Glycobiology vol. 18 no. 6 pp. 456–462, 2008
doi:10.1093/glycob/cwn022
Advance Access publication on March 10, 2008

The *O*-linked glycosylation of secretory/shed MUC1 from an advanced breast cancer patient's serum

Sarah J Storr[1,2,5], Louise Royle[3,6], Caroline J Chapman[5], Umi M Abd Hamid[4,6], John F Robertson[5,7], Andrea Murray[7], Raymond A Dwek[6], and Pauline M Rudd[4,6]

[5]Tumour Immunology Group, University of Nottingham, Division of Breast Surgery, Nottingham City Hospital, Hucknall Road, Nottingham NG5 1PB; [6]Department of Biochemistry, Oxford Glycobiology Institute, Oxford University, South Parks Road, Oxford OX1 3QU; and [7]Oncimmune Ltd, Clinical Sciences Building, Nottingham City Hospital, Hucknall Road, Nottingham NG5 1PB, UK

Received on November 26, 2007; revised on February 11, 2008; accepted on March 4, 2008

MUC1 is a high molecular weight glycoprotein that is overexpressed in breast cancer. Aberrant *O*-linked glycosylation of MUC1 in cancer has been implicated in disease progression. We investigated the *O*-linked glycosylation of MUC1 purified from the serum of an advanced breast cancer patient. *O*-Glycans were released by hydrazinolysis and analyzed by liquid chromatography-electrospray ionization-mass spectrometry and by high performance liquid chromatography coupled with sequential exoglycosidase digestions. Core 1 type glycans (83%) dominated the profile which also confirmed high levels of sialylation: 80% of the glycans were mono-, di- or trisialylated. Core 2 type structures contributed approximately 17% of the assigned glycans and the oncofoetal Thomsen–Friedenreich (TF) antigen (Galβ1-3GalNAc) accounted for 14% of the total glycans. Interestingly, two core 1 type glycans were identified that had sialic acid α2-8 linked to sialylated core 1 type structures (9% of the total glycan pool). This is the first *O*-glycan analysis of MUC1 from the serum of a breast cancer patient; the results suggest that amongst the cell lines commonly used to express recombinant MUC1 the T47D cell line processes glycans that are most similar to patient-derived material.

Keywords: α2-8 linked sialic acid/breast cancer/MUC1/*O*-linked glycosylation

Introduction

MUC1 is a high molecular weight transmembrane glycoprotein found on numerous epithelia. During breast cancer an atypical expression pattern is exhibited, changing from the apical border of epithelial cells to apolar cell-surface distribution (Heyderman et al. 1979). In addition to the altered cell surface location MUC1 can be found in the cytoplasm and at higher levels in the circulation of patients (Robertson et al. 1999). The majority of the extracellular domain of MUC1 consists of a variable number of tandem repeats (VNTR) (30–100) (Gendler et al. 1990). Each repeat consists of 20 amino acids with five serine and threonine residues, all of which can act as sites for *O*-linked glycosylation (Müller et al. 1999).

MUC1 is initially expressed as a precursor protein and is autoproteolytically cleaved in the endoplasmic reticulum (Hilkens and Buijs 1988; Levitin et al. 2005; Macao et al. 2006). The extracellular subunit remains associated with the transmembrane and cytoplasmic domain throughout intracellular processing and is transported to the cell surface as a stable heterodimeric complex (Ligtenberg et al. 1992; Baruch et al. 1999; Julian and Carson 2002). The extracellular domain of the MUC1 heterodimeric complex can be shed into bodily fluids and in addition a secreted isoform of MUC1 (MUC1/sec) that lacks the transmembrane domain and cytosolic components of MUC1 is also present in the circulation (Wreschner et al. 1990; Boshell et al. 1992; Smorodinsky et al. 1996). The sialylation of MUC1 is incomplete when the glycoprotein is first observed on the cell surface and the addition of sialic acid only takes place following trafficking and successive rounds of recycling through the *trans*-golgi (Hilkens and Buijs 1988). The sialic acid content of secreted MUC1 differs from that of the transmembrane-bound MUC1 as the former only goes through the golgi once and is not subjected to further rounds of sialylation (Engelmann et al. 2005).

