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Glycosylation pathways of human corneal and conjunctival epithelial cell mucins

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Abstract

Mucin glycoproteins on the ocular surface are rich in O-glycans and have important roles in the protection from physical, chemical and microbial impact. In this work, we have cultured human corneal and conjunctival epithelial cells to examine the glycosyltransferase activities that synthesize the O-glycans of mucins. The results indicate that ocular surface epithelial cells have active enzymes that synthesize O-glycans with sialylated core 1, Gal β 1–3GalNAc α , and core 2, GlcNAc β 1–6(Gal β 1–3)GalNAc α structures which corresponds to previous structural studies. Eye cells also have enzymes that synthesize complex N-glycans that are found on mucins. Results from treatment of eye cells with TNF α suggest that epithelial O-glycosylation changes in a dynamic fashion during inflammatory stimuli of the eye surface.

Graphical Abstract

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The authors do not have competing interest to declare.

Appendix A. Supplementary data related to this article can be found

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Keywords

human corneal epithelial cells; human conjunctival epithelial cells; glycosyltransferases; mucins; O-glycans; N-glycans

1. Introduction

The surface and tear fluid of the human eye contains glycoproteins and mucins produced in epithelial cells of the cornea, conjunctiva, and lacrimal and meibomian glands. Normal vision depends on the continuous synthesis of mucins and glycoproteins on the eye surface. Highly O-glycosylated mucins in the epithelial glycocalyx and tear fluid have an important barrier function and protect the eye from adverse environmental conditions such as dehydration, injury and infection [1–6]. The role of O-glycans on epithelial cell mucins includes control of antigenicity, chemical properties and the conformation of the mucin molecules. O-glycans contribute to the attachment of microbes thus preventing them from invading the underlying cell, including viruses [7] and bacteria, e.g. *Staphylococcus aureus* as a major contributor to eye infections [8]. Mucin O-glycosylation is also an important regulator of cell surface functions and immunity [9–11]. On the ocular surface, MUC1 is a docking protein for cell surface receptors and contributes to cell signaling and the innate immune responses [12–15]. In conditions of dryness of the eye and salivary glands (dry eye disease and Sjögren's syndrome) mucin expression, secretion and their glycan structures are affected, associated with inflammation [16–18].

Human ocular surface epithelium produces transmembrane mucins, primarily MUC1, MUC4 and the large MUC16. The most highly expressed transmembrane mucin on eye conjunctival surfaces is MUC20 [19]. The goblet cells on the conjunctival surface contribute the large secreted mucin MUC5AC [1] while the lacrimal glands and conjunctival cells produce the small soluble MUC7. About 75% of the mucin weight is carbohydrate. Typically, O-glycans are densely attached to Ser/Thr residues in tandem repeat sequences [6,19,20]. N-glycosylation sites (Asn-x-Ser/Thr) and N-glycans have also been found in mucins [21,22] and the cell surface-bound MUC1 associates with Tyr-kinase receptors that are N-glycosylated [13,14]. Mucin N-glycosylation plays a role in epitope exposure, barrier function and intracellular transport of mucins [23,24].

Both N- and O-glycans of glycoproteins can carry sulfate esters, and mucins from bronchial, intestinal and reproductive tissues have been shown to be sulfated [20]. Sulfotransferases are ubiquitously expressed and selected sulfotransferases play a role in inflammation. Human corneal tissues produce sulfated glycosaminoglycans, such as Keratan sulfate, as well as mucins. Sulfotransferase Gal6ST that adds a sulfate ester to Gal as well as sulfotransferase GlcNAc6ST-3 that acts on GlcNAc acceptors are expressed in cornea. Patients with the hereditary condition macular corneal dystrophy type I have mutations in corneal GlcNAc6ST [25,26]. We previously showed that Gal-sulfotransferase activities were reduced in labial salivary glands from patients with Sjögren's syndrome [18].

Glycan analyses by HPLC and glycosidase digestion analyses suggested that mucin-type O-glycans from human tear film include modified core 1 structures [27,28] Gal β 1-3GalNAc (TF antigen). Targeted disruption of the core 1 β 1,3-Gal-transferase (C1GalT) gene in human corneal keratinocytes suggested that the O-glycan core 1 structure is important in the maintenance of the barrier function by limiting endocytosis [29]. The small sialylated O-glycans on the eye surface include sialyl α 2-3Gal β 1-3GalNAc α , Gal β 1-3[sialyl α 2-6]GalNAc and sialyl α 2-3Gal β 1-3[sialyl α 2-6]GalNAc [28,30]. In addition, human conjunctival mucin O-glycans include sialylated core 2 structures and those extended by a Gal residue. Sialylated Lewis epitopes were also found in conjunctiva [31]. The O-glycan structures suggest that polypeptide GalNAc-transferases (ppGalNAc-Ts) are active in eye cells as well as C1GalT that synthesizes core 1 and sialyltransferases. Based on O-glycan structures, core 2 β 1,6-GlcNAc-transferase (C2GnT) is also predicted to be active in conjunctival cells.

