Mucin-type O-glycans in Tears of Normal Subjects and Patients with Non-Sjögren’s Dry Eye

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PURPOSE. O-linked carbohydrates (O-glycans) contribute to the hydrophilic character of mucins in mucosal tissues. This study was conducted to identify the repertoire of O-glycans in the tear film and the glycosyltransferases associated with their biosynthesis, in normal subjects and patients with non-Sjögren’s dry eye.

METHODS. Human tear fluid was collected from the inferior conjunctival fornix. O-glycans were released by hydrazinolysis, labeled with 2-aminobenzamide, and analyzed by fluorometric, high-performance liquid chromatography (HPLC) coupled with exoglycosidase digestions. O-glycan structures identified in tears were related to potential biosynthetic pathways in human conjunctival epithelium by using a glycan microarray database. Lectin-binding analyses were performed with agglutinins from Arachis hypogaea, Maackia amurensis, and Sambucus nigra.

RESULTS. The O-glycan profile of human tears consisted primarily of core 1 (Galβ1-3GalNAcα1-Ser/Thr)–based structures. Mono-sialyl O-glycans represented approximately 66% of the glycan pool, with α2-6-sialyl core 1 being the predominant O-glycan structure in human tears (48%). Four families of glycosyltransferases potentially related to the biosynthesis of these structures were identified in human conjunctiva. These included 13 polypeptide-GalNAc-transferases (GALNT), the core 1 β3-galactosyltransferase (T-synthase), three α2-6-sialyltransferases (ST6GalNAc), and two α2-3-sialyltransferases (ST3Gal). No significant differences in total amount of O-glycans were detected between tears of normal subjects and patients with dry eye, by HPLC and lectin blot. Likewise, no differences in glycosyltransferase expression were found by HPLC and lectin blot. Likewise, no differences in glycosyltransferase expression were found by HPLC and lectin blot.

CONCLUSIONS. This study identified the most common mucin-type O-glycans in human tears and their expected biosynthetic pathways in ocular surface epithelia. Patients with non-Sjögren’s dry eye showed no alterations in composition and amount of O-glycans in the tear fluid. (Invest Ophthalmol Vis Sci. 2009;50:4581–4587) DOI:10.1167/iovs.09-3563

The tear film covering the ocular surface epithelia consists of two distinct layers: an outer lipid layer produced primarily by meibomian glands, and an inner layer containing a mixture of mucin and lacrimal fluid, adjacent to the goblet cells of apical epithelial cells.1 The mucin component of the tear film contains both secreted and cell-surface–associated mucins,2 the latter being constitutively shed from the apical glyocalyx of the ocular surface epithelia. The goblet-cell–specific MUC5AC is the major secreted mucin in tears, whereas MUC1, -4, and -16 constitute the major cell-surface–associated mucins.2

Structurally, both types of mucins are large and very densely glycosylated proteins. O-glycosylation is the predominant posttranscriptional modification of mucins, conferring on them unique physicochemical properties essential for their functions on the ocular surface. These functions include hydrophobicity and an antiadhesive property that provide boundary lubrication and protection against pathogen invasion.3,5 Mucin-type O-linked glycosylation is initiated by a family of enzymes known as UDP-N-acetylgalactosamine:polypeptide N-acetylgalactosaminyltransferases (GALNT) that transfer α-N-acetylgalactosamine (GalNAc) to Ser/Thr residues in the protein backbone to form the Tn-antigen. The Tn-antigen can be elongated by other glycosyltransferases to generate a series of inner core O-linked glycans. To date, eight different core structures have been described, with core 1 (Galβ1-3GalNAcα1-Ser/Thr), synthesized by core 1 β3-galactosyltransferase (T-synthase), being among the most common in mucins.6 Core structures can be further modified by addition of other carbohydrates, such as poly-lactosamine, and may be terminated by blood group or tissue antigens, or by sialic acid and sulfate. The composition and sequence of carbohydrates in the mucin O-glycan chain is governed by cell type and tissue-specific expression of mucin-type glycosyltransferases. Recent work by Royle et al.7 has elegantly demonstrated the structure of mucin O-glycans isolated from conjunctival tissue, but the detailed nature of mucin O-glycans in tears (where they may interact with lectin-like proteins or microorganisms) and the glycosyltransferases associated with their biosynthesis remain mostly uncharacterized.

