MUC5AC Mucin Is a Component of the Human Precorneal Tear Film

Marcia M. Jumblatt, Richard W. McKenzie, and James E. Jumblatt

PURPOSE. Mucins are important structural and functional components of the precorneal tear film, yet little is known of their composition and synthesis. The mRNAs of MUC1, MUC4, and MUC5AC have previously been identified in human conjunctiva. Of these, only MUC5AC mRNA appears to be associated with goblet cells. The purpose of the this study was to quantify MUC5AC transcript levels, to identify MUC5AC protein in conjunctiva, tears, and goblet cells and to determine whether this mucin is secreted in response to the calcium ionophore ionomycin.

METHODS. MUC5AC mRNA from normal human conjunctiva was identified, quantified, and compared with β2-microglobulin levels using a competitive reverse transcription-polymerase chain reaction (RT-PCR) method. An antibody to a MUC5AC peptide was used to localize this mucin in conjunctival sections by immunohistochemistry. Anti-MUC5AC antiserum was used to label western blot analysis of conjunctiva and tears. Conjunctival tissues were incubated with ionomycin, and secreted mucins were detected with Helix pomatia agglutinin conjugated to horseradish peroxidase and with anti-MUC5AC antiserum.

RESULTS. MUC5AC and β2-microglobulin transcripts were expressed at a ratio of approximately 1:500. Immunohistochemical labeling showed that MUC5AC was localized in conjunctival goblet cells and at the apical surface of the conjunctival epithelium. MUC5AC protein was present in conjunctiva and in the tear film. Ionomycin stimulation of conjunctival secretion resulted in a fourfold increase in total mucin secretion and in a corresponding increase in secreted MUC5AC.

CONCLUSIONS. MUC5AC is synthesized by goblet cells of the normal human conjunctiva, and this mucin is a component of conjunctival secretions and of normal human tears. (Invest Ophthalmol Vis Sci 1999;40:43-49)

The precorneal tear film consists of aqueous and proteinaceous secretions of the lacrimal gland, hydrated mucus of conjunctival origin, and a superficial lipid layer produced by the meibomian glands. The mucus layer, consisting primarily of soluble mucin glycoproteins, serves to lubricate and protect the superficial ocular epithelium, to retard fluid evaporation, and to anchor the tear film to the ocular surface.5-8 Recent studies indicate that the hydrated mucus layer occupies 70% to 90% of the tear film thickness, suggesting that soluble mucins are an important structural component of the precorneal tear film.5,8 Biochemical evidence suggests that the mucins of the normal human tear film are heterogeneous, although the functional significance of this complexity is not clear.5,6 In gastrointestinal and respiratory tissues, mucins function as buffers, antioxidants, and inhibitors of bacterial adhesion, although the roles of specific mucins in mediating these effects are not fully understood.7,9

Mucins are a family of high-molecular-weight glycoproteins secreted by goblet and nongoblet epithelial cells of mucosal tissues. Molecular studies of mucins derived from human intestinal respiratory and reproductive tissues have identified nine distinct mucin genes (MUC1, 2, 3, and 4 MUC5AC, MUC5B, MUC6, 7, and 8), each of which is expressed in a tissue-specific manner.7-10 Variations in the pattern of mucin expression in various tissues suggests that individual mucins serve unique protective functions at the apical surfaces of specialized epithelial cells. Although mucins are a major constituent of the precorneal tear film, little is known of the regulation of their synthesis and secretion. Histologic and molecular studies suggest that tear film mucins arise from goblet and nongoblet conjunctival epithelial cells.11,12 Of the secretory mucin species identified to date and characterized at the nucleic acid level, transcripts of MUC2, MUC4, and MUC5AC have been identified in mRNA isolated from human conjunctiva.11,13-15 MUC1 is also present and seems to be associated with the cell membrane of conjunctival, but not corneal, epithelial cells.14 Of the conjunctival mucins, only MUC5AC transcripts have been localized to conjunctival goblet cells, the presumptive source of most tear film mucin.11

