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Glycan structures of ocular surface mucins in man, rabbit and dog display species differences

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Abstract The composition of the mucus gel of the tear film reflects the competing needs for transparency, stability, hydration, and protection of the ocular surface. Mucins form the macromolecular scaffolding of this hydrated gel, and glycans decorating these glycoproteins represent a rich source of binding ligands that may both modulate microbial binding and regulate the physicochemical characteristics of the gel. This study compares the structure of O-linked glycans derived from the ocular mucins of three species, to determine whether the ocular surface microenvironment dictates the need for a common pattern of O-linked carbohydrate structures. Ocular mucus aspirates were collected from healthy humans, rabbits and dogs. Mucins were purified using standard protocols. O-glycans were released by hydrazinoloysis and subsequently analysed by a combination of HPLC, exoglycosidase digestions and LC–MS/MS. A total of 12 different O-glycans were identified. In human ocular mucin, the majority were negatively charged and terminated in sialic acid, whilst those from rabbit or dog were mainly neutral and terminated in α 1-2 fucose and/or α 1-3 *N*-acetylgalactosamine. The glycans were short: the most common structures being tetra-, tri- or disaccharides. Less elaborate glycan structures are encountered at the ocular surface than at many other mucosal surfaces. Species-specific glycan expression is a feature of ocular surface mucins, and has implications for their defensive properties where different microbial and environmental challenges are encountered.

Keywords Ocular · Mucin · Glycans · Comparative study

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Introduction

The pre-ocular tear film protects the anterior surface of the eye. This unique barrier is adapted to defend against continuous assault from the external environment. It functions to combat desiccation, microbial colonisation and the effects of toxins, abrasive particles and a variety of environmental chemicals. It also maintains transparency and ensures the smooth optical quality of the cornea as a refracting surface. To perform these functions it must be continually secreted, removed, and renewed [1]. In mammals the tear film comprises a thin superficial layer of

meibomian lipid; an intermediate aqueous layer containing dissolved mucins; and an inner secreted mucus network, which probably derives mainly from conjunctival goblet cells. The latter both surmounts and integrates with the underlying epithelial glycocalyx [2].

The mucus network constitutes a gel layer [3] whose characteristics are imparted principally by secreted mucins. These are very high molecular weight glycoproteins, with up to 80% of their mass as O-linked glycan chains. The high carbohydrate content of mucins contributes to the physico-chemical properties required for protective roles at mucosal surfaces [1], and is highly adaptable. Mucins are capable of blocking microbial binding to the ocular surface [4]. Thus, their carbohydrate content represents a bank of glycan structural sequences with the potential to create specific ligands by manipulation of existing structures [5].

Twenty mucin genes (MUC genes) have been identified. These are abundantly expressed in epithelial and glandular tissues. Seven of these genes have been identified at the human ocular surface and in the lacrimal glands [6, 7]. The supramucosal gel layer of the ocular surface is mainly composed of MUC5AC, which is an apocrine secretion of the conjunctival goblet cells [8].

The great majority of mucin glycans are O-linked to repeating (VNTR) domains rich in the linkage amino acids serine and threonine. Typically, these glycan chains comprise galactose, *N*-acetylgalactosamine and *N*-acetyl-glucosamine, fucose, the sialic acids and sulphate. Their size and branching varies considerably and depends on the tissue location [9–11]. The low mannose content and absence of uronic acids serves to discriminate the mucins from other glycoproteins and the proteoglycans, but it is important to note that many of the O-glycan structures carried by mucins are also common to other glycoproteins and glycolipids. The small proportion of N-linked oligo-saccharides in mucins are associated with the correct folding, subcellular transport, and polymerisation of the mucin peptide [12, 13].

O-glycans are linked to serine and threonine residues through *N*-acetyl-galactosamine, and a series of core structures form the basis for branched or linear glycans. Eight core structures have been identified, of which Galβ1-3GalNAc-ser/thr (core 1) and Galβ1-3(GlcNAcβ1-6)Gal-NAc-ser/thr (core 2) are the most common. The core structures are then extended, by the sequential action of a series of glycosyltransferases organised along established pathways, to generate the wide variety of O-glycan chains [14, 15].

Due to the extensive capacity for diversity represented by protein glycosylation, it is likely that species have responded to selective pressures in different ways [16–19]: thereby achieving a functional ocular defence. This may also be reflected in the nature of disease susceptibility to specific microbial strains. Evidence has come from studies implicating glycan ligands containing sialic acids, fucose, *N*-acetylhexosamines and galactose in the attachment of *Pseudomonas aeruginosa* and *Escherichia coli* strains to ocular epithelial cells, epithelial mucins [20–22], and other tissue targets [23–26]. Bacteria have been shown to adapt to the library of glycan structures produced by the mucosal barriers of their hosts by evolving families of glycan binding lectins that facilitate colonization [27]. These data emphasise the importance of individual glycan structures in host defence and bacterial recognition. They indicate the need to understand tissue specific glycan expression in order to identify bacterial binding partners associated with the disease mechanism.

Previous analyses of canine ocular mucin O-glycans [28] and of human ocular mucins [29–31] provided compositional information, and showed that canine ocular mucin glycans are small compared with those found at other mucosal surfaces. However, no direct sequence information was provided.