Different glycoforms of ppGalNAc-T are expressed in different cell types [32]. Gene expression analyses by glycan gene microarrays showed that indeed a number of ppGalNAc-Ts are expressed in human conjunctival epithelium [27,32,33]. Especially ppGalNAc-T2 and 4 were shown to be expressed in eye cells but the expression of ppGalNAc-T6 was only found in mucin producing conjunctival goblet cells. In most cell types including corneal cells, GalNAc can be extended to form core 1 (Fig. 1) by core 1 β 3-Gal-transferase [20,29]. Two β 1,6-GlcNAc-transferases are expressed in selected human epithelial cells that synthesize core 2. The leukocyte-type Core 2 β 1,6-GlcNAc-T synthesizes only core 2 [10,34], while the mucin-type Core 2/4 β 1,6-GlcNAc-T synthesizes both, core 2 from core 1, and core 4 from core 3 [35,36]. The expression of mucin-type core 2/4- β 1,6-GlcNAc-transferase (C2GnT-M) has been found in eye cells [27]. However, only core 2 and not core 4 has been found among O-glycan structures and the enzyme activities have not been confirmed [27,30] (Fig. 1). The reason for the absence of core 4 can be explained by the lack of core 3 which is the intermediate structure in core 4 synthesis. However, Gal-transferase B4Gal-T5 is expressed in conjunctiva [26] and is especially active using a core 3 acceptor substrate [37]. Previous microarray studies also suggested the expression in conjunctiva of three α 2,6-sialyltransferases (ST6GalNAc-I, -II and -IV) and two α 2,3-sialyltransferases (ST3Gal-I and -IV) [27,28].

The glycosyltransferase (GT) activities responsible for the synthesis of mucin type O-glycans and N-glycans have not yet been determined in ocular cells. The current study had the goal to establish the GT activities in corneal and conjunctival epithelial cells that are

involved in O- and N-glycosylation. Since TNF α is a common cytokine associated with inflammation in the eye [38] we also examined the effect of TNF α on GT activities. Our combined results suggest that ocular epithelial cells can synthesize biantennary N-glycans and sialylated O-glycan with core 1 and 2 structures, and that TNF α can impact on biosynthetic pathways.

2. Results

2.1. Biosynthesis of O-glycans in human corneal epithelial cells

Human corneal epithelial cells (HCLE) were grown to confluence and stratified for seven days in the presence of serum. GTs were assayed in homogenates from these cells with radioactive sugar-donor substrates and synthetic acceptor substrates which have the advantage of being defined in structures and concentrations in the assays. In contrast, natural acceptors such as mucins are heterogeneous in structure and are difficult to isolate. Having hydrophobic aglycone groups, synthetic acceptors and reaction products can be isolated by hydrophobic (C18) chromatography. The radioactive nucleotide sugar donors can also be removed by anion exchange chromatography (AG1 $\times$ 8 or Dowex1 $\times$ 8). In addition, negatively charged sulfated and sialylated reaction products are separated from their donor substrates by high voltage electrophoresis on paper. The negative control assays lacked the acceptor substrate and yielded a low background for all assays. As a positive control, purified GTs were used as well as homogenates from mucosal tissue of the rat colon that had high activities of GTs and sulfotransferases involved in the synthesis of mucin O-glycans. Because the amounts of cellular homogenates from eye cells were limited, duplicate assays were carried out and the results are shown as averages of duplicates.

The activity of polypeptide GalNAc-transferase (ppGalNAc-T) was assayed using a broad spectrum ppGalNAc-T acceptor peptide (AQPTPPP) as well as peptide acceptors derived from MUC1 (VTSAPDTRPAPGST) and MUC2 mucin tandem repeat sequences (TTVTPTPTG, TVTPTPTPTG), and Dowex 1 $\times$ 8 assays to remove UDP-[3 H]GalNAc. The ppGalNAc-Ts have overlapping specificities and recognize short peptides and glycopeptides with existing O-glycosylation, favouring the TxxP sequence as the acceptor site [34]. The chosen MUC1 and MUC2 acceptors have been shown to be useful to assess cell-derived and purified ppGalNAc-T activities [34,37,39,40] and are likely to encompass the combined activities in eye cells. All four peptides were excellent acceptor substrates (13.7 to 33.7 nmol/h/mg) for ppGalNAc-T, indicating that HCLE can efficiently O-glycosylate mucin glycoproteins (Table 1).

Homogenates from these corneal cells were found to have activities that synthesize O-glycans with core 1 and core 2 structures (Fig. 1). The activities determined with the Dowex1 $\times$ 8 assays were routinely confirmed after separation of reaction products by HPLC where the benzyl group of the acceptors and products is well retained by a C18 column and detected by absorbance at 195 nm. The activity of C1GalT was 2.8 nmol/h/mg and C2GnT had an activity of 1.9 nmol/h/mg. However, core 3 β 3-GlcNAc-transferase and core 4 β 6-GlcNAc-transferase (C2GnT-M) activities were below detection levels. This suggests that mucins produced in corneal cells have O-glycan core 1 and 2 structures and that the gene encoding core 2 β 6-GlcNAc-transferase (C2GnT-L) is expressed in these cells.