Mucin secretion from goblet cells occurs by calcium-dependent compound exocytosis.16 In the conjunctiva, several agents including prostanoids, autonomic transmitters, and neuropeptides have been identified as potential mucin secretagogues.17-21 Qualitative and quantitative analyses of conjunctival mucin secretion have been hampered because there are no specific probes for individual mucins, and little is known of...
the regulatory mechanism governing secretion of any of the major mucins. MUC2 and MUC5AC have been localized to goblet cells and may be secreted in response to various mediators; it is unlikely, however, that the secretion of membrane-associated MUC1 is acutely regulated. 6,16

In the present study we examined the expression and secretion of MUC5AC in human conjunctival tissue and in the precorneal tear film. We confirmed that MUC5AC mRNA transcripts are abundant in human conjunctival tissue and showed, in addition, that the corresponding protein was present in conjunctival goblet cells and in the precorneal tear film. Furthermore, we showed that secretion of MUC5AC from human conjunctiva can be elicited by stimulation of calcium influx.

METHODS

Collection of Human Conjunctiva

Human donor conjunctivae were obtained from the Kentucky Lions Eye Bank in accordance with protocols approved by the University of Louisville Human Studies Committee. Conjunctival tissue was collected from eye bank donors (identified by age, gender, race, and cause and time of death) within 3 hours after death. Tissue (approximately 200 mg) was collected by excision and included conjunctiva from the superior and inferior culbar regions and conjunctiva from the nasal fornix (plica semilunaris). Excised conjunctiva was placed in ice-cold tissue culture medium (Dulbecco’s modified Eagle’s medium/F12 containing 5% fetal bovine serum). Tissue destined for RNA analysis was transferred within 1 hour to ice-cold RNA lysis buffer (Qiagen, Santa Clarita, CA) and stored at -70°C before homogenization. Tissues used for western blot analysis of mucin and for secretion studies were stored at 4°C and used within 10 hours after death.

RNA Isolation and Characterization

Total RNA was isolated from human conjunctiva using a guanidinium isothiocyanate protocol (RNAasy; Qiagen, CA). The resultant RNA was treated with RNase-free DNase, extracted twice with phenol-chloroform-isomyl alcohol (24:24:1), precipitated with ethanol, dissolved in RNase-free water, and quantified spectrophotometrically. Average yields observed were approximately 1 μg RNA per milligram tissue (wet weight). Each RNA preparation was characterized qualitatively precipitated with ethanol, dissolved in RNase-free water, and quantified spectrophotometrically. Average yields observed were approximately 1 μg RNA per milligram tissue (wet weight). Each RNA preparation was characterized qualitatively.

Competitive Reverse Transcription–Polymerase Chain Reaction of MUC5AC mRNA

Quantitative analysis of MUC5AC mRNA was carried out as described by Guzman et al. using a competitive polymerase chain reaction (PCR) technique (MIMIC; Clontech, Palo Alto, CA) based on target-specific primers and internal competitive standards. 22 The MUC5AC competitive template, was a double-stranded DNA construct with ends complementary to the MUC5AC primers but containing a nonhomologous central sequence. Preliminary studies were conducted to show that target and competitive sequences amplified with linear kinetics. Coamplification of a constant amount of conjunctival cDNA in the presence of serial dilutions of this internal standard allowed for quantification of MUC5AC mRNA. A ubiquitously expressed protein, β2-microglobulin, was used as an internal, quantitative control.

After reverse transcription, 1 μl conjunctival cDNA was amplified and analyzed. The PCR parameters consisted of an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 2 minutes. MgCl2 was included at 2 mM. After electrophoresis in 1% agarose, bands corresponding to MUC5AC mRNA and its MIMIC were stained with ethidium bromide, photographed, and analyzed by digital image processing. The identity of the MUC5AC product was confirmed by sequencing of cDNA isolated from the agarose gel. The abundance of MUC5AC mRNA was determined by densitometric analysis and compared with the MIMIC standard. Results of MUC5AC amplification were compared with those obtained from β2-microglobulin amplification to determine the relative abundance of MUC5AC mRNA transcripts.