Altered mucin O-glycosylation has been frequently observed with several pathologies, including Tn syndrome, IgA nephropathy, cystic fibrosis, inflammatory bowel disease, and cancer.8–12 In the eye, several reports have described alterations in the carbohydrate composition of the apical glyocalyx of conjunctival epithelial cells in patients with dry eye. These include a reduction in lectin and antibody binding to cell surface carbohydrate epitopes such as sialic acid and core 1, and alteration in the distribution of glycosyltransferases involved in mucin-type O-glycosylation.13–17 As determined by rose bengal staining, alteration in cell surface mucin O-glycosylation in aqueous-deficient dry eye correlates with epithelial damage at the ocular surface.15 It remains unknown, however, whether the O-glycosylation of tear film mucins is altered in dry eye and if so, whether it contributes to an increased susceptibility to ocular surface damage. This study was designed to determine the profile of O-glycans in the human tear film and to analyze the expression of mucin-type O-glycosyltransferases involved in their biosynthesis in normal subjects and patients with dry eye.
O-glycans were fluorescently labeled with 2-aminobenzamide (2-AB) with a labeling kit (Signal 2-AB; Prozyme). Briefly, released O-glycans were dissolved in 5 μL of a freshly prepared solution containing 5 mg 2-AB and 6 mg sodium cyano borohydride in 150 μL acetic acid and 350 μL dimethyl sulfoxide. The mixture was incubated at 65°C for 5 hours in a heating block. For subsequent analysis by HPLC, excess dye and other labeling reagents were removed with a cartridge for cleanup of fluorescently labeled glycans (Glyco-Gen S cartridge; Prozyme). The 2-AB-labeled O-glycans were eluted in water, lyophilized, and stored wrapped in foil at −20°C until analysis.

**Fluorometric HPLC Analysis**

2-AB-labeled O-glycans were analyzed by fluorometric normal-phase HPLC using an NH₂ analytical column (4.6 × 250 mm; Zorbax; Agilent Technologies, Palo Alto, CA) connected to a chromatography system (BioLC; Dionex Corp., Sunnyvale, CA). Samples were eluted in 50 mM formic acid (pH 4.4) and acetonitrile, in gradient conditions previously described. The total amount of O-glycans in normal and dry eye samples was expressed in picomoles after normalization of fluorescence intensity values to those obtained for a 2-AB-labeled core 1 O-glycan standard (Prozyme).

**Exoglycosidase Digestions**

2-AB-labeled O-glycans were subject to exoglycosidase digestion with 1 U/mL α2-3-sialidase from *Streptococcus pneumoniae* (NAN1; Prozyme) and 1.5 U/mL α2-3,6-sialidase from *Arthrobacter ureafaciens* (ABS; Prozyme). Digestions were performed in 50 mM sodium phosphate (pH 6.0), for 16 hours at 37°C. To remove enzymes before HPLC analyses, samples were passed through protein-binding filters (MicroPure-EZ; Millipore, Bedford, MA).

**Lectin Blot Analysis**

Tear fluid (7.5 μg of total protein) was diluted in Laemmli buffer and separated by 1% (wt/vol) agarose gel electrophoresis, as described by Thornton et al.24 For lectin blot, the proteins were transferred to nitrocellulose membranes (Millipore) by vacuum. The membranes were then blocked with 1% polyvinylpyrrolidone in 0.1% Tween-Tris-buffered saline (pH 7.5), for 1 hour, and incubated with biotin-labeled peanut agglutinin (PNA, 25 μg/mL) to the mucin-associated T-antigen epitope, *Sambucus nigra* agglutinin (SNA, 100 μg/mL) to terminal α2-3 sialic acid, and *Mausiella amurensis* agglutinin (MAA, 100 μg/mL) to terminal α2-3 sialic acid at 4°C and 65°C for 1.5 hours at room temperature. Membranes were developed with an ABC kit (Vectastain; Vector Laboratories, Burlingame, CA), and lectin binding was visualized with chemiluminescence (SuperSignal West Pico; Pierce). Films were scanned and densitometric analysis was performed with image-analysis software (Digital Science 1D; Eastman Kodak, New Haven, CT).

**Statistical Analysis**

Statistical comparisons of HPLC data were performed with commercial software (InStat3 software; GraphPad Software, La Jolla, CA). P-values were determined using the unpaired t-test with Welch correction. For microarray data, statistical comparisons were performed using analysis of variance (ANOVA). P < 0.05 was considered statistically significant.