Previous studies comparing the *O*-linked glycans of MUC1 from breast cancer cell lines and nonmalignant breast epithelial cell lines demonstrated a variation in the *O*-glycan population. These studies have mainly focused on various cancer cell lines due to limiting sample amounts for glycan analysis. A shift from branched di-sialylated core 2 type *O*-glycans on epithelial cell line MUC1 to shorter mono-sialylated core 1 type *O*-glycans on MUC1 from the T47D tumor cell line has been observed (Lloyd et al. 1996). However, MUC1 from the MCF-7 cell line has been shown to consist principally of neutral core 2 type *O*-glycans (Müller and Hanisch 2002). Interestingly, Engelmann et al. (2005) demonstrated that a MUC1 probe secreted by MCF-7 cells contained predominantly neutral core 2 type *O*-glycans in contrast to a membrane-bound MUC1 probe in the same cell line, which contained mainly sialylated core 1 glycans. Thus the glycosylation varies depending on the location of the mucin.

In addition to altered glycan processing, cancer-associated MUC1 may also be more extensively glycosylated in comparison to MUC1 from a healthy tissue. Müller et al. (1999) demonstrated that T47D MUC1 contained an average of 4.8 glycans of a possible 5 per 20-amino-acid repeat. This indicated a

[1]To whom correspondence should be addressed: Tel: +44-11582-31871; e-mail: sarah.storr@nottingham.ac.uk
[2]Present address: Oncimmune Ltd, Clinical Sciences Building, Nottingham City Hospital, Hucknall Road, Nottingham NG5 1PB, UK.
[3]Present address: Ludger Ltd, Culham Science Centre, Abingdon, Oxfordshire OX14 3EB, UK.
[4]Present address: Dublin-Oxford Glycobiology Laboratory, NIBRT, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland.

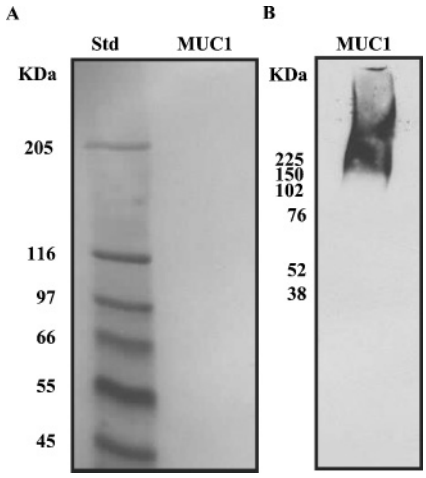

Fig. 1. Analysis of advanced breast cancer serum (ABC) MUC1 on a reducing 10% SDS–polyacrylamide gel. (**A**) 10% SDS–polyacrylamide gel stained with silver; Std: sigma wide range MWt markers; MUC1: ABC serum MUC1 contains no detectable contaminating proteins. MUC1 was not visualized by silver staining which is possibly due to high levels of glycosylation. (**B**) Western blot of the ABC serum MUC1 with a murine C595 monoclonal antibody indicating the presence of high molecular weight MUC1 compared against GE Healthcare full-range MWt Rainbow markers.

significantly higher level of glycosylation than the 2.7 glycans per repeat that was observed on lactation-associated MUC1 (Müller et al. 1997).

In this study we analyzed the *O*-linked glycosylation of MUC1 purified from the serum of an advanced breast cancer (ABC) patient using high performance liquid chromatography (HPLC), sequential exoglycosidase digestion, and liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). This combination of methods enabled us to identify the *O*-linked glycans present on the secretory/shed isoform of MUC1 in breast cancer.

