行政院國家科學委員會專題研究計畫　期中進度報告

Muc5 AC-Diphtheria Toxin 基因轉殖鼠之黏膜黏液素表現

(1/3)

計畫類別：個別型計畫
計畫編號：NSC93-2314-B-002-090-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：國立臺灣大學醫學院眼科

計畫主持人：王一中

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國　94 年 6 月 9 日

The tear film is composed of an outer lipid layer and an inner aqueous phase, which contains a variety of mucin, adjacent to the glycocalyx of the apical cells of the epithelium. Mucins are a heterogenous group of O-linked glycoproteins, synthesized and secreted primarily by goblet cells, which coat and protect mucosal epithelia. Ocular surface mucin adheres to the glycocalyces of conjunctival and corneal epithelial cells and enhances wetability of the cornea by serving as an interface between the hydrophobic corneal epithelium and the aqueous tear fluid.[1,2] Therefore, mucin can stabilize the tear film, provide a smooth and refractive surface of high optical quality over the cornea, lubricate the corneal and conjunctival epithelial surfaces during eye blinking, and prevent desiccation of the ocular surface through water retention. It also serves as a barrier to microbial invasion and shields conjunctival and corneal cells from surface debris and noxious substances.[3,4] As a consequence of their extensive glycosylation, mucins offer a wide range of terminal carbohydrate-related epitopes that can act as receptors for ligands expressed in certain microorganisms and viruses. Consequently, ocular mucins may also prevent pathogen penetrance by inhibiting bacterial adhesion to the ocular surface epithelium.

From previous studies, 15 epithelial mucin genes have been completely or partially sequenced. They are MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6 to 9, MUC11 to 13 and MUC16.[5,6,7,8,9,10,7] According to their physiological functions, they can further be classified as gel-forming mucins (MUC2, MUC5AC, MUC5B, and MUC6), soluble mucins (MUC7 and MUC9), and membrane-spanning mucins (MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, and MUC16). To date, 4 mucins have been found in ocular surface epithelium. Conjunctival goblet cells secrete the gel-forming mucin MUC5AC, whereas the conjunctival and corneal epithelium produces the membrane-spanning mucins MUC1 and MUC4.[11,12] Low expression of MUC2 by the conjunctival epithelium is also found in tears.

The mucus secretion is often found in keratoconjunctivitis sicca and ocular surface inflammation and allergy, but we still don’t know whether these secretions are due to over production of mucin or decreased tear secretion. For example, the role of conjunctival goblet cell mucin in keratitis conjunctiva sicca (dry eye syndrome) remain controversial, our previous clinical experience showed that some cases of dry eye are secondary to a mucus deficiency leading to decreased tear breakup time. Destabilization of the tear film occurs on the ocular surface of eyes in patients with keratoconjunctivitis sicca or other dry eye diseases. Although several reports have shown alterations of mucin products in the ocular surface in dry eye,[13,14,15,16,17] to date, there is no information correlating expression of specific mucin genes with their

protein products. The density of conjunctival goblet cell is found to decrease by 86% in keratitis conjunctiva sica and even more in extreme types of dry eye, such as ocular pemphigoid and Stevens–Johnson syndrome.[18,19] In patients with Sjögren syndrome, a systemic autoimmune condition characterized by a combination of keratoconjunctivitis sicca and dry mouth. But in a recent study using an antibody against a mucinlike glycoprotein present on the surface of stratified ocular epithelia including conjunctiva found an increased staining of goblet cells in patients with dry eye. The increase in the number of positively stained cells is believed to reflect altered mucin glycosylation in a diminished population of conjunctival goblet cells.[20]