Two product standards, Gal β 1–4GlcNAc β -Bn and Gal β 1–3GlcNAc β -Bn were used for HPLC assays of Gal-transferase activities towards GlcNAc β -Bn acceptor substrate. This allowed us to distinguish between β 4-Gal-transferase (β 4Gal-T, 7.2 nmol/h/mg) and β 3-Gal-transferase activities (β 3Gal-T, 2.6 nmol/h/mg) in HCLE homogenates. These enzyme activities may extend the GlcNAc residue of the core 2 structure (Fig. 1).

α 3-Sialyl-transferase activity using 2 mM core 1 acceptor Gal β 1–3GalNAc α -*p*-nitrophenyl, was relatively high in HCLE homogenates as measured by high voltage electrophoresis (7.8 nmol/h/mg). In spite of the negative charge of sialic acid, the reaction product using 1 mM Gal β 1–3GalNAc α -Bn acceptor was well retained by hydrophobic chromatography but showed a lower activity (0.5 nmol/h/mg). The activity of α 6-sialyltransferase activity towards GalNAc-mucin (desialylated ovine submaxillary mucin) was not detected. Sulfotransferase activities using core 1 or GlcNAc β -Bn substrates were too low to be measured reliably (Table 1).

2.2. Biosynthesis of N-glycans in human corneal epithelial cells

HCLE homogenates also had high activities of GlcNAc-transferases that synthesize complex types of N-glycan chains. GlcNAc-transferase I (GnT I) activity was 8.5 nmol/h/mg and GlcNAc-transferase II (GnT II) activity was 5.3 nmol/h/mg (Table 1). Compounds containing the octyl group could be well separated by HPLC using a C18 column. The HPLC analysis of reaction products indicated that about 2 % of the product in GnT I assays have radioactive bi-antennary structures which are formed by GnT II in HCLE that utilizes the GnT I reaction product as acceptor. In GnT II assays, about 9 % of the reaction products had a mono-antennary structure which could have been formed by N-acetylglucosaminidase action on the bi-antennary GnT II product. Using a bi-antennary acceptor substrate, low activities of GnT III, IV and V were detected only in a small number of HCLE batches. Once the N-glycan antennae are formed, Gal-transferase(s) that have high activity in HCLE can extend these chains.

2.3. Glycosyltransferase activities in human conjunctival epithelial cells

As with corneal cells, human conjunctival epithelial cells (HCjE) were grown to confluence and stratified for seven days in the presence of serum. The total yield of cells was much lower than that of HCLE, thus limiting the number of enzymes to be assayed. Homogenates from conjunctival cells also had high activity of ppGalNAc-T using the AQPTPPP acceptor (26.7 nmol/h/mg) and C1GalT (14.4 nmol/h/mg), indicating that core 1 can be efficiently synthesized in these cells (Table 2). However, core 3 β 3-GlcNAc-transferase and core 2/4 β 6-GlcNAc-transferase activities were below detection levels. These results suggest that in conjunctival cells the main O-glycan structures are based on core 1 (Fig. 1). α 3-Sialyltransferase acting on core 1 substrate was very active in human conjunctival cells (5 nmol/h/mg). Thus, O-glycans are expected to have primarily the sialyl-T structure Sialyl α 2–3Gal β 1–3GalNAc α –. GnT I activity was high in HCjE cell homogenates (12.3 nmol/h/mg) indicating that mucins or other glycoproteins carry complex or hybrid types of N-glycans. The combined β 3/4-Gal-transferase activities towards GlcNAc β -Bn acceptor varied between batches of cells between 1.0 to 8.1 nmol/h/mg. These enzymes could extend both O-glycan

and N-glycan chains. In the presence of β 4GalT inhibitor N-butrylglucosamine β -thio-2-naphthyl (**612**) [39], conjunctival cell β 3/4Gal-T activities were reduced to 8 %.

2.4. Effect of TNF α on glycosyltransferase activities in ocular surface epithelial cells

Treatment of corneal and conjunctival epithelial cells with TNF α for 24 h or 48 hr did not affect viability and proliferation as compared to no treatment control (Fig. S1). However, cell viability in HCjE cells was generally lower than in HCLE cells under all conditions studied, as conjunctival cells are more susceptible to serum-starvation. Treatment of HCLE with TNF α maximally caused a four-fold increase in C1GalT activity at 40 ng/ml TNF α for 24 h. Variable but consistent increases in Gal-transferase activities towards GlcNAc β -Bn were also observed. The maximal increase in activity was seen after 24 h treatment with 40 ng/ml TNF α . TNF α treatment of HCjE cells resulted in a 3-fold increase of C1GalT activity at 40 ng/ml after 48 h. The β 3/4Gal-T activities were also increased up to two-fold after TNF α treatment, with maximal stimulation at 20 ng/ml and 24 h incubation time.

