The Cell-Layer– and Cell-Type–Specific Distribution of GalNAc-Transferases in the Ocular Surface Epithelia Is Altered during Keratinization

Pablo Argüeso,1 Ann Tisdale,1 Ulla Mandel,2 Erik Letko,3 C. Stephen Foster,3 and Ilene K. Gipson1

PURPOSE. It has been hypothesized that the biosynthesis of O-linked glycans on proteins, particularly on the highly O-glycosylated mucins, by the corneal and conjunctival epithelium is necessary for the protection and maintenance of a healthy ocular surface. The initial step in O-glycosylation is the enzymatic addition of N-acetylgalactosamine (GalNAc) to serine and threonine residues by a large family of polypeptide GalNAc-transferases (GalNAc-Ts). The purpose of this study was to determine the cellular distribution of GalNAc-Ts in the normal ocular surface epithelia and to compare their distribution with that in pathologically keratinized conjunctival epithelia.

METHODS. Five conjunctival biopsy specimens and 5 corneas from normal individuals, and 14 conjunctival specimens from patients with ocular cicatricial pemphigoid (OCP) were used. Based on the histologic characteristics of their epithelia, OCP specimens were divided into two groups: less advanced, non-keratinized (n = 6), and late-stage, keratinized (n = 8). Five monoclonal antibodies raised against the GalNAc-T1, -T2, -T3, -T4, and -T6 isoenzymes, were used for immunofluorescence microscopic localization according to standard protocols.

RESULTS. Immunohistochemical studies revealed the presence of GalNAc-T2, -T3, and -T4 isoforms within the stratified epithelium of the cornea and the conjunctiva. The GalNAc-T4 isoenzyme was found in the apical cell layers, whereas GalNAc-T2 was found in the supranuclear region of the basal cell layer of the cornea and conjunctiva. GalNAc-T5 was distributed throughout the entire ocular surface epithelium, whereas GalNAc-T1 was found in scattered cells in conjunctiva only. Binding of antibody to GalNAc-T6 was restricted exclusively to conjunctival goblet cells. There were distinct alterations in expression patterns of GalNAc-T2, -T6, and -T1 in nonkeratinized OCP epithelia compared with normal epithelia. Both GalNAc-T2 and -T6 were expressed in the apical stratified epithelia, and T1 was detected in all cell layers in five of six biopsy specimens. By comparison with nonkeratinized OCP epithelia, a marked reduction in the binding of GalNAc-T1 antibody was observed in the late-stage keratinized conjunctival epithelia of patients with OCP. In all samples, apical GalNAc-T2 was absent, and GalNAc-T6 was entirely absent. Only one of eight samples was positive for GalNAc-T1.

CONCLUSIONS. The presence of GalNAc-T isoenzymes in the human corneal and conjunctival epithelia is cell-layer and cell-type specific. The increased distribution of GalNAc-Ts observed in early stages of the keratinization process in patients with OCP suggests a compensatory attempt of the ocular surface epithelium to synthesize mucin-type O-glycans to maintain a wet-surface phenotype. This early increase in isoenzymes in nonkeratinized OCP epithelia is reduced as keratinization proceeds in the disease. (Invest Ophthalmol Vis Sci. 2003;44:86–92) DOI:10.1167/iovs.02-0181

Epithelial mucins are high-molecular-weight glycoproteins that are major components of the mucus secretions and apical cell membranes in the wet-surfaced epithelia of the ocular surface.1–2 Their main function is to lubricate and protect the underlying epithelium from desiccation and invasion of pathogens. Structurally, mucins have been defined by the presence of tandem repeat domains containing heavily O-glycosylated serine and threonine residues.1 Several of the properties of mucins derive from their carbohydrate content, which may represent up to 80% of the mass of the mature molecule. The structural diversity found in mucin-type oligosaccharides plays a major role in determining the protein structure and stability, as well as conveying specific physicochemical properties to these complex molecules (for review, see Van den Steen et al.3). Glycosyltransferases are the enzymes responsible for the initiation and elongation of glycan chains by transfer of an activated sugar residue to the proper acceptor on proteins, carbohydrates, or lipids. In mucins, the initial transfer involves primarily the posttranslational addition of N-acetylgalactosamine (GalNAc) to serine or threonine residues of the protein backbone, producing an O-linked sugar. The family of enzymes that catalyze this initial step, uridine diphosphate (UDP)-GalNAc-polypeptide N-acetylgalactosaminyl transferases (GalNAc-Ts), regulate the density and the position of O-linked sugar chains in the protein’s backbone.4 To date, nine different GalNAc-T isoenzymes have been described in humans6–14. The members of this family of genes have different chromosomal localization15 and encode amino acid sequences that have the highest homology (greater than 80%) within the catalytic domains of the enzyme.16