Immunopathological changes that have been reported to occur in the conjunctival epithelium of dry eyes include inflammatory cell infiltration, abnormal expression of immune activation markers (HLA-DR
and ICAM-1 antigens) and elevated levels of the inflam-matory cytokine interleukin-6 (IL-6).19–24 Furthermore, elevated concentrations of inflammatory proteins, such as b2-microglobulin and IL-6, have been reported in the tear fluid of patients with Sjögren's syndrome.[21] Consistent with these findings are observations that ocular irritation symptoms and ocular surface disease improve following treatment of eyes with keratoconjunctivitis sicca with anti-inflammatory agents such as corticosteroids and cyclosporin A.[22] The concept that inflammation may alter normal epithe-lial differentiation is well recognized in other organ systems. For example, elevated levels of the pro-inflammatory cytokines IL-1, IL-6 and IL-8 have been detected in the epidermis of patients with psoriasis.[23] Treatment of psoriasis with anti-inflammatory therapies, such as corticosteroids and cyclosporin, improves clinical manifestations of the disease, decreases levels of the chemotactic cytokine IL-8 and returns the epidermis to a more normal phenotype.[23]

The transgenic techniques of microinjection of cloned DNA into fertilized mouse eggs is first accomplished nearly simultaneously in the laboratories of Brinster, Costantini, Ruddle, Mintz, and Wagner. Since then numerous applications of transgenic animal technology have been reported. Altered phenotypes resulting from transgene expression demonstrated that introduced genes can exert profound effects on animal physiology. Transgenic mice have been important for the study of gene expression by hormonal and developmental control. Transgenic mice have also been invaluable for producing animal models for cancer and other diseases, as well as testing the efficacy of gene therapy through the use of cellular overexpression of functional gene products, or negative dominant mutations of transgenes driven by cell type-specific promoters. In addition, cell-cell interactions and cell lineage relationships have been explored using the cell-specific expression of toxin genes in

transgenic mice. Recent studies suggest that attenuated and inducible toxins hold promise for future transgene ablation experiments.

Methods

Preparation of human MUC 5AC-DTA promoter

The 680 bp attenuated diphtheria toxin A cDNA (Tox176) was released from plasmid Ktnpr-DTa/BpA (a generous gift from Dr. Winston Kao) by Hind III digestion and replaced with the luciferacse reporters cDNA in plasmids containing the 3806 bp human Muc5AC promoter in a PGL3 luciferase reporters vector (Promega, Madison,WI) also digested by HindIII digestion to generate the human Muc5AC-DTA/SV 40 constrcuts.{Maxwell, 1987 270 /id}{Li, 1998 269 /id} The constructed plasmid contained the human Muc5AC promoter, attenuated diphtheria toxin A cDNA, and a SV40 poly A site were inserted into plasmid pb-gal-Basic (Clontech, Palo Alto, CA) by replacing the LacZ gene with Tox176, placing GNAT2 promoter upstream of Tox176 and placing the IRBP enhancer downstream of the SV40 intron and poly A site to create a new plasmid, pGNAT2-Tox176-IRBP. The 2.4 kb Trc-Tox176 minigene was then isolated from pGNAT2-Tox176-IRBP by Hind III/Sal I digestion.

Generation of Transgenic Mouse Lines-

Transgenic mice overexpressing human MUC5AC promoter are constructed as described previously, using a transgene containing the human MUC5AC promoter-diphtheria toxin cDNA(Tox176) and an 850-base pair cassette (SVPA) containing the polyadenylation and splice site sequences of SV40.(Liu et al., 2000; Ying et al., 2000) Animal experiments are performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The 2.4 kb MUC5AC-Tox176 minigene is gel purified and microinjected into single-cell stage FVB/N embryos by the transgenic core facility in the Medical College, National Taiwan University. Transgenic mice are identified by PCR of mouse tail DNA with a primer pair chosen from within Tox176 gene, 5'-AACTTTTCTTCGTACCACGG-3' and 5'-ACTCATACATCGCATCT TGG-3'. Since FVB/N mice have a recessive mutation at the rd locus, the founders are bred with C57BL/6J mice to establish stable Trc-Tox176 transgenic mouse lines. Both C57BL/6J mice and C57BL/6J x FVB/N F1 hybrids have normal ocular surface. Male mice heterozygous for the MUC5AC-Tox176 transgene are bred with female mice heterozygous for the MUC5AC-Tox176 transgene to produce mice of four separate genotypes: those carrying both transgenes, those carrying one of the two,

and those carrying none of the transgenes. The presence of the transgenes was determined by PCR analysis of genomic DNA obtained from tail biopsy and prepared using the QiAmp tissue isolation kit (Qiagen, Valencia, CA). Sex-matched littermate mice between the ages of 2 and 4 months are used for all experiments.