3. Discussion

In order to understand the biochemical basis of glycans that are essential for the protection of the human eye surface, several approaches have been taken, i.e. mucin glycan structural analysis [27,28,30,41–43], gene expression of mucins [19,24] and of enzymes involved in the biosynthesis of O- and N-glycans [32,33,44,45], and disruption of gene expression [29]. None of these methods alone can reveal mechanisms of glycosylation and a combination of approaches is essential to fully understand the biosynthesis. The activities of GTs measured in this study largely correlate with these previous results and help to understand the prevalence of specific glycan structures found on the surface of the eye. In addition, the current study detected a number of enzymes that have not previously been addressed in eye cells. The synthetic acceptors used here are structural analogs of natural substrates they are not entirely representative of all the acceptors (mucins and glycoproteins) found in the cell. Natural mucin and glycoprotein substrates are highly heterogeneous, complex and difficult to obtain in a purified form. In contrast, synthetic substrates have the advantage that structures and concentrations are well defined, they have functional groups that help to isolate the reaction products, and they have been verified using purified enzymes. The activities obtained are ‘in vitro activities’ while the in vivo activities are estimated by the glycan structures of glycoproteins, another approach. The presence of natural acceptors and enzymes (e.g. isotypes of ppGalNAc-T) changes according to the state of the cell and this would be difficult to mimic in vitro.

We have shown that human corneal epithelial cells have the enzyme activities to synthesize O-glycan core 1 and 2 structures, but not core 3 and 4 structures which are common in human lung and colonic tissues [20]. The enzymes were similar in conjunctival cells except that the enzyme activity that synthesizes core 2 was below detection levels.

Glycan analysis by lectin binding, glycosidase digestion and HPLC are often not specific or sensitive. Mass spectrometry (LCMS) analyses can be extremely sensitive but lack the ability to determine exact linkages between sugars. To determine GT expression, RNA analyses have great sensitivity. However, a comparison of RT-PCR and microarray method

[44] to detect GT expression levels showed that the quantification from these analyses in some instances is not reliable. In studies of prostate cancer cells we also observed that the RNA expression levels determined by RT-PCR cannot necessarily be used to predict enzyme activities [37]. On the other hand, enzyme assays can prove that the expressed enzyme is active but are often not sensitive enough to unequivocally quantify low enzyme activities. Thus, all available methods have inherent inaccuracies and should to be combined to come to a definite conclusion as to the structures formed and their biochemical basis. Another difficulty of glycomic studies is that GT expression and activity varies according to the growth conditions and differentiation status of cells [45].

Based on the relative GT activities, we expect that core 1 structures are prevailing in both HCLE and HCjE cells which supports the structural analyses [27,28]. We found that ppGalNAc-T activity is the highest of all GT activities which could explain why mucins are almost completely O-glycosylated with at least one sugar (GalNAc). The best acceptor substrate for ppGalNAc-T was derived from MUC1, expressed in eye cells. However, MUC2-derived substrates were also highly active although MUC2 is not expressed [19]. This suggests that ppGalNAc-Ts do not distinguish *in vitro* between Thr residues of small mucin-derived substrates as long as Pro is present in the +3 position [34].

Since in ocular mucins virtually all GalNAc residues are substituted by Gal, the *in vivo* efficiency activity of C1GalT must be high due to intracellular organization. O-glycan structures are also efficiently sialylated, and the sialyltransferase activities using core 1 acceptors confirmed this. However, the specific sialyltransferases could not be determined by enzyme assays but were suggested by RNA analyses [44].

C2GnT activities in corneal and conjunctival cells were relatively low. In addition, the sialylation of core 1 would prevent the synthesis of core 2 [46]. Thus only a small amount of core 2 is expected in the mixture of O-glycans. Core 2 can be efficiently galactosylated and then provide substrate for additional sialyltransferases expressed in conjunctiva. The GlcNAc-transferase responsible for core 3 synthesis (which is the prerequisite for core 4 synthesis) could not be detected in corneal or conjunctival cells, explaining the absence of both core 3 and core 4 structures. Although the expression of core 2/4 β 1,6-GlcNAc-transferase (C2GnT-M) was found in conjunctival epithelium [27], no core 4 synthesis activity could be detected. This may be explained by a low expression level and GT assays that were not sufficiently sensitive to detect low activities. However, the activity of core 2 β 1,6-GlcNAc-transferase was detected which allows the prediction that C2GnT-L was also expressed [20]. Di-sialylated and galactosylated core 2 structures have been found in human conjunctiva. However, there has not been a comprehensive linkage analysis on eye mucin O-glycans.

Clearly, the branching enzymes GnT I and II in the pathway to complex N-glycans are active in HCLE cells. We can therefore predict that corneal cells synthesize mainly biantennary complex N-glycan structures that have Gal and sialic acid termini that could be attached to eye mucins. The structures and functions of these N-glycans have not yet been fully elucidated although they appear to be critical to barrier function at the ocular surface [24].

