Identification of two highly sialylated human tear-fluid DMBT1 isoforms: the major high-molecular-mass glycoproteins in human tears

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Human open eye tear fluid was separated by low-percentage SDS/PAGE to detect high-molecular-mass protein components. Two bands were found with apparent molecular masses of 330 and 270 kDa respectively. By peptide-mass fingerprinting after tryptic digestion, the proteins were found to be isoforms of the DMBT1 gene product, with over 30% of the predicted protein covered by the tryptic peptides. By using gradient SDS/agarose/polyacrylamide composite gel electrophoresis and staining for glycosylation, it was shown that the two isoforms were the major high-molecular-mass glycoproteins of > 200 kDa in human tear fluid. Western blotting showed that the proteins expressed sialyl-Le^a. After the release of oligosaccharides by reductive β-elimination from protein blotted on to PVDF membrane, it was revealed by liquid chromatography-MS that the O-linked oligosaccharides were comprised mainly of highly sialylated oligosaccharides with up to 16 monosaccharide units. A majority of the oligosaccharides could be described by the formula $dHex_{0\rightarrow 2}NeuAc_{1\rightarrow x}Hex_xHexNAc_x$(-ol), $x = 1$–6, where Hex stands for hexose, dHex for deoxyhexose, HexNAc for *N*-acetylhexosamine and NeuAc for *N*-acetylneuraminate. The number of sialic acids in the formula is less than 5. Interpretation of collision-induced fragmentation tandem MS confirmed the presence of sialic acid and suggested the presence of previously undescribed structures carrying the sialyl-Le^a epitopes. Small amounts of neutral and sulphated species were also present. This is the first time that O-linked oligosaccharides have been detected and described from protein variant of the DMBT1 gene.

Key words: eye, glycosylation, mucin, O-linked oligosaccharide, sialyl-Le^a, tear film.

INTRODUCTION

High-molecular-mass glycoproteins in tear fluid is an area of research that has generated some interest, since it has been shown that mucus is a major component of the tear film [1]. A major protein component of mucus is the mucin glycoproteins, which are highly glycosylated (up to 80% of the mass), have high molecular mass and the molecular mass of cloned apomucins is usually above 100 kDa. Common for the protein backbone of mucins is the high amount of serine and threonine. These amino acids serve as scaffolds for mucin-type glycosylation, where oligosaccharides are linked via GalNAcα1-. The secretion of mucins into tear fluid is mainly via goblet cells, which are located in the conjunctiva, but shedding of membrane-bound mucins from the underlying epithelial cells could also contribute to the mucin component of tear fluid [2,3]. The secretion from these cells, together with the lacrimal-gland aqueous components and the meibomian-gland lipid secretion, constitute the tear film of the human eye, which also contains smaller proteins, glycoproteins and salt.

Mucins are considered to be responsible for properties such as viscosity and lubrication. By interaction with various microbes, possibly via the densely glycosylated surface of the mucins, they also have a role in the protection of tear fluid against pathogens [4]. All these properties are desirable in tear fluid, as the conjunctiva and cornea need to be protected from drying out, and the eyes are under a constant threat from exposure to airborne pathogens and noxious agents.

Many attempts have been made to identify the mucins expressed in tear fluid. The secreted MUC2, MUC4 and MUC5AC gene products have been identified by antibody reactivity in the human tear fluid [5–7], and all these mucins have also been shown to be expressed by Northern blotting or competitive PCR in conjunctiva or in the corneal epithelium [6–8]. A presumed membrane-bound MUC1 gene product has been identified in the conjunctival epithelium [7,9], but it is unclear whether a secreted version is present in tear fluid [7].

Information on the glycosylation of tear glycoproteins is scarce. The epithelial and goblet cells of conjunctiva express antigens of various blood groups from the *ABO*, *H*, *secretor* and the *Lewis* systems and also the sialyl-Le^a epitope, as detected by immunohistochemistry [10]. This is in contrast with the simple oligosaccharides found on conjunctival mucin components, mainly the Tn (GalNAcα1-Ser/Thr) and the sialyl-Tn (NeuAcα2-6GalNAcα1-Ser/Thr) antigens [11]. Recently, a major sialoglycoprotein of 450–500 kDa was found in the reflex tear fluid [12,13]. This protein reacted strongly with an anti-sialyl-Le^a antibody, and it appeared to be predominantly of an epithelial origin, since it could be pelleted together with epithelial cells from the collected tear fluid.

In the present study, two isoforms of a high-molecular-mass glycoprotein from human open eye tears were separated and isolated by SDS/PAGE and SDS/agarose/polyacrylamide composite gel electrophoresis (SDS/AgPAGE), and they were stained with Coomassie Blue (for protein content) or periodate–Schiff reagent (PAS; for oligosaccharide content). Peptide-mass fingerprinting identified them as the gene product variants of the human DMBT1 gene, which we believe is the 450–500 kDa sialoglycoprotein already detected in tear fluid [12,13]. The glycosylation of both isoforms was characterized by liquid

Abbreviations used: AgPAGE, agarose/polyacrylamide composite gel electrophoresis; LC-MS, liquid chromatography-MS; LC-MS/MS, LC-tandem MS; MALDI, matrix-assisted laser-desorption ionization; MS/MS, tandem MS; PAS, periodate–Schiff; SRCR, scavenger receptor cysteine-rich.

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chromatography (LC)-MS and LC-tandem MS (MS/MS) after the release of O-linked oligosaccharides by reductive β-elimination, and a high degree of sialylation was confirmed. Oligosaccharides with up to 16 monosaccharide units and up to 4 sialic acids were found. Fucosylated oligosaccharides with one or two sialic acids gave sequence information from MS/MS indicative of previously unknown small oligosaccharide structures carrying the sialyl-Le^a epitope