In severe ocular surface diseases, the abnormal progression from a healthy wet-surfaced epithelium into a keratinized epithelium may cause opacity of the cornea. Although not yet completely understood, the keratinization process is accompanied by loss of goblet cells, epithelial hyperproliferation, and the anomalous synthesis of proteins known to be present in the keratinized epithemis of the skin.17–19 Ocular cicatricial pemphigoid (OCP) is a systemic autoimmune disease that causes chronic cicatrizing conjunctivitis and progressive subepithelial fibrosis of the conjunctiva.20 In the later stages of the...

From the 1Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts; the 2Department of Oral Diagnostics, School of Dentistry, Faculty of Health Sciences, University of Copenhagen, Denmark; and the 3Immunology and Uveitis Service, Massachusetts Eye and Ear Infirmary, Boston, Massachusetts.

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Corresponding author: Ilene K. Gipson, Schepens Eye Research Institute, Department of Ophthalmology, Harvard Medical School, 20 Staniford St., Boston, MA 02114; gipson@vision.eri.harvard.edu.

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disease, these alterations result in complete keratinization of the epithelium and dry eye. It has been proposed that patients with OCP have an overproduction of ocular mucins in the early stages of the disease that diminishes progressively during the later stages. Scanning electron microscopy studies have shown that the ocular mucin in these patients has an altered morphology and appears as homogeneous granular sheets that cover extensive areas of the conjunctiva.

We hypothesize that alterations in the expression and distribution of GalNAc-T isoenzymes in the keratinizing epithelium result in the aberrant synthesis of mucin O-glycans and thus in an alteration of the physicochemical properties of mucins. Using a panel of monoclonal antibodies against the different human GalNAc-T isoforms, we have identified potential candidates responsible for initiating mucin O-glycosylation in normal human corneal and conjunctival epithelia and in the goblet cells. This study demonstrates the alteration in the specific distribution and in the expression of GalNAc-Ts during the keratinization process in patients with OCP.

METHODS

Tissue Collection

The study was conducted in compliance with Good Clinical Practices, institutional review board regulations, informed consent regulations, and the Declaration of Helsinki. Human conjunctival biopsy specimens were obtained from the superior bulbar conjunctiva at the incision sites in five patients (all women, age range, 47–67 years) undergoing cataract surgery (normal conjunctiva). Biopsy specimens were obtained from an inflamed area in the inferior bulbar region of 14 patients with OCP. The gender and ages of the patients is shown in Table 1. Five corneas unsuitable for transplantation (from three women, two men; age range, 50–85 years) were obtained from the Lions Eye Bank of Nebraska.

Selection Criteria for Normal and OCP Specimens

Specimens of normal conjunctiva were obtained from individuals who did not have a history of OCP, chronic cicatrizining conjunctivitis, dry eye, or other ocular surface disorders. The clinical profile of the patients with OCP from whom biopsy specimens were obtained is summarized in Table 1. Diagnosis was based on the demonstration of immunoglobulin and/or complement deposition at the epithelial basement membrane zone of conjunctival specimens in all patients. Two different grading systems of the severity of the disease were used. In one, the activity of OCP was graded based on conjunctival injection, as previously described. At the time of conjunctival biopsy, a single observer (CSF) graded the degree of disease activity in each eye (i.e., conjunctival injection), using a scale of 0 to 4 in increments of 0.5. In a second grading, the stage of OCP was evaluated according to the previously published staging system for OCP. Briefly, stage I is characterized by chronic conjunctivitis with subepithelial conjunctival fibrosis, stage II by foreshortening of conjunctival fornices, and stage III by the presence of symblepharon. Stage IV is the end stage of OCP, consisting of a dry eye with complete keratinization of the cornea and ankyloblepharon. As reported previously, the histologic manifestations in the conjunctiva of these patients included patches of ocular surface squamous metaplasia with loss of goblet cells. None of the patients was treated with an immunosuppressive agent at the time of conjunctival biopsy.