The current study aims to identify and compare the ocular glycan profile for ocular mucins in man, dog, and rabbit. It is intended to provide a basis to illuminate the structural constraints placed upon mucin glycans at the ocular surface, and to facilitate future studies into host microbial interactions in ocular infectious disease. We show that species-specific glycan expression characterises ocular surface mucins, and has implications for the defensive properties of ocular mucus. We also show that ocular mucins present less elaborate glycan structures than those found at other mucosal surfaces. This information has the capacity to provide new insights into host pathogen interactions and the development of novel strategies to combat colonisation by pathogens.

Methods

Collection of mucus samples and purification of mucins

Five normal rabbits (four female and one male, all <6 months old) and seven normal dogs (two female, five male between 2 and 6 years of age) were culled by barbiturate overdose for reasons unconnected with this study. The eyelids were then ‘tented’ manually before the application of two drops of a chatropic solution (4 M guanidine hydrochloride in PBS containing the following protease inhibitors: 5 mM EDTA disodium salt, 5 mM *N*-ethylmaleimide, 1 mM phenylmethylsulfonylfluoride, 0.01 mg/ml soybean trypsin inhibitor, and 10 mM benza-midine). This solution was used to solublise both extracel-lular and intracellular mucins before aspiration of the ocular surface using a water-powered vacuum pump. All samples

were collected within 4 h of death. Immediately after collection, mucus was dispersed into plastic universal containers containing a further 2–3 ml of chaotrope. Human conjunctival mucins were obtained by extraction of conjunctival tissue from 16 eye bank eyes (eight male and eight female, 17–78 years) with the same chaotrope. The tissue was obtained following donation of the eyes for transplantation and research; its use adhered to the tenets of the Declaration of Helsinki, and was approved by the local ethics committee.

After a minimum of 24 h dispersal in the chaotrope, mucins were purified from the samples using methods as previously described, for dog, rabbit [32] and human samples [33]. Briefly, mucins were isolated from proteins, glycoproteins, and nucleic acids by caesium chloride density gradient centrifugation at a starting density of 1.4 g/ml, Fractions were pooled between 1.3 and 1.47 g/ml, and subjected to gel filtration on Sepharose CL-4B columns. The Vo fraction from the column (representing undegraded mucin) was detected using slot blotting with PAS, Wheat Germ Agglutinin, or anti-mucin antibodies, and pooled. This material was then desalted on Sephadex G10, before freeze drying and subsequent analysis.

Release and fluorescent labeling of glycans

O-linked glycans were released from lyophylised, cryogenically dried samples by manual hydrazinolysis [34]. The released glycans were then fluorescently labeled with 2-aminobenzamide (LudgerTag 2-AB labelling kit, Ludger Ltd, Oxford, UK) [35]. Excess label was removed by ascending paper chromatography in acetonitrile on Whatmann 3MM paper strips. After drying, the glycans were eluted from the origin with water.

Normal phase HPLC

Aliquots of the 2-AB labelled glycan pools were analysed by normal phase (NP) HPLC using a 4.6×250 mm TSK gel Amide-80 column (Anachem, Luton, Beds, UK) [34]. A 2-AB labeled dextran hydrolysate was used as a ladder of standard glucose oligomers to calibrate the system. The number of glucose residues (GU units) of the dextran peaks was used to calculate the GU value of the unknown glycan structures that were then compared to known O-glycan GU values in our database [34].

Exoglycosidase digestions

Aliquots of the O-glycan pools were incubated overnight with arrays of exoglycosidases at 37°C and then subjected to NP-HPLC. The enzymes, obtained from Prozyme (San Leandro, CA, USA), and their specificities, are presented in Tables 1 and 2. Following digestion, enzymes were removed using protein binding Millipore Micropure-EZ filters (Millipore).

Mass spectrometry

Liquid chromatography electrospray ionisation mass spectrometry (LC–ESI-MS) was performed using a Waters CapLC interfaced with a Q-TOF mass spectrometer (Waters-Micromass, Wythenshawe, Manchester, UK). Chromatographic separation was achieved using a 2× 250 mm, microbore NP-HPLC TSK gel Amide-80 column (Hichrome) with the same gradient and solvents as used with the standard NP-HPLC but at a lower flow rate of 40 μL/min. The mass spectrometer was operated in positive ion mode with 3 kV capillary voltage; cone voltage 30 V; source temp 100°C; desolvation temp 150°C; desolvation gas flow 300 L/h; and collision energy range 14–28 V. Results from the different collision energies were combined for data evaluation [34].

Results

Release of glycans by hydrazinolysis and separation by normal phase HPLC

O-Glycans released from ocular mucin fractions by hydrazinolysis and tagged with 2-aminobenzamide (AB) were separated by NP-HPLC. Glycan elution positions were expressed as glucose units (GU). The results are shown in Fig. 1. The human major O-glycans structures were peaks *d*, *h* and *k* (GU 2.26, 2.98 and 4.40 respectively), which were found at only low levels in the rabbit and dog samples. The rabbit and dog O-glycans were dominated by a peak at 2.5GU (*g*), which was not detected in the human samples. The rabbit O-glycan mixture showed a significant peak eluting at 3.9GU, suggesting that it would consist of four monosaccharides. However, its structure was not identified as it was not digested by exoglycosidases and we were unable to identify it by LC–MS.