EXPERIMENTAL

Separation of human tear fluid by gel electrophoresis

Open-eye tear fluid was collected in a capillary and stored at −20 °C until enough material was collected (20–200 μl). For SDS/PAGE and SDS/AgPAGE, tear fluid was reduced by adding an equal amount of a two times concentrated sample loading buffer [20 % glycerol; 0.75 M Tris/HCl, pH 8.8 (SDS/PAGE) or pH 8.1 (SDS/AgPAGE); 2 % SDS; 0.01 % Bromophenol Blue and 10 mM tri-n-butylphosphine] and kept at 95 °C for 30 min. The sample was then alkylated with iodoacetamide (12.5 mM) and kept at 20–25 °C for 1 h before loading an equivalent of 20 μl of tear fluid into each lane. The low-percentage polyacrylamide (3–8 %) pre-made gels were run according to the manufacturer's instructions (Novex, San Diego, CA, U.S.A.). SDS/AgPAGE gels were made by boiling two solutions with 0.5 % agarose and 0.375 M Tris/HCl (pH 8.1), one also containing 6 % T (percentage of acrylamide and piperazine diacrylamide in the gel), 2.5 % C (percentage of piperazine diacrylamide of total amount of acrylamide and piperazine diacrylamide) and 10 % glycerol. The 0–6 % gradient polyacrylamide/0.5 % agarose gradient gels were cast in the mini-Protean gel-casting apparatus (Bio-Rad, Hercules, CA, U.S.A.) at 50 °C after adding *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (0.0125 %) and ammonium peroxide (0.005 %) to each solution. The gels were polymerized for 1 h at 50 °C and agarose was allowed to set at 20–25 °C overnight in a humidified environment. The anode and cathode buffer was 192 mM Tris/borate (pH 7.6) with 1 mM EDTA and 0.1 % SDS. Gels were run at 100 V for 2–3 h until the dye front migrated out of the gels. The apparent molecular mass was calculated using pre-stained high-molecular-mass markers of 218, 126 and 90 kDa (Kaleidoscope; Bio-Rad). Gels were stained using Coomassie Blue [14] or PAS [15]. For release of the oligosaccharides and the amino acid composition, the human tear fluid (100 μl) was reduced and alkylated in the sample loading buffer as described above, and the sample was concentrated to 20 μl using a Microcon centrifugal filter device (Millipore, Bedford, MA, U.S.A.) with a 100 kDa cut-off. The retained material was loaded on to the gel. The sample was blotted on to Immobilon-P^{SQ} (Millipore) essentially by the semi-dry method described by Khyse-Anderson [16] but omitting the methanol in the cathode solution, and then transferred at 4 mA/cm^2 for 1 h. The blot was stained with 0.125 % Alcian Blue in 25 % ethanol/10 % acetic acid for 10 min and destained in 100 % methanol for 20 min.

Immunochemical detection of oligosaccharide epitopes

Tear fluid (equivalent of 20 μl) separated by SDS/AgPAGE was blotted on to Immobilon-P^{SQ} and the membranes were blocked overnight at 4 °C using 1 % BSA and 0.1 % (v/v) Tween 20 in PBS (pH 7.4). The membranes were then incubated for 1 h in the same solution with antibodies raised against sialyl-Le^a (C241, 17 μg/ml) [17], sialyl-Le^x (CSLEX-1, culture supernatant diluted 1/250) from the A.T.C.C. (Manassas, VA, U.S.A.) or biotinylated wheat-germ agglutinin lectin (1 μg/ml; Sigma). After washing the membranes thrice for 5 min with PBS, they were incubated for 2 h with secondary antibody [horseradish peroxidase-labelled sheep anti-mouse (Silenus, Melbourne, Australia) diluted 1/500], or horseradish peroxidase-labelled streptavidin (0.25 μg/ml; Sigma), in the blocking solution. After washing five times with PBS for 5 min, the blots were developed for 30 min in 1 mg/ml chloronaphthol in methanol/water (1:2, v/v) containing 0.0375 % of H_2O_2.

MS identification of proteins by trypsin digestion

The two Coomassie Blue-stained SDS/PAGE bands with apparent molecular masses of 330 and 270 kDa were destained and digested with trypsin [18]. A portion of the extracted peptides (10 %) was desalted using a C18 Zip-Tip (Millipore) and analysed by matrix-assisted laser-desorption ionization (MALDI)–time-of-flight-mass spectrometry (Biflex III, Bruker, Bremen, Germany) in positive reflectron mode after eluting the sample from the Zip-Tip with 50 % acetonitrile, 0.2 % formic acid and 5 mg/ml α-cyano-4-hydroxycinnamic acid. The remaining sample was analysed by LC-MS and LC-MS/MS on a reversed-phase column (PepMap C18, 3 μm, 0.3 mm × 150 mm; LC-Packings, San Francisco, CA, U.S.A.). Flow of solvent through the column at the rate of 5 μl/min was provided by a Surveyor-LC pump (ThermoFinnigan, San Jose, CA, U.S.A.) with flow splitting from 100 μl/min. The gradient was formed from 0.1 % formic acid up to 50 % acetonitrile/0.1 % formic acid for 40 min, followed by a 10-min column wash with 90 % acetonitrile/0.1 % formic acid. MS was performed on an LCQ Deca (ThermoFinnigan). The mass spectrometer was scanned from 400 to 2000 atomic mass units (a.m.u.), and charge state (zoom scan window of 10 a.m.u.) and MS/MS scans were recorded for the most intense ions in each scan. The capillary temperature was 200 °C, the capillary voltage was 3.00 V and the electrospray voltage was 4.50 kV. For MS/MS, the normalized collision energy was 35 % with an activation time of 30 min. Search of the database was performed using Protein Prospector (http://prospector.ucsf.edu), MS-FIT for MALDI data and MS-TAG for LC-MS/MS for matches in the NCBI protein database.

Release of O-linked oligosaccharides

O-linked oligosaccharides, attached to the glycoproteins, separated by SDS/AgPAGE and blotted to the membrane were released by miniaturized reductive β-elimination. Alcian Blue-stained bands corresponding to the 330 and 270 kDa proteins were excised from the membranes, wetted with methanol and incubated at 50 °C for 16 h in 20 μl of 50 mM NaOH and 0.5 M $NaBH_4$. The resulting solutions were neutralized by the addition of 1 μl acetic acid, before being desalted with 25 μl of AG50WX8 cation-exchange resin (Bio-Rad) in a Zip-Tip (Millipore) and dried in a Savant SpeedVac. Borate was removed as its methyl ester by repeated (five times) addition and evaporation of 50 μl of 1 % acetic acid in methanol to the sample. Finally, the samples were resuspended in 10 μl of MilliQ water for LC-MS analysis.

MS identification of the released oligosaccharides

Oligosaccharides were analysed by LC-MS as described above. Separation with LC was performed using a home-made graphitized carbon column (7 μm Hypercarb particles) (Thermo-

