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Decreased Levels of the Goblet Cell Mucin MUC5AC in Tears of Patients with Sjögren Syndrome

Pablo Argüeso,¹ Mini Balaram,^{1,2} Sandra Spurr-Michaud,¹ Henry T. Keutmann,³ M. Reza Dana,^{1,2} and Ilene K. Gipson¹

PURPOSE. To determine whether the relative amounts of mucin mRNA in the conjunctival epithelium and mucin protein in the tears are altered in patients with Sjögren syndrome compared with healthy individuals.

METHODS. Tear fluid was collected from the inferior fornix of normal subjects ($n = 17$) and patients with Sjögren syndrome ($n = 11$) after instillation of 60 μ L sterile water onto the ocular surface. Immediately after tear fluid collection, conjunctival epithelium was obtained by filter paper-stripping from the bulbar temporal region for mRNA isolation. Primers to nontandem repeat sequences of the gel-forming mucin MUC5AC and the membrane-spanning mucins MUC1 and MUC4 were used in real-time RT-PCR to determine relative abundance of MUC mRNA in patients with Sjögren syndrome in relation to that of normal subjects. Enzyme-linked immunosorbent assay was performed on neuraminidase-treated tears, using a polyclonal antibody against a synthetic peptide mimicking the deduced amino acid sequence from the D3 region of MUC5AC.

RESULTS. The number of RNA transcripts for the goblet cell-specific mucin MUC5AC in the conjunctival epithelium of patients with Sjögren syndrome was significantly lower than in normal individuals. No significant changes were detected when analyzing the mRNA levels of the mucins expressed by the stratified epithelium of the conjunctiva, MUC1 and MUC4. Protein levels of the goblet cell mucin MUC5AC were significantly reduced in the tear fluid of patients with Sjögren syndrome, corroborating mRNA data obtained using real-time RT-PCR.

CONCLUSIONS. The tear fluid of patients with Sjögren syndrome has reduced levels of the goblet cell-specific mucin MUC5AC, which correlates to decreased levels of conjunctival MUC5AC mRNA. The authors propose that deficiency of MUC5AC mucin in tears constitutes one of the mechanisms responsible for tear film instability in Sjögren syndrome. (*Invest Ophthalmol Vis Sci.* 2002;43:1004-1011)

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The tear fluid covering the ocular surface epithelium is composed of a thin outer lipid layer and an inner aqueous phase adjacent to the glycocalyx of the apical cells of the epithelium. Epithelial mucins are a heterogeneous group of high-molecular-weight glycoproteins present as major protein components of the aqueous phase of the tear fluid.¹ Mucins have been ascribed several roles: stabilizing the tear film, providing a smooth and refractive surface of high optical quality over the cornea, lubricating the corneal and conjunctival epithelial surfaces during eye blinking, and preventing desiccation of the ocular surface through water retention, due to their hydrophilic character.^{2,3} Mucins also participate in defense of the underlying epithelium. 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 micro-organisms and viruses.⁴ Consequently, ocular mucins may also prevent pathogen penetrance by inhibiting bacterial adhesion to the ocular surface epithelium.⁵

To date, 15 epithelial mucin genes have been completely or partially sequenced. They have been named and numbered chronologically in order of discovery as MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6 to 9, MUC11 to 13 and MUC16.⁶⁻¹¹ Based on the presence of common structural motifs within the amino acid sequence they have been 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). The ocular surface epithelium expresses at least three major mucin genes. In conjunctiva, goblet cells are responsible for secreting the gel-forming mucin MUC5AC, whereas the stratified epithelium produces the membrane-spanning mucins MUC1 and MUC4.^{12,13} The expression of MUC2 by the conjunctival epithelium is still controversial. Recent quantitative analysis using the highly sensitive polymerase chain reaction (PCR) has shown MUC2 mRNA expression in conjunctival tissue. The number of MUC2 transcripts was, however, approximately 6000 times lower than the number of MUC5AC transcripts¹⁴ and are below the level of detection by Northern blot analysis and in situ hybridization.¹² The corneal epithelium produces only membrane-spanning mucins MUC1 and MUC4.¹³ Using Northern blot analysis, the expression of MUC4 mRNA has been demonstrated in limbal and peripheral corneal epithelia,¹⁵ with decreasing levels toward the central cornea, as demonstrated by in situ hybridization.¹²

Several methods have been used to detect and quantify mucin proteins at the ocular surface. Mucin histochemistry, with PAS, Alcian blue, and lectin stains, has been used in the past to localize sites of mucin synthesis and secretion.¹⁶ However, because these methods are primarily based on the recognition of common carbohydrate moieties on mucins, they neither differentiate between members of the mucin family nor allow mucin quantitation, because of varying glycosylation of mucins. Recent progress in the development of antibodies against specific mucin proteins has allowed more precise localization of the different types of mucins in mucosal epithelia and ocular surface epithelia. Some groups have reported the

use of mucin antibodies in combination with immunohistochemistry and flow cytometry techniques to detect mucins at the ocular surface.^{17,18} Nevertheless, these methods are time consuming and require the use of human tissue, which is difficult to obtain. The use of the highly sensitive enzyme-linked immunosorbent assay (ELISA) to quantify specific mucin products in tears constitutes an alternative to these other methods, although it has been infrequently used.¹⁹

Destabilization of the tear film occurs on the ocular surface of eyes in patients with keratoconjunctivitis sicca or other dry eye diseases.²⁰ Tear-breakup time, a measure of tear stability, is decreased in dry eye.²¹ The most severe symptoms of this disease are often seen in patients with Sjögren syndrome, a systemic autoimmune condition characterized by a combination of keratoconjunctivitis sicca and dry mouth.²² Although several reports have shown alterations of mucin products in the ocular surface in dry eye,^{17-19,23} to date, there is no information correlating expression of specific mucin genes with their protein products.