Determination of glycan structure

The structures of the O-glycans were assigned by a combination of the following: (a) their GU value and reference to a database of GU values [34]; (b) the shift in glucose units after glycosidase digestion with specific exoglycosidases; (c) LC–MS that gives a possible composition for a specific peak; and, (4) the MS/MS fragmentation pattern from LC–ESI-MS/MS which combines peak retention time (GU), mass composition information and fragmentation. All the identified structures are listed in

Table 1 Exoglycosidases and their activity

Enzyme	Origin	EC	Removes	Abbreviation
Sialidases	*Arthrobacter ureafaciens*	3.2.1.18	α2-3/6/8 sialic acids	ABS
	Streptococcus pneumoniae[a]	3.2.1.18	α2-3 sialic acids	NAN1
α-Fucosidases	Bovine kidney	3.2.1.51	Fuc α1-2/6≫3/4	BKF
	Almond meal	3.2.1.111	Fucα1-3/4	AMF
β-Galactosidase	Bovine testes[a]	3.2.1.23	Galβ1-3/4	BTG
β-*N*-acetylglucosaminidase	*Streptococcus pneumoniae*	3.2.1.52	β1-2,3,4,6 GlcNAc	GUH
α-*N*-acetylgalactosaminidase	Chicken liver	3.2.1.49	GalNAc α1-3	CLH

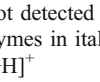
[a] Where a sugar is directly linked to the core GalNAc (*e.g.* Galβ1-3GalNAc, Neu5Acα2-6GalNAc) these enzymes do not remove the sugar efficiently because the core GalNAc is in an open ring form [34]

Table 2 using symbols (Fig. 2) to represent the individual monosaccharides and their glycosidic linkages.

The table indicates that, for example, peak g moved after digestion with CLH, which removes α galactose, but only partially after treatment with the α-fucosidase BKF. Thus we can distinguish between the two structures e and g, because although they have the same *m*/*z*, they elute at different GU values. These two different GU peaks follow different digestion patterns as well as having different fragmentation patterns.

The identity of the human O-glycans is shown in Fig. 3. The major peak *h* together with peaks *d*, *k* and *l* were

Table 2 Structures Identified in ocular mucin samples

Peak	Structure	Name	GU	Human NP HPLC % Area[$]	Rabbit NP HPLC % Area[$]	Dog NP HPLC % Area[$]	Peak Digested[&] by Exoglycosidase	Composition Hex	HexNAc	Fuc	NeuNAc	m/z* Calc	Human m/z Obs	Rabbit m/z Obs	Dog m/z Obs
a		Fucosyl galactose [#]	1.57	nd	3.9	7.6	BKF	1	0	1	0	447.2	nd	447.1	447.4
b		Core 1	1.80	4.4	6.7	1.8	*BTG*	1	1	0	0	504.2	504.2	504.1	504.4
c		alpha GalNAc galactose [#]	1.90	nd	7.0	5.7	CLH	1	1	0	0	504.2	504.2	504.1	504.4
d		sialyl galactose [#]	2.26	14.4	3.8	1.4	ABS	1	0	0	1	592.2	592.2	592.1	592.4
e		Fucosyl core 1	2.28	nd		nd	BKF	1	1	1	0	650.3	nd	650.2	650.4
f		sialyl Tn	2.44	nd	2.8	nd	ABS	0	1	0	1	633.3	nd	633.1	nd
g		Blood group A trisaccharide	2.50	nd	17.2	29.9	CLH *BKF*	1	1	1	0	650.3	nd	650.2	650.4
h		mono 2-3 sialyl core 1	2.98	47.4	1.3	3.2	ABS	1	1	0	1	795.3	795.3	795.2	795.4
i		mono 2-6 sialyl core 1	3.26	3.0	2.1	3.8	ABS	1	1	0	1	795.3	795.3	795.2	795.4
j		galactosyl core2	3.57	1.5	1.9	0.8	BTG	2	2	0	0	869.4	nd	869.1	869.4
k		disialyl core 1	4.40	16.1	0.6	nd	ABS	1	1	0	2	1086.4	1086.4	nd	1086.4
l		di 2-3 sialyl galactosyl core 2	5.39	4.1	nd	nd	ABS	2	2	0	2	737.27**	737.3	nd	nd

nd Not detected
[a] Enzymes in italic only partially digested the peak
[b] $[M+H]^+$
[c] % Areas calculated from integration of all peaks with >0.5% area. Unidentified peaks not shown in table
[d] Possible peeling products
[e] $[M+H+Na]^{2+}$