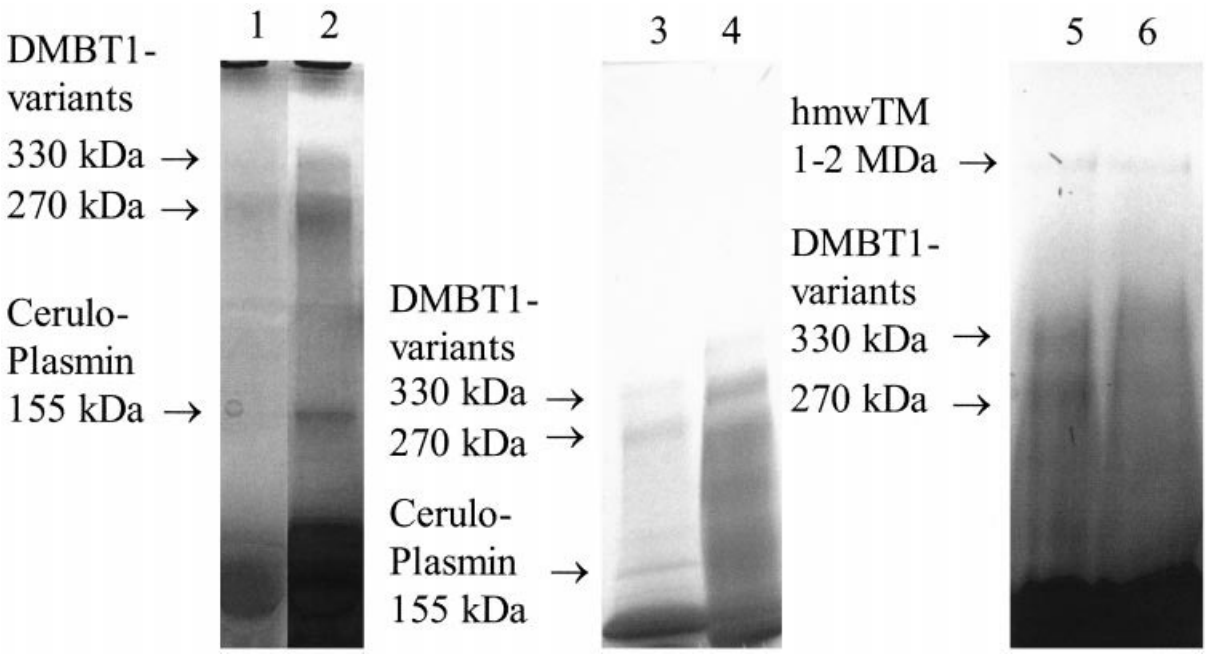

Figure 1 SDS/PAGE and SDS/AgPAGE of human tear fluid

Human tear fluid (20 μl) was reduced and alkylated, and loaded on to 3–8% polyacrylamide gels and stained with PAS (**1**) or Coomassie Blue (**2**), Coomassie Blue stained SDS/AgPAGE gels of reduced and alkylated (**3**) and non-reduced (**4**) human tear fluid (20 μl). PAS-stained SDS/AgPAGE gels of reduced and alkylated (**5**) and non-reduced (**6**) human tear fluid (20 μl). Arrows represent the localization of the human tear-fluid DMBT1 variants and indicate the position of high-molecular-mass tear mucin components.

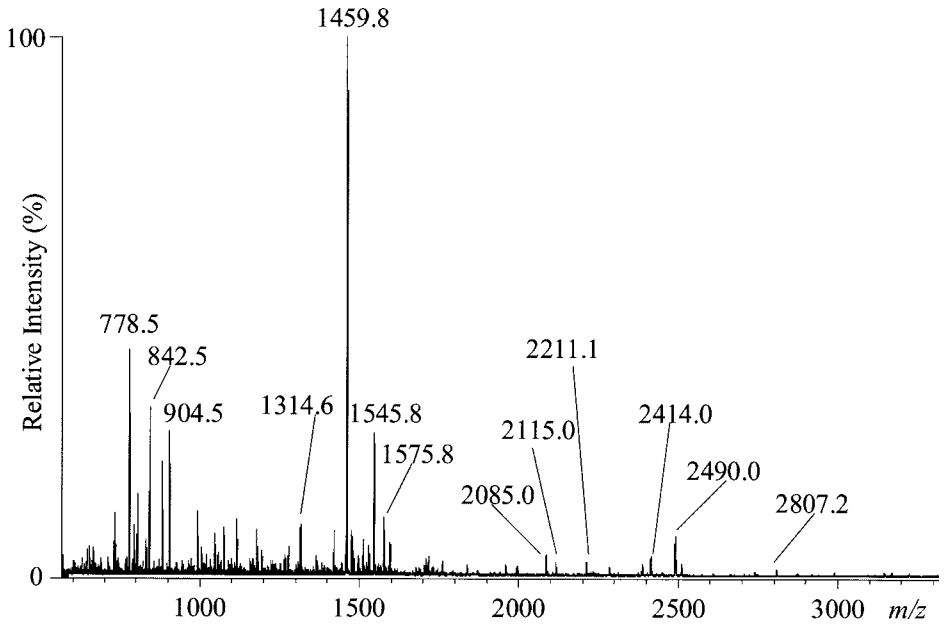

Figure 2 MALDI-MS of tryptic peptides recovered from the 270 kDa protein

Peptide-mass fingerprinting of the lower SDS/PAGE band stained with Coomassie Blue in Figure 1(**2**) and analysis by MALDI-MS. Identified tryptic peptide masses are shown in Table 1.

Hypersil, Runcorn, U.K.) in a 250 μm internal diameter column, and eluted with a water–acetonitrile gradient (0–40 % acetonitrile for 30 min, followed by a 3 min wash with 90 % acetonitrile) containing 10 mM NH_4HCO_3. MS was performed in negative-ion mode, with three scan events: full scan with mass range 320–2000 m/z, dependent zoom scan and dependent MS/MS scan after collision-induced fragmentation. For MS/MS, the normalized collision energy was 40 % with an activation time of 30 min. Dynamic exclusion of ions for zoom scan for 30 s was introduced after three selections within the previous 30 s.

Table 1 Peptide coverage by the tryptic peptides of the high-molecular-mass glycoproteins in human tear-fluid matched against the sequence of DMBT1

The tryptic digests of glycoproteins with apparent molecular masses of 270 and 330 kDa respectively matched against the NCBI-protein database entry 5912464. n.d., not detected.