Collection of Human Tear Samples

Collection of tear samples followed a protocol approved by the University of Louisville Human Studies Committee. Human tear samples were collected by absorption to Schirmer strips (Alcon, Fort Worth, TX). Schirmer strip samples were collected from unanesthetized eyes of normal volunteers, air dried and, in some cases, cut into 3-mm sections representing the bulb portion of the strip and two proximal 5-mm sections. To elute mucins, each strip or section was placed in a tube containing 40 μl sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled for 5 minutes, and centrifuged to compact the paper strip.

Production of MUC5AC Antiserum

A polyclonal antibody recognizing MUC5AC epitopes was produced by QCB (Hopkinton, MA). Two peptides (KT-THSQPVRTD and PSPGPHGKEYNN), corresponding to non-glycosylated sequences flanking the tandem repeat domains of the human MUC5AC sequence (National Center for Biotechnology Information accession number 2135766) were synthesized, coupled to keyhole limpet hemocyanin, and injected into a goat from which preimmune serum had been collected. Antiserum was collected and titered after the initial injection and subsequent injections. After four boosts, the immune serum was collected and stored as frozen aliquots.

Immunohistochemical Detection of MUC5AC in Human Conjunctival Tissue

Freshly isolated human conjunctival tissue was fixed in 10% neutral buffered formalin overnight, embedded in paraffin, cut into 10-μM sections, and mounted on glass slides. Sections were deparaffinized and incubated overnight with 1:500 MUC5AC antisera. Bound antibody was detected using an anti-goat avidin–biotin complex horseradish peroxidase (HRP) protocol according to the manufacturer’s directions (Vector, Burlingame, CA).

SDS-PAGE and Western Blot Analysis

Human conjunctival tissue, tears, and porcine stomach mucin (PSM), at approximately 1 mg/ml, were homogenized in SDS-PAGE sample buffer. All samples and standards were boiled for 5 minutes before loading onto 4% to 12% polyacrylamide gradient gels (Bio-Rad, Hercules, CA). Gels were run for 1 hour at
RESULTS

Identification of MUC5AC Transcripts in Human Conjunctival Tissues

The presence of MUC5AC transcripts in human conjunctiva was confirmed and quantified by competitive reverse transcription-polymerase chain reaction (RT-PCR) of conjunctival RNA. The primer pairs used for amplification of MUC5AC mRNA were selected from sequences outside the tandem repeat region and do not resemble sequences encoding the related mucins MUC5B or MUC2. After amplification and electrophoretic separation, a product of the predicted size (680 bp) was present in ethidium bromide-stained gels (Fig. 1). This product was present only when reverse transcriptase was present in the incubation mixture, indicating that RNA, not genomic DNA, served as an amplification template. The PCR product was identified as a MUC5AC fragment by sequence analysis of the gel-isolated product.

Competitive RT-PCR was used to determine the abundance of MUC5AC transcripts in normal human conjunctival tissue (Fig. 1). RNA from a pooled sample of five normal donors (three male, two female; aged 44–69 years) was DNase-treated, reverse transcribed, and amplified under conditions generating

membrane was blocked with the same buffer containing 1% bovine serum albumin. Blots were incubated (2 hours, 22°C) with Helix pomatia agglutinin coupled to horseradish peroxidase (HPA-HRP, 12.5 µg/ml; Sigma, St. Louis, MO) and rinsed extensively with TTBS. Binding was detected with 3,3′-diaminobenzidine and quantified by densitometry.