The increased C1GalT and β 4Gal-T activities observed in TNF α treated HCLE cells suggest that mucin O-glycosylation may change during inflammation. This corresponds to the increase of C1GalT activity observed in porcine endothelial cells after TNF α treatment [47]. The data also correspond to results from a canine model of dry eye where ocular mucins from dogs with dry eye express more core 1 structures than controls [41]. The effects of this cytokine may vary in different cell types [42]. β 4-Gal-T activity or mRNA expression has also been found to increase after treatment with TNF α in a number of primary cell types studied [40,48–51]. Although the mechanisms and biological consequences of this regulation are still poorly understood, β 4GalT may be a target for therapeutic re-engineering of cell surfaces to restore the normal non-inflammatory phenotype.

4. Experimental

4.1. Materials

All reagents were purchased from Sigma unless stated otherwise. Gal β 1–3GalNAc α -pnp was from Toronto Research Chemicals. Gal β 1–4GlcNAc β -Bn was enzymatically synthesized from GlcNAc β -Bn using bovine milk β 1,4-Gal-transferase [39]. Other substrates and substrate analogs were obtained as reported earlier [37,39,40,50–52]. [14 C]Sialyl α 2–3Gal β 1–3GalNAc α -pnp was produced as a standard compound using rat colon homogenate, Gal β 1–3GalNAc α -pnp acceptor substrate and CMP-[14 C]sialic acid as described [47]. Radioactive nucleotide sugar donor substrates and radioactive 3'-phosphoadenosine-5'-phospho [35 S]sulfate (PAPS) were purchased from New England Nuclear and American Radiolabeled Chemicals. Peptide AQPTPPP was synthesized by Queen's University. Human recombinant TNF α was from PeproTech, Inc. (Rocky Hill, NJ). Dowex 1 $\times$ 8 (200–400 mesh) was purchased from Sigma. Sep-Pak C18 cartridges were from Bio-Rad.

4.2. Cell culture and treatments

Immortalized human corneal-limbal (HCLE) and conjunctival (HCjE) epithelial cells were plated at 5×10^4 cells/cm 2 and cultured to confluence in keratinocyte serum-free medium (Gibco-Invitrogen Corp., Rockville, MD). After reaching confluence, cells were switched to stratification medium containing 10% calf serum (Invitrogen, Rockville, MD), 1 mM CaCl $_2$ and 10 ng/ml EGF (Hyclone, Logan, UT) in DMEM/F12 (Gibco-Invitrogen Corp.) as described [43]. Cells were maintained in this medium for 7 days to promote stratification and optimal mucin biosynthesis [5]. HCLE and HCjE cells were then serum-starved for 1 h followed by treatment with 20 or 40 ng/ml TNF α in DMEM/F12 medium for 24 and 48 h (medium with fresh TNF α replaced at 24 h). Cell viability was estimated directly on cells grown on 24-well culture plates using the Vybrant $^{\text{®}}$ MTT cell proliferation assay kit (Molecular Probes, Eugene, Oregon, USA) as described by the manufacturer.

4.3. Enzyme assays

For enzyme assays, cells were washed in Phosphate-buffered saline, frozen, thawed and hand homogenized in five volumes of 0.25 M sucrose. Homogenates were stored in aliquots at -80°C . Protein content was determined by the Bio-Rad (Bradford) assays using bovine serum albumin as the standard. As a positive control for all mucin transferases, rat colon

mucosal homogenate was used which has high activities of GTs that synthesize O-glycan core 1 to 4 structures, and GTs that extend and terminate glycan chains such as sialyltransferases and sulfotransferases [18,20,46,52]. Purified GTs also served as positive controls. GT activities in cell homogenates were assayed using specific acceptor substrates and radioactive nucleotide sugar donor substrates at concentrations indicated in the Tables and conditions reported earlier [37,40]. The detailed assay conditions for each enzyme are described below.

The ppGalNAc-T assay mixtures contained in a total volume of 40 μ l: 20 μ l HCLE homogenate in 0.25 M sucrose (6 mg protein/ml) or HCjE homogenate in 0.25 M sucrose (1.5 mg protein/ml), 0.125 M MES pH7, 0.125 % Triton X-100, 10 mM MnCl_2 , 0.88 mM UDP[6- ^3H]GalNAc as the donor substrate (1100 cpm/nmol) and 1 mM AQTPPPP acceptor substrate which is a general ppGalNAc-T acceptor. Other acceptor peptides that have previously been shown to be active were derived from mucins MUC1 (VTSAPDTRPAGST) and MUC2 (TTTVTPTPTG and TVTPTPTPTG) (Table 1). Mixtures were incubated for 1 h at 37°C. Reaction product was isolated by anion exchange chromatography (AG1 $\times$ 8 or Dowex1 $\times$ 8, Cl⁻-form) to remove the radioactive nucleotide sugar. 600 μ l water were added after the incubation and the mixture was applied to an AG1 $\times$ 8 or Dowex 1 $\times$ 8 column (0.4 ml). The column was washed 2 times with 600 μ l water. Radioactivity (cpm) in the eluates was determined by scintillation counting. At least duplicate determinations were carried out and the average was calculated. Radioactivity from control assays lacking acceptor substrate was subtracted. The control assays contained a small amount of free GalNAc from breakdown of UDP-GalNAc, and possibly a small amount of radioactive product from endogenous glycoprotein substrates (390 cpm for HCLE and 620 cpm for HCjE). Activities (nmol/h/mg) were calculated based on the incubation time, protein content and the specific radioactivity (cpm/nmol) of the donor substrate.

