Expression of Secretory Mucin Genes by Human Conjunctival Epithelia

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Purpose. To determine whether human conjunctival epithelium expresses any of the human mucin genes designated MUC2 through MUC7.

Method. Northern blot analysis was performed using total RNA isolated from surgically removed conjunctival tissues. Complementary DNA or oligonucleotides to the tandem repeat region of each mucin gene were labeled and hybridized to conjunctival RNA. In situ hybridization also was performed to determine the distribution of mucin mRNA.

Results. Only MUC4 and MUC5 probes hybridized to conjunctival RNA by Northern blot analysis. Both probes bound in a polydispersed pattern, which is characteristic of mucin genes. Using in situ hybridization, MUC4 mRNA was detected in the cells of the stratified conjunctival epithelium, whereas MUC5 mRNA expression was limited to goblet cells. MUC4 or MUC5 probes did not hybridize to sections of corneal epithelium.

Conclusions. The mucins MUC4 and MUC5 are expressed by the human conjunctiva. These mucins may play an important role in forming the tear-film layer at the air and ocular surface epithelium interface. Invest Ophthalmol Vis Sci. 1996;37:1684–1692.

The surfaces of the corneal and conjunctival epithelia are covered by tear film, which has a variety of components, including mucus. Mucus constitutes the innermost layer of the tear film and interfaces with the apical surface of the epithelium.[1] This relationship of mucus to the apical surface of epithelium is present along all other mucosal surfaces, including those of the gastrointestinal, tracheobronchial, and reproductive tracts. The most prominent components of the mucus layer are mucins. Mucins are glycoproteins in which at least 50% of mass is O-linked carbohydrate.[2] Mucins are notoriously difficult glycoproteins to study; their high carbohydrate content impedes many conventional assays.

Although the mucins are essential for the integrity of the tear film, in which they form a viscoelastic gel,[3] the characteristics of mucins on the ocular surface are not well known. Several studies[4–7] have detailed approximate molecular weight, carbohydrate content, and amino acid composition of glycoproteins and plasma proteins of ocular mucus. These studies have provided the initial data suggesting the presence of a heterogeneous mixture of glycoproteins in the tear film.

Generally, the mucins on the ocular surface have been considered to be derived from the goblet cells in the conjunctiva. Histologically, vesicles packed with periodic acid-Schiff or Alcian blue staining material can be detected inside the conjunctival goblet cells.[8] These staining data suggest the presence of acidic and neutral carbohydrates in mucins. It has been proposed that the stratified squamous cells of the conjunctiva provide a second source of mucus for the tear film.[9,10] These cells were shown to contain vesicles that bind dyes with affinity for carbohydrate, and, by electron microscopy, the vesicles appeared to be emptying into the tear film. In support of that hypothesis, the conjunctival apical cell vesicles, as well as vesicles in apical cells of corneal epithelium, bind a monoclonal antibody (H185) to mucin-like glycoproteins.[11] Thus, conjunctival and corneal stratified squamous epithelia may provide a source of mucins for the ocular surface.

A major advance in understanding mucin structure, function, and heterogeneous character has come

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TABLE 1. Human Mucin Genes

Designation	*Type if Sequence Verified*	*cDNA Clone Source*	*Chromosomal Mapping*	*Amino Acids in Tandem Repeat*	*References*
MUC1	Membrane-spanning	Mammary/pancreatic tumor	1q21q24	20	15, 16
MUC2	Gel-forming–secretory	Intestine	11p15	23	18, 19
MUC3		Intestine	7	17	24
MUC4		Trachea	3	16	25
MUC5AC	Gel-forming–secretory	Trachea	11p15	8	19, 20
MUC5B		Trachea	11p15	29	21
MUC6		Stomach	11p15	169	27
MUC7	Soluble monomer–secretory	Salivary gland	4	23	23
MUC8		Trachea	12	13/41	26

from the recent cloning of mucin genes.[2,12–14] To date, nine human mucin genes have been cloned; they have been designated MUC1 through MUC8 (two MUC5 genes have been described), in order of their discovery (Table 1). Besides the fact that the biochemical property of a high percentage of their mass (>50%) is O-linked oligosaccharides, each mucin gene provides a unique amino acid sequence that is repeated tandemly in the protein backbone. The number and sequence of amino acids of each mucin tandem repeat unit is different for each mucin gene (Table 1).[14] The only common feature in the repetitive sequences are that they contain a high percentage of serine and/or threonine residues,[14] which provide the O-glycosylation sites on the protein. The number of amino acids in the tandem repeats of the eight mucin genes cloned to date varies from 8 to 169,[14] but the number of tandem repeats per mucin also can vary in individuals as a result of genetic polymorphism.[15]