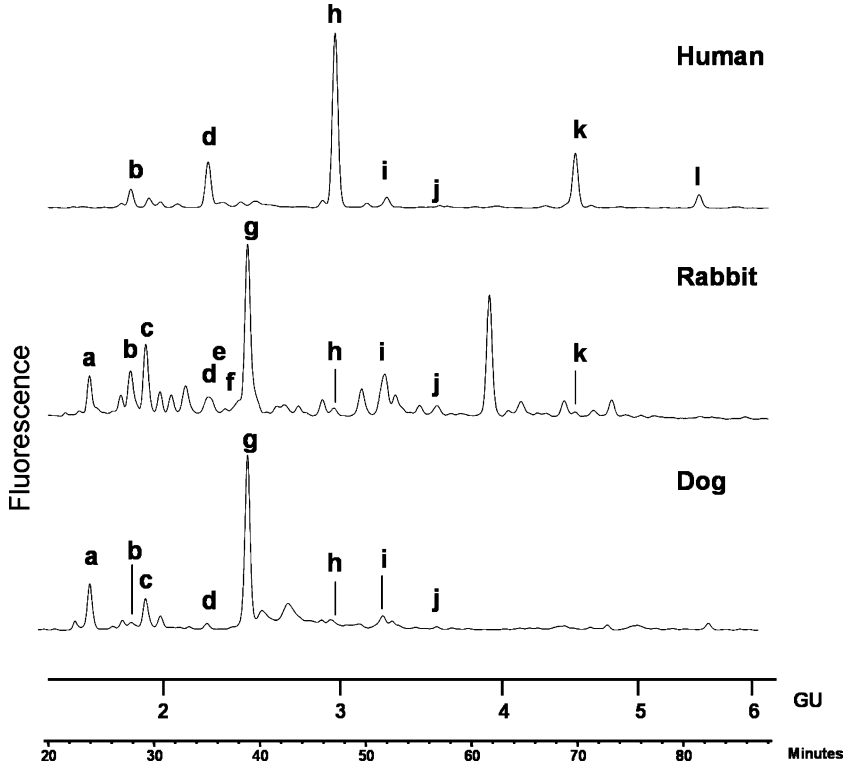


Fig. 1 NP-HPLC profile of 2-AB fluorescently labeled O-glycans released by hydrazinolysis from human, rabbit and dog ocular mucins. The larger the number of monosaccharides in a glycan the later it elutes. Peaks labeled *a*–*l* are those identified and listed in Table 2. Other peaks were not identified

susceptible to the α2-3 specific sialidase (NAN1). This digestion confirms an α2-3sialyl core 1 at peak *h*; an α2-3sialyl-galactose at peak *d* (resistant to digestion), and a disialyl core 1 at peak *k*. The latter digests to an α2-6sialyl core 1 product after ABS (peak *i*, Fig. 3). The disialyl core 2 glycan, peak *l*, contained only α2-3 sialic acid yielding peak *j* as a product of NAN1. Peaks *d* and *i*, resistant to the NAN1 sialidase, were cleaved by ABS. BTG converted peak *j* to the core 2 trisaccharide Galβ1-3(GlcNAcβ1-6) GalNAc (Fig. 3) which was in turn converted into peak *b*, core 1 by the action of GUH with specificity for β1-6 linked GlcNAc in O-glycans.

Human ocular mucin glycans contained most sialic acids in α2-3 linkage and β1-4 linked galactose attached to the β1-6 *N*-acetylglucosamine in core 2. Over 80% of them were sialylated structures with 66.5% sialylated core 1, 4.1% sialylated-galactosylated core 2 and 14.4% sialylated galactose. Only 4.4% unsialylated core 1 and 1.5% unsialylated core 2 were found.

The glycosidase digestion data for rabbit O-glycan structures is shown in Fig. 4. The dog data was similar to the rabbit glycans (data not shown). The minor sialylated peaks *0*, *f*, *h*, *i* and *k* were all desialylated by the action of the ABS. The major peak *g* was resistant to this sialidase, but was digested by α-*N*-acetylgalactosaminidase (CLH) to yield peak *a*, indicating the presence of α1-3GalNAc. Peak *a* and peak *g* were further digested by BKF, which releases fucose α1-2 to Gal, but not by AMF, which removes terminal α1-3/4 fucose. The digestion of peak *g* by BKF was slow (Fig. 4), confirming the reduction in the reaction rate of this enzyme with substrates containing an α1-3GalNAc linked to the same galactose as the fucose.

The glycan in the minor peak *e* had the same mass and composition as peak *g* (Table 2), but was resistant to CLH, but readily digested by BKF (Fig. 4). This different fragmentation pattern corresponds to a reducing GalNAc in a core 1 structure with an α1-2 linked fucose.

The action of the β-galactosidase BTG released one galactose from the minor peak *j* resulting in the core 2 trisaccharide Galβ1-3(GlcNAcβ1-6)GalNAc-AB (Fig. 4).

The major epitopes identified on the rabbit and dog O-glycans were fucose α1-2 linked to galactose (6% in rabbit and 7% in dog) and fucose α1-2 plus GalNAc α1-3 linked to the same galactose (17% in rabbit and 28% in dog).