Animal Care and Tissue Preparation-- Animals are housed in a room maintained at 23 °C with a fixed 12-h light/dark cycle (lights on 6 a.m. to 6 p.m.) and given free access to Purina chow and water. On the morning of the experiment, animals are anesthetized with pentobarbital sodium (5 mg/100 g body weight by intraperitoneal injection).

Generation of Inducible Transgenic Mouse Lines

The transgenes containing tTA under the control of the human MUC5AC (MUC5AC-tTA) promoter are constructed. For construction of the MUC5AC-tTA transgene, the *Eco*RI-*Bam*HI fragment containing the tTA sequence from pUHD15.1 is cloned into the *Eco*RI and *Bam*HI sites of the pBSIIKS$^+$ vector (Stratagene). The tTA fragment released by digestion of the resulting plasmid with *Not*I and *Xho*I is inserted into the pGEMAlbSVtpA vector. The resulting construct is digested with *Nhe*I and *Nsi*I sites to generate the MUC5AC-tTA transgene (~4.5 kilobase pairs). The MUC5AC-tTA transgenic construct is generated the following way. The *Pst*I-*Kpn*I fragment containing the MUC5AC promoter is inserted into the *Nsi*I and *Kpn*I sites of pSVSPORT1 (Life Technologies, Inc.), replacing the SV40 promoter. The *Eco*RI-*Hpa*I fragment containing tTA and SV40tIVSpA from pUHD15.1 is inserted into the *Sal*I (treated with Klenow for blunt end) and *Eco*RI sites of the previous construct. The resulting plasmid is then digested with *Cla*I and *Mlu*I to generate the MUP-tTA transgenic fragment (~4.0 kilobase pairs). These transgenes are introduced into fertilized eggs of FVB/n mice using standard transgenic techniques. Mice transgenic for SV40 T antigen (*tetO*-TAg;) or β-galactosidase (*lacZ*; from the Jackson Laboratory, Bar Harbor, ME) linked to the cytomegalovirus minimal promoter fused to the tTA-binding sequences (*tetO*) have been described previously. Genomic DNA isolated from tail clips of the offspring is used for genotyping. Genotyping of the MUC5AC-tTA transgenic mice is performed using standard Southern blotting, and genotyping of the TAg mice is performed using the polymerase chain reaction (PCR) with TAg-specific primers (sense, 5'-ggaatagtcaccatgaatgagtacag-3'; and antisense, 5'-ggacaaaccacaactagaatgcagtg-3').

Administration of Tetracycline-- Slow-release tetracycline pellets (Innovative Research of America, Sarasota, FL) are implanted subcutaneously in the shoulder regions of mice using a trochar as described by the manufacturer. These pellets release 0.7 mg of tetracycline hydrochloride/day for 21 days. As negative controls, placebo tablets obtained from the same manufacturer are similarly implanted. Expression of the transgene is measured on day 20. A group of mice are killed and analyzed for their transgene expression on day 28 (7 days after the dissolution of the tetracycline pellets) to evaluate the reversibility of the transgene expression

Visualization and Quantitation of Filled Goblet Cells

To visualize and quantitate the filled conjunctival goblet cells, a conjunctival wholemount technique is used.(Gipson and Tisdale, 1997) One eye from each animal is fixed for 5 to 10 minutes in situ with 4% paraformaldehyde in PBS. Eyes are enucleated, and wholemounts of superior and inferior conjunctiva are dissected and fixed overnight in 4% paraformaldehyde. Tissues are then rinsed three times (10 minutes per rinse) in PBS and incubated with rhodamine-conjugated phalloidin, which primarily binds to the filamentous actin at the cell periphery (dilution 1:100; Molecular Probes, Eugene, OR). With this technique, only filled goblet cells are visualized. Conjunctival wholemounts are postfixed for 30 minutes in 4% paraformaldehyde and rinsed three times for 10 minutes each in PBS. Tissues are flatmounted on a glass slide in mounting medium (Vectashield; Vector, Burlingame, CA).