Classification of the OCP Specimens

Six-micrometer cryostat sections of the conjunctival specimens were stained with periodic acid–Schiff (PAS) reagent and hematoxylin–eosin (HE) to determine the morphologic appearance of the conjunctiva. Specimens containing areas of stratified epithelial cells with round nuclei, only zero to two layers of flattened apical cells, and intermixed goblet cells were classified as “nonkeratinized” epithelia. Specimens containing more than two layers of flattened apical cells with PAS-positive cytoplasm, flattened nuclei, and no goblet cells were classified as “keratinized” epithelia that had undergone squamous metaplasia. Six OCP specimens were categorized as nonkeratinized epithelia and eight were categorized as keratinized epithelia. Three pathologic specimens in the nonkeratinized group contained some areas considered to be keratinized.

Antibody Specificity

Five monoclonal antibodies, designated UH3, -4, -5, -6, and -7 and directed against the purified recombinant GalNAc-T1, -2, -3, -4, and -6 isoenzymes, respectively, were used for immunolocalization in tissue sections. Their specificity has been confirmed by immunocytoLOGY on S9 cells expressing different recombinant GalNAc-T enzymes and by measuring the enzymatic activity of differentially immunoprecipitated GalNAc-T constructs. The specificities of the UH3, -4, and -5 antibodies were additionally tested by coating ELISA plates with serial dilutions of the purified recombinant enzymes.

Immunofluorescence Localization

Six-micrometer cryostat sections of human conjunctival and corneal tissue were placed on gelatin-coated slides and dried overnight at 37°C. Sections were rehydrated in PBS (pH 7.2) and blocked in PBS with 1% bovine serum albumin (BSA) for 10 minutes. Primary antibodies were applied undiluted and then incubated for 1 hour at room temperature in a moist chamber. Sections were rinsed with PBS followed by 10 minutes in PBS with 1% BSA. The secondary antibody fluorescenti

### Table 1. Clinical Profile of Patients with OCP

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Eye</th>
<th>Stage</th>
<th>Activity</th>
<th>Other Manifestations</th>
<th>Other Conditions</th>
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<td>2</td>
<td>63</td>
<td>M</td>
<td>OD</td>
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<td>Psoriasis, atopy</td>
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<td>F</td>
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<td>Rosacea</td>
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<tr>
<td>4</td>
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<td>OS</td>
<td>II</td>
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<tr>
<td>5</td>
<td>72</td>
<td>F</td>
<td>OS</td>
<td>III</td>
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<td>6</td>
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</table>

NA = not available.
isothiocyanate (FITC) donkey anti-mouse IgG (Jackson Immunoresearch; West Grove, PA) was similarly applied for 1 hour at room temperature. After a PBS wash, coverslips were applied using antifade mounting medium with propidium iodide (Vectorshield; Vector Laboratories, Burlingame, CA) to visualize the nuclei of the cells, thereby elucidating the position of the transferase in relation to the nuclei. Incubation with the primary antibody was routinely omitted in control experiments. The sections were viewed on a photomicroscope (Carl Zeiss, Thornwood, NY) equipped for epi-illumination. To identify goblet cells within the conjunctival epithelia, phase-contrast images were obtained for each conjunctival area photographed for immunolocalization of GalNAc-T enzymes.