The goal of this study was to compare the amount of specific mucin transcripts in conjunctival cells of normal individuals with those of patients with Sjögren syndrome and to correlate mucin mRNA expression with the amount of specific mucin protein product found in the tear fluid of the same two groups.

METHODS

The study was conducted in compliance with good clinical practice, institutional review board regulations, informed-consent regulations, and the tenets of the Declaration of Helsinki.

Subject Selection

All prospective subjects completed an institutional review board-approved questionnaire regarding the presence, type, and frequency of symptoms of dry eye and dry mouth, the use of dry-eye therapy (e.g., supplemental lubrication or punctal plugs), current medications, and history of ocular allergies.

Based on this questionnaire two groups of subjects were studied. The first group consisted of 17 normal subjects (14 females and 3 males; age range, 23-63 years; mean, 40) who had no dry eye symptoms, no eye diseases, and no history of eye surgery or contact lens wear. The second group consisted of 11 women (ages range, 44-67 years; mean, 54) who fit our inclusion criteria for Sjögren syndrome. We adopted a modified version of the San Diego Criteria for Sjögren Syndrome, which includes the following: symptomatic xerophthalmia (dry eyes), symptomatic xerostomia (dry mouth), and underlying autoimmune disease, as evidenced by positive binding of antinuclear antibody (ANA), rheumatoid factor, or anti-Ro or -La antibodies at a titer of 1:160, with or without clinical features diagnostic of any of the following connective tissue disorders: rheumatoid arthritis, systemic lupus erythematosus, polymyositis, or scleroderma. None of the patients with Sjögren syndrome was treated by punctal occlusion. None of the study subjects in either group was receiving any type of xerogenic medication, and none had a history of ocular allergies. We collected tear samples and impression cytology specimens from both eyes of all subjects. Of these, 23 tear samples and 15 conjunctival epithelial specimens from the normal group and 11 tear samples and 11 conjunctival epithelial specimens from the Sjögren group were suitable for analysis.

Sample Collection

Tear fluid and conjunctival epithelium from normal subjects and patients with Sjögren syndrome were collected to determine MUC5AC protein levels and mucin mRNA expression, respectively. Tears were collected from unanesthetized eyes by micropipette after instillation of 60 μ L sterile water in the cul-de-sac, followed by movement of the eyes

to mix the tear fluid content. A drop of topical anesthetic (0.5% proparacaine HCl, Alcaine; Alcon Inc., Fort Worth, TX) was then applied to the eye. A sterile disc of nitrocellulose filter paper, 10 mm in diameter, was placed on the temporal bulbar conjunctiva, gentle pressure was applied to the disc for 15 seconds, and the disc was carefully transferred into a tube (Eppendorf, Fremont, CA) containing RNA extraction reagent (TRIzol; GibcoBRL, Grand Island, NY). All samples were promptly frozen at -80°C until the time of analysis.

Individual tear samples were centrifuged for 30 minutes at 14,000 rpm at 4°C . Protein concentration was determined with a protein assay reagent kit (MicroBCA; Pierce, Rockford, IL), using a dilution series of bovine serum albumin as the standard. The soluble fraction of the centrifuged sample contained 50 times more protein than the SDS-solubilized pellet. The amount of protein in the pellets was insufficient for assay.

RNA Isolation and Reverse Transcription

Total RNA was isolated from impression cytology samples using the extraction reagent (TRIzol; GibcoBRL), according to the manufacturer's protocol. Briefly, conjunctival cells were homogenized by adding 1 mL reagent per filter paper sample. After passing the cell lysate through a pipette several times, samples were incubated at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes. A volume of 0.2 mL chloroform was added to the reagent solution. Tubes were vigorously shaken by hand and centrifuged for 15 seconds. The colorless upper aqueous phase containing the RNA was recovered. Samples were then incubated with 0.5 mL isopropanol and centrifuged. After removing the supernatant, the RNA pellet was washed once with 75% ethanol. The RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water and incubated for 10 minutes at 60°C . Residual genomic DNA from the samples was eliminated by DNase I digestion of the RNA preparation.

One microgram total RNA prepared from conjunctival epithelium obtained by filter paper stripping and treated with DNase I was used for cDNA synthesis (SuperScript II Reverse Transcriptase; GibcoBRL), as described in the manufacturer's protocol. Briefly, random hexamer primers were added to each sample, and tubes were heated to 70°C for 10 minutes. After a brief centrifugation, a mixture containing PCR buffer, MgCl_2 , dNTPs, and dithiothreitol (DTT) was added to the samples and incubated for 5 minutes at room temperature. The reverse transcription was performed by adding 1 μ L reverse transcriptase to the mixture and incubating the tubes at 25°C for 10 minutes, 42°C for 50 minutes, and 70°C for 15 minutes. Samples were placed in ice for 1 minute, and 1 μ L RNase H was added to each tube and incubated at 37°C for 20 minutes. Reverse-transcription products were then ready for use in real-time PCR reactions. Of the total reverse transcription volume of 20 μ L, 0.8 μ L was used for each PCR. The PCR was performed in triplicate in a total volume of 50 μ L, using *Taq* polymerase chemistry according to the manufacturer's recommendations (*Taq*-Man; PE-Applied Biosystems, Foster City, CA).

