β is a pleiotropic

Figure 2. The activity of MUC5 AC-luciferase upon Gene Gun

Figure 2 Proinflammatory cytokines induce MUC5AC gene expression in rabbit conjunctiva in vivo. Rabbit conjunctival tissues were transfected with a pGL3-control luciferase vector and the 3.7-kb MUC5AC luciferase construct by Helios Gene Gun System (Bio-Rad Lab.) and challenged with different kinds of proinflammatory cytokines (TNF-α 1000 pg/ml, IL-1β 1000 pg/ml, IL-2 1000 pg/ml, IL-6 1000 pg/ml, and IL-8 1000 pg/ml) at the frequency of q6h for 24 hours. Bars represent means ± SEM (n = 3).
Abbreviations: IL, interleukin; RLU, relative luciferase units; SEM, standard error of mean; TNF-α, tumor necrosis factor-α.

and an early-response cytokine in the pulmonary inflammatory cascade, and is produced by many cell types; however, resident and migratory macrophages are considered to be the principal source of IL-1β during inflammatory episodes (Dinarello and Wolff 1993). Increased levels of IL-1 β have been reported in inflammatory airway diseases associated with the hypersecretion of mucins (Monton et al 1999) and signal molecules involved, especially those downstream of the mitogen-activated protein (MAP) kinases (Song et al 2003). Song and colleagues (2003) indicated that the activation of mitogen- and stress-activated cascades, such as the protein kinase 1 (MSK1), the cAMP-response element-binding protein, and the cAMP-response element signaling cascades via ERK and p38 MAP kinases, are crucial aspects of the intracellular mechanisms that mediate MUC5AC gene expression. Fischer and others have also shown that TNF-α can trigger the secretion of mucin by the airway epithelium, and they have demonstrated that TNF-α induces mucin secretion via an intracellular pathway that appears to involve endogenously produced nitric oxide (NO) (Fischer et al 1995; Levine et al 1995). Many other studies have focused on the association between Th2 cytokines (such as IL-4, IL-9, and IL-13) and MUC genes expression (Temann et al 1997; Longphre 1999; Kim et al 2002).

In the present study, we have demonstrated that proinflammatory cytokines such as TNF-α and IL-1β increase the expression of MUC5AC in cultured conjunctival epithelial cells in vitro and in vivo. These results are compatible with previous findings. However, IL-2, IL-6, and IL-8 had no effect on the activity of the human MUC5AC promoter in in vitro studies. In gene gun studies, IL-2, IL-6, and IL-8 could increase the promoter activity of human MUC5AC promoter. These findings suggested that proinflammatory cytokines could induce mucin gene expression by direct (interaction of cytokine receptors with the regulatory regions of the mucin gene) and indirect (immune-mediated) mechanism. As we know, proinflammatory cytokines such as TNF-α, IL-1β, IL-2, IL-6, and IL-8 have a wide variety of activities on many cell types. They may be produced by conjunctival cells, macrophages, lymphocytes, or fibroblasts in response to stimuli (Gamache et al 1997; Hingorani et al 1998). TNF-α, IL-1β, and IL-2 are considered primary cytokines because they initiate a cascade of events in the inflammatory process. They also induce the production of secondary cytokines such as IL-6 (Sironi et al 1989; Sanceau et al 1990). IL-8 is a member of the C-X-C family of chemokines, which has an important role in inflammation through the ability to recruit and activate leukocytes (Albelda et al 1994). IL-8 can induce

the integrin expression on neutrophils, promoting their adhesion to vascular endothelial cells, promote neutrophil degralation, chemotaxis of basophils and eosinophils (Tanimoto et al 1992; Collins et al 1993). This may be the reason why IL-2, IL-6, and IL-8 can induce MUC5AC gene expression in rabbit conjunctival tissue in vivo, while in cultured human conjunctival cell in vitro they cannot. It also explains why the effects of proinflammatory cytokines on MUC5AC gene expression were augmented in conjunctiva in vivo.

The other cause of the discrepancy of in vivo and in vitro results may be because the conjuctival cells were cultured in KSFM which is a calcium-free medium, but exposed to Dulbecco's Modified Eagle Medium (DMEM) which contains high calcium (1.8 M) and results in the changes of conjunctival cell morphology and phenotype. Cells in KSFM were uniformly small and formed a monolayer on confluence (data not shown). In contrast, cells in DMEM were large and squamous, with many vacuoles and became stratified at the center of the dish after confluence. These morphological changes indicate a possible change of phenotype and different response to different cytokines as compared with in vivo assays.

In conclusion, these results demonstrate the differential effects of cytokines on MUC5AC gene expression and suggest that proinflammatory cytokines can induce mucin gene expression by direct and indirect mechanisms. The exact role of these inflammatory cytokines in the regulation of gene expression remains to be elucidated. The current results also implied that ocular mucin gene expression can be affected through the production of proinflammatory and chemotactic cytokines following stimuli.

Disclosure

The authors have no proprietary or financial interest in any product mentioned herein.

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