Tissue is viewed by confocal microscopy using a confocal microscope (TCS 4D; Leica, Heidelberg, Germany). Optical sections are taken of 3 forniceal fields using a 40x PL Fluotar oil immersion objective. In this manner, the entire forniceal area is usually captured.

Images are imported into image management software (Photoshop, ver. 5.0; Adobe, San Jose, CA), and measurement of the entire epithelial area is achieved by tracing the area by using the "lasso" tool in the software program. The total data area, measured in pixels, is acquired through the "image: histogram" command in the program. Two independent counts are recorded for filled, phalloidin-labeled goblet cells. Goblet cells per unit area of pixels are adjusted to real unit area or cells per square millimeter of real epithelial area, based on 28.346 pixels/cm in the software and a calibration factor of 1 mm = 4096 pixels at x40 magnification on the confocal microscope. Data are recorded as goblet cells per

square millimeter, and the results are analyzed on computer with ANOVA (StatView software, ver. 5.0; Abacus Concepts; Berkeley, CA).

Goblet Cell Involution and Apoptosis.

Goblet cells are visualized using periodic acid-Schiff/Alcian blue/hematoxylin staining. Depth of mucin goblets are measured using a Zeiss Universal Research Microscope fitted with a Hitachi video camera using Scion Image software. Apoptosis is visualized after H&E staining using standard morphological criteria and using the TUNEL assay. For quantitative analysis of TUNEL-positive cells, a cell is scored TUNEL positive only if it is in the epithelial layer and had apoptotic morphology. Goblet cells are scored only if they occupied at least 50% of a x400 microscopic field, ensuring reasonable longitudinal orientation. Immunohistochemical staining with monoclonal antibody against single-stranded DNA is also used to assess apoptosis in tissue sections. Monoclonal antibody F7–26 is obtained from Chemicon International Inc., and single-stranded DNA is stained using the protocol suggested by the manufacturer.

Northern Blot Analysis

Northern blot analysis is performed by AlkPhos Direct (Amersham Pharmacia Biotech, Uppsala, Sweden). Complementary DNA (cDNA) of MUC2 and MUC5AC mRNA is obtained by RT-PCR using RNA extracted from fresh mouse colon or stomach as template, respectively (MUC1 5' actactaccaagagctg 3' [J05582: 3264-3280; M84683: 1339-1355] and MUC1 5' ctcataggatggtaggt3' [J05582: 3693-3677; M84683: 1762-1746]), (MUC2: sense 5'-acgatgcctacaccaaggtc-3', antisense 5'-ccatgttattggggcatttc-3', annealing temperature 52°C, 40 cycles, product size 509 bp; MUC5AC: sense 5'-tcatgagtgcaaacaggagc-3', antisense tgacccagatcctccatctc-3', annealing temperature 55°C, 40 cycles, product size 683 bp). Alkaline phosphatase-labeled cDNA probes are obtained by incubating of each PCR product with reaction buffer, labeling reagent, and cross-linker for 30 minutes at 37°C. These three solutions are included in AlkPhos Direct. Forty µg of total RNA are subjected to electrophoresis on 1.0% agarose gel in the presence of formaldehyde. RNA is transferred by capillary blotting onto an Hybond+ N nylon membrane (Amersham Pharmacia Biotech) and cross-linked with ultraviolet light using Spectrolinker (Tomy Digital Biology Co., Ltd., Tokyo, Japan). The membrane is incubated with the prehybridization buffer included in AlkPhos Direct for 30 minutes at 50°C. After that, the labeled probe is added to the prehybridization buffer at a

concentration of 15 ng/ml, and then hybridized at 50°C for 18 hours. The membrane is ished twice with primary ish buffer [2 mol/L urea, 0.1% sodium dodecyl sulfate (SDS), 50 mmol/L sodium phosphate buffer, pH 7.0, 1 mmol/L NaCl, and 10 mmol/L $MgCl_2$] for 10 minutes at 50°C, and then twice more with secondary ish buffer (50 mmol/L Tris base and 100 mmol/L NaCl) for 5 minutes at room temperature. Signals are detected with CDP-Star chemiluminescent detection reagent (Amersham Pharmacia Biotech). The membrane is incubated with detection reagent (40 μl/cm^2) for 5 minutes at room temperature and then placed in a detection bag with Hyperfilm (Amersham Pharmacia Biotech) for 3 hours, for image development. After the detachment of probes by incubation with 5% SDS for 30 minutes at 50°C and 0.1% SDS for 5 minutes at 100°C, the membrane is reused.