			330 kDa isoform	270 kDa isoform
Percent coverage			31%	37%
Peptide sequence	Start–end	$[M+H]^+_{Calc}$	Δm (Da)	Δm (Da)
LVNGDGR	104–110, 236–242, 365–371, 496–502, 604–610, 864–870, 995–1001, 1124–1130, 1253–1259, 1382–1388, 1511–1517, 1642–1648	730.4	−0.02	0.02
VTVIFR	2045–2050	734.5	0.01	0.00
SSPLIAR	2072–2078	743.4	0.02	−0.03
IHVSCR	2213–2218	771.4	0.02	0.02
VEVLYR*	247–252, 376–381, 507–512, 615–620, 746–751, 875–880, 1135–1140, 1264–1269, 1393–1398, 1653–1658	778.5	0.02	0.01
ICNDTR	1840–1845	778.5	0.11	0.11
VEILYR	115–120	792.5	0.00	0.00
MTIHFR	1855–1860	804.4	−0.01	−0.01
FPSVYLR	2352–2358	881.5	0.02	0.02
AFHFLNR	2345–2351	904.5	0.03	0.03
INLGFSNLK	1804–1812	1005.6	0.04	0.04
FISDHSITR	2098–2106	1075.6	0.06	0.06
QIFTSSYNR	1846–1854	1115.7	0.06	0.11
ASTAGSESTLALR	1498–1510	1263.7	0.01	0.06
GWFSHNCNHR	1969–1978	1314.6	0.20	0.06
GSFTSSSNFMSIR	2085–2097	1420.7	0.06	0.06
FGQGSGPIVLDDVR*	418–413, 549–562, 657–670, 788–801, 917–930, 1177–1190, 1306–1319, 1435–1448	1459.8	0.06	0.06
CVWEIEVNSGYR	1792–1803	1511.7	0.07	0.01
QLGCGWATSAPGNAR	403–417, 773–787, 902–916, 1291–1305, 1420–1434	1545.8	0.04	0.04
QLGCGWAMSAPGNAR	642–656, 1033–1047, 1162–1176, 1680–1694	1575.8	0.04	0.04
CVWDIEVQNNYR	2033–2044	1595.8	0.03	0.03
GSWGAVCDDSWDTNDANVVCR	121–141	2384.0	−0.01	0.04
GSWGTVCDDSWDTSDANVVCR	382–402, 881–901	2387.0	−0.01	−0.01
GSWGTVCDDSWDTNDANVVCR	513–533, 621–641, 752–772, 1659–1679	2414.0	−0.02	0.03
GSWGTVCDDYWDTNDANVVCR	253–273, 1270–1290, 1399–1419	2490.0	0.00	0.00
DVQLEGGCNYDYIEVFDGPYR	2051–2071	2509.1	0.01	0.01
SDISFQNTGFLAWYNSFPSDATLR	1861–1884	2737.3	n.d.	0.02
QLGCGWAMSAPGNAQFGQGSGPIVLDDVR	274–302	2988.4	n.d.	−0.05
QLGCGWAMSAPGNAQFGQGSGPIVLDDVR	1549–1577	2988.4	n.d.	−0.05
VEVLYQGSWGTVCDDSWDTNDANVVCR	1006–1032	3145.2	n.d.	−0.17
VEVLYQGSWGTVCDDYWDTNDANVVCR	1522–1548	3221.5	n.d.	0.11

* Sequence confirmed by LC-MS/MS.

RESULTS

Separation of high-molecular-mass glycoproteins in human tear fluid by SDS/PAGE

Tear fluid from one individual (Blood group O, Lewis^{a-b+}) was reduced and alkylated before being applied to low-percentage SDS/PAGE (3–8%). Two bands were found in the high-molecular-mass region. Both bands stained with Coomassie Blue (protein content) and PAS (oligosaccharide content) (Figure 1). Their apparent molecular masses were estimated to be 330 and 270 kDa respectively, although these sizes were uncertain, given that they lie outside the range of the molecular-mass markers used.

Using gradient Ag/PAGE, it was found that these two bands were the major high-molecular-mass protein components of tear fluid. No other higher-molecular-mass proteins were detected by Coomassie Blue under either reducing or non-reducing conditions (Figure 1), but it looked like the two bands of 330 and 270 kDa found on SDS/PAGE were migrating somewhat slower in non-reducing condition. Additonal weak bands with high molecular masses (> 1 MDa) were also seen by staining with PAS (Figure 1). The high-molecular-mass tear mucin components, staining with PAS but not with Coomassie Blue, are characteristic of high-molecular-mass, highly glycosylated mucin-type molecules, and their positioning in the gel corresponds to the size of other mucin-type molecules separated on such gels (rat Muc2 and human MUC5B; results not shown). The data from the SDS/AgPAGE indicate that the two bands found by low-percentage SDS/PAGE are the major components of the high-molecular-mass proteins in human tear fluid with respect to both reducible and non-reducible complexes.

Identification of the two major high-molecular-mass glycoproteins

Trypsin digestion and peptide-mass fingerprinting of the 330 and 270 kDa bands gave almost identical MALDI-MS data. The mass spectrum of the 270 kDa band is shown in Figure 2. The 330 kDa band gave a somewhat low-intensity mass spectrum. The similarity of the two spectra indicated that they were isoforms of the same protein. When peptide-mass data was matched with predicted tryptic peptides of proteins in the NCBI protein database,

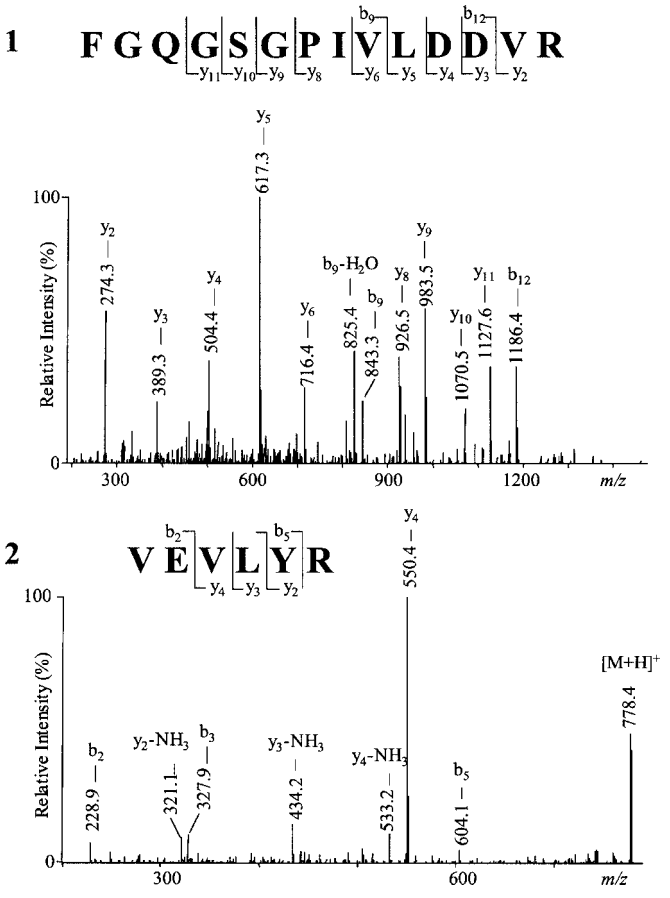

Figure 3 Collision-induced fragmentation of two peptides from LC-MS/MS of tryptic peptides from the 270 kDa isoform of DMBT1

Fragmentation of the peptide $[M+H]^+$ ion at m/z 1459.8 (observed as doubly charged at $m/z = 730.5$ in LC-MS) eluted at 24.79 min (**1**). Fragmentation of the peptide $[M+H]^+$ ion at $m/z = 778.5$ (observed as singly charged at $m/z = 778.5$) eluted at 16.62 min (**2**).

both matched the DMBT1 gene product (Table 1). The coverage of the protein was somewhat lower for the 330 kDa isoform (31 % compared with 37 %). This is probably because of the lower abundance of the 330 kDa isoform. Some low-intensity high-mass ions detected in the 270 kDa isoform could not be detected in the 330 kDa isoform (Table 1).

The identification of the DMBT1 gene products was substantiated by LC-MS/MS of the tryptic digest, where two peptides (Figure 3) were identified and matched to the repeat sequences within the gene product. Again, the 330 kDa isoform gave weaker but still interpretable MS/MS data. The 155 kDa protein (Figure 1), not staining with PAS but with Coomassie Blue, was also identified with the same approach to be human ceruloplasmin (results not shown).

Amino acid analyses of the 330 and 270 kDa proteins also supported the conclusion that the proteins were isoforms of the DMBT1 gene product (Table 2). A previously reported sialoglycoprotein from the reflex tear fluid [12] also had an amino acid composition similar to the two proteins described in the present study and the described sequence of the glycoprotein gp-340 variant of the DMBT1 gene product. This protein was previously described as a MUC1-like protein present in human tear fluid, but the data presented here suggest that it is actually a DMBT1 gene product variant.

Table 2 Amino acid composition of the human tear-fluid DMBT1 variant

Composition compared with predicted composition of the DMBT1 gene and reported composition of a high-molecular-mass glycoprotein isolated from human tear fuid.

Amino acid	DMBT1*	330 kDa isoform	270 kDa isoform	Tear sialoglycoprotein†
Asx	12.3	11.2	12.0	10.8–13.0
Glx	7.3	11.6	9.8	8.8–11.9
Ser	12.8	11.8	11.7	13.3–17.0
Gly	11.3	14.4	12.6	12.1–20.1
His	3.3	0.0	1.9	2.9–3.0
Arg	6.3	6.9	6.7	4.2–5.5
Thr	6.0	3.0	3.9	6.1–6.7
Ala	6.4	7.3	7.6	6.7–7.7
Pro	5.6	6.7	6.7	2.3–5.8
Tyr	3.3	2.3	2.3	2.2–2.7
Val	7.5	7.0	7.5	4.2–6.3
Met	0.7	0.0	0.6	0–0.6
Ile	3.2	3.5	3.4	2.1–2.7
Leu	7.4	8.5	8.4	5.1–8.3
Phe	2.2	3.2	2.9	2.1–2.7
Lys	0.4	2.6	1.9	1.3–2.9

* NCBI entry 5912464.
† From ref. [12].

Identification of carbohydrate epitopes of the human tear-fluid variant of the DMBT1 protein

Previous reports have shown that the high-molecular-mass sialoglycoprotein in human tear fluid expresses the sialyl-Le^a epitope [10,12] and interacts with the wheat germ agglutinin (WGA)–lectin (sialic acid and GlcNAc recognition) [5]. Western and lectin blotting showed that the human tear-fluid variants of the DMBT1 protein found in this report also express these epitopes (Figure 4). Other proteins, with both lower and higher molecular masses than the DMBT1 variants, also express these epitopes, indicating that the glycosylation of the DMBT1 variants could be generalized to some extent to many glycoproteins in the tear fluid. Interestingly, a DMBT1 variant (gp-340) found in human parotid saliva expresses the sialyl-Le^x epitope instead of sialyl Le^a [19]. However, sialyl-Le^x has not been found to be expressed in human tear fluid [12], and no sialyl-Le^x activity was found by Western blotting of the high-molecular-mass human tear-fluid proteins in the present study. This indicates that the human tear-fluid variants of the DMBT1 protein are differently glycosylated than the DMBT1 gene product found in saliva.

Identification of oligosaccharides in the human tear-fluid variant of the DMBT1 protein

To gain more information about the glycosylation of the DMBT1 isoforms, O-linked oligosaccharides were released from the 330 and 270 kDa DMBT1 variants, then blotted on to the PVDF membrane and stained by Alcian Blue. Excised bands were subjected to alkaline β-elimination in the presence of sodium borohydride. The samples were then analysed by negative-ion LC-MS and LC-MS/MS, using a capillary column of graphitized carbon and ion trap MS (Figures 5 and 6). The two isoforms of the human tear-fluid DMBT1 variant gave similar oligosaccharide profiles with only minor differences in their relative intensity (Table 3 and Figure 6). Oligosaccharides were identified as either their $[M-H]^-$ ions or their $[M-2H]^{2-}$ ions. In addition, some high-molecular-mass oligosaccharides with more than three sialic acids gave $[M-3H]^{3-}$ ions.

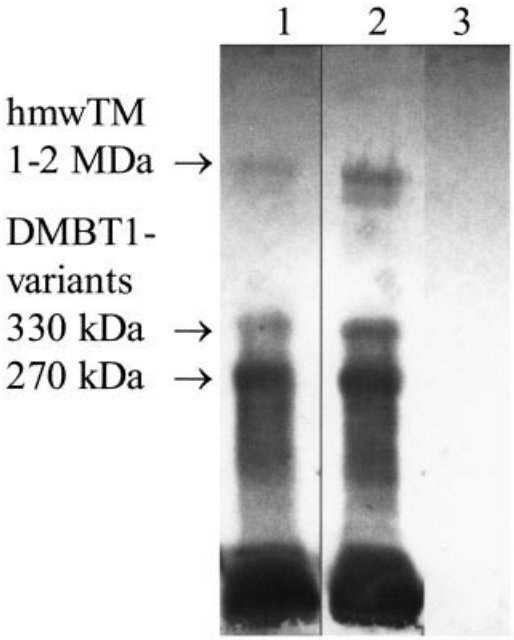

Figure 4 Identification of sialylated oligosaccharide epitopes on human tear-fluid DMBT1 variant protein

Human tear fluid (20 μl) was separated by SDS/AgPAGE and blotted on to Immobilon-P^{SQ} membrane. Oligosaccharide epitopes were reacted with anti-sialyl-Le^a antibody (**1**), WGA–lectin-binding epitope (sialic acid and GlcNAc) (**2**) and anti-sialyl-Le^x antibody (**3**). Arrows indicate the localization of high-molecular-mass sialyl-Le^a-containing glycoproteins.

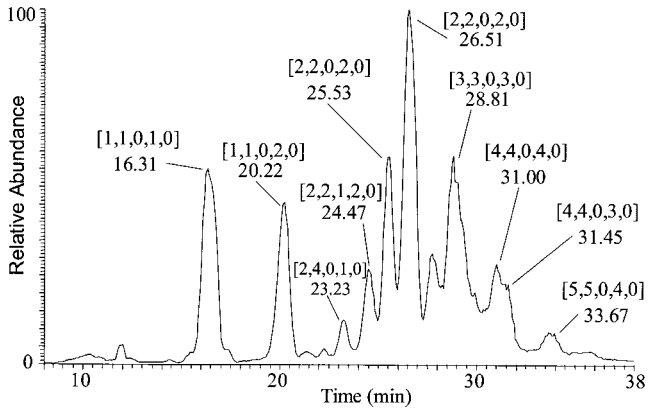

Figure 5 Base peak chromatogram from LC-MS of O-linked oligosaccharides released from the 270 kDa isoform of the human tear-fluid DMBT1 variant

Oligosaccharides were eluted from a graphitized carbon column and detected by negative electrospray ionization MS. Numbers in brackets refer to the monosaccharide composition (Hex, HexNAc, dHex, NeuAc, sulphate) of the major component in each peak. GalNAcol is included as a HexNAc unit in the composition.

Sialylated oligosaccharides on the human tear-fluid DMBT1 variant

The data show that the majority of the released oligosaccharides were sialylated (Table 3). The detected oligosaccharides ranged from monosialylated trisaccharides up to a maximum of four sialic acid residues on to oligosaccharides with up to 16 monosaccharide units. A generalized formula covering the composition of most of the sialylated oligosaccharides can be written as

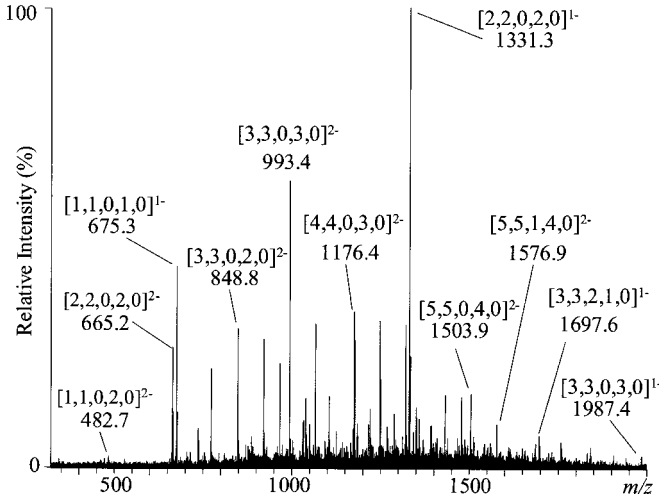

Figure 6 Combined mass spectrum of detected O-linked oligosaccharides from the 270 kDa isoform of the human tear-fluid DMBT1 variant

Oligosaccharides detected from 15 to 34 min by LC-MS. Some major $[M-xH]^-$ pseudo-molecular ions are labelled with charge state and monosaccharide composition (Hex, HexNAc, dHex, NeuAc, sulphate). Relative percentage of predicted compositions are found in Table 3. GalNAcol is included as a HexNAc unit in the composition.

$dHex_{0\rightarrow2}NeuAc_{1\rightarrow x}Hex_xHexNAc_x$, $1 < x < 7$, where Hex stands for hexose, HexNAc for *N*-acetylhexosamine and NeuAc for *N*-acetylneuraminate. The highest degree of sialylation in any one molecule was 4. The HexNAc in the formula also includes the GalNAc, converted into a GalNAcol during the release of the oligosaccharide. One could predict from the data that the oligosaccharides consist of *N*-acetyl-lactosamine units with up to one sialic acid per unit, but sparingly fucosylated. The presence of terminating fucose as an epitope in a sialylated *N*-acetyl-lactosamine chain could account for the sialyl-Le^a epitopes [NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-] as found by Western blotting. Searching GlycoSuiteDB (www.glycosuite.com) (a database containing previously reported oligosaccharides) for the compositions of the detected oligosaccharides from DMBT1 shows that many may be previously unreported oligosaccharide structures (Table 3).

Further structural information on the oligosaccharides was obtained from collision-induced dissociation MS. An example of the MS/MS fragmentation is shown by the $[M-H]^-$ ion at m/z 1477.5, corresponding to a composition of $NeuAc_2dHex_1Hex_2HexNAc_2$(-ol) eluted at 24.47 min (Figure 7). The sialic acid linkages appear to be quite vulnerable towards fragmentation. This could be used in many cases to confirm the amount of sialic acid present in a structure, due to the strong fragment ions from the loss of one or more sialic acids (-291 a.m.u.). Since most of the negative charge would be expected to be held by the sialic-acid residues, fragmentation would only give limited amount of information after the sialic acids have been lost. This was particularly true for the high-molecular-mass, highly sialylated species, where sequencing beyond the loss of sialic acid was quite difficult. However, fragmentation of smaller and less-sialylated species gave additional information. The trisaccharide $NeuAc_1Hex_1HexNAc_1$(-ol) and the tetrasaccharide $NeuAc_2Hex_1HexNAc_1$(-ol) correspond to the sialyl-T (NeuAcα2-3Galβ1-3GalNAcα1-Ser/Thr) and di-sialyl-T [NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAcα1-Ser/Thr] epitopes respectively, commonly found as mucin-type O-linked

Table 3 Identification by LC-MS of O-linked oligosaccharides found on human tear-fluid DMBT1 variant isoforms

Composition of oligosaccharides of the two isoforms of DMBT1 variants and their relative intensity from the MS data. x = charge, Sulph = sulphate.

		Composition (unreduced)*					330 kDa isoform			270 kDa isoform		
$[M-xH]^{x-}_{Calc}$	x	Hex	HexNAc	dHex	Sulph	GlycoSuiteDB†	Δm (Da)	Int (%)‡	Isomers§	Δm (Da)	Int (%)‡	Isomers§
Neutral												
530.22	1	1	1	1		Yes	0.28	1.8	1	0.28	3.6	1
749.29	1	2	2			Yes	0.21	0.9	1	−0.09	6.6	1
1406.54	1	3	3	2		Yes	−0.14	2.2	2	−0.14	3.2	2
Monosialylated												
675.25	1	1	1			Yes	0.05	73.3	1	0.05	33.9	1
755.21	1	1	1		1	Yes	0.19	2.1	2	0.11	2.4	2
878.33	1	1	2			Yes	−0.03	3.9	1	−0.03	3.4	1
1040.39	1	2	2			Yes	−0.09	16.6	3	0.01	17.7	3
1186.44	1	2	2	1		Yes	0.06	11.2	3	−0.04	11.3	3
1332.50	1	2	2	2		Yes	0	7.4	2	−0.00	6.1	2
1697.63	1	3	3	2		Yes	−0.13	5.4	2	−0.03	7.1	2
848.32	2						−0.03	20.4		−0.03	17.5	
1843.69	1	3	3	3		Yes	−0.09	0.6	1	−0.09	0.8	1
921.35	2						−0.09	3.8		−0.09	2.6	
Disialylated												
966.35	1	1	1			Yes	−0.15	20.6	1	−0.15	28.9	1
482.67	2						0.05	1.6		0.05	2.6	
1128.40	1	2	1			No	0	1.9	1	−0.20	3.8	1
1331.48	1	2	2			Yes	−0.18	100.0	2	−0.18	100.0	2
665.24	2						0.12	24.6		0.12	27.7	
1477.54	1	2	2	1		Yes	−0.24	16.3	2	−0.24	19.2	2
738.27	2						0.06	9.1		0.06	11.0	
1696.61	1	3	3			Yes	−0.11	4.5	1	−0.11	2.5	1
847.81	2						−0.01	14.5		−0.01	19.2	
1842.67	1	3	3	1		Yes	−0.17	1.6	2	−0.17	0.8	1
920.84	2						−0.07	13.6		−0.07	13.1	
1030.37	2	4	4			Yes	0.05	3.5	1	0.05	2.5	1
1103.40	2	4	4	1		No	−0.00	4.0	1	−0.00	3.8	1
1176.43	2	4	4	2		No	−0.06	8.4	1	−0.06	4.1	1
Trisialylated												
1987.71	1	3	3			No	−0.31	2.1	1	−0.31	3.1	1
993.35	2						−0.11	48.8		−0.11	54.1	
1033.33	2	3	3		1	No	−0.27	6.9	1	−0.27	7.7	1
1066.38	2	3	3	1		No	−0.17	24.9	1	−0.17	23.0	1
1094.89	2	3	4			Yes	−0.39	3.5	1	−0.39	3.9	1
1175.92	2	4	4			No	−0.04	27.4	2	−0.04	16.4	1
1215.90	2	4	4		1	No	−0.2	2.1	1	−0.20	3.3	1
1248.95	2	4	4	1		No	−0.10	15.8	1	−0.10	18.0	1
1321.98	2	4	4	2		No	−0.36	3.2	1	−0.36	4.3	1
1358.49	2	5	5			No	−0.37	4.1	1	−0.17	2.9	1
1431.52	2	5	5	1		No	−0.23	3.9	1	−0.23	3.8	1
Tetrasialylated												
1321.47	2	4	4			No	0.06	26.1	1	0.06	20.0	1
1394.50	2	4	4	1		No	−0.39	7.0	1	−0.19	9.0	1
1504.03	2	5	5			No	−0.27	10.6	1	−0.27	11.5	2
1002.36	3						−0.47	3.0		−0.47	2.8	
1577.06	2	5	5	1		No	−0.33	3.9	1	−0.33	4.9	1
1051.04	3						−0.43	3.5		−0.43	8.2	
1686.60	2	6	6			No	−0.00	2.5	1	0.00	1.6	1
1124.07	3						−0.20	1.3				

* GalNAcol is included as a HexNAc unit in the composition.
† Presence of composition among human entries in GlycoSuiteDB.
‡ Intensity standardized against the major $[M-H]^-$ ion corresponding to the composition $Hex_2HexNAc_2NeuAc_2$.
§ Based on the number of peaks found by LC-MS.

oligosaccharides. Confirmation of the identity of these structures was supported by findings that the elution time and MS/MS fragmentation were consistent with the identical released structures isolated from other sources (results not shown). For other smaller sialylated compositions, matching human entries in the GlycoSuiteDB generally contain the type 2 chains (Galβ1-4GlcNAcβ1-). However, since sialyl-Le^a [NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-] and not sialyl-Le^x [NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-] is expressed in human tear fluid (the present study and [12]), type 1 chains (Galβ1-3GlcNAcβ-) are most probably the dominant ones among DMBT1 tear-fluid oligosaccharides. The only human type 1 chain that matched fragmentation data was the NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-3GalNAcol, previously found only in the colonic cancer cell line, Cl.16E [20]. The summary of data (Table 4) from the sialylated oligosaccharides indicates a possible difference in the glycosylation of eye proteins compared with that reported from any other tissue.

Table 4 Structural elements of O-linked oligosaccharides from human tear fluid

Based on compositional data, MS/MS fragmentation and antibody reactivity

Structural element	Structure detected
Terminating complete structures	
Sialyl-T	NeuAcα2-3Galβ1-3GalNAcol
Disialyl-T	NeuAcα2 \ 6 NeuAcα2-3Galβ1-3GalNAcol
Terminating or repeated units	
Sialylated *N*-acetyl-lactosamine	$NeuAc_{0\rightarrow1}$ \ $(-Gal-3/4GlcNAc-)_{0\rightarrow5}$
Terminating units	
Sialyl Le[a]	Fucα1 \ 4 NeuAcα2-3Galβ1-3GlcNAc-

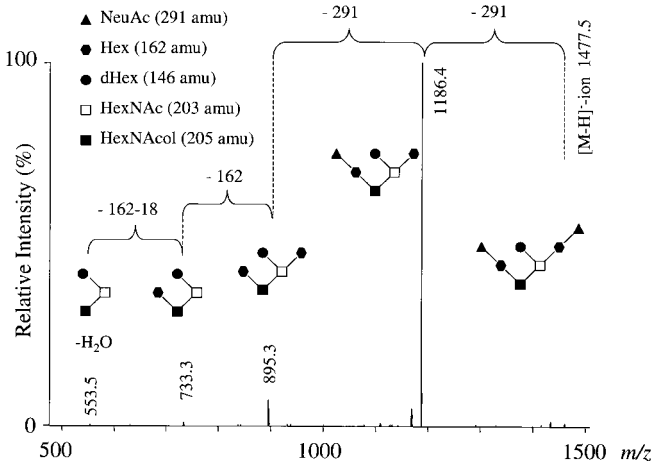

Figure 7 MS/MS of an O-linked disialylated oligosaccharide component with the $[M-H]^-$ ion of 1477.5

The component with composition $NeuAc_2dHex_1Hex_2HexNAc_2$(-ol) eluting at 24.47 min in Figure 5 together with proposed fragmentation.

Neutral oligosaccharides on the human tear-fluid DMBT1 variant

Since LC–MS was performed at high pH, neutral oligosaccharides were also detected as $[M-xH]^{x-}$ ions by negative electrospray ionization MS. Their compositions could be summarized as $dHex_{0\rightarrow2}Hex_xHexNAc_x$, where $1 < x < 3$ (HexNAc also includes GalNAcol). This formula is similar to the formula described for the sialylated species, indicating that structural features found in the sialylated oligosaccharides are also found among the neutral oligosaccharides. MS/MS spectra of these ions gave weak Z-type fragment ions (fragmentation according to Domon and Costello [20a]) confirming their assignment as oligosaccharides (results not shown). However, the quality of the spectra did not allow any further predictions of linkages and sequences.

Sulphated oligosaccharides on human tear-fluid DMBT1 variant

It has been indicated that conjunctival mucins contain sulphated oligosaccharides [5]. Since some of the human tear-fluid variants of the DMBT1 protein probably have an epithelial origin from

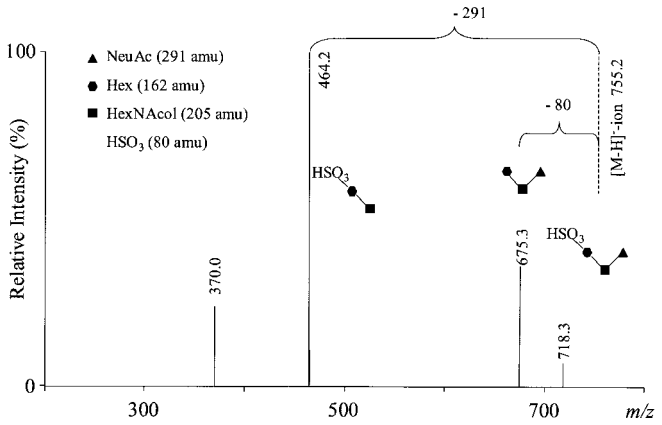

Figure 8 MS/MS of a sulphated O-linked oligosaccharide component with the $[M-H]^-$ ion of 755.5

A component with the composition $HSO_3NeuAc_1Hex_1HexNAc_1$(-ol) eluted at 19.52 min in Figure 5 together with proposed fragmentation.

cells shed from the conjunctiva (see above and [12]), it is likely that some of the sulphated conjunctival glycoproteins could be found in tear fluid. Low-intensity pseudomolecular ions from sulphated species were detected in the DMBT1 oligosaccharides (Table 3) with one or two sulphates per oligosaccharide. Collision-induced dissociation confirmed that the parent ions were oligosaccharides, due to the presence of Y- and Z-sequence ions. A minor component with an $[M-H]^-$ ion at $m/z = 755.21$ (eluted at 19.52 min), corresponding to a composition of $HSO_4NeuAc_1Hex_1HexNAc_1$(-ol), showed fragmentation corresponding to the loss of both sulphate (-80 a.m.u.) and sialic acid (-291 a.m.u.) (Figure 8), indicating that the ionization process in the mass spectrometer distributed the charge to either the sialic acid or the sulphate. Two human oligosaccharide structures were found in GlycoSuiteDB that matched with this composition, both of which have been found on human respiratory mucins from a patient suffering from cystic fibrosis [21]. The isomers could be described as HSO_4-4/6Galβ1-3(NeuAcα2-6)GalNAcol. The composition and structural data from the sialylated oligosaccharides described above indicate that some oligosaccharides contained a sialic acid-linked α2-6 to the GalNAcol. Further structural characterization of the sulphated oligosaccharides in human tear fluid has to be performed to establish the nature of the sulphate linkage. A characteristic of the sulphated oligosaccharides found is that they are all unfucosylated and have low intensity.

DISCUSSION

The DMBT1 gene product belongs to a family of proteins that contain the scavenger receptor cysteine-rich (SRCR) domains. These molecules have been proposed to be involved in the innate immune response [22]. Recently [19], the gp-340 variant of DMBT1 was shown to be identical with the salivary agglutinin that strongly binds *Streptococcus mutans* and *Helicobacter pylori*, probably from the interaction of these bacteria with the oligosaccharide part of the protein.

Identification and characterization of two major high-molecular-mass glycoprotein isoforms in human tear fluid as DMBT1 variants emphasizes that the tear surface is different

from other mucosal surfaces. Although MUC1, MUC2, MUC4 and MUC5AC mucins have been detected by antibodies in human tear fluid, the mucin component is shown to be only a minor component compared with the human tear-fluid DMBT1 variant, as judged by the staining intensity on blots and gels from SDS/AgPAGE (Figures 1 and 4). The reason why there are two human tear-fluid variants of DMBT1 is still unclear. However, the DMBT1 gene products from lungs also separate as two isoforms by SDS/PAGE [23], whereas the salivary gene product is a single migrating band [19]. The difference in the O-linked glycosylation profiles of the two tear-fluid isoforms is probably not sufficient to explain the difference in migration, since the only obvious difference is an increase in the NeuAcα2-3Galβ1–3GalNAcol content (Tables 3 and 4). There is also the possibility that N-linked glycosylation may account for the difference in migration. However, two splice variants have been found for the human DMBT1 gene [24,25], one encoding ten SRCR domains (total product 6 kbp) and another encoding 14 SRCR domains (8 kbp). The difference of four SRCR domains (11.7 kDa each), together with the SRCR interspersed domains (2.1 kDa each), agrees completely with the difference in size (60 kDa) between the two human tear-fluid isoforms detected by SDS/PAGE. Thus the presence of two splice variants is a likely explanation. The difference in the amino acid composition between these types of isoforms is small that it would not be detected by analysis of the amino acid composition (Table 2).

A small serine-/threonine-rich region in the C-terminus of the DMBT1 protein could be one major site of O-linked glycosylation, similar to mucins, where glycosylation is located in serine-/threonine-/proline-rich 'mucin domains'. These glycosylated tandem repeat sequences in mucins render these molecules resistant to various proteases. With DMBT1, the repeated region (SRCR domains) is less rich in serines and threonines. The data from the tryptic map in the present study illustrate that at least some of the serines and threonines in the SRCR domains are not fully glycosylated, since they are detected as unmodified peptides. Despite the indication that the human tear-fluid DMBT1 variants are less glycosylated than mucins, and as such will be more accessible to proteases, they could still be protected from degradation by high levels of protease inhibitors, such as tear lipocalin present in tear fluid [26].

The DMBT1/gp-340 gene has been found using reverse transcriptase–PCR to be highly expressed in mucosal tissues as much as in trachea, salivary gland, small intestine, lungs and stomach [24]. All these are tissues that also express high levels of mucins. It may therefore be possible that the DMBT1 gene products are significant contributors to the protective role of the mucous layer that is traditionally assigned to the mucins. Interestingly, another member of the SRCR family is the bovine gall-bladder mucin, where regions with high serine and threonine content are interspersed with the SRCR domain [27].

Tear fluid is a continuously regenerated, initial protective barrier, which is used for cleaning the underlying epithelia and providing clear eyesight. The finding that the O-linked oligosaccharides on the major high-molecular-mass glycoproteins in tear fluid are highly charged implies that it could be the charge itself that is necessary for maintaining the milieu in the tear film. Ionic interactions of small particles or pathogens with the human tear-fluid variants of DMBT1, possibly connected to free-floating epithelial cells in the tear fluid [12], may embed foreign materials and allow their clearance by the blinking reflex. This process will of course be assisted by any specific interaction with a pathogen, such as the one demonstrated for salivary gp-340 [19]. The DMBT1 gene products could also bind pathogens indirectly via interactions with the pathogen-binding surfactant protein-A and -D, or other smaller proteins via its unglycosylated parts [23,28,29]. One can also postulate a protective role for the human tear-fluid variants of DMBT1 in mediating leucocyte interactions, similar to that proposed for the sialyl-Le^x expressing salivary MUC7 and gp-340 in the oral cavity [19,30].

The methodology outlined in the present study represents a non-traditional, but efficient, approach for studying the high-molecular-mass glycoproteins including mucins. Mucins are usually isolated by a combination of repeated isopycnic density centrifugation and chromatography steps (exemplified in [5]). The information obtained from samples separated by SDS/AgPAGE is extensive due to the use of sensitive analytical methods, including MS and miniaturization of preparation procedures. The approach outlined in the present study for isolating oligosaccharides from high-molecular-mass tear glycoproteins could be applied to other samples for studying glycosylation differences, both between tissues and within the high-molecular-mass glycoprotein subpopulations of the same tissue.

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