**RESULTS**

**Immunolocalization of GalNAc-Ts in Stratified Corneal and Conjunctival Epithelia**

All the GalNAc-T isoenzymes analyzed in this study were present in the human ocular surface epithelium (Fig. 1). Interestingly, their distribution in the corneal and conjunctival epithelia was cell-layer and cell-type specific. The monoclonal antibody UH6 (GalNAc-T4) localized primarily in terminally differentiated apical cells of cornea and conjunctiva (Figs. 1G, 1H). The binding was progressively reduced toward the central layers of the epithelium, with weak or no binding in the basal cells of either the corneal or conjunctival epithelia. Binding of the UH4 antibody (GalNAc-T2) was found exclusively in the undifferentiated basal layer of the corneal epithelium (Fig. 1C). Basal cells of the conjunctival epithelium also bound the UH4 antibody, although weak staining was also observed in suprabasal cells (Fig. 1D). All cell layers of the cornea and conjunctiva expressed GalNAc-T5, although binding of the UH5 antibody was stronger in the apical cells (Figs. 1E, 1F). The GalNAc-T1 isoenzyme was found in scattered cells of the stratified conjunctival epithelium of some but not all specimens, with little or no expression in corneal cells (Figs. 1A, 1B). The GalNAc-T1, -T2, -T3, and -T4 isoenzymes localized to the position of the Golgi apparatus in the supranuclear region of the conjunctival cells. The position of the nucleus was visualized with propidium iodide. In the cornea, the isoenzymes localized to the supranuclear region in basal cells and to the perinuclear region in apical cells.

**Immunolocalization of GalNAc-Ts in Conjunctival Goblet Cells**

In addition to finding that the distribution of GalNAc-T2 and -T4 are cell-layer specific, one isoenzyme was found to be cell-type specific. Binding of the UH7 antibody (GalNAc-T6) was restricted exclusively to the conjunctival goblet cells in the nonpathologic conjunctival epithelia (Figs. 1I, 1J). The GalNAc-T6 enzyme localized along perinuclear ropelike strands of Golgi apparatus in the conjunctival goblet cells. There was no antibody binding to GalNAc-T6 in the stratified corneal and conjunctival epithelia. Some of the goblet cells identified in these sections did not bind to the goblet-cell-specific GalNAc-T6 antibody used in this study (Fig. 1J). This may have been due to the absence of Golgi in the plane of section, or alternatively, to a subpopulation of goblet cells that do not contain GalNAc-T6, indicating heterogeneity in the goblet cell population. Goblet cells also expressed the GalNAc-T1, -T3, and -T4 isoenzymes (Figs. 1B, 1F, 1H, respectively). No binding of the UH4 antibody (GalNAc-T2) was observed in goblet cells (Fig. 1D). Expression of GalNAc-Ts within goblet cells is not surprising, because goblet cells are known to express the highly O-glycosylated mucin MUC5AC.

**Expression of GalNAc-Ts in Patients with OCP**

The presence of GalNAc-T1, -T2, -T3, -T4, and -T6 was evaluated in both nonkeratinized and keratinized conjunctival epithelium of OCP specimens. The most remarkable observation made when analyzing the spatial distribution of the transferase isoenzymes in nonkeratinized epithelium of OCP specimens was the detection of the goblet cell–associated GalNAc-T6 and the basal cell–associated GalNAc-T2 in the upper cell layers of the stratified squamous epithelium (Figs. 2C, 2G; Table 2). Their expression was observed in the apical and subapical layers of the conjunctiva with decreasing levels toward the basal cells. Binding of the UH7 antibody (GalNAc-T6) to nonkeratinized epithelium was noted in all the samples included in this study (5/5) and the UH4 antibody (GalNAc-T2) was found in four of five samples (Table 2). Along with GalNAc-T6 and GalNAc-T2, increased binding of the antibody to GalNAc-T1 was observed throughout all cell layers in the nonkeratinized stratified epithelium of OCP specimens when compared with normal subjects (Fig. 2A). Additionally, the number of patients whose conjunctival specimens tested positive for GalNAc-T1 was higher than that of normal subjects, with specimens from five of six patients expressing the GalNAc-T1 isoenzyme (Table 2). The expression of GalNAc-T3 and -T4 seemed not to be affected in early stages of the disease. In some nonkeratinized OCP epithelia, the distribution of GalNAc-T4 was observed in the lower half of the conjunctival epithelium (Fig. 2E).