Real-Time RT-PCR to Determine Relative MUC1、MUC2、Muc5AC and Muc4 Expression

The contralateral eye is taken for RNA isolation to use for relative quantitation of Muc5AC and Muc4 mRNAs using real-time RT-PCR. Mouse conjunctivas are snap frozen in liquid nitrogen, and total RNA is isolated by a single-step extraction technique with reagent (TRIzol; Gibco-Life Technologies, Grand Island, NY). The first strand of cDNA is synthesized from 1 μg of DNase-treated RNA with random primers, using reverse transcriptase (SuperScript II; Gibco-Life Technologies), followed by RNase H treatment.

Real-time RT-PCR is performed using a sequence-detection system (*Taq*Man PCR GeneAmp 5700; (PE Applied Biosystems, Foster City, CA), essentially as previously described.(Fink et al., 1998; de Cremoux et al., 2000) The Muc5AC-specific primers (based on GenBank accession number L42292; provided in the public domain by the National Center for Biotechnology and available at http://www.ncbi.nlm.nih.gov)(Shekels et al., 1995)are sense 5'-aaagacaccagtagtcactcagcaa-3' (nucleotides [nt] 745-769) and antisense 5'-ctgggaagtcagtgtcaaacca-3' (nt 883-862), and the *Taq*Man probe sequence is 5'-TCACACACAACCACTCAACCAGTGACCA-3' (nt 802-829). The Muc4-specific primers (based on GenBank accession number AF218265) are sense 5'-ctccaagaaatgtagtggctttcag-3' (nt 2925-2949), antisense 5'-cacggtcttgggctggagta-3' (nt 883-862), and *Taq*Man probe 5'-aacatccccagaagcgtgtaccctgg-3' (nt 802-829). 5 The Muc1-specific primers (based on GenBank accession number J05581) are sense 5'-accatcctatgagcgagtacc-3', and 5'-gccaccattacctgcagaaac-3'. The Muc2 specific

primiers are sense 3' primer, 5'-cacacagcgacctttctcat-3' and anti-sense-Muc2 5' primer, 5'-accctcctcctaccacattg-3'

For relative quantitation as used in this study, a comparative threshold (C_T) method normalizes the amount of target to an internal standard control—for example, a suitable housekeeping gene. The relative amount of target gene in different samples is determined and compared with the amount in the naïve control specimens (calibrator specimen). The internal standard control gene is amplified using rodent GAPDH (*Taq*Man Control Reagents; PE Biosystems). To verify the validity of using GAPDH as the internal calibration standard, the efficiencies of the Muc5AC-Muc4 and GAPDH amplifications are compared and found to be equivalent. The identities of the Muc5AC and Muc4 PCR products are verified by sequencing performed by the DNA Sequencing Core Facility of the Massachusetts General Hospital (Boston, MA).

Each PCR reaction contained equivalent amounts of cDNA. Assays are performed in triplicate using a kit according to the manufacturer's recommendations (*Taq*Man PCR Reagent Kit; PE Biosystems). Relative quantitation of amounts of Muc 1, Muc5AC and Muc4 mRNAs at 0, 6, 24, and 48 hours after final antigen challenge is determined as previously described.(Fink et al., 1998) Results are analyzed using the unpaired *t*-test .