**RESULTS**

**Identification of Mucin-type O-glycans in Normal Tear Fluid**

Mucin-type O-glycans in normal tear fluid were fluorescently labeled and analyzed by normal-phase fluorometric HPLC (Fig. 1). The identity of each O-glycan structure in the chromatographic profile was assigned by comparison to retention times of the core 1 standard and O-glycans derived from bovine fetuin. Three major O-glycan structures were detected in normal tear film and included core 1 (Galβ1-3GalNAc), α2-6 sialylated biantennary glycans, and other complex glycans.
core 1 (Galβ1-3[NeuNacα2-6][GalNac]), and α2-3 sialyl core 1 (NeuNacα2-3Galβ1-3GalNac). In addition to these structures, α2-3 sialyl galactose (NeuNacα2-3Gal), a peeling product from the α2-3 sialyl core 1,7 was also detected. Analyses of the relative amounts of structures revealed that monosialylated core 1 constituted 66% of the O-glycan pool in tears. α2-6 Sialyl core 1 was the most abundant O-glycan in tears (48%), followed by core 1 (16%), α2-5 sialyl galactose (10%), and α2-3 sialyl core 1 (8%).

The identities of the different structures in the chromatogram were further confirmed by exoglycosidase digestions (Fig. 1). The NAN1 sialidase, specific for α2-3 sialic acid linkages, hydrolyzed the α2-3 sialyl core 1 structure to yield core 1 alone. Similarly, the peeling product α2-3 sialyl galactose was also hydrolyzed after NAN1 treatment. The α2-6 sialyl core 1, on the other hand, was resistant to NAN1, but was cleaved by ABS, a generic sialidase that removes α2-3,6,8-linked sialic acids (α2-8 sialic acids are not found in monosialylated structures, but mainly in di- or tri-sialylated O-glycans25).

**Pathways of Mucin O-glycosylation at the Ocular Surface**

Analyses of the O-glycan profile in normal tears provided the basis for delineating the pathways of mucin-type O-glycosylation at the ocular surface epithelia, taking advantage of public data on the expression of glycosyltransferases identified by glycogene microarray in conjunctival epithelium. Four families of glycosyltransferases potentially associated with the biosynthesis of O-glycan structures identified in normal tears were detected in human conjunctiva (Fig. 2). These included 13 UDP-GalNac:polypeptide N-acetylgalactosaminyl-

**Figures:**

- **Figure 1.** HPLC elution profile of mucin O-glycans in normal tear fluid. Tear samples containing 300 μg of total protein were subjected to hydrazinolysis. O-glycans were fluoroexcantly labeled with 2-AB and analyzed by fluorometric normal-phase HPLC. O-glycan structures were identified by comparison of their retention times with those of the core 1 standard and fetuin. Top: a representative O-glycan profile of normal tear fluid. Bottom: the O-glycan profile after exoglycosidase digestion with NAN1 and ABS. (●) Galactose, (□) N-acetylgalactosamine, and (●) N-acetylglycosaminic acid.

- **Figure 2.** Pathways of O-glycosylation on the ocular surface epithelia. Glycosyltransferases potentially involved in the biosynthesis of mucin-type O-glycans present on tear mucins were identified using a public microarray database. These included 13 polypeptide GALNTs involved in the initiation of mucin-type O-glycosylation, the core 1 β3-galactosyltransferase (T-synthase), three α2-6-sialyltransferases (ST6GalNAc-I, II, and IV) and two α2-3-sialyltransferases (ST3Gal-I and IV). In addition to these, six additional sialic acid glycosyltransferases were detected by microarray; three α2-6-sialyltransferases (ST6GalNAc-III, V, and VI), and three α2-3-sialyltransferases (ST3Gal-III, V, and VI). However, acceptor substrate specificity assays have shown that these transferases act preferentially to nonmucin substrates.26,27