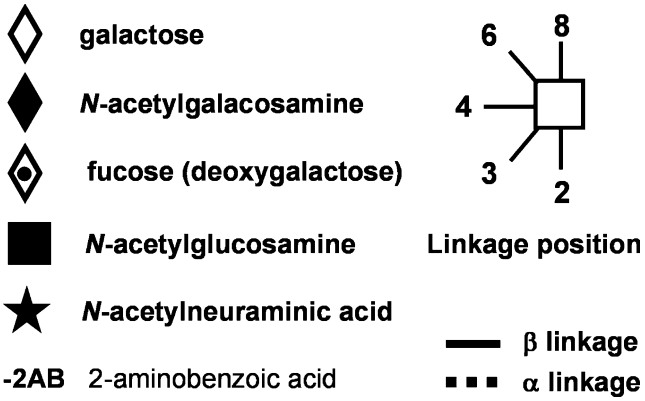


Fig. 2 Key to symbols used to represent glycans

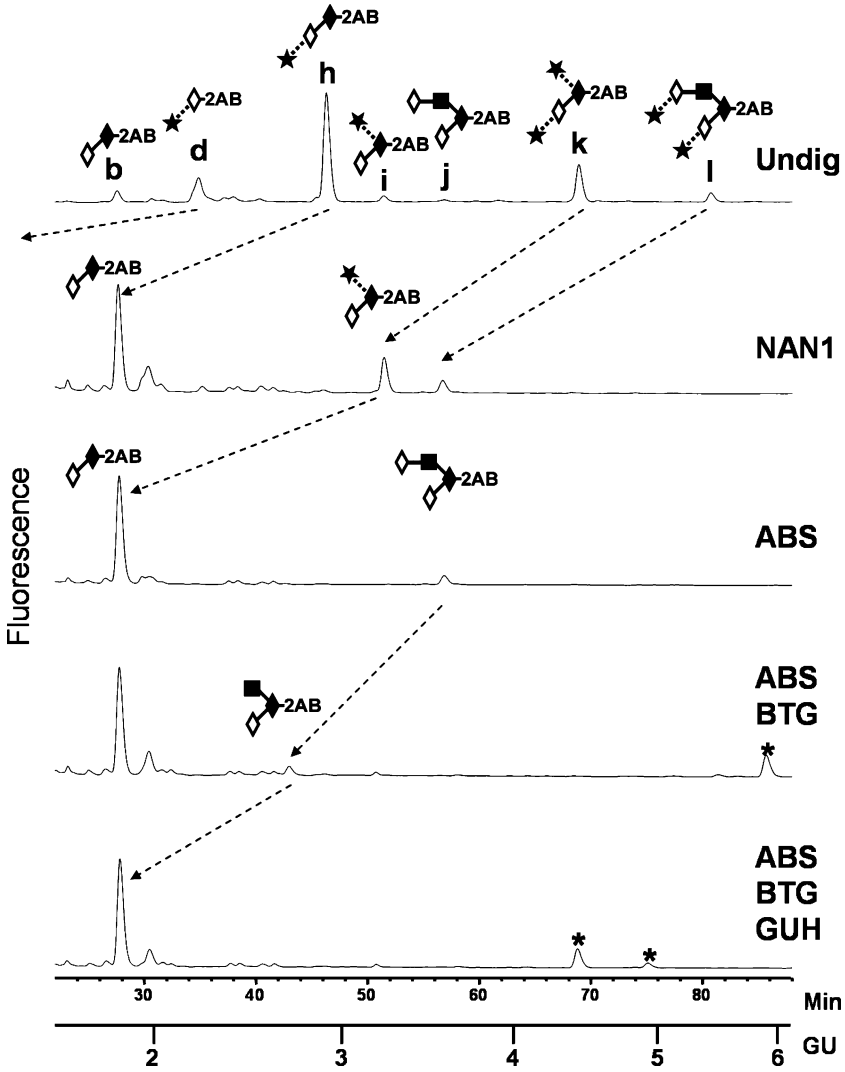


Fig. 3 NP-HPLC digestion profiles of human ocular mucin O-glycans. The *top panel* shows the undigested whole O-glycan pool with profiles shown below following digestion with exoglycosidases. *Dashed arrows* indicate movement of peaks following digestion. *Peaks annotated with asterisk* come from the digestion of N-glycans present in the sample. Abbreviations as in Table 1, symbols as in Fig. 2

There were several structures from the rabbit O-glycans which were not identified; the major unidentified peak eluted with a GU value of 3.9 suggesting that it contained four to five monosaccharides and accounted for 13% of the whole pool. This glycan was resistant to the panel of exoglycosidases used, and no *m*/*z* data was obtained. In contrast to the human O-glycans, less than 9% of the O-glycans in rabbit and less than 8% in dog were sialylated. Sialyl Tn was only found in the rabbit sample and comprised less than 3% of the whole pool.

The structures with galactose at the reducing terminus (*a*, *c*, *d* and *g*) may be generated by loss of GalNAc from the reducing terminus through the peeling reaction during release of the glycans by hydrazinolysis [36]. It is, however, possible that the galactose was directly linked to serine or threonine residues of the mucin. Nevertheless, these structures (αFuc1-2Gal, GalNAcα1-3Gal, NeuNAcα2-3Gal and Fucα1-2Gal[GalNAcα1-3]Gal) are clearly present in these samples as recognisable epitopes.

The α2-3 sialylated galactose (peak *d*) in the human samples is most likely a peeling product of the major sialylated core 1 peak *h*. The peak *g*, abundant in the rabbit and dog samples seems to be susceptible to peeling and together with the minor peaks *a* and *c* and the core 1 linked α1-2 fucose in peak *e* suggest that a major structure may correspond to the tetrasaccharide GalNAcα1-3(Fucα1-2) Galβ1-3GalNAc-ser/thr with a terminal blood group A trisaccharide epitope.

The mass spectrometric data for the glycans separated by NP-HPLC (Fig. 1) is shown in Fig. 5. The complete dataset is presented in Table 2. The value of separating glycans of the same mass by LC before MS analysis is demonstrated clearly for the two glycans having the same *m*/*z* value of 650.1. The fragmentation patterns for each of these two structures are different confirming that these are different isomers: peak *g* has the GalNAc as a terminal sugar bound to Gal, whilst in peak *e* the GalNAc is at the core and directly attached to the 2AB label.

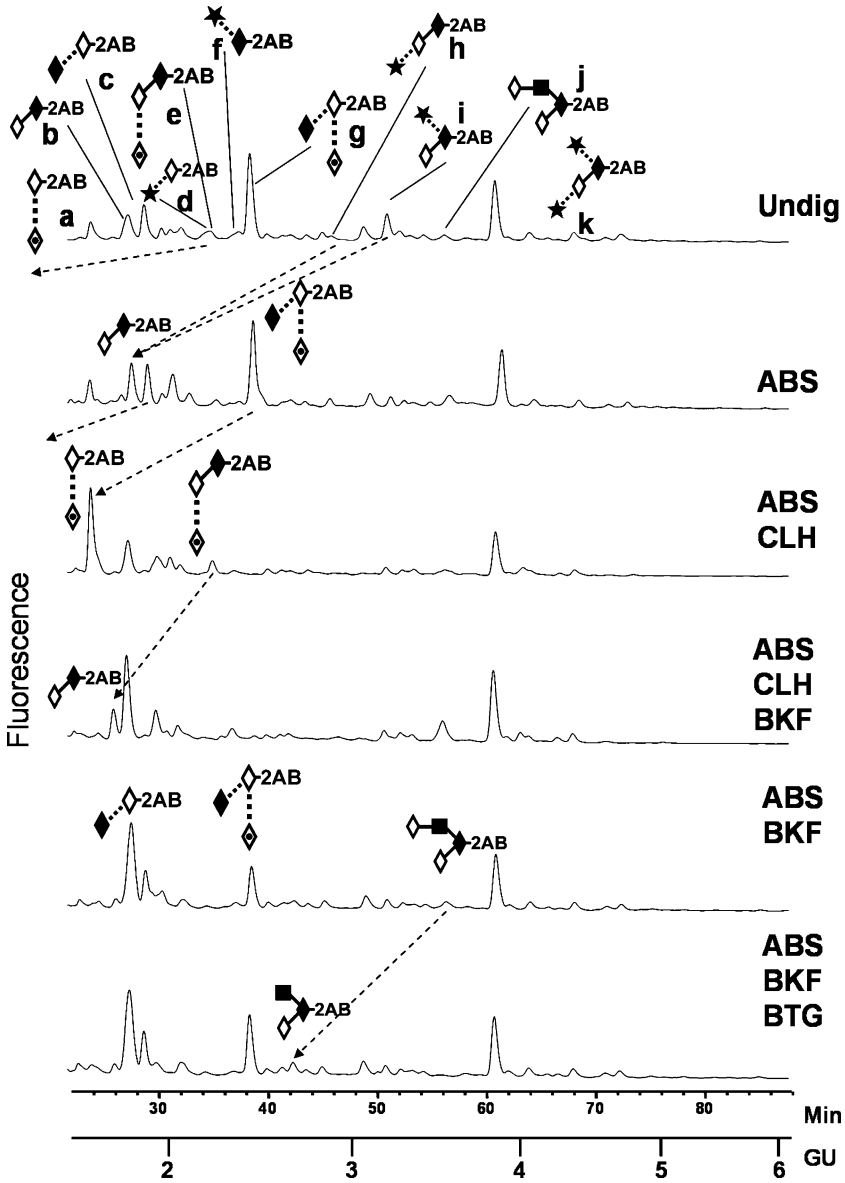


Fig. 4 NP-HPLC digestion profiles of Rabbit ocular mucin O-glycans. The *top panel* shows the undigested whole O-glycan pool with profiles shown below following digestion with exo-glycosidases. *Dashed arrows* indicate movement of peaks following digestion. Abbreviations as in Table 1, symbols as in Fig. 2

Discussion

This is the first time that detailed comparative O-glycan sequence data has been obtained for ocular mucins. This work has confirmed that these glycans are consistently short: containing between two and six monosaccharides. The most significant structural difference between the human, rabbit and dog ocular glycan complements is that the sialylated glycans predominate in humans, as opposed to the high percentage of fucosylated structures in both rabbit and dog.

The O-glycans from human ocular mucins were predominantly (>70%) core 1 based structures with less than 6% as core 2. The peeled product Neu5Acα2-3Gal (14%) could be derived from either core. The importance of sialylation in human ocular mucin is underlined by the detection of only 6% of non-sialylated glycans. Di-sialylated forms of core 1 and core 2 were found, while the α2-3 mono-sialylated core 1 comprises almost half of the total glycan pool. These results suggest that the major glycans in human ocular mucin are mono- and di-sialylated core 1 and di-sialylated core 2 tetrasaccharide Galβ1-3 (Galβ1-4GlcNAcβ1-6)GalNAc. The structures identified do not include sialyl-Le^a, which has previously been detected by antibody binding [37, 38]. This structure may therefore constitute only a small proportion of the overall glycan profile. No ABH, Lewis- blood group glycans or other fucosylated structures were detected, confirming Garcher's previous immunohistological conclusions [37].

The predominantly sialylated O-glycans in human ocular mucins have the potential to modulate host pathogen interactions, and also the interaction between leukocytes

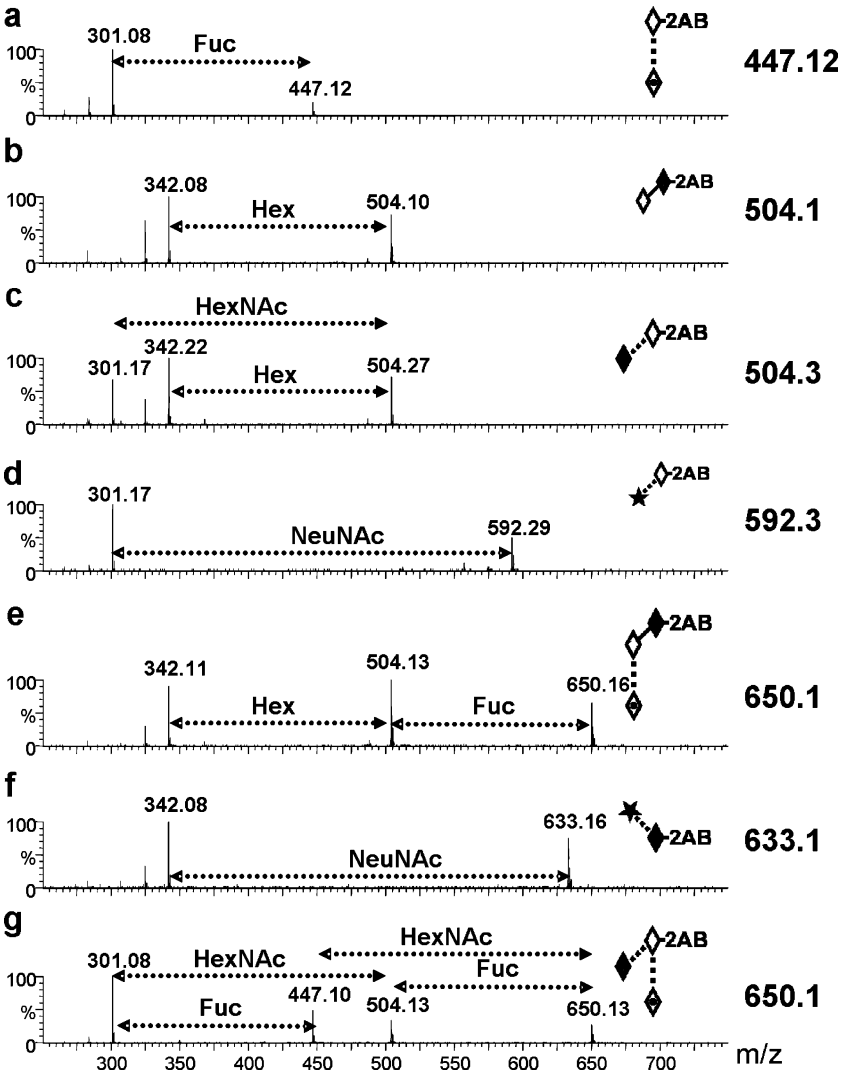


Fig. 5 MS/MS spectra of 2AB-glycans separated by LC–MS/MS. Panels a, e, f & g are from Rabbit; b from Human; and c & d from Dog O-glycans. The two structures with *m/z* 650.1 were clearly separated by the LC and the fragmentation of these two structures shows a difference in the arrangement of the monosaccharides. Symbols as in Fig. 2. Different isomers are determined by the different fragmentation patterns. The 2AB label is attached to the reducing end of the glycan. Thus, the presence of *m/z* 301.17 in (**c**) indicates the presence of structure with a core Hex attached to 2AB in addition to the structure with a core HexNAc attached to the 2AB (*m/z* 342.08) shown in (**b**). Similarly the presence of *m/z* 301.08 in (**g**) indicates that there is a core Hex labelled with 2AB, whereas in (**e**) *m/z* 342.11 corresponds to a core HexNAc labelled with 2AB

and the ocular surface [39]. Their abundance may be predicated on local suppression of FUT2 expression in human conjunctival goblet cells [37]. The family of sialic acids contributes negative charge to many glycoconjugates, and cell-types. They are an integral part of many cell–cell and cell microbe recognition phenomena [27, 40–46]. Sialic acids, may occur with α2-3 or α2-6 linkages to other sugars, or in α2-8 form as di- or polysialyl motifs. The differential expression of different linkages on cellular receptors as opposed to secreted mucins may have relevance for the ability of these mucins to act of molecular decoys capable of blocking pathogen binding [47]. Many ocular pathogens utilize α2-3 linked sialic acids as cellular receptors. These include adenovirus serotype 37, enterovirus serotype 70 and avian influenza A [48–50]. Sialic acids in the α2-6 linkage participate in the binding of *P. aeruginosa* pili to membrane bound corneal glycoproteins [20], and tear glycoproteins [51]. We show the predominance of α2-3 linked structures in human ocular mucins, with a lower content of α2-6 linkages. Thus, variability in sialic acid linkages in ocular mucins of different individuals may influence infectivity rates.

During sleep (closed eyes), high numbers of leukocytes accumulate on the ocular surface, and may provide defense against microbial multiplication in stagnant nocturnal tears [52, 53]. Sialic acids can influence leukocyte dynamics through binding to selectins and siglecs. Indeed, closed-eye tears have an increased sialoglycoprotein content [54]. Thus, sialylated structures on ocular mucins have the potential to affect local leukocyte dynamics.

In contrast to the human conjunctiva, where fucosylated blood group structures are locally suppressed [37], the O-glycan repertoire in the canine and lapine eye has selected for a common pattern of fucosylated structures. The majority are related to a fucosylated tetrasaccharide similar to blood group A GalNAcα1-3(Fucα1-2)Galβ1-3GalNAc−, and only low levels of sialylated core 1 and core 2 are found. The marked differences in the sialylation between human

and rabbit or dog ocular mucins may be partly responsible for differences in the species specificity of ocular pathogens. In man, the binding of *Helicobacter pylori* to Lewis^b on gastric epithelial cells is abrogated by the extension of the Le^b structure to form blood group A-GalNAcα1-3 and correlates with a reduced incidence of peptic ulcers [46]. The proposed structure of the major fucosylated glycan in rabbit and dog has a related structure, and provides a new target for future studies on ocular microbial infection in these species.

Despite the predominance of fucosylated glycans in the dog and the rabbit, approximately 10% of sialylated structures were also detected. Sialic acids exist in a variety of forms in nature, including mono- and oligo-*O*-acetyl esters. These are known to influence lectin binding and glycan degradation [55, 56]. 9-O-acetylated esters of *N*-acetylneuraminic acid are known to represent a significant proportion of the total sialic acid found in healthy canine ocular mucin, but are depleted in Keratoconjunctivitus sicca [57]. They are also present in human ocular mucins [58]. Neuraminic acid O-acetylation may therefore be a common cross-species modification for ocular mucins.

Ocular mucin glycosylation may be a factor in differentiating ocular pathogens from commensals. The typical microflora observed in a healthy eye is dependant on seasonal, geographical, climate, age and species variation [59]. Dramatic differences are observed in overall composition of commensal flora and the capacity of individual microbes to act as pathogens. *Moraxella* is a pathogen in the human eye, but is a commensal in both the canine and rabbit eyes [60–62]. This disparity may be partly attributable to differences in ocular mucin glycosylation. While we show a common theme of fucosylation in the ocular mucin glycans of rabbits and dogs, there are also inter-species differences. These may have relevance to differences in pathogen susceptibility. *Pasturella multocida* [59], is prevalent on the healthy ocular surface of dogs and rabbits. In rabbits, however, this organism is a facultative pathogen associated with chronic conjunctivitis and dacryocystitis [63, 64]. It is suggested that *P. aeruginosa* binds to other elements of the human tear fluid other than the sialoglycoprotein fraction [65]. Significantly, ocular mucus purified from rabbits, whilst unable to bind or inhibit *Staphylococcus aureus* and *S. pyogenes*, did bind and modulate the adherance of *P. aeruginosa* to the ocular surface [66]. Both *P. aeruginosa* and *Haemophilus influenza* have binding proteins that recognize fucose [67]. Thus, ligands, presented on the ocular mucins of dogs and rabbits, may provide better structural defense against invasion by these pathogens. *S. aureus* was the most frequent microbial isolate in the eye of all three species under study. It preferentially binds specific carbohydrate moieties on the mucin molecules, and shows strain differences in mucin binding capacities [68].

The ability of the ocular mucus layer to act as a barrier to microbial invasion will vary with both the O-glycan complement of the ocular surface and the mechanisms employed by the microorganism to modify and colonize this barrier. We have demonstrated the short length and relatively low diversity of O-linked mucin glycans within individual species: possibly reflecting the specialised requirements of the ocular surface microenvironment including tear film transparency. The need to resist pathogenic microbes and form a symbiotic relationship with commensal bacteria is a strong selective pressure leading to glycan diversity [16–19]. Thus, marked inter-species differences in sialylation may reflect host-related variations in ocular microbial flora.

The major glycan structures identified here could be used as the foundation of a glycoarray to screen for carbohydrate-dependent host–microbial interactions at the ocular surface. They also provide a baseline for studies of disease-related changes in ocular mucin glycosylation, and have potential as novel ‘mucomimetic’ glycotherapeutics in topical ophthalmic preparations.

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