Core 1 β 3-Gal-transferase was assayed in a total volume of 40 μ l containing 0.92 mM UDP[6- ^3H]Gal (670 cpm/nmol) as the donor substrate, 1 mM GalNAc α -Bn acceptor substrate, 10 mM MnCl_2 , 0.125 % Triton X-100, 0.125 M MES pH 7 and 10 μ l cell homogenate. Mixtures were incubated for 1 h at 37°C and UDP-[^3H]Gal was separated by AG1 $\times$ 8 as described above. The control assays (without acceptor) showed a background ([^3H]Gal and endogenous products) of 360 cpm. These assays were confirmed by HPLC analyses as described below. The β 3 and β 4-Gal-transferase (β 3Gal-T and β 4Gal-T) activities were assayed similarly by AG1 $\times$ 8 assays with 2 mM GlcNAc β -Bn acceptor. For HPLC separations, UDP-[^3H]Gal was removed by AG1 $\times$ 8 and eluants were lyophilized. 120 μ l water were added and 80 μ l were injected into HPLC, using a C18 column with acetonitrile-water mixtures as the mobile phase [37,40]. The absorbance at 195 nm was measured from assays containing acceptor, and 2 min fractions were collected and the radioactivity determined by scintillation counting. For comparison, the elution times of standard compounds (acceptor and the reaction product standards Gal β 1-3GlcNAc β -Bn and Gal β 1-4GlcNAc β -Bn) were determined by measuring the absorbance at 195 nm. At acetonitrile/water = 10/90, free [^3H]Gal eluted at 4 to 10 min, GlcNAc β -Bn eluted at 47 min, Gal β 1-4GlcNAc β -Bn at 42 min and Gal β 1-3GlcNAc β -Bn at 46 min. The corresponding reaction products eluted in 2 to 3 fractions. The cpm values of these fractions were added

and the background cpm values were subtracted. Control assays (not containing acceptor) showed no radioactivity above background.

Core 1 Sialyl-transferase assay mixtures contained 20 μ l HCLE or HCjE homogenate, 0.125 M Tris-HCl pH7, 0.125 % Triton X-100, 10 mM MnCl_2 , 1.1 mM CMP[6- ^3H]sialic acid (5300 cpm/nmol) and 1 mM Gal β 1-3GalNAc α -Bn or 2 mM Gal β 1-3GalNAc α -*p*-nitrophenyl acceptor. Mixtures were incubated for 1 h at 37°C. Reaction products were separated by Sep-Pak C18 chromatography, and by high voltage electrophoresis. For Sep-Pak C18 assays, 700 μ l water were added after the incubation and mixtures applied to Sep-Pak C18 columns under a slow flow rate of (1 drop per 2 seconds). Columns were washed 3 times with 1 ml water. The eluants contained >99 % of CMP-[^3H]sialic acid and free [^3H]sialic acid. The reaction product was then eluted with 3 ml MeOH. Radioactivity was quantified by scintillation counting. The background (220 cpm) from control assays (lacking the acceptor) was subtracted to calculate activities. Sialyltransferase ST3Gal1 donated by D. Jarvis, University of Wyoming, was used for positive control assays.

For high voltage paper electrophoresis assays, CMP-[^{14}C]sialic acid was used since [^3H]-labeled compounds exhibit low efficiency of scintillation counting on paper. The assay mixtures contained in a total volume of 40 μ l: 10 μ l HCLE homogenate, 2 mM Gal β 1-3GalNAc α -pnp, 10 mM AMP, 0.125 M Tris-HCl, pH 7, 0.5 % Triton X-100 and 0.5 mM CMP- [^{14}C]sialic acid (3000 cpm/nmol). Mixtures after the incubation were applied to a lane (2 cm wide) on Whatman #1 paper, with lanes containing controls (lacking acceptor) and standard compounds (CMP-[^{14}C]sialic acid, [^{14}C]sialic acid and product standard [^{14}C]sialyl α 2-3Gal β 1-3GalNAc α -pnp synthesized using rat colon homogenate [52]. High voltage electrophoresis was carried out in 1 % tetraborate buffer to separate CMP-[^{14}C]sialic acid and [^{14}C]sialic acid from reaction products that remained close to the origin.

Sialyltransferase ST6GalNAcI activity was assayed by high voltage electrophoresis on paper as described above using de-sialylated ovine submaxillary mucin as acceptor that carries mainly GalNAc (4 mM in the assay).