Real-Time PCR

Real-time PCR amplification was performed in the presence of double-labeled fluorogenic probes (*Taq*Man probes; PE-Applied Biosystems) that allow the relative quantitation of gene expression in real time.²⁴ Assays were performed on a sequence detection system (Prism 5700; PE-Applied Biosystems). Primers and probes used in this study (Table 1) were designed with the assistance of computer software (Primer Express; PE-Applied Biosystems), had been previously published (MUC1),²⁵ or were purchased from PE-Applied Biosystems (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). All the primers and probes used for mucin gene amplification in this study were selected from areas flanking the tandem repeat domains. BLASTN searches against nucleotide databases were performed to confirm the sequence specificity of the chosen nucleotide sequences (BLAST is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and is available at <http://www.ncbi.nlm.nih.gov/>

TABLE 1. Sequence of Primers and Probes Used for Gene Amplification in Real Time RT-PCR

Gene (amplicon size)	Sequence	Reference
<i>MUC1</i> (123 bp)	Sense: 5'-GTGCCCTAGCAGTACCG-3' Antisense: 5'-GACGTGCCCTACAAGTTGG-3' Probe: 5'-AGCCCCTATGAGAAAGTTTCTGCAGGTAATG-3'	de Cremoux et al. ²⁵
<i>MUC4</i> (102 bp)	Sense: 5'-GCCCAAGCTACAGTGTGACTCA-3' Antisense: 5'-ATGGTGCCGTTGTAATTTGTTGT-3' Probe: 5'-CGGCCACATCCCCATCTTCTTCAC-3'	GenBank accession No. AF058803
<i>MUC5AC</i> (103 bp)	Sense: 5'-TCCACCATATACGCCACAGA-3' Antisense: 5'-TGGACCGACAGTCACTGTCAAC-3' Probe: 5'-CTCGCTGGCCATTGCTATTATGCC-3'	GenBank accession No. Z48314
<i>GAPDH</i> (226 bp)	Sense: 5'-GAAGGTGAAGGTCGGAGTC-3' Antisense: 5'-GAAGATGGTGATGGGATTTTC-3' Probe: 5'-CAAGCTTCCCCTTCTCAGCC-3'	Manufacturer's protocol (PE-Applied Biosystems)

GenBank is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and is available at <http://www.ncbi.nlm.nih.gov/genbank/>.

blast). Conventional RT-PCR experiments were performed to confirm that only a single band is obtained when amplifying conjunctival cDNA with the *MUC4* and *MUC5AC* primers used in this study. As shown in Figure 1, a unique band corresponding to the predicted size for *MUC4* and *MUC5AC* was obtained after 40 cycles of cDNA amplification. PCR products were electrophoresed in 1.0% agarose gels containing ethidium bromide. To verify the identity of the *MUC4* and *MUC5AC* PCR products, the bands in the agarose gel were excised, and the extracted DNA was sequenced at the DNA Sequencing Core Facility of Massachusetts General Hospital (Boston, MA).

The average threshold cycle (C_T) values for *GAPDH* were used as an endogenous reference to correct for differences in the amount of total RNA added to each reaction. To validate the relative quantitation, the efficiency of the target gene amplification was compared with the efficiency of the *GAPDH* amplification, as described in the manufacturer's protocol (PE-Applied Biosystems). The comparative C_T method was used for relative quantitation of the number of mucin transcripts in normal subjects and patients with Sjögren syndrome, with the relative mucin mRNA levels in normal subjects selected as the calibrator. A nontemplate control was included in all the experiments performed with real-time PCR to evaluate DNA contamination of the

reagents used for amplification. None of the experiments resulted in a nontemplate control-positive signal, indicating that there was no DNA contamination in the RNA used for the assays.

MUC5AC Antibody Production

A polyclonal antibody, designated 791, was raised in chickens against a synthetic peptide mimicking the deduced amino acid sequence from the D3 region of the goblet cell-specific mucin *MUC5AC* (CDFATRSRSVVGVDVLEFGNS). The peptide was synthesized by solid-phase procedure using 9-fluorenylmethylcarbonyl (Fmoc) chemistry and conjugated to keyhole limpet hemocyanin (KLH) with glutaraldehyde by the Peptide Synthesis Core Facility of the Reproductive Endocrine Sciences Center, Massachusetts General Hospital. Antibody against the KLH-791 peptide was produced in chickens by Rockland Immunochemicals (Gilbertsville, PA). The specificity of the 791 antibody was tested by coating ELISA plates with 200 ng of its respective synthetic peptide and other peptides corresponding to the cysteine-rich and D4 domains of *MUC5AC* (peptides designated 715, 769, and 771, respectively). Additional specificity controls were peptides mimicking regions in *MUC4* (peptide 527) and *MUC5B* (peptide 798) mucins. Protein extracts obtained from human cervical mucus secretions known to express *MUC5AC* mRNA were used as a positive control.²⁶ Human cervical mucus was obtained as described previously.²⁷ Specificity and titers of the antibody were assessed by ELISA using peroxidase-conjugated rabbit anti-chicken IgY (1:10,000) and color development with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma; St. Louis, MO).