Results

Isolation of MUC1

MUC1 was isolated from the serum of an advanced breast cancer patient using a double antibody affinity matrix (C595/NCRC-11) followed by size exclusion chromatography. The murine monoclonal antibodies, C595 and NCRC-11, were used for the purification of MUC1 because they are directed against epitopes within the VNTR, and C595 binds MUC1 irrespective of the number of repeats or its glycosylation (Karsten et al. 2004). The efficiency of purification was monitored by enzyme-linked immunosorbant assays (ELISA) and the CA15.3 assay values of the serum pre- and post-affinity chromatography. Following reduction and alkylation the sample was subject to separation by size exclusion chromatography to remove copurified antibodies, albumin, and other protein contaminants. The purity of purified MUC1 was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and is shown in Figure 1A. MUC1 could not be visualized by silver staining which is possibly due to high levels of glycosylation. Western blot analysis of the purified ABC serum MUC1 using a C595 monoclonal antibody confirms the presence of high molecular weight MUC1 (Figure 1B).

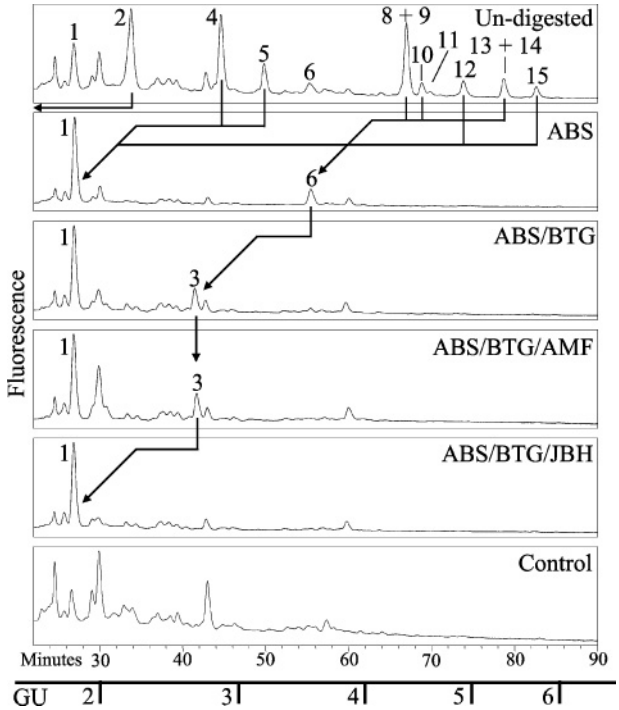

Fig. 2. Structural analysis of advanced breast cancer (ABC) patient MUC1. *O*-Glycans were released from MUC1 by hydrazinolysis, fluorescently labeled with 2-aminobenzamide (2-AB), and analyzed by normal phase (NP)-HPLC. Glycans were determined by their elution position measured in glucose units (GU) in comparison to a database of GU values of glycans, their GU value following exoglycosidase digestion and their weak anion exchange (WAX)-HPLC elution time. Peaks are identified by numbers defined in Table 1. The control was 2-AB-labeled glycans from fetuin. ABS removes α2-3-, α2-6-, and α2-8-linked sialic acid, BTG removes β1-3- and β1-4-linked galactose, JBH removes GalNAc, and AMF removes α1-3- or 4-linked fucose.

Analysis of O-linked glycans by HPLC

O-glycans were cleaved from MUC1 isolated from a breast cancer patient with metastatic disease and labeled with 2-aminobenzamide (2-AB). The glycan pool was analyzed by normal phase (NP)-HPLC, in combination with sequential digestion using an array of exoglycosidases, and weak anion exchange (WAX)-HPLC. Calibration of the NP-HPLC was performed using an external standard of 2-AB-labeled glucose oligomers to create a dextran ladder which was used to convert the retention times of glycans to glucose units (GU). Preliminary structures were assigned by comparing the glucose unit (GU) values of the experimental data with our 'in-house' structural database for *O*-glycans and confirmed from the GU values of digestion products and the known specificities of the enzymes (Royle et al. 2002) (Figure 2). The data were consistent with glycan compositions obtained from the MS analysis. Hydrazinolysis, predictably, gave rise to base catalyzed β-elimination (peeling) resulting in the presence of NeuNAcα2-3Gal (25% of all glycans). This fragment remains after the elimination of terminal GalNAc from susceptible *O*-glycans (both core 1 and core 2 type glycans) and was therefore excluded from determining the percentage of individual glycans, calculating the ratios of core 1 type to core 2 type glycans and sialylated to neutral glycans. A list of the assigned *O*-linked glycans attached to MUC1 and their behavior following exoglycosidase