Sulfotransferase activities [52] were assayed in a total volume of 40 μ l containing 10 μ l HCLE homogenate, 2 mM Gal β 1-3GalNAc α -pnp or GlcNAc β -Bn, 6.25 μ M 3'-phosphoadenosine-5'-phospho-[^{35}S]sulfate (2400 cpm.pmol), 40 mM Tris-HCl pH 6.3, 10 mM NaF, 5 mM Mg-acetate, 0.125% Triton X-100, 2.5 mM ATP. Mixtures were incubated for 1 h at 37°C. 10 μ l 20 mM EDTA /2 % Na-tetraborate were added and mixtures applied to paper for high voltage electrophoresis as described above to isolate reaction products and separate the donor substrate and free [^{35}S]sulfate. Control assays lacked the acceptor substrate (90 cpm).

Core 2 β 6-GlcNAc-transferase assay mixtures contained 20 μ l HCLE or HCjE homogenate, 0.125 M MES pH7, 0.125 % Triton X-100, 0.8 mM UDP[6- ^3H]GlcNAc (4037 cpm/nmol) and 1 mM Gal β 1-3GalNAc α -Bn or 2 mM Gal β 1-3GalNAc α -*p*-nitrophenyl acceptor while core 4 β 6-GlcNAc-transferase assays contained 1 or 2 mM GlcNAc β 1-3GalNAc α -Bn acceptor. Mixtures were incubated for 1 h at 37°C. The reaction product was isolated by anion exchange chromatography (Dowex1 $\times$ 8), and cpm determined by scintillation counting

of eluates. CPM of eluates of control assays lacking acceptor were subtracted from eluates of assays containing acceptor. To confirm the activities, aliquots of assays containing acceptor were analyzed by HPLC using a C18 column and acetonitrile/water mixtures (12:88) as the mobile phase [37]. Core 3 β 3-GlcNAc-transferase activity was assayed similarly, but 5 mM MnCl_2 was present in the assay mixtures. As a positive control, core 2 β 6-GlcNAc-transferase expressed in insect cells was used [34].

GnT I assay mixtures contained 10 μl HCLE or HCJE homogenate, 0.125 M MES pH7, 0.125 % Triton X-100, 10 mM MnCl_2 , 0.85 mM UDP[6- ^3H]GlcNAc (4037 cpm/nmol) and 1 mM Mana1–6 (Mana1–3)Man β -octyl acceptor. GnT II was assayed similarly with 1.2 mM Mana1–6 (GlcNAc β 1–2Mana1–3)Man β -octyl as the acceptor substrate, which was synthesized from Mana1–6 (Mana1–3)Man β -octyl with GnT I (donated by H. Schachter, Hospital for Sick Children, Toronto), followed by HPLC purification. Assay mixtures for GnT I and II were incubated for 1 h at 37°C. Control assays lacked the acceptor substrates. The reaction products were isolated by anion exchange chromatography (Dowex 1 $\times$ 8) and activity calculated after subtracting the cpm of controls (1870 cpm). To confirm the activities, eluates were lyophilized and aliquots analyzed by HPLC using a C18 column [37] run in 14 % acetonitrile / 86 % water at 1 ml/min (Fig.S2). The absorbance at 195 nm indicated the presence of octyl-derivatives containing GlcNAc. Biantennary product eluted at 23 min and mono-antennary product at 42 min. The N-glycan branching enzymes GnT III, IV and V were assayed similarly by Dowex 1 $\times$ 8 and HPLC using the biantennary compound 1 mM GlcNAc β 1–2Mana1–6 (GlcNAc β 1–2Mana1–3)Man β -octyl as the acceptor (donated by H. Paulsen, University of Hamburg, Germany). The electrospray ionization mass spectrum in the positive mode showed that this compound was intact (m/z 1023.46 for $[\text{M}+\text{H}]^+$ and 1045.45 for $[\text{M}+\text{Na}]^+$). GnT V was also assayed in the absence of Mn^{2+} using GlcNAc β 1–2Mana1–6 Man β -methoxycarbonyloctyl (donated by O. Hindsgaul, University of Alberta, Canada) by the C18 Sep-Pak assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Human corneal and conjunctival epithelial cells were grown to study mucin glycosylation pathways.
2. Eye cells have the enzymes that synthesize O-glycans with core 1 and 2 but not core 3 and 4.
3. Eye cells have GlcNAc-transferases involved in N-glycan branching.
4. TNF α treatment of cells leads to dynamic changes of glycosyltransferase activities.
5. The results supplement those from structural and RNA expression studies.

Figure 1.