Functionally, mucins have been subdivided into two types, transmembrane mucins and secretory mucins.[2] Only one transmembrane mucin has been cloned. This mucin, MUC1, is found on the surfaces of various epithelial cells and carcinomas.[15,16] Recently, we reported[17] that MUC1 mucin is expressed by corneal and conjunctival epithelial cells but not by goblet cells. We hypothesize that this transmembrane mucin plays a role in tear film spread and association of the mucus gel on the epithelial surface. Its antiadhesion property may prevent adhesion of foreign debris to the ocular surface.

Of the remaining mucins, MUC2 to MUC8, the best characterized are MUC2, MUC5, and MUC7. Complete cDNA sequence is available for MUC2 and MUC7. The MUC2 cDNA contains two tandem repeat domains and four cysteine-rich D domains that are homologous to von Willebrand factor.[18,19] These cysteine-rich D domains appear to be necessary for the disulfide linking of mucin molecules, which leads to polymerization into gels. Initially, partial sequences of a number of MUC5 cDNAs were cloned.[20,21] They were placed into three groups, MUC5A, MUC5B, and MUC5C. All localize to chromosome 11p15. Recent data suggest that MUC5A and MUC5C are at precisely the same gene locus.[22] Thus, MUC5A and MUC5C are part of the same gene and have been designated MUC5 or MUC5AC. MUC5B appears to be distinct from MUC5AC. Although the full length of MUC5 cDNA is unknown, MUC5 has cysteine-rich regions with a high degree of homology to MUC2 cysteine-rich domains. The presence of this conserved domain of MUC5 suggests that MUC5, like MUC2, may be a gel-forming mucin. The second secretory mucin for which full-length cDNA sequence is known was cloned from the salivary gland and designated MUC7.[23] In contrast to other larger secretory mucins, the full-length cDNA of MUC7 is 2350 bp. This small mucin, which exists as a monomer, lacks the cysteine-rich domain found in MUC2 and MUC5.[23]

The other mucin genes MUC3, MUC4, MUC6, and MUC8 are not as well characterized.[14] MUC3 was cloned from a small intestine cDNA library and is expressed in small intestine and colon.[24] MUC4 and MUC8 were cloned from a tracheobronchial cDNA library; however, only tandem repeat sequences are available.[25,26] Even though various tissues express MUC4, this mucin is relatively uncharacterized. MUC6, cloned from a stomach cDNA library, has the longest tandem repeat sequence reported.[27]

It is unknown whether ocular surface epithelium expresses any of the cloned, so-called secretory mucins. To understand better the characteristics of the ocular surface mucins, we determined the expression of MUC2 through MUC7 by Northern blot analysis of conjunctival RNA and then, by in situ hybridization, determined the cellular origin of the expressed mucins.

MATERIALS AND METHODS

Tissue Samples

All investigations followed the tenets of the Declaration of Helsinki, and informed consent and full institutional review board approval were obtained. Small segments (approximately 1 × 2 mm) of forniceal con-

TABLE 2. Mucin cDNA and Oligonucleotide Probes Corresponding to Tandem Repeat Region

Mucin Gene	*cDNA/oligo*	*Designations*	*Length (bp)*	*References*
MUC2	cDNA	SMUC41	836	18
	cDNA	HAM-1	90	32
MUC3	cDNA	SIB124	387	24
MUC4	oligo	MUC4oligo	48	25
MUC5	cDNA	4F	494	31
MUC6	cDNA	MUC6.2T	1014	27
MUC7	oligo	MUC7oligo	45	23

bp = base pair; oligo = oligonucleotide; cDNA = complementary DNA.