In Situ hybridization techniques for MUC4 and MUC5AC

In Situ hybridization is performed on sections from normally cycling mouse tissue, using ^{35}S-labeled anti-sense oligonucleotide probes corresponding to the tandem repeat regions of Muc4 and Muc5AC. The sequence used for the Muc4 antisense oligonucleotide, 5' tgtagaaccttgagtccttactgctgttgtgtgtcctgtg 3', is obtained from GenBank, accession #AF441786, and that for the Muc5AC antisense oligonucleotide, 5' ggttgtagagatggtgctggtctttcctgtattgggtgagctggtttg 3', from GenBank, accession #L42292. Techniques used are as previously described.(Lange et al., 2003) Briefly, paraffin sections are deparaffinized and treated with proteinase K. The samples are acetylized and hybridized overnight with 2-5 x10^6 cpm ml^{-1}^{35}S-labeled probe. After ishing, the sections are dipped in Kodak NTB-2 emulsion (Eastman Kodak; Rochester, NY, USA) and developed after one week. The sections are counterstained with hematoxylin and eosin. *In Situ* hybridization using a sense oligonucleotide probe for Muc4, 5' cacaggacacacaacagcagtaaggactcaaggttctaca 3' and for muc5ac, 5' caaaccagctcacccaatacaggaaagaccagcaccatctctacaacc 3', is performed on all tissues

as a control.

In Situ Hybridization for MUC2

Tissues were fixed in Bouin's fixative (for *Klf4*) or 4% PFA (for *Muc2*), embedded in paraffin, and 5 μm sections were applied to Probe-on Plus^{TM} slides (Fisher). Sections were deparaffinized in xylene, then rehydrated in ethanol. Digoxigenin-labeled sense and antisense probes were transcribed in vitro from the following plasmids: a 220 bp fragment of the mouse *Klf4* full-length cDNA (GenBank Accession Number, U20344) containing the nucleotides 1360-1574 cloned into the pBluescript SK (Stratagene) and a 357 bp fragment of the mouse *Muc2* partial cDNA (GenBank Accession Number, AJ010437) containing the nucleotides 337 to 694 cloned into pCRII (Invitrogen). In situ hybridization was performed as described by Wilkinson (Wilkinson, 1992□). Anti-digoxigenin antibody (Roche Diagnostics) was used at a dilution of 1:2000. The slides were developed overnight using NBT/BCIP substrate (Roche Diagnostics), counterstained with 0.1% nuclear Fast Red for 5 seconds or less, dehydrated and mounted with Cytoseal^{TM} 60 (Stephens Scientific). Images were captured on a Nikon Microphot FX microscope.

Immunohistochemical Staining

For all mucin detections, antigen retrieval is performed by microwaving rehydrated sections in 10 mM citric acid (pH 6.0) four times for 5 min each with 1 min between heatings. Monoclonal antibody against MUC1、MUC2、MUC4、MUC5AC and cytokeratin-7 (Santa Cruz Biotechnology, Santa Cruz, CA) is applied at a 1:40 dilution at 4°C overnight. Histochem-Plus kits are used to detect bound primary antibody. Lysozyme immunohistochemistry is performed by the Moffitt Hospital pathology laboratory using polyclonal antibody obtained from Cell Marque.

Electron Microscopy

Samples are fixed in 2.7% glutaraldehyde, 0.8% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at room temperature. They are rinsed in distilled water and postfixed in 2% osmium tetroxide for 1 h, dehydrated through graded alcohols to 100% ethanol, and embedded in Eponate resin (Polysciences, Inc., Warrington, PA). Seventy-nanometer sections are cut and stained with uranyl

acetate (10% in 50% ethanol) and Reynold's lead citrate (microwave method). A Philips Bioscan Techni 10 is used at 80 kV for photography.

Results

Effect of proinflammatory cytokines on MUC5AC gene expression in cultured human conjunctival cell line

The luciferase activities were measured upon the effects of proinflammatory cytokines on MUC5AC gene expression in cultured human conjunctival cell line. TNF-α (>1 pg/ml) and IL-1β (>1 pg/ml) induced 2 and 3.5-fold increases in the activities of MUC5AC gene expression compared to controls, respectively (Figure 1A and 1B). However, IL-2, IL-6, and IL-8 did not increase the activities of MUC5AC gene expression from the concentration of 1 pg/ml to 1000 pg/ ml (Figure 1C, 1D and 1E).