Table 1. Aliquots of the 2-aminobenzamide (2-AB)-labeled glycan pool from advanced breast cancer (ABC) serum MUC1 were incubated with different exoglycosidases and the products analyzed by normal phase (NP)-HPLC. Liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) data are also displayed and was performed on the total glycan pool from ABC serum MUC1. NAN1 and ABS, ABS/BTG, ABS/BTG/BKF, ABS/BTG/AMF, and ABS/BTG/JBH exoglycosidase digestions are compared against the whole glycan pool where ABS removes α2-3-, α2-6-, and α2-8-linked sialic acid, NAN1 removes α2-3-linked sialic acid, BTG removes β1-3- and β1-4-linked galactose, JBH removes GalNAc, AMF removes α1-3- or 4-linked fucose, and BKF removes α1-2- and α1-6-linked fucose.

	LC-ESI-MS unlabeled glycans							NP-HPLC 2-AB-labeled glycans									
Glycan	Peak No.	Mass		Composition			Retention time LC-ESI-MS	GU	Undigested whole glycan pool (%)	Adjusted against peak 2 (%)	Exoglycosidase Digestions						
		[M+Na]+										NAN1					
		Detected	Calculated	Hex	HexNAc	NeuNAc					BTG		ABS	ABS BTG	ABS BTG BKF	ABS BTG AMF	ABS BTG JBH
Galβ1-3GalNAc	1	406.1	406.1	1	1	0	76.1	1.8	10.7	14.3	=	↑	↑	↑	↑	↑	↑
NeuNAcα2-3Gal	2	494.1	494.2	1	0	1	80.7	2.2	25.0	0.0	=	=	↓	↓	↓	↓	↓
Galβ1-3[GlcNAcβ1-6] GalNAc	3	nd	609.2	1	2	0	nd	2.7	0.0	0.0	=	=	=	↑	↑	↑	↓
NeuNAcα2-3Galβ1-3GalNAc	4	697.1	697.2	1	1	1	83.4	2.9	19.7	26.3	=	↓	↓	↓	↓	↓	↓
Galβ1-3[NeuNAcα2-6]GalNAc	5	697.1	697.2	1	1	1	88.2	3.3	7.2	9.6	=	↑	↓	↓	↓	↓	↓
Galβ1-4GlcNAcβ1-6 [Galβ1-3]GalNAc	6	771.2	771.3	2	2	0	91.5	3.6	3.8	5.1	↓	↑	↑	↓	↓	↓	↓
NeuNAcα2-3Galβ1-3 [GlcNAcβ1-6]GalNAc	7	nd	900.4	1	2	1	nd	3.7	0.0	0.0	↑	=	=	=	=	=	=
NeuNAcα2-3Galβ1-3 [NeuNAcα2-6]GalNAc	8	988.2	988.3	1	1	2	90.9	4.4	18.4	24.5	=	↓	↓	↓	↓	↓	↓
NeuNAcα2-3Galβ1-4GlcNAcβ1-6 [Galβ1-3]GalNAc	9	1062.2	1062.4	2	2	1	93.4										
Galβ1-4GlcNAcβ1-6 [NeuNAcα2-3Galβ1-3]GalNAc	10	1062.2	1062.4	2	2	1	93.4	4.5	3.0	4.0	↓	↓	↓	↓	↓	↓	↓
NeuNAcα2-6Galβ1-4GlcNAcβ1-6 [Galβ1-3]GalNAc	11	1062.2	1062.4	2	2	1	95.1	4.6	1.0	1.3	=	↑	↓	↓	↓	↓	↓
NeuNAcα2-8NeuNAcα2-3 Galβ1-3GalNAc*	12	988.2	988.3	1	1	2	92.2	4.9	4.2	5.6	=	↓	↓	↓	↓	↓	↓
NeuNAcα2-6Galβ1-4GlcNAcβ1-6 [NeuNAcα2-3Galβ1-3]GalNAc	13	1353.3	1353.5	2	2	2	95.1	5.3	4.5	6.0	=	↓	↓	↓	↓	↓	↓
NeuNAcα2-3Galβ1-4GlcNAcβ1-6 [NeuNAcα2-3Galβ1-3]GalNAc	14	1353.3	1353.5	2	2	2	95.1										
NeuNAcα2-8NeuNAcα2-3Galβ1-3 [NeuNAcα2-6]GalNAc*	15	1279.3	1279.4	1	1	3	96.7	5.7	2.4	3.2	=	↓	↓	↓	↓	↓	↓