junctiva were excised at the time of cataract surgery. All conjunctiva appeared normal at the time of preoperative examination. None of the patients from whom samples were obtained were receiving chronic topical eye medications. Tissues for Northern blot analysis were frozen in liquid nitrogen immediately after removal and stored at −70°C until RNA isolation. Four pieces of conjunctiva (three women and one man, 56 to 77 years of age) were used to obtain sufficient RNA for analysis. Conjunctival tissues for in situ hybridization were fixed immediately after removal and processed as described below. Six samples were obtained; they included tissues from three women (70 to 75 years of age) and three men (21, 67, and 70 years of age). Corneas with adjacent limbus and adjacent conjunctiva used for in situ hybridization were obtained from eye banks and were fixed less than 24 hours after death.

Isolation of RNA and Northern Blot Analysis

Total RNA was isolated with an RNA Isolation Kit (Stratagene, La Jolla, CA) using an acid guanidinium thiocyanate phenol-chloroform single-step extraction.[28] Conjunctival RNA, isolated from two biopsies of forniceal conjunctiva, were pooled to obtain enough for one lane on each Northern blot. The pooling of several samples decreases the possibility of a false-negative result caused by tissue variation. Total RNA was isolated from cultured human corneal epithelium as described previously.[17] Total RNA from human trachea, stomach, salivary gland, and small intestine, purchased from Clontech (Palo Alto, CA), were used for positive controls; a BT-20 breast carcinoma cell RNA, cultured rabbit corneal fibroblast RNA, and human umbilical vein endothelial cell RNA were used as negative controls.

RNA samples of 10 μg were separated on 1% agarose gels in the presence of formaldehyde. The integrity and amount of RNA loaded on each gel was determined by staining with ethidium bromide. The RNA was then transferred to Gene Screen Plus membrane (DuPont, Boston, MA).

The membranes were prehybridized in a solution of 50% formamide, 5 × saline–sodium phosphate–EDTA buffer, 5 × Denhardt's solution, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA for 2 hours at 42°C. Hybridization was performed in the same hybridization solution with 2×10^6 cpm/ml of ^{32}P-labeled probe at 42°C overnight. After hybridization, the membranes that were hybridized with cDNA probes were washed using high-stringency conditions; two washes with 2 × saline-sodium citrate (SSC) buffer for 15 minutes at room temperature, two washes with 2 × SSC, 1% SDS at 55°C for 20 minutes, followed by two washes with 0.1 × SSC, 0.1% SDS at 55°C for 20 minutes. For the oligonucleotide probes, high-stringency washes were decreased to 1 × SSC, 0.1% SDS at 42°C. After autoradiography, each membrane was stripped and reprobed three times.

cDNA and Probe Preparation

Because tandem repeat sequences are specific for each mucin gene[14] and probes corresponding to this region may hybridize to a number of the repeats of these sequences per mRNA, we chose to use cDNAs or oligonucleotides corresponding to tandem repeat regions as probes for Northern blot analysis and in situ hybridization (Table 2). The sequence of cDNAs for MUC2, MUC3, and MUC5 were described in the references cited in Table 2. MUC6.2T cDNA clone contains two repeats of MUC6 tandem repeat sequence. Because cDNA probes for MUC4 and MUC7 were unavailable, the most frequently found repetitive sequences of each tandem repeat were selected for oligonucleotide probes. MUC4 oligo corresponds to antisense sequence of the 48-bp fragment of the 69-bp tandem repeat (5′-GTC-GGTGACAGGAAGAGGGGTGGCGTGACCTGT-GGATGCTGAGGAAGT-3′), and MUC7 oligo corresponds to the antisense sequence of a 45-bp tandem repeat (5′-GGTGTGGGTGGGGCAGCTGTGGTC-TCTGGTGGAGCTGAGGAAGAT-3′). These oligonucleotides, purified by reverse-phase chromatography, were purchased from BioServe (Laurel, MD) and labeled with γ-^{32}P adenosine triphosphate by