Immunofluorescence

Cellular localization of *MUC5AC* antibody binding was determined by immunofluorescence microscopy on paraffin-embedded sections of human conjunctiva, cornea, and intestine, using standard protocols.²⁷ Conjunctival tissue obtained from C. Stephen Foster, Massachusetts Eye and Ear Infirmary (Boston, MA), was excised at the time of cataract surgery. Corneal tissue was obtained from the Florida Eye Bank. Colonic tissue was obtained from the National Disease Research Interchange (NDRI; Philadelphia, PA). Sections of these tissues were incubated for 1 hour at room temperature with the anti-*MUC5AC* as primary antibody (1:500) and fluorescein isothiocyanate-conjugated donkey anti-chicken IgY (Jackson ImmunoResearch, West Grove, PA) as secondary antibody. Slides were coverslipped with fluorescence mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) and viewed under a fluorescence microscope.

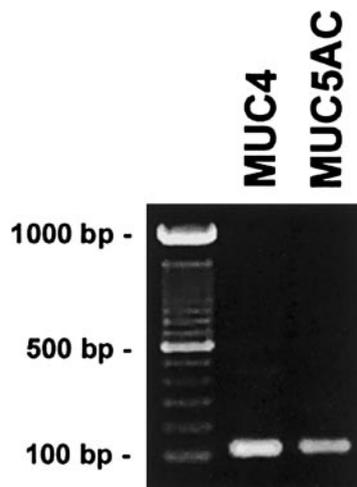


FIGURE 1. Conventional RT-PCR analysis demonstrates that the *MUC4* and *MUC5AC* primers used for relative quantitation in real-time PCR produced the expected amplicon size. Amplified products were electrophoresed on a 1% agarose gel and visualized with ethidium bromide.

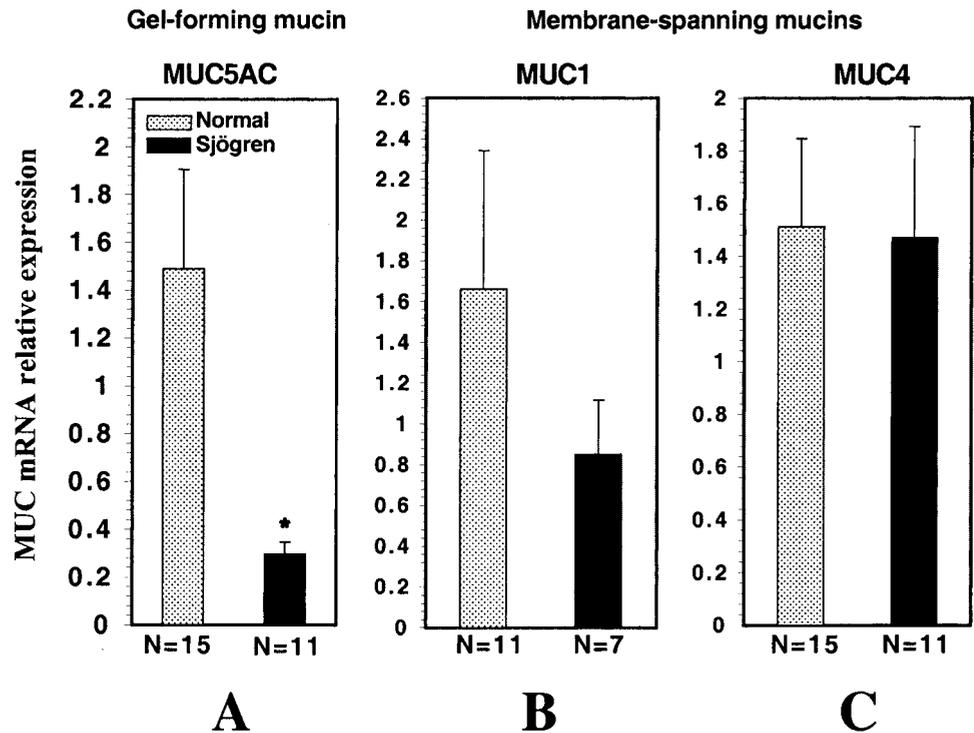


FIGURE 2. Relative expression of MUC transcripts in the conjunctival epithelium of normal subjects and patients with Sjögren syndrome. (A) A significant reduction in the goblet cell-specific mucin MUC5AC mRNA was detected in patients with Sjögren syndrome ($P = 0.02$). (B) There was a trend toward decreased levels of the membrane-spanning mucin MUC1 in these patients compared with the normal group ($P = 0.37$). (C) No changes were detected in the expression of the membrane-spanning mucin MUC4 mRNA ($P = 0.94$). Error bars, SEM; * $P < 0.05$.

Electrophoresis and Immunoblot Analysis

To determine whether the MUC5AC antibody binds to an appropriately high-molecular-weight protein in conjunctival cell extracts, the antibody was used in immunoblot experiments. Total protein (100 μg) from conjunctival biopsy specimens was diluted with Laemmli sample buffer and processed by SDS-polyacrylamide gel electrophoresis (PAGE; 6% separating, 4% stacking), using the Laemmli buffer system.²⁸ After SDS-PAGE, proteins were transferred to nitrocellulose, using standard protocols.²⁹ After the membrane was washed with Tris-buffered saline (pH 7.5), immunostaining was performed, with the MUC5AC antibody as the primary antibody (1:200) and peroxidase-conjugated rabbit anti-chicken IgY (1:1000) as the secondary antibody. Color was developed using 3,3'-diaminobenzidine (DAB; BioRad Laboratories, Hercules, CA).