↑ denotes increased % peak area; ↓ denotes decreased % peak area and = shows % peak area remained the same following digestion. Adjusted against peak 2 denotes the percent contribution of individual glycans, when the base catalyzed β-elimination (peeling) glycan (peak 2) is removed from analysis; *Proposed structure. The α2-8 sialic acid is allocated as such because sialic acids have previously been reported to be linked to each other in this way. However, due to the ABS sialidase having specificity for both α2-3 and α2-8 sialic acids, it is possible that the sialic acid could have either linkage.
nd: not detected.

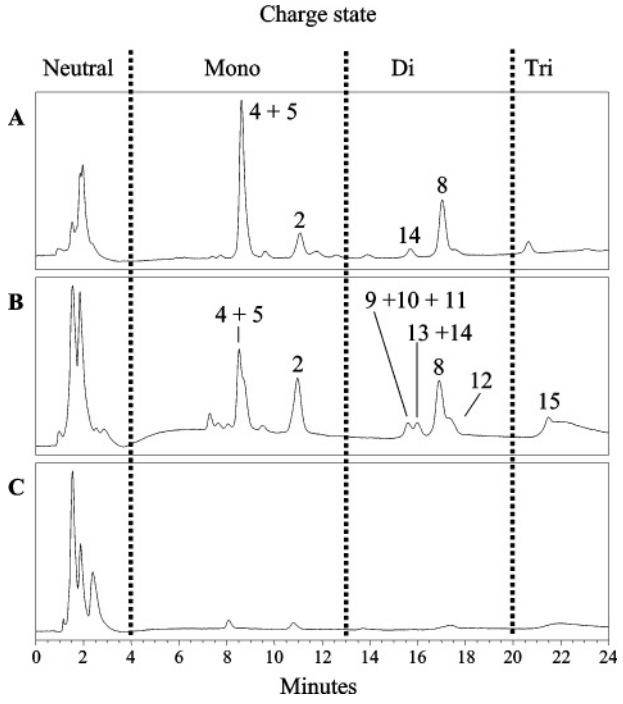

Fig. 3. Structural analysis of advanced breast cancer (ABC) patient MUC1 by weak anion exchange (WAX)-HPLC. The fluorescently 2-aminobenzamide (2-AB)-labeled *O*-glycan pool of the ABC serum MUC1 was subjected to charge-based separation by WAX-HPLC. Peaks are identified by numbers defined in Table 1. (**A**) The *O*-linked profile of a fetuin *O*-glycan control. (**B**) The undigested *O*-glycan profile of the ABC serum MUC1; the major charged structures are visible. (**C**) The α2-3-, α2–6-, and α2-8-linked sialidase (ABS) digested *O*-glycan profile of the ABC serum MUC1.

digestion together the masses detected by LC-ESI-MS are detailed in Table 1. In addition, Table 1 details the percentages of the whole undigested glycan pool and the percentages once the base catalyzed β-elimination (peeling) glycan was removed from the analysis. Charged structures present on the ABC serum MUC1 were also investigated by WAX-HPLC fractionation of the total glycan pool based on total charge (Figure 3).