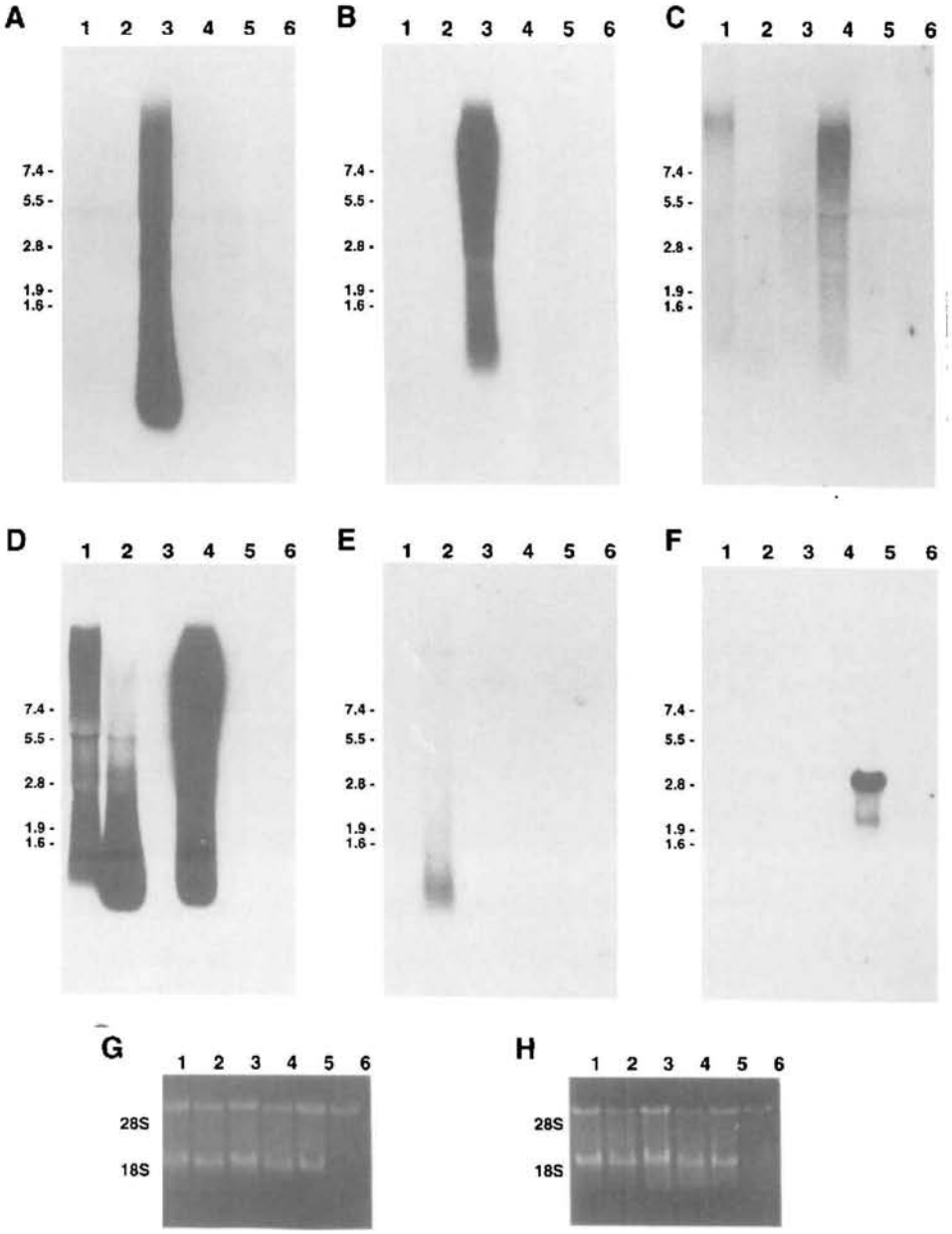

FIGURE 1. Northern blot analysis demonstrating tissue-specific expression of mucin mRNA in conjunctiva and other tissues. RNA samples of 10 μg were separated on 1% agarose gels in the presence of formaldehyde. Two membranes, blots 1 and 2, were prepared, respectively. Blot 1 was hybridized serially with probes for MUC2 (**A**), MUC4 (**C**), and MUC6 (**E**), and blot 2 was hybridized with probes for MUC3 (**B**), MUC5 (**D**), and MUC7 (**F**). Ethidium bromide staining of each gel show the integrity of RNA blot 1 (**G**) and blot 2 (**H**). Note that probes for only MUC4 and MUC5 bound to the conjunctival RNA. RNA ladders were used as size markers: lane 1 = conjunctiva; lane 2 = stomach; lane 3 = small intestine; lane 4 = trachea; lane 5 = salivary gland; lane 6 = human umbilical vein endothelial cell HUVEC.

T4 polynucleotide kinase (BioLabs, Richmond, CA). cDNA probes were labeled with α-^{32}P deoxycytidine triphosphate by Random Primer Plus kit (DuPont). Labeled probes were purified using a Sephadex-G25 or Sephadex-G50 column (Boehringer–Mannheim, Indianapolis, IN) before Northern blot analysis.

In Situ Hybridization

Because MUC4 and MUC5 mRNA were detected in conjunctival RNA by Northern blot analysis, their distribution was determined by in situ hybridization. Because recent ARVO abstracts[29,30] suggest that MUC2 is present at the ocular surface and because a faint band was detected for MUC7 by Northern blot analysis with extended exposure, in situ hybridization of MUC2 and MUC7 mRNA also was performed. Techniques were as previously described.