In addition to the O-glycan structures detected in tear fluid in the present study, a recent report showed core 2 (Galβ1-3(GlcNAcβ1-6)GalNAc) O-glycans in protein extracts from human conjunctival epithelium.7 Core 2 is synthesized by β1,6 N-acetylglucosaminyltransferases acting on core 1. Analysis of the microarray data revealed two enzymes with β1,6 N-acetylglucosaminyltransferase activity in conjunctiva: the mucin-type core 2 β1,6 N-acetylglucosaminyltransferase (C2GnT-M), and the I-branching β1,6 N-acetylglucosaminyltransferase. C2GnT-M is implicated in the formation of core 2, core 4, and the blood group I structure, whereas the I-branching transferase catalyzes the formation of blood group I.28-31 Since blood group I was not detected on mucin-type O-glycans from human conjunctival epithelium or tear fluid, the I-branching transferase was not included as a component in the proposed pathways of mucin-type O-glycosylation at the ocular surface (Fig. 2).
Mucin-type O-glycosylation in Patients with Dry Eye

By fluorometric HPLC, the chromatographic profile of O-glycans from tear samples of patients with dry eye was similar to that from normal subjects: core 1 and monosialylated core 1 O-glycans were detected in the chromatograms from both normal and dry eye samples (data not shown). Quantitative analysis revealed no differences in the amount of core 1 structures between both groups (Fig. 3). In these experiments, α2-3 sialyl core 1 exhibited a downward trend in dry eye, but the difference was not statistically significant.

Lack of alteration of glycan structures in the tear fluid of patients with dry eye was further confirmed by lectin blot after separation of the tear proteins in agarose gels (Fig. 4). These analyses revealed several bands with different electrophoretic mobilities, which correspond to different glycoproteins present in human tear fluid. In normal samples, PNA bound to core 1 structures on a high-molecular-weight band that exhibited an electrophoretic migration typical of mucins (>250 kDa). Binding to low-molecular-weight glycoproteins was also detected and could correspond to proteolytically degraded mucins or to other O-linked glycoproteins present in tear fluid. PNA binding in non-Sjögren’s dry eye samples was comparable in both mobility and intensity to that observed in normal samples (Fig. 4). SNA and MAA also revealed the presence of several bands corresponding to tear glycoproteins carrying either individual or combined α2-3 and α2-6 sialic acid-terminated glycans. SNA and MAA can recognize sialic acid epitopes in both N-glycans and O-glycans, compared with PNA, which primarily recognizes O-glycans. The mobility and intensity of the SNA and MAA bands was comparable in normal and dry eye samples.

The expression of glycosyltransferases in the conjunctival epithelium of patients with dry eye was analyzed with a microarray database, as reported in the Materials and Methods section. As shown in Table 1, no significant differences were observed in the overall expression of glycosyltransferases associated with the biosynthesis of O-glycan structures identified in the tear fluid of normal subjects and those with dry eye.

DISCUSSION

Mucin-type O-glycans have been ascribed multiple functions in mucosal secretions, which include hydration and protection of underlying epithelial cells, providing resistance to protease degradation, and trapping and removing bacteria via specific receptor sites within the O-glycan chains of the mucin.32 In this study, we have determined the structure and relative amounts of mucin-type O-glycans in human tear fluid, as well as expression of glycosyltransferases involved in their biosynthesis in conjunctival epithelium, in normal subjects, and in patients with non-Sjögren’s dry eye.

Our findings on the composition of O-glycans in human normal tears are comparable to those reported recently by Royle et al.7 on human mucins extracted from human conjunctival tissue; core 1-based structures are the major mucin-type O-glycans at the ocular surface. It is of interest, however, to note three major differences between tissue-associated O-glycans in conjunctiva and those found in tears. First, the conjunctival epithelium contains core 2-based structures, including galactosyl core 2 and di α2-3 sialyl galactosyl core 2. Second, α2-3 sialyl core 1 is the predominant O-glycan in human conjunctival tissue (47.4%), whereas in tears the prominent O-glycan is α2-6 sialyl core 1 (48%). And third, disialyl core 1 is present in conjunctival mucin but not in tears. These
discrepancies could be due to several factors. First, Royle et al. evaluated O-linked glycans in high-molecular-weight fractions of mucin isolates (representing nondegraded mucin) from conjunctival tissue, whereas our study included all O-linked glycans in tears. Second, differences in cellular trafficking may influence the O-glycosylation profiles in mucins. Engelmann et al. have shown that breast cancer epithelial cells transfected with membrane-bound and secretory-recombinant MUC1 produce a transmembrane form that contains mainly sialylated core 1 structures and a secreted form containing predominantly neutral core 2 O-glycans. Third, it is also possible that specific mucin-type O-glycans in conjunctival tissue are associated with intracellular or cell surface glycoproteins and, therefore, are not secreted into the tear film. And fourth, it is possible that the difference in O-glycan composition between the two methods.38 In our hands, no changes were detected by HPLC in the total amount of mucin O-glycans in tears of patients with dry eye. Similarly, no changes were detected by HPLC in the total amount of mucin O-glycans in conjunctival mucin. By contrast, ST3GalIII exhibits preference for Galβ1-3GlcNAC present on glycoproteins and glycolipids, therefore, constituting potential candidates to synthesize α2-3 sialyl core 1 in conjunctival mucin. By contrast, ST3GalIII exhibits preference for Galβ1-3GlcNAC structures11; the ST3Gal-VI mainly uses Galβ1-4GlcNAC as an acceptor substrate12; and the ST3Gal-V only acts on glycolipids. Enzymatic assays using suitable substrates for the glycosyltransferases identified in this study would provide valuable information toward further characterization of the acceptor specificity and kinetic mechanisms of these enzymes.