An increase in MUC5AC secretion from ionomycin-stimulated conjunctival tissue was demonstrated qualitatively using a dot-blot immunoassay. Conjunctival secretions from control and ionomycin-stimulated tissues were blotted onto nitrocellulose membranes, blocked for 1 hour with TTBS containing 1% ovalbumin, and incubated with a 1:500 dilution of anti-MUC5AC antibody. Blots were rinsed extensively and incubated with HRP-coupled rabbit anti-goat IgG (Vector, Burlingame, CA) and developed with 3,3′-diaminobenzidine.

Ionomycin Stimulation of Mucin Secretion

Freshly isolated human conjunctival pieces (approximately 100 mg) were incubated in oxygenated Kreb’s Ringer buffer for 30 minutes at 37°C and subsequently transferred to 5 ml of the same buffer containing 10 µM ionomycin. After 30 minutes, a 1-ml aliquot was removed and placed on ice. Mucin content of the sample was determined by a dot-blot modification of the previously described enzyme-linked lectin assay. Briefly, 50 µl aliquots of the incubation fluid were blocked to a nitrocellulose membrane using a 96-well dot-blot apparatus (Bio-Rad). Standards, consisting of PSN (0–500 ng/ml) were prepared and loaded in an identical manner. After gravity filtration for 1 hour, wells were washed with Tris-buffered saline (pH 7.5) containing 0.1% Tween-20 (TTBS) three times, and the

100 mV, washed in transfer buffer, and electobotted onto nitrocellulose membranes. Blots were blocked overnight at 4°C with Tris-buffered saline (TBS) containing 1% dried milk and 2% ovalbumin, then incubated with the anti-MUC5AC antiserum (1:500). Control lanes were incubated with the diluted serum, preabsorbed with agarose beads conjugated to the immunizing peptides (1 mg/ml for each peptide) or with preimmune serum at the same concentration. After three rinses, blots were incubated with HRP-conjugated rabbit anti-goat IgG (Vector, Burlingame, CA) and developed with 3,3-diaminobenzidine and quantified by densitometry.
a linear target-to-product ratio. Analysis of the data obtained from Figure 1 indicates that messenger RNA encoding MUC5AC was present at $64,2 \times 10^4$ transcripts per nanogram of starting cDNA compared with $\beta_2$-microglobulin at $64,1 \times 10^3$ transcripts in an equivalent amount of starting material.

**Presence of MUC5AC Glycoprotein in Human Conjunctival Tissue and Tears**

Immunoperoxidase labeling of human conjunctival sections revealed that anti-MUC5AC antiserum (MUC5AC Ab) labels conjunctival goblet cells but not nongoblet epithelium (Fig. 2). Considerable apical staining of the epithelial surface of the conjunctiva was observed and may represent MUC5AC adherence to the most superficial conjunctival cells.

Western blot analysis of human conjunctiva, human tears, and PSM (Fig. 3) revealed specific binding of MUC5AC Ab to extremely high-molecular-mass (~600 kDa) components in all samples. In human tears, MUC5AC Ab labeled predominantly high-molecular-mass material, which barely entered the gel, and smaller, diffuse bands at less than 200 kDa.

The specificity of MUC5AC Ab labeling of human tears is shown in Figure 4, in which negligible labeling was seen after preabsorption of the MUC5AC Ab with 1 mg/ml combined peptide antigens or with 100 $\mu$g/ml PSM.

To identify MUC5AC in the soluble (aqueous) fraction of human tears, samples were collected on paired Schirmer strips. These strips were cut into 5-mm segments corresponding to the proximal (bulb) portion directly in contact with the conjunctiva and more distal regions wetted by aqueous tears through capillary action. As revealed by western blot analysis (Fig. 5, MUC5AC was present in all segments but appeared more concentrated in the proximal section contacting the conjunctiva (Fig. 5A) than in distal sections (Figs. 5B, 5C) containing predominantly the aqueous tear fraction.
**Figure 4.** Specificity of MUC5AC antiserum binding to tear proteins. Tear samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to western blot analysis. *Lane A* shows the labeling pattern obtained with whole antiserum. *Lane B* shows labeling after preincubation of the antiserum to MUC5AC peptide antigens coupled to agarose beads, which abolished antibody binding. *Lane C* shows that preincubation of the antiserum with 1 μg/ml porcine stomach mucin also abolished labeling.