In vivo effect of pro-inflammatory cytokines on MUC5AC gene expression in rabbit conjunctiva

Next, we still used luciferase assay system to investigate the effects of pro-inflammatory cytokines on MUC5AC gene expression in rabbit conjunctiva *in vivo*. TNF-α (1 pg/ml), IL-2 (1 pg/ml), and IL-8 (1pg/ml) significantly increased the activities of MUC5AC gene expression (about 6-fold increase compared to controls) in the rabbit conjunctival tissue *in vivo*, IL-1β(1 pg/ml) and IL-6 (1 pg/ml) also up-regulated the gene to lesser extent (about 3-fold increase compared to controls).

Effect of proinflammatory cytokines on MUC5AC gene expression in mouse conjunctiva in vivo

Figure 1 proinflammatory cytokines induce MUC5AC gene expression in cultured conjunctival epithelial cells in vitro. ATCC CCL-20.2 cells were transfected with 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 pg/ml) at the frequency of q6h for 24 hours. RLU, relative luciferase units. Bars represent means ± SEM (n = 3 wells in triplicate). *Significantly different from SFM control; $P < 0.05$.

Figure 2 Proinflammatory cytokines induce MUC5AC gene expression in rabbit conjunctiva in vivo. Rabbit conjunctival tissues were transfected with 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 100 pg/ml, IL-1β 100 pg/ml, IL-2 100 pg/ml, IL-6 100 pg/ml, IL-8 1000 pg/ml) at the frequency of q6h for 24 hours. RLU, relative luciferase units. Bars represent means ± SEM ($n = 3$ in triplicate). *Significantly different from SFM control; $P < 0.05$.

Figure 3 mMuc5ac and β-actin mRNA were quantified by RT-PCR in mouse conjunctiva treated with different kinds of cytokines (TNF-α 100 pg/ml, IL-1β 100 pg/ml, IL-2 100 pg/ml, IL-6 100 pg/ml, IL-8 1000 pg/ml).

Discussion

The alteration of a protective mucus layer in the ocular surface disorders initiated the question, whether proinflammatory cytokines act on the expression of MUC genes. There had been many studies focusing on the association between the Th2-associated cytokines (such as IL-4, IL-9, IL-13) and MUC genes expression.[9, 10,14] Dabbagh and coworkers demonstrated IL-4 can up-regulate MUC2 and MUC5AC gene expression and goblet cell metaplasia in cultured human respiratory epithelial cells and mouse airway.[10] The Th2 cytokine IL-9 was also demonstrated to act directly on the respiratory epithelial cells to stimulate mucin transcription.[11]

In the present study we demonstrate that proinflammatory cytokines such as TNF-α, IL-1β may be responsible for increased expression of MUC5AC gene directly in cultured conjunctival epithelial cells, while IL-2, IL-6, and IL-8 had no effect. Contrary to ATCC CCL-20.2 cells, TNF-α, IL-2, and IL-8 significantly up-regulated MUC5AC gene expression in the rabbit conjunctival tissue in vivo, IL-1β and IL-6 also up-regulated the gene to lesser extent. Mucin gene expression showed large variations in levels and patterns from conjunctival cell line to conjunctival tissue. These findings suggested that proinflammatory cytokines could induce mucin gene expression by direct (cytokine receptor interaction with the regulatory regions of mucin gene) and indirect (immune-mediated) mechanism. As all 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[16,17], macrophages, lymphocytes or fibroblasts in response to stimuli. 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.[18. 19] 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. IL-8 induces integrin expression on neutrophils, promoting their adhesion to vascular endothelial cells.[20] Willems and coworkers demonstrated that IL-8 could promote neutrophil degralation.[21] Chemotaxis of basophils and eosinophils is also induced by IL-8.[22, 23] This may be the reason why IL-2, IL-6, and IL-8 can induce MUC5AC gene expression in the rabbit conjunctival tissue in vivo, while they can not in cultured human conjunctival cell line (ATCC CCL-20.2) in vitro. It also explained why the effects of proinflammatory cytokines on MUC5AC gene expression were augmented in conjunctiva in vivo.