In several studies, the expression of carbohydrate epitopes has been evaluated in dry eye, both in the tear fluid and the ocular surface epithelial glycocalyx. Using HPLC and the CA 19-9 ELISA test, Garcher et al. and Nakamura et al. have shown a decrease in sialic acid and sialyl-Lea in the tear fluid of patients with dry eye. This decrease, however, could be attributed to changes to glycolipids and N-linked glycans present in the tear film; no sialyl-Lea was detected in our analysis of tear O-glycans or in conjunctival mucin O-glycans. In our hands, no changes were detected by HPLC in the total amount of mucin O-glycans in tears of patients with dry eye. Similarly, no differences were detected in PNA, SNA, and MAA binding to tear glycoproteins in dry eye, indicating lack of alterations in core 1 O-glycosylation and glycoprotein sialylation in the disease. In the epithelial glycocalyx, several authors have reported changes in the distribution of mucin-associated O-glycans. These included alteration in (1) core 1, as determined by PNA staining, and (2) O-acetyl sialylation on MUC16, as determined by H185 antibody binding, and (3) sialylation of MUC1, as determined by KL-6 antibody staining. These alterations in distribution suggest a compensatory attempt by the ocular surface epithelium to preserve a wet-surfaced phenotype in dry eye by modifying mucin-type O-glycosylation on membrane-

### Table 1. Glycosyltransferases Associated with Mucin O-Glycosylation Expressed in the Conjunctival Epithelium of Normal Subjects and Patients with Non-Sjögren’s Dry Eye

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<tr>
<th>Common Name</th>
<th>Accession Number</th>
<th>Normal</th>
<th>Dry Eye</th>
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<tr>
<td>GALNT1</td>
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<td>99.87</td>
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Expression values represent the geometric mean of intensities corresponding to nine normal subjects and nine patients with dry eye, as described by Mantelli et al.
associated mucins on the epithelial glycocalyx. Our results suggest that these alterations in carbohydrate distribution on the epithelial glycocalyx in dry eye do not result in major changes in O-glycan composition and amount in the tears of these patients.

We sought to identify genes with altered expression in patients with dry eye by analyzing microarray data from conjunctival samples collected by impression cytology. The results revealed no differences in mRNA expression of mucin-type glycosyltransferases between normal subjects and patients with dry eye. These results are in agreement with those obtained by Imbert et al.,

by real-time RT-PCR, showing no alterations in GALNT expression in conjunctival epithelium harvested by brush cytology in patients with aqueous-deficient dry eye. In these studies, however, the conjunctival—stratified epithelium containing both goblet and non-goblet cells—was homogenized previous to analysis. Using immunofluorescence, our laboratory has reported changes in the cell-type and cell-layer distribution of GALNT in pathologically keratinized conjunctival epithelium. Although no localization studies have been performed with other mucin-type glycosyltransferases, we hypothesize that alteration in the local distribution of these enzymes, not in their overall expression, might correlate with altered cell surface O-glycosylation and epithelial damage.

In summary, this study provides data on the composition of mucin-type O-glycans in human tear film and on the expression of mucin-type O-glycosyltransferases associated with their biosynthesis in conjunctival epithelium. Using a combination of techniques that included HPLC, lectin-blot, and glycogene microarray analysis, we found no detectable differences in mucin-type O-glycosylation in tears or in mucin-type O-glycosyltransferase expression in conjunctiva between patients with non-Sjögren’s dry eye and normal subjects.

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