**Figure 5.** Detection of MUC5AC in tear proteins eluted from Schirmer strips. Schirmer strip samples were collected from a healthy volunteer, cut into 5-mm segments, eluted in Laemmli sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subjected to western blot analysis using MUC5AC antiserum. *Lane A* is a sample eluted from the bulbous portion of the strip, which contacted the conjunctival surface. *Lanes B* and *C* represent sequential, distal sections of the same strip containing only soluble tear components.

**Figure 6.** Ionomycin-stimulated secretion of Helix pomatia agglutinin-binding mucin from isolated human conjunctiva. Tissue samples were incubated for 30 minutes in KRH buffer (control) followed by 30 minutes in HEPES-modified Krebs Ringer buffer containing 10 μM ionomycin. Aliquots of the incubation media were collected, and the total mucin content was quantified by a dot-blot assay based on *Helix pomatia* agglutinin-horseradish peroxidase binding. Mean ± SEM (*n* = 4-6).

**Figure 7.** Ionomycin-stimulated secretion of MUC5AC from human conjunctiva. Tissue samples were incubated in the absence and presence of 10 μM ionomycin for 30 minutes as described in Figure 6. Duplicate aliquots of the incubation media were subjected to dot-blot analysis using MUC5AC antiserum. Spots were visualized by indirect immunoperoxidase labeling.

**Discussion**

Evidence presented in this study shows that the MUC5AC protein is a secreted product of the normal human conjunctiva.

**Ionomycin-Stimulated MUC5AC Secretion**

Stimulation of isolated human conjunctiva with ionomycin (10^{-3} M) resulted in a three- to fourfold increase in total mucin secretion as measured with the HPA-HRP dot-blot assay (Fig. 6). A comparable increase in MUC5AC mucin secretion was observed when the dot blots were labeled with specific MUC5AC Ab (Fig. 7). These results indicate that MUC5AC mucin is among the mucin glycoproteins released subsequent to intracellular influx of calcium.

MUC5AC transcripts are abundantly present in conjunctival tissue, and the corresponding protein is present in conjunctival goblet cells and in the tear film. In addition, stimulation of calcium influx in vitro triggers discharge of soluble MUC5AC mucin into the incubation media, suggesting that, as in other tissues, a regulated pathway governs secretion of this goblet cell mucin.

Results obtained using competitive RT-PCR confirm and extend those of Inatomi et al., which showed by in situ

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hybridization that MUC5AC mRNA is expressed predominantly by human conjunctival goblet cells. The present data indicate that MUC5AC transcripts are expressed by conjunctival tissues at a level (2 × 10^5 transcripts/ng cDNA) approximately 500 times lower than that of the abundant-message β2-microglobulin (∼10^6 transcripts/ng cDNA). If MUC5AC transcripts are present only in goblet cells, as suggested by Inatomi et al., and if goblet cells represent approximately 1% to 5% of conjunctival epithelial cells, then it is reasonable to assume that MUC5AC message is relatively abundant in these cells. For comparison, Guzman et al. reported a similar ratio of MUC5AC to β2-microglobulin transcripts (1:200) in a mucin-secreting human tracheobronchial cell line (NHBE cells).

Tissue samples used in the present study consisted of superior and inferior bulbar conjunctiva and the fornical tissue of the plica semilunaris. These regions vary greatly in goblet cell density, and it is not known at present whether MUC5AC message shows a similar regional variation. Because the present study used pooled RNA samples from several normal donors, it is unclear whether the relative abundance of MUC5AC transcripts varies as a function of age or gender. However, preliminary results from our laboratory suggest that MUC5AC expression varies little between normal, age-matched adults.