Identification of core-type glycans

The *O*-glycans present on the ABC serum MUC1 revealed a major population of core 1 type glycans; core 2 type glycans accounted for only around 17% of the patient-derived glycans. The percentage of core 1 and core 2 type glycans was estimated using the peak areas in Figure 2. The exact contribution of core 1 and core 2 type glycans could not be calculated due to the co-elution of peaks 8 (di-sialylated core 1) and 9 (mono-sialylated core 2) on NP-HPLC (Figure 2). These two glycans were successfully separated by WAX-HPLC indicating that the major component was the di-sialylated core 1 type glycan (Figure 3). However, the percentage of core 2 type glycans could still not be determined accurately as peak 9 coeluted with peaks 10 and 11 in WAX-HPLC (Figure 3).

Identification of sialylated glycans

NP-HPLC was used to identify sialylated *O*-linked glycans by comparing the undigested total glycan pool with its ABS-digested counterpart. Provisional structures were assigned using the *O*-glycan database, and then confirmed by sequential exoglycosidase digestion and comparison of the resultant digestion products against a structural database and known digestion pathways (Royle et al. 2002). WAX-HPLC fractionation was also used to confirm the identity of sialylated glycans. LC-ESI-MS was used to confirm the mass and hence composition of each structure. LC separation prior to mass spectral analysis helped to separate the glycans from the background contamination that would interfere with MS analysis. In addition, the LC column allowed the masses to be cross-checked with elution time (GU) to confirm that individual masses correlated with the glycans identified by NP-HPLC for the 2-AB-labeled glycans.

Exoglycosidase digestion with the sialidase ABS revealed that the majority of *O*-linked glycans present on the ABC serum MUC1 were sialylated (Figure 2). When quantified as a percentage of the total undigested glycan pool, 80.5% of the *O*-linked glycans were found to be either mono-, di-, or tri-sialylated structures. The principal *O*-glycan species on the ABC serum MUC1 was sialylated core 1 type glycans. NeuNAcα2-3Galβ1-3GalNAc (peak 4, Table 1) was the most abundant glycan. In addition, the oncofetal Thomsen–Friedenreich (TF) antigen (Galβ1-3GalNAc) accounted for 14.3% of the glycans.

Two new *O*-glycan structures that were not in the structural database were identified (Royle et al. 2002) and have not previously been reported on MUC1. These were peaks 12 and 15 which we have identified as core 1 type glycans containing 2 and 3 sialic acids, respectively. The tri-sialylated peak 15 eluted in the tri-sialylated region on WAX-HPLC, eluted last by NP-HPLC (GU 5.7) and also last by LC-ESI-MS where a clear peak of *m/z* 1279.3 indicated a composition of three sialic acids, one Hex, and one HexNAc. The sialidase used to investigate the sialic acid linkage of these structures has specificity for both α2-3 and α2-8 linkages. We propose an α2-8 sialic acid linkage to the structure as it is well documented that sialic acids add on to each other in the α2-8 position and have been identified previously on both human (Fukuda et al. 1987) and mouse (Yasukawa et al. 2006) serum glycoproteins. In addition, it is known from measuring the GU values of glycans released from glycosphingolipids that the addition of a sialic acid α2-8 to a sialic acid increases the GU by 1.3 (Wing et al. 2001). The difference between peaks 8 and 15 is 1.3 GU. There is a higher GU difference between peaks 12 and 4 (2 GU) but the evidence that α2-8-linked sialic acid adds more GU than α2-6-linked sialic acid and that this peak elutes after peak 8, as well as eluting in the di-sialylated fraction of WAX-HPLC, suggests that it is also an α2-8-linked sialic acid containing structure.

Identification of fucosylated glycans

The fucosylation of *O*-linked glycans on ABC MUC1 was investigated by NP-HPLC and exoglycosidase digestion with the fucosidases AMF and BKF. No change in the NP-HPLC profiles was observed and demonstrated the absence of fucosylated glycans (Figure 2).

Discussion

The glycosylation status of breast cancer-associated MUC1 has long been of interest because of the altered immunogenicity that the MUC1 VNTR exhibits as a consequence of glycosylation. Many monoclonal antibodies have demonstrated alternate

binding characteristics in response to the glycosylation status of MUC1 (Karsten et al. 2004). A shift from core 2 to smaller core 1 type glycans on MUC1 is proposed to make the protein backbone more accessible to immune surveillance.