Enzyme-Linked Immunosorbent Assay

Tear samples containing 4 μg total protein were treated for 2 hours at 37°C with 2.5 mU neuraminidase from *Arthrobacter ureafaciens* (Calbiochem, La Jolla, CA) to enhance MUC5AC mucin recognition by the 791 antibody. Microtiter plates (CoStar EIA plates; Corning Glass Co., Corning, NY) were coated with neuroaminidase-treated tear samples containing 0.5 μg protein in carbonate-bicarbonate buffer overnight at 4°C. After the plates were blocked for nonspecific binding with PBS containing 3% wt/vol fish gel (Sigma), the MUC5AC antibody (1:800) was applied and incubated for 1 hour at room temperature. Detection of the MUC5AC was performed using rabbit peroxidase-anti-chicken IgY at 1:10,000 dilution, as described previously.²⁷ Color development was performed with TMB (Sigma).

Statistical Analysis

Statistical comparisons of results obtained by real-time PCR and ELISA were performed with the Fisher protected least-significant difference (PLSD) test (Statview 5.0 for Macintosh; Abacus, Berkeley, CA).

RESULTS

Comparison of Mucin Transcripts between Normal Subjects and Patients with Sjögren Syndrome

The amount of total RNA obtained by filter paper stripping of human conjunctiva varied from approximately 1 to 10 μg , and there were no apparent differences in recovery of total RNA from normal subjects and patients with Sjögren syndrome. Reverse transcription followed by real-time PCR was used in these studies, because it is an especially valuable technique for comparing multiple gene transcripts from a limited amount of RNA. Comparative amplification curves using MUC1, MUC4, MUC5AC, and GAPDH primers were obtained with differing amounts of conjunctival cDNA template (data not shown). Similar efficiencies of amplification, as determined by the slope of the standard curve for each primer, allowed relative quantitation of mucin gene expression profiles in the various impression cytology samples used in this study.

By using the same conditions described in the Methods section, two groups—normal individuals and patients with Sjögren syndrome—were compared (Fig. 2). Data obtained from analysis of the normal population were considered the basal condition or calibrator. The amount of conjunctival MUC5AC transcripts in 11 Sjögren syndrome samples was significantly lower than in normal subjects (Fig. 2A). Although there was a tendency toward reduced levels of the membrane-spanning mucin MUC1 in the conjunctival epithelium of patients with Sjögren syndrome, the difference was not statistically significant (Fig. 2B). Levels of the membrane-spanning mucin MUC4 remained essentially unaltered in both groups (Fig. 2C). Taken together, these data suggest an alteration in the transcription of the goblet cell-specific mucin MUC5AC in patients with Sjögren syndrome, with little to no effect on the membrane-spanning mucins MUC1 and MUC4 expressed by the conjunctival epithelium.

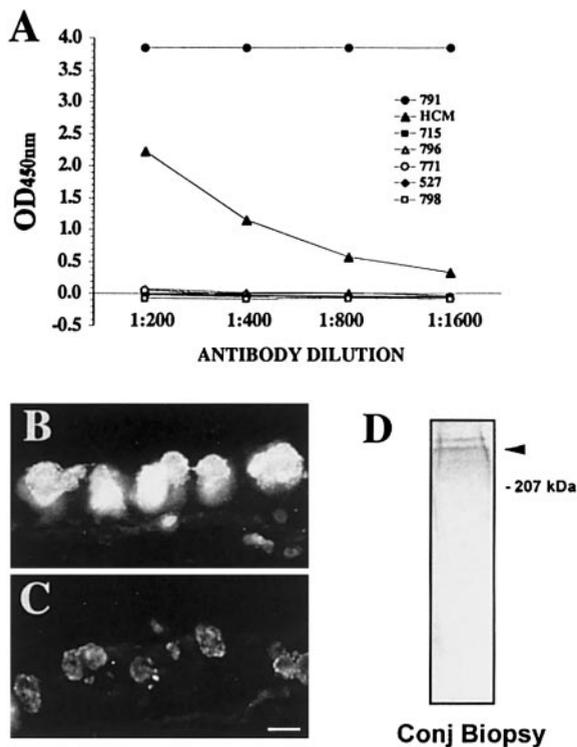


FIGURE 3. Demonstration of the affinity of the 791 antibody. (A) Data from ELISA showing binding of the MUC5AC antibody in serial dilutions to its own peptide (791) and to protein extracts from human cervical mucus (HCM) and to protein extracts from human conjunctival goblet cells. (B) Immunoreactivity of the MUC5AC antibody to the conjunctival goblet cells. (C) MUC5AC immunoreactivity can be diminished by incubating the antibody with 1.5 mg/mL of 791 synthetic peptide, verifying the affinity of this antibody for the MUC5AC mucin. (D) The anti-MUC5AC antibody recognized high-molecular-weight components in protein extracts of human conjunctival biopsy specimens. *Arrowhead*: positive binding of the antibody to high-molecular-weight proteins separated by SDS-PAGE. Myosin was used as the 207-kDa protein marker. Bar, 50 μ m.

Demonstration of MUC5AC Antibody Specificity

Although antibodies to mucins have been useful in determining the spatial distribution of mucin products in wet-surfaced epithelia, they can lead to confusing results if their specificity is poorly understood. An important problem in raising antibodies against mucins is whether they cross-react with other members of the mucin family expressed concomitantly in the same tissue.³⁰ In the ocular surface, the major gel-forming mucin expressed by the ocular surface is the goblet cell-specific mucin MUC5AC, although other mucin species are present, such as the membrane-spanning MUC1 and MUC4, and potentially low levels of the gel-forming mucin MUC2.