Pathways of O-glycosylation in human corneal epithelial cells. The glycosyltransferase activities, together with expression levels in corneal cell homogenates allow the construction of O-glycosylation pathways, as shown. Corneal epithelial cells have the enzymes to synthesize core 1, Gal β 1–3GalNAc-, and core 2, GlcNAc β 1–6[Gal β 1–3]GalNAc-, structures but not core 3, GlcNAc β 1–3GalNAc-, or core 4, GlcNAc β 1–6[GlcNAc β 1–3]GalNAc-. The O-glycan structures shown can be further modified by Gal residues and terminated by sialic acid residues. A small amount of sulfate could also be added to core 1 structures. Red bars indicate lack of activity. ppGalNAc-T, polypeptide GalNAc-transferase; Core 1 GalT, core 1 β 1,3-Gal-transferase (C1GalT); Core 2 GlcNAcT, core 2 β 1,6-GlcNAc-transferase (C2GnT); B3GalT, B4GalT, β 1,3- and β 1,4-Gal-transferases; ST3Gal, α 2,3-sialyl-transferase; ST6GalNAc, α 2,6-sialyl-transferase; -T, -transferase.

Table 1.
Glycosyltransferase activities in human corneal epithelial cell homogenates

Enzyme assays were performed as described in the Experimental section, using homogenates prepared from human corneal epithelial (HCLE) cells as the enzyme source. Enzyme products were isolated by anion exchange (AG1), or by AG1 and HPLC, or by hydrophobic chromatography using C18 Sep-Pak columns (C18). Sialyltransferases and sulfotransferases were also assayed by high voltage electrophoresis. Results were reported as the average of at least duplicate determinations that differed by less than 10 %. All of the results reported in the Tables were confirmed in assays using alternative assay methods. Bn, benzyl; DS-OSM, acid treated ovine submaxillary mucin; HVE, high voltage electrophoresis; mco, methylcarbonyloctyl; pnp, *p*-nitrophenyl; -T, -transferase. The numbers show the average of at least duplicate determinations.

Enzyme	Substrate	Assay	Activity (nmol/h/mg)
Polypeptide GalNAc-T (ppGalNAc-T)	1 mM AQTPPPP	AG1	8.2
	1 mM TTTVTPTPTG		31.7
	1 mM TVTPTPTPTG		13.7
	1 mM VTSAPDTRPAGST		33.7
Core 1 β 3-Gal-T (C1GalT)	1 mM GalNAc α -Bn	AG1	2.8
Core 2 β 6-GlcNAc-T (C2GnT)	1 mM Gal β 3GalNAc α -pnp	AG1	1.9
Core 3 β 3-GlcNAc-T	1 mM GalNAc α -Bn	AG1	<0.1
Core 2/4 β 6-GlcNAc-T (C2GnT-M)	2 mM GlcNAc β 3GalNAc α -pnp	AG1	<0.1
β 3Gal-T	2 mM GlcNAc β -Bn	AG1/HPLC	2.6
β 4Gal-T	2 mM GlcNAc β -Bn	AG1/HPLC	7.2
Core 1 Sialyl-T	mM Gal β 3GalNAc α -Bn	C18 HVE	0.5
	mM Gal β 3GalNAc α -pnp		7.8
α 6- Sialyl-T	DS-OSM (4 mM GalNAc)	HVE	<0.1
Core 1 Sulfo-T	2 mM Gal β 3GalNAc α -Bn	HVE	<0.003
GlcNAc Sulfo-T	2 mM GlcNAc β -Bn	HVE	<0.001
GlcNAc-T I (GnT I)	1 mM Mana α 6(Mana α 3)Man β -octyl	AG1/HPLC	8.5
GlcNAc-T II (GnT II)	1.2 mM Mana α 6 (GlcNAc β 2Mana α 3) Man β -octyl	AG1/HPLC	5.3
GlcNAc-T III,IV,V	1 mM GlcNAc β 2Mana α 6 (GlcNAc β 2Mana α 3) Man β -octyl	AG1/HPLC	<0.1
GlcNAc-T V (GnT V)	0.5 mM GlcNAc β 2Mana α 6 Man β -mco	AG1/HPLC	<0.1
		C18	<0.1

Table 2.
Glycosyltransferase activities in homogenates from human conjunctival epithelial cells

Enzyme assays were performed as described in the Experimental section, using homogenates prepared from human conjunctival epithelial cells as the enzyme source, as explained in Table 1.

Enzyme	Substrate	Assay	Activity (nmol/h/mg)
Polypeptide GalNAc-T (ppGalNAc-T)	1 mM AQPTPPP	AG1	26.7
Core 1 β 3-Gal-T (C1GalT)	1 mM GalNAc α -Bn	AG1	14.4
Core 2 β 6-GlcNAc-T (C2GnT)	1 mM Gal β 3GalNAc α -pnp	AG1	<0.2
Core 3 β 3-GlcNAc-T	1 mM GalNAc α -Bn	AG1	<0.1
Core 2/4 β 6-GlcNAc-T (C2GnT-M)	2 mM GlcNAc β 3GalNAc α -pnp	AG1	<0.1
β 3/4-Gal-T	2 mM GlcNAc β -Bn	AG1/HPLC	1.0 – 8.1
Core 1 Sialyl-T	1 mM Gal β 3GalNAc α -Bn	C18	5.0
GlcNAc-T I (GnT I)	1 mM Mana6(Mana3)Man β -octyl	AG1	12.3