To characterize the expression of MUC5AC protein, we used a goat antiserum prepared against two synthetic oligopeptides corresponding to predicted amino acid sequences that flank the tandem repeat domains of MUC5AC. The specificity of the antiserum for conjunctival MUC5AC mucin was shown by immunohistochemical staining of conjunctival tissues (Fig. 2) and by western blot analysis of conjunctival tissues and tears (Figs. 3, 4). Our results suggest that MUC5AC protein is localized primarily in the apical, secretory bodies of conjunctival goblet cells and confirm that goblet cells are the primary source of this secreted mucin. However, we cannot exclude the possibility that other, nongoblet conjunctival epithelial cells may synthesize and secrete minor amounts of MUC5AC mucin. The presence of mucin-containing granules in nongoblet epithelial cells has been shown by electron microscopy and our results show some labeling of MUC5AC protein at the conjunctival surface (Fig. 2). Alternatively, this epithelium-associated MUC5AC may represent mucin that has been secreted into the tear film and subsequently remains adherent to the epithelial glycocalyx.

The presence of MUC5AC in the tear film was verified by western blot analysis of electrophoretically separated tear proteins. Tears collected without anesthesia contained a major immunoreactive band with a molecular mass of 600 kDa or more. Several smaller immunoreactive bands seen in some samples may represent MUC5AC variants and/or degradation products. Western blot analysis of Schirmer strips shows that MUC5AC is concentrated on the proximal (bulb) portion of the strip that directly contacts the conjunctiva, with lesser amounts in distal sections containing soluble components of the tear fluid. These results suggest that MUC5AC is present in cell-associated and soluble forms and are consistent with a predominantly conjunctival origin of MUC5AC protein in tears.

To assay secretion of mucins from isolated human conjunctival tissues, we used commercially obtained PSM as a standard. Previous studies have shown that MUC5AC is present in human gastric mucin and is similar in all species thus far studied. Thus, it was not surprising to find that antiserum to human MUC5AC mucin cross-reacted with PSM (Figs. 3, 4). Although PSM was used in the present study to estimate total, HPA-reactive mucin in conjunctival secretions, we are currently using PSM as a standard for quantitative immunooassay of MUC5AC mucin in human tear samples absorbed onto Schirmer strips.

The present results confirm, and extend to human tissue, our previous studies of ionomycin-stimulated mucin secretion in rabbits. As in rabbits, ionomycin stimulates total mucin secretion, shown by the HPA-based ELLA assay. It is likely that this stimulation, which causes rabbit goblet cells to empty, also results in extensive goblet cell exocytosis in human conjunctiva. In support of this conclusion, the results shown in Figure 7 show that ionomycin stimulated secretion of MUC5AC, a specific goblet cell mucin. This is the first demonstration of regulated secretion of a specific mucin from human conjunctival tissue. Exocytosis of mucin from goblet cells is known to be dependent on calcium influx secondary to stimulation by various mediators. For example, we have reported that mucin secretion from rabbit conjunctiva is triggered by muscarinic receptor activation through a calcium-dependent mechanism. Recent findings indicate that secretion of rabbit and human conjunctival mucins (including MUC5AC) is strongly stimulated by nucleotides (adenosine triphosphate and uridine triphosphate) acting at the P2Y nucleotide receptors.

Further studies are necessary to determine whether calcium signaling represents a common intracellular pathway for secretion of MUC5AC and other mucins in response to nucleotides and other secretagogues.

In summary, the present study confirmed the expression of MUC5AC mRNA in human conjunctiva and suggests that MUC5AC transcripts are relatively abundant in this tissue. MUC5AC protein was localized to human conjunctival goblet cells and was a major soluble mucin in the precorneal tear film. The secretion of MUC5AC was acutely regulated by intracellular calcium and paralleled the secretion of total mucin. The detection and comparison of MUC5AC and other specific mucins in the conjunctiva and tear film in relation to age, gender, and ocular surface disease are a high priority for future study.

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References