The *O*-linked glycosylation profiles of MUC1 purified from a number of breast cancer cell lines differ significantly from one another, indicating a cell-specific rather than a cancer-defined pattern of *O*-glycans. MUC1 purified from the T47D breast cancer cell line contains predominantly core 1 type glycans, with less than 5% of core 2 type structures. In contrast, MUC1 purified from the MCF-7 breast cancer cell line contains primarily core 2 type glycans (83%) (Müller and Hanisch 2002).

The *O*-linked glycans attached to the ABC serum MUC1 consist of core 1 type glycans (83%), sialic acid α2-8 linked to sialylated core 1 type glycans (8.8%), the TF antigen (Galβ1-3GalNAc) (14.3%), and core 2 type glycans (17%). The principal glycans present on the ABC serum MUC1 are similar to those present on MUC1 secreted from the T47D breast cancer cell line, being sialylated core 1 type structures, having high levels of Galβ1-3GalNAc (peak 1), and lacking fucosylated structures. In addition, although the ABC serum MUC1 has 17% core 2 type structures, it resembles MUC1 from the T47D breast cancer cell line more closely in its level of core 2 type glycans (less than 5%) than MUC1 purified from other cell lines analyzed by Müller and Hanisch (2002). The ABC serum MUC1 does differ from T47D MUC1 in an increased diversity of structures, including the sialic acid α2-8 linked to sialylated core 1 type structures identified here.

The *O*-glycan pool of the ABC serum MUC1 was heavily sialylated; 80.5% of the total assigned structures were sialylated. This level of sialylation is similar to that observed on MUC1 from the breast cancer cell lines T47D, MDA-231, and ZR-75-1 (93.2%, 70.3%, and 88.4%, respectively). MCF-7 shows a dramatically different level of sialylation, as only 5.2% of its *O*-linked glycans contain sialic acid (Müller and Hanisch 2002).

The oncofetal antigen TF (Galβ1-3GalNAc) (14.3% of the total *O*-glycan pool) was released from the ABC serum MUC1. An interaction between the TF antigen on MUC1 and galectin-3 has recently been demonstrated. This interaction has been proposed to increase the adhesion between cancer and endothelial cells, suggesting a role in cancer progression (Yu et al. 2007). Sialic acid α2-8 linked to sialylated *O*-glycan structures on serum glycoproteins have not been described in detail previously although *N*-linked polysialylation is a feature of glycoproteins of the brain, such as neural cell adhesion molecule (N-CAM) (Hoffman et al. 1982) and the sodium channel α subunit (Zuber et al. 1992). The *N*-linked polysialylation of *N*-CAM has been shown to modulate cell adhesion (Fujimoto et al. 2001). *O*-Linked polysialylation has been identified on CD36 from human milk (Yabe et al. 2003) and on unidentified proteins in MCF-7 and RBL basophilic leukemia cells (Martersteck et al. 1996). In addition a Neu5Gcα2-8Neu5Gc2→ reactive monoclonal antibody has identified disialic acid on carbonic anhydrase II, an immunoglobulin light chain, vitronectin, and plasminogen in mouse serum, which has been confirmed by fluorometric analysis and mild acid hydrolysates-fluorometric anion exchange chromatography (Yasukawa et al. 2006). It is unclear as to the effects that the sialic acid α2-8 linked to sialylated structures may have on the functions or properties of MUC1. In addition, it would be of interest to know if these structures are present during the early stages of disease and if they have diagnostic/prognostic significance, however this would be difficult due to low amounts of MUC1 and the glycan structure.

We have analyzed the *O*-glycans attached to MUC1 circulating in the serum of an advanced breast cancer patient. High levels (14.3%) of the oncofetal antigen TF were detected. This antigen has been implicated in a number of interactions, such as increased cancer cell endothelial adhesion by interaction with galectin-3 (Yu et al. 2007). Interestingly, 8.8% of ABC MUC1 *O*-linked glycans had sialic acid α2-8 linked to sialylated core 1 type structures. The glycan pool was dominated by core 1-based structures most of which were sialylated. This is similar to the *O*-linked glycosylation present on the T47D cell line MUC1 which may therefore be the most appropriate cell line for the study of 'cancer state' *O*-linked glycosylation of MUC1.

Materials and methods

Materials

The monoclonal antibody hybridoma cell lines NCRC-11 and C595 were kindly provided by Professor Alan Perkins at the University of Nottingham, UK. Serum samples were taken from patients under informed consent at the Breast Institute, University of Nottingham, UK. MUC1 from an individual with advanced breast cancer (ABC) was investigated.

Purification of NCRC-11 and C595 mouse monoclonal antibodies

NCRC-11 and C595 hybridoma cell lines were grown at 37°C in the presence of 5% CO_2. RPMI 1640 containing 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal calf serum, 1 μg/mL fungizone, and 3.6 ng/mL β-mercaptoethanol (all Sigma-Aldrich, MO) was used for both C595 and NCRC-11 hybridoma cell line culture. Cell culture supernatant was isolated by centrifugation at 400 × *g* and was 0.45 μm filtered (Minisart, Sartorius, Goettingen, Germany) prior to the circulation through activated CNBr sepharose 4B (GE Healthcare, Uppsala, Sweden) coupled to a 9-mer VNTR peptide; APDTRP (Peptide Protein Research Ltd, Fareham, UK) at 4°C over 17–24 h. The unbound protein was washed from the column, with 10 column volumes of phosphate-buffered saline (PBS) containing 0.2 M sodium chloride. Bound antibody was eluted from the column using 2 column volumes of 3 M sodium thiocyanate, and fractions were immediately desalted using a PD10 column (GE Healthcare). The reactivity of a purified antibody was confirmed in ELISA.

Purification of MUC1

C595 and NCRC-11 were bound to activated CNBr Sepharose 4B (GE Healthcare) at 1.7 mg of protein per mL of matrix. Five milliliters of antibody matrix was used for MUC1 purification. Serum was diluted 1:10 in PBS with 0.2 M sodium chloride, and 0.45 μm Minisart filtered and circulated through the C595/NCRC-11 affinity matrix for 17–24 h at 4°C prior to elution. MUC1 was eluted from the column using three elution buffers (0.25 M glycine/HCl, pH 2.5; 25 mM diethylamine, pH 11; and 100 mM diethylamine, pH 11), and eluted column fractions were assayed using C595 mouse monoclonal antibody and peroxidase-conjugated affinipure rabbit anti-human IgG and

IgM (H+L) (Jackson Immunoresearch, PA) in an ELISA. The affinity purification was repeated five times. Positive fractions were pooled and concentrated using Amicon Ultra-15 centrifugal filter units (30 000 MWCO) (Millipore, MA).

Concentrated serum MUC1 was reduced using 50 mM *threo*-1,4-dimercapto-2,3-butanediol (DTT) for 1-h rolling at room temperature. The reduced sample was then alkylated using 100 mM iodoacetamide (IAA) for 30 min protected from light at room temperature prior to size exclusion chromatography using an HiPrep 26/60 Sephacryl S-300 (GE Healthcare) with a flow rate of 0.5 mL/min. Following size exclusion chromatography fractions were assayed by ELISA and fractions positive for MUC1 were again pooled and concentrated using Amicon Ultra-15 centrifugal filter units (30 000 MWCO). Purity was assessed by SDS–PAGE by resolution in a 10% gel using a Mini-Protean 3 Cell (Bio-Rad Laboratories, Hercules, CA) for 50 min at a constant voltage of 150 V. Gels were stained with silver using a Silver Stain Kit (Bio-Rad Laboratories). CA15.3 levels were determined using the Bayer Centaur automated CA15.3 immunoassay and glycoprotein was stored at −20°C until analysis.

Western blot analysis

Purified MUC1 was resolved in a 10% SDS–polyacrylamide gel for 50 min at a constant voltage of 150 V, followed by gel equilibration in a Tris–glycine buffer. Protein transfer from gels to a 0.2-μm nitrocellulose membrane (Bio-Rad Laboratories) was performed with a Bio-Rad Trans blot system (using a constant voltage of 40 V over 5