As determined by ELISA, the MUC5AC antibody 791 used in this study showed high binding affinity to the synthetic peptide used for immunization (Fig. 3A). Binding to secretions of human cervix known to express MUC5AC mRNA was also observed. None of the synthetic peptides corresponding to other domains in MUC5AC nor peptides mimicking regions in the membrane-spanning mucin MUC4 and the gel-forming mucin MUC5B were recognized by the 791 antibody (Fig. 3A). Immunohistochemical localization of the 791 antibody showed strong binding within cytoplasmic vesicles in conjunctival goblet cells, corresponding to mucous granules (Fig. 3B). The specificity of the antibody to its immunizing peptide was confirmed by a decrease in binding to goblet cells after preadsorp-

tion of the antibody with the unconjugated peptide before immunohistochemistry on conjunctival sections (Fig. 3C). Immunoblot analysis results demonstrated that the antibody 791 recognized high-molecular-weight components in protein extracts from conjunctival biopsy specimens (Fig. 3D).

Binding of the 791 antibody to conjunctival goblet cells is in agreement with in situ hybridization experiments, which demonstrate that MUC5AC antisense riboprobe binds specifically to conjunctival goblet cells.¹² As seen in Figure 4A, the 791 antibody recognized conjunctival goblet cells, but not the stratified epithelium, which expressed the membrane-spanning mucins MUC1 and MUC4. Preimmune serum did not bind to sections of human conjunctiva that contained goblet cells (Fig. 4B). No binding was detectable in sections of human corneal tissue (Fig. 4C) or human colonic epithelium, which is known to express the gel-forming MUC2 and MUC5B mucins, but not MUC5AC (Fig. 4D). These data demonstrate specific affinity of the 791 antibody to MUC5AC and not to other gel-forming or membrane-spanning mucins of the ocular surface.

Measurement of MUC5AC Protein Levels in Human Tears of Normal Subjects and Patients with Sjögren Syndrome

In normal subjects, the amount of tear fluid recovered after instillation of 60 μ L sterile water ranged from 12 to 55 μ L ($n = 23$), with an average protein concentration of 3.95 mg/mL. In patients with Sjögren syndrome, the volume ranged from 14 to 80 μ L ($n = 11$), and the average protein concentration was 3.27 mg/mL. There was no correlation between collection volume and protein concentration.

Serial dilution experiments using decreasing amounts of total tear protein were performed by ELISA to determine the range of linear response of the 791 antibody. MUC5AC mucin was detectable in tears in all samples when plates were coated with at least 50 ng of total tear protein (data not shown). The linear response of the antibody to the MUC5AC mucin in individual samples was determined to range between 0.16 μ g and 1.25 μ g total protein. Coating the ELISA plates with higher amounts of protein did not produce linear responses to the 791

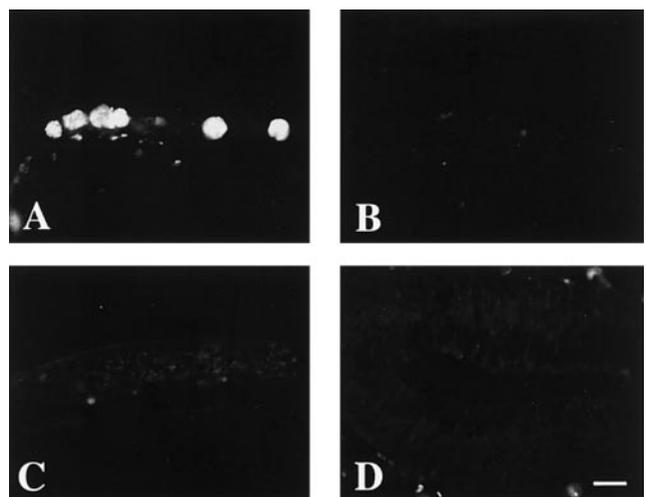


FIGURE 4. Immunolocalization of MUC5AC mucin using the 791 antibody. (A) Binding of antibody was specific to the conjunctival goblet cells but not to the stratified epithelium. (B) Preimmune serum control showing no binding to goblet cells. (C) Human corneal epithelium did not bind the 791 antibody. (D) Human colonic epithelium showing no antibody binding to the goblet cell content. These cells produce the gel-forming mucins MUC2 and MUC5B but not MUC5AC. Bar, 50 μ m.