[17] Oligonucleotide probes to MUC4 and MUC7 were labeled with ^{35}S-dATP by terminal deoxynucleotidyl transferase (Gibco BRL, Gaithersburg, MD). Antisense and sense riboprobes, generated from the MUC5 cDNA (designated F4)[31] and from the MUC2 cDNA (designated HAM-1),[32] were labeled with ^{35}S-uridine triphosphate by T3 or T7 RNA polymerase (Boehringer Mannheim, Indianapolis, IN).

Tissues prepared by two different methods were available. In one, frozen conjunctival sections were mounted on gelatin-coated slides and fixed with 4% paraformaldehyde. In the second, sections from 4% paraformaldehyde-fixed, paraffin-embedded tissues were mounted on gelatin-coated slides. Proteinase K treatment and acetylation were performed before hy-

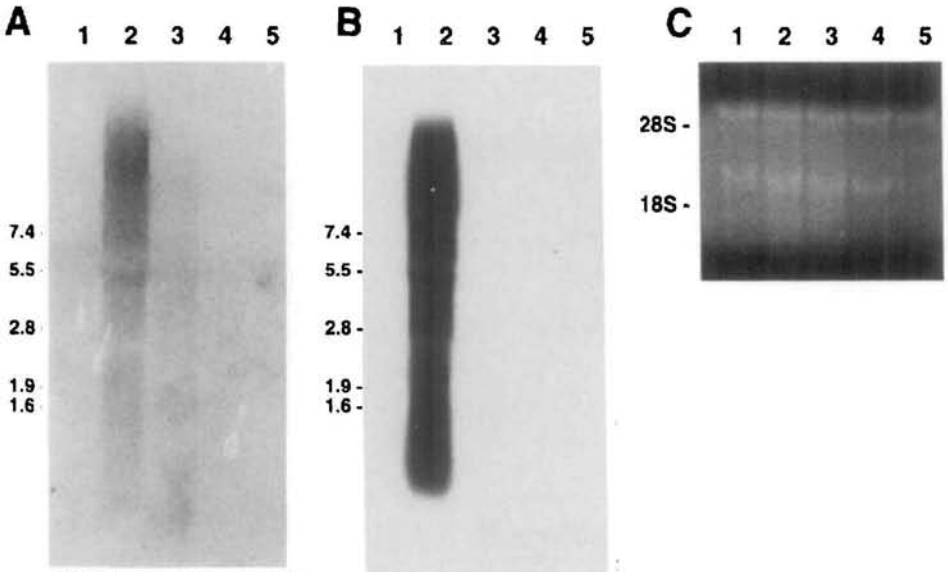

FIGURE 2. Northern blot analysis demonstrated lack of expression of MUC4 (**A**) and MUC5 (**B**) mRNA in cultured human corneal epithelium. RNA samples of 10 μg were separated on a 1% agarose gel in the presence of formaldehyde. Ethidium bromide staining shows the integrity of RNA (**C**). Lane 1 = cultured human corneal epithelium; lane 2 = trachea; lane 3 = small intestine; lane 4 = BT-20 breast cell carcinoma; lane 5 = cultured rabbit corneal fibroblast.