**Histology with PAS-HE staining of nonkeratinized sections in patients with OCP revealed subepithelial infiltration of lymphocytes and the presence of PAS-positive conjunctival goblet cells (Fig. 2I).** The goblet cells in these patients tested positive for all GalNAc-T antibodies used in the study (Fig. 2, Table 2). There were increased levels of GalNAc-T1 and -T2 in goblet cells in specimens from patients with OCP compared with those from normal subjects (Table 2). Progression of OCP is characterized by an early-stage reduction in the number of and then the complete loss of goblet cells and keratinization of the conjunctival epithelium (Fig. 2J). This process is accompanied by a reduction in the expression of GalNAc-Ts by the stratified epithelium compared with the nonkeratinized OCP epithelia (Figs. 2B, 2D, 2F, 2H). Of these, GalNAc-T2 and -T6 were the isoenzymes most severely affected during the keratinization process, followed by GalNAc-T1 (Table 2). The specimens from patients 9 and 4 were particularly useful in demonstrating the progressive reduction of GalNAc-T2 and -T6 associated with the disease, because they contained two areas with both nonkeratinized and partially keratinized epithelia (data not shown). In the keratinized samples, there was a reduction of GalNAc-T3 in two specimens and GalNAc-T4 in four (Table 2; Fig. 2F).

**DISCUSSION**

This study demonstrates that there is a cell-layer and cell-type specific distribution of several members of the GalNAc-T family in the stratified epithelium of the human ocular surface. The highly ordered distribution of GalNAc-Ts observed in the conjunctiva of normal individuals is altered during the keratinization process that leads to severe ocular surface dryness. The use of Northern blot techniques with transcript-specific RNA probes provided preliminary evidence showing that the repertoire of mammalian GalNAc-Ts varies in its levels and distribution among a variety of organ systems. These experiments, however, have led to ambiguous results when analyzing the expression of GalNAc-Ts in individual cell types, because they were performed with tissue homogenates that contained a mixture of cell populations. The recent develop-
Figure 1. Distribution of GalNAc-T isoenzymes in the normal human ocular surface. Sections of corneal and conjunctival epithelium were incubated with antibodies to GalNAc-T1 (A, B1), -T2 (C, D1), -T3 (E, F1), -T4 (G, H1), and -T6 (I, J1) and visualized with a fluorescein-labeled anti-mouse antibody (green). Phase-contrast images (B2, D2, F2, H2, J2) corresponding to the same conjunctival area shown in (B1, D1, F1, H1, and J1), respectively, were obtained to determine the position of goblet cells (white and yellow arrows) in relation to the enzyme localization. Propidium iodide was included in the mounting medium to localize the position of the nuclei in all sections (red). Scale bar, 25 μm.
ment of specific monoclonal antibodies has helped to determine more precisely the cellular distribution of GalNAc-Ts in human tissues. Thus, it has been shown that the distribution of GalNAc-T1, -T2, and -T3 in the stratified epithelium in oral mucosa varies with respect to the different cell layers of the epithelium. GalNAc-T1 has been found in the apical-most cell layers of the epithelium of the mouth, whereas GalNAc-T2 and -T3 were found in the basal cell layers and throughout the entire epithelium, respectively. The same pattern of expression has also been found in skin, although with less pronounced differences. No expression of GalNAc-T6 has been found in normal oral epithelium, although it is present in oral squamous cell carcinomas (Mandel et al., personal communication). Our results show that the distribution of GalNAc-T2 in basal cells and -T3 in all layers of the stratified ocular surface epithelium is similar to previous epithelial tissues analyzed. In contrast, there were low levels of GalNAc-T1 localized in the apical cell layers of the conjunctiva and none in cornea, compared with the strong expression observed in the apical cell layers of the oral mucosa. The detection of GalNAc-T4 in the apical epithelium of cornea and conjunctiva suggest that additional isoenzymes may be responsible for specific mucin-type O-glycosylation in the apical cells of the ocular surface epithelium.

The full significance of the cell-layer and cell-type differences in distribution of the GalNAc-T isoenzymes in the stratified epithelium is not clear. Because these enzymes have distinct, although partly overlapping, substrate specificities, it has been hypothesized that specific distribution may be necessary to ensure proper O-glycosylation of proteins and mucins present in different cell types. Alternatively, a given protein may require differential glycosylation as the cell moves from the basal cell layer to the surface of the epithelium to maintain specific functions, such as adhesion in basal cells or


**FIGURE 2.** Distribution of GalNAc-T isoenzymes in the conjunctival epithelium of OCP specimens. Sections of nonkeratinized and keratinized conjunctival epithelium were incubated with antibodies to GalNAc-T1 (A, B), -T2 (C, D), -T4 (E, F), and -T6 (G, H), and visualized with a fluorescein-labeled anti-mouse antibody (green). Propidium iodide was included in the mounting medium to localize the position of the nuclei in all sections (red). (I, J) A representative section of each group stained with PAS to localize mucin-containing goblet cells. White arrowheads: position of goblet cells. Dotted lines: border between the upper stratified epithelium and conjunctival stroma. Scale bars: (A-H) 25 μm; (I, J) 50 μm.
GalNAc-Ts in Normal and Keratinized Ocular Surface

Table 2. GalNAc-T Isoenzyme Expression in the Conjunctival Epithelium of Normal Subjects and Patients with OCP

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<tr>
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<th>Apical Cells of Stratified Epithelium</th>
<th>Goblet Cells</th>
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<tr>
<td></td>
<td>Normal Subjects</td>
<td>Nonkeratinized Epithelium</td>
</tr>
<tr>
<td>GalNAc-T1</td>
<td>2/5*</td>
<td>5/6</td>
</tr>
<tr>
<td>GalNAc-T2</td>
<td>0/5†</td>
<td>4/5</td>
</tr>
<tr>
<td>GalNAc-T3</td>
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<td>GalNAc-T4</td>
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</tr>
<tr>
<td>GalNAc-T6</td>
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<td>5/5</td>
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</table>

Ratio of specimens that expressed the isoenzyme to the total number of specimens examined.
* Weak binding of the antibody.
† Positive binding of the GalNAc-T2 antibody to basal cells only.

The pathologic transition from nonkeratinized stratified epithelium into keratinized epithelium is accompanied by changes in gene expression that finally lead to the aberrant differentiation of the epithelial cells. Examples of these alterations have been reported in the human cervical and gastrointestinal tracts, where the pattern of cytokeratins present in the keratinized tissue is distinctively different from the cytokeratin pattern expressed by the normal nonkeratinized epithelium. In the ocular surface, the presence of proteins also known to be expressed during differentiation in upper layers of the epidermis (i.e., transglutaminase, keratins 1 and 10) has been associated with the severely keratinized epithelium in Stevens-Johnson syndrome, OCP, and chemical injuries. The observation in the current study that there is increased expression of GalNAc-T1, -T2, and -T6 (T6 is found only in goblet cells in normal epithelium) in the nonkeratinized conjunctival epithelium of patients with OCP, in addition to GalNAc-T3 and -T4, suggests that there is an initial attempt by the epithelium to maintain a wet-surface phenotype by upregulating mucin-type O-glycosylation. Whether this is a compensatory upregulation that results in an increase in the density of O-glycan residues attached to membrane-spanning mucins of the epithelium and/or in the glycosylation of additional proteins synthesized as a consequence of the disease remains unknown. Alternatively, as previously suggested, hyperproliferation in patients with OCP may prevent the normal differentiation of the conjunctival epithelial cells, resulting in epithelial abnormalities. The presence of GalNAc-T6 in the nonkeratinized epithelium of patients with OCP may be a consequence of the inability of the progenitor cells of the conjunctiva to differentiate as non goblet stratified epithelia, reflecting an attempt of these cells to mimic the goblet cell phenotype. The alteration in the pattern of expression of GalNAc-Ts may lead to mucin-type glycan abnormalities and morphologic changes in the appearance of mucins on the ocular surface of patients with ocular surface diseases. Several studies have demonstrated an altered expression of carbohydrates in dry eye syndrome as well as the presence of amorphous mucin-like material in the conjunctival surface of patients with OCP. Based on immunohistochemical studies in which an antibody against a carbohydrate epitope on a mucin molecule was used, Danjo et al. reported an alteration in its distribution in patients with dry eye syndrome compared with normal individuals. Although it is not clear whether there is an alteration in mucin distribution or mucin glycosylation in these patients, our results showing an altered distribution of GalNAc-T isoenzymes suggest the latter. Similarly, it is not clear whether the alteration of mucin glycosylation may affect terminal carbohydrates or core carbohydrate structures. Thus, the definitive answer to these questions awaits the identification of the carbohydrate epitopes present on mucins at the ocular surface. We envision that strategies focused on the modulation of glycosyltransferases by specific gene targeting, in combination with the structural analysis of O-glycan chains on individual mucin molecules at the ocular surface, will provide information regarding the biological significance of these glycoconjugates in the normal tear surface and in dry eye diseases.

References