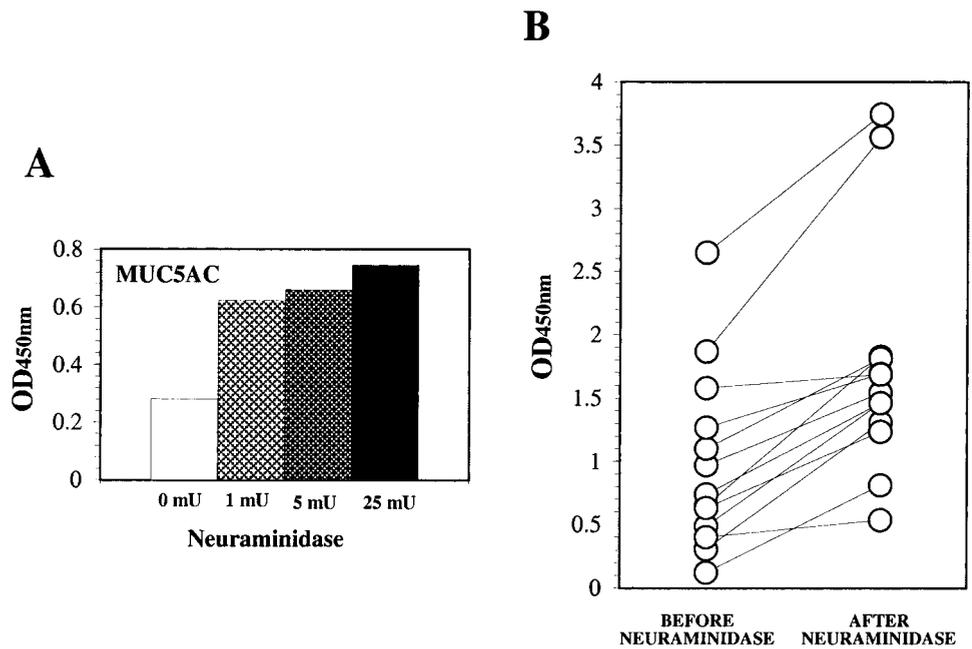


FIGURE 5. Effect of neuraminidase on the 791 antibody binding to the MUC5AC mucin in human tears, as determined by ELISA. **(A)** Addition of increasing amounts of neuraminidase (0–25 mU) to 0.5 µg tear proteins enhanced the recognition of the 791 antibody for the MUC5AC mucin. **(B)** Effect of neuraminidase treatment on tear samples from 13 normal subjects included in this study. The line connects the data from individual subjects before and after treatment with 2.5 mU neuraminidase. The binding of the anti-MUC5AC antibody was enhanced in all tear fluid samples after treatment. OD, optical density.

antibody. For the data reported herein, the MUC5AC levels are from ELISA plates coated with 0.5 µg total protein. The release of neuraminic acid from glycoconjugates present in the tear samples efficiently enhanced recognition of the MUC5AC protein backbone by the 791 antibody (Fig. 5). All the samples were, therefore, individually treated with neuraminidase before the ELISA analysis.

The tear fluid sampling and assay were performed on several healthy donors three times during the span of a year and analyzed by two different investigators to assure reproducibility of the ELISA. No significant differences were found in the relative amount of MUC5AC protein obtained in tear samples collected in different seasons and by different investigators, indicating the reproducibility of the assay.

Protein levels of the gel-forming mucin MUC5AC were significantly reduced in tears of patients with Sjögren syndrome compared with the amount in tears of healthy individuals (Fig. 6). The difference in mucin content at the protein level between the two groups was more significant than that observed by measuring mRNA transcripts, as determined using the Fisher PLSD statistic (lower probabilities when protein levels were compared).

DISCUSSION

The National Eye Institute/Industry Workshop classification of dry eye distinguished two main categories of dry eye states: aqueous-deficient and evaporative (lipid-deficient).³¹ Although mucin deficiency was originally proposed as a leading cause of dry eye,²⁰ lack of information on mucin expression in ocular surface disorders led to the elimination of mucin deficiency as a definitive clinical entity in dry eye syndrome. In this study, we investigated alterations of mucins in the aqueous layer of the human tear film in patients with clinical symptoms of dry eye. We found that levels of the goblet cell-specific mucin MUC5AC were reduced in the ocular surface of patients with Sjögren syndrome. There was a consistent correlation between the amount of MUC5AC mucin transcripts in conjunctival samples collected by filter paper stripping and the levels of secreted mucin protein found in tears of the same patients.

The presence of MUC5AC mucin transcripts in the human ocular surface epithelium was initially demonstrated and as-

cribed specifically to conjunctival goblet cells, using Northern blot analysis and in situ hybridization.¹² The recent application of competitive RT-PCR to the quantitation of gene expression

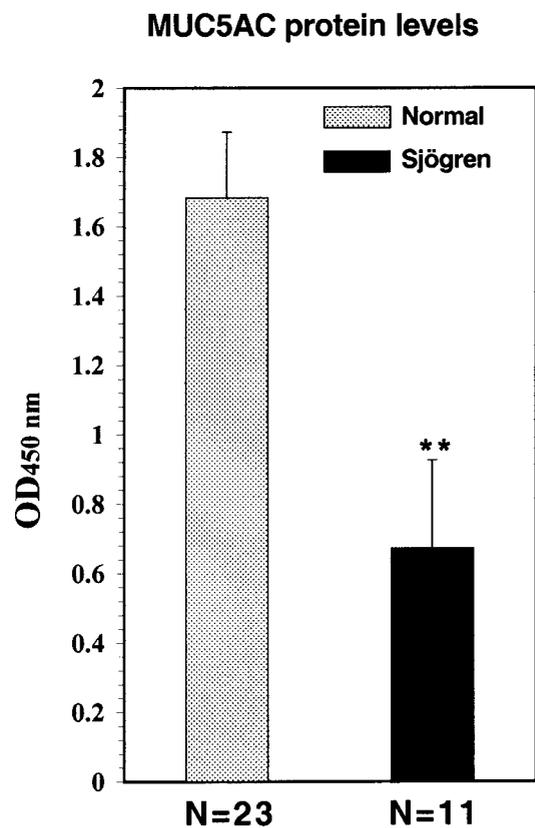


FIGURE 6. Comparison of MUC5AC protein content in tear fluid samples of normal subjects and patients with Sjögren syndrome. The levels of protein of the gel-forming mucin MUC5AC were also reduced in tears of patients with Sjögren syndrome ($P = 0.004$), thus corroborating the RNA data demonstrating a decrease in MUC5AC transcripts in the conjunctival epithelium. OD, optical density. Error bars, SEM; $**P < 0.005$.

of gel-forming mucins has demonstrated MUC5AC to be the major mucin in human normal conjunctiva, rather than MUC2.¹⁴ Evidence showing MUC5AC mucin protein as a secreted product of normal conjunctival goblet cells has been made possible by the development of antibodies raised against synthetic peptides corresponding to nonglycosylated domains flanking the central tandem repeats in the MUC5AC molecule.^{32,33} Data from the present study are in agreement with these previous studies but extend prior results to a comparison between MUC5AC protein and mRNA levels in normal subjects and patients with Sjögren syndrome.

In preliminary ELISAs assessing the amount of MUC5AC protein in tears, we found that some healthy individuals had reduced levels of the protein. However, further experimentation revealed that treatment of the tears by neuraminidase facilitated the access of the antibody to the mucin protein backbone. Similar masking of the antibody binding by carbohydrate has been demonstrated in assays detecting MUC1.³⁴ It is important that masking be taken into account when assessing individual variations in tear mucin content by immunoassay with the 791 antibody.

By using a combination of real-time RT-PCR and the highly sensitive ELISA, we have demonstrated a significant reduction of MUC5AC mucin mRNA expression and a concomitant reduction in mucin protein content on the ocular surface of eyes in patients with Sjögren syndrome. Several reports have shown that rMuc4 mucin expression in the rat is regulated by post-translational mechanisms, resulting in a discordance between protein and mucin mRNA levels.^{35,36} Based on previous studies showing a concordance in the expression of the gel-forming mucin MUC5B and protein levels in endocervical epithelium during the menstrual cycle,²⁷ we speculate that posttranslational regulation of gel-forming mucins is less complex than that of the membrane-spanning mucins.

Although we did not obtain data on the number of goblet cells in the two groups analyzed, we hypothesize that the MUC5AC mucin reduction observed in patients with Sjögren syndrome results from a depletion in the number of MUC5AC mucin-producing goblet cells, which is associated with the disease.³⁷⁻³⁹ This presumption is supported by recent data showing a reduction in the percentage of MUC5AC-positive conjunctival cells in patients with dry eye, as determined by flow cytometry.¹⁸ Evidence correlating a reduction in MUC5AC mucin gene expression with goblet cell density is shown in an animal model of ocular surface keratinization using vitamin A-deficient rats. As demonstrated by Tei et al.,³ the number of rMuc5AC transcripts in the ocular surface epithelium of rats deprived of vitamin A for 20 weeks decreases, as did identifiable goblet cells. A concomitant reduction of MUC5AC RNA transcripts and goblet cells appears to directly affect the level of MUC5AC protein in the tear fluid.

In addition to the expression of MUC5AC, we evaluated the expression of MUC1 and MUC4 transcripts in the normal and Sjögren syndrome groups. Our data suggest that the expression of membrane-spanning mucins in the surface of the eye is minimally affected or is unaffected in the disease. Although there was a tendency toward a reduction in the expression of MUC1 mRNA in patients with Sjögren syndrome, there was no statistical significance when compared with the normal group. Recently, Jones et al.¹⁷ reported a decrease in MUC1 protein levels in patients with Sjögren syndrome, detected by immunofluorescent staining of conjunctival epithelium, using an antibody raised against the VNTR domain of MUC1. However, interpretation of these results is of concern, because the number of tandem repeats per mucin molecule varies due to genetic polymorphism, and probes against regions within the VNTR domains of mucins are not useful as quantitative tools. Pflugfelder et al.,⁴⁰ using an antibody recognizing an unknown

epithelial membrane-spanning mucin expressed in human conjunctiva, found less binding to the bulbar and tarsal conjunctiva in patients with Sjögren syndrome than in normal subjects. Whether this mucin corresponds to MUC1, MUC4, or another uncharacterized membrane-spanning mucin remains to be determined. Thus, the development of well-characterized specific antibodies against defined protein domains in membrane-spanning mucins is needed to verify the discordance between mucin mRNA expression and protein levels on the ocular surface.

The decrease in the amount of mucin molecules on the ocular surface may affect the stability of the tear film. Mucins, mainly through the hydrophilic O-linked oligosaccharide side chains, contribute to the formation of a highly hydrated gel structure that prevents the ocular surface from drying—mucus gels contain more than 90% water, with the concentration of mucins at 0.5% to 5%.⁴¹ A diminution in the amount of mucin in the tear fluid of patients with dry eye may lead to decreased water retention, with loss of water through the nasolacrimal outflow tract or evaporation.

In summary, we report that the use of specific antibodies raised against the MUC5AC mucin, in combination with the highly sensitive ELISA, has been a useful and reproducible method to study MUC5AC protein levels in individual samples of tears. Our data show that patients with Sjögren syndrome have reduced levels of the gel-forming mucin MUC5AC in tears, which correlates with a reduced number of MUC5AC transcripts in conjunctival epithelia. Because gel-forming mucins, through their O-linked hydrophilic carbohydrates, are responsible for maintaining the fluid on wet-surfaced epithelia, we propose that a depletion of MUC5AC mucin in the tears of patients with Sjögren syndrome could compromise tear film stability and normal ocular surface physiology.

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