bridization. Hybridization was carried out in a solution of 50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1 × Denhardt's solution, 10 mM Tris, pH 8, 5 mM EDTA, 0.5 mg/ml tRNA, and 10 mM dithiothreitol. Sections were hybridized at 42°C for MUC4 and MUC7 oligoprobes and at 57°C for MUC 2 and MUC5 riboprobes. After washing, autoradiography was performed, and the sections were counterstained with hematoxylin and eosin. To verify the cellular distribution of MUC4 mRNA, colorimetric, nonradioactive in situ hybridization was performed. MUC4 oligoprobes were labeled with digoxigenin (DIG)-dUTP (Boehringer Mannheim) by terminal deoxynucleotidyl transferase. Sections were pretreated according to the protocol used with the ^{35}S probe. MUC4 hybridization was performed at 37°C in a solution containing 50% formamide, 10% dextran sulfate, 20 μg/ml tRNA. After hybridization, washes were performed for 30 minutes serially in each of the following: 2 × SSC at room temperature, 1 × SSC at room temperature, 0.5 × SSC at 37°C, and 0.5 × SSC at room temperature. MUC4-hybridized probes were detected by DIG detection kit (Boehringer Mannheim) according to the manufacturer's instruction.

RESULTS

Northern Blot Analysis

To examine the expression of secretory mucin genes MUC2 through MUC7, Northern blot analysis (Fig. 1) was performed with probes to each MUC tandem repeat region (Table 2). MUC2 and MUC3 mRNA were detected only in small intestine RNA and not in other RNA samples (Figs. 1A, 1B). Positive signals of MUC2, MUC3, MUC4, MUC5, and MUC6 showed the typical smeared, polydispersed pattern previously reported as characteristic of these mucin genes.[18,24,25,27]

MUC4 mRNA expression was observed in conjunctiva as well as in trachea; the latter was used as positive control (Fig. 1C). Although the binding to conjunctiva is less intense than to trachea, both signals in conjunctiva and trachea show the typical polydispersed pattern. In the MUC5 Northern blot (Fig. 1D), an intense signal was detected in the positive controls of trachea and stomach, similar to that reported by Toribara et al.[27] The same polydispersed intense binding was detected in conjunctiva (Fig. 1D, lane 1).

MUC6 mRNA expression was detected only in stomach RNA, as reported by Toribara et al.[27] No binding was observed in the conjunctival sample. Intense MUC7 mRNA signal, approximately 2.4 kbp, was detected in the salivary gland only (Fig. 1F). A faint band of the same size appeared in conjunctiva only after an extended 4-day exposure of the autoradiogram (data not shown). The integrity and amount of RNA loaded on each gel was verified by staining with ethidium bromide, as shown in Figures 1G and 1H.

To determine whether MUC4 or MUC5 mRNA expression was limited to the conjunctival region of the ocular surface epithelia, Northern blot analysis using total RNA from cultured human corneal epithelium, known to express MUC1,[17] was performed. MUC4 and MUC5 probes bound to positive control RNA from trachea (Fig. 2, lanes A2 and B2) in the same fashion as shown in Figure 1C, lane 4, and Figure 1D, lane 4. Figure 2, lane A3, shows weak binding of MUC4 probe to small intestine RNA as previously reported[14] and as shown in Figure 1C, lane 3. Neither MUC4 nor MUC5 mRNA was detected in cultured corneal epithelial RNA (Fig. 2). To verify these results, in situ hybridization was performed on corneal tissues (see next section).

In Situ Hybridization

The stratified conjunctival epithelium showed positive binding of the ^{35}S-labeled MUC4 oligoprobe (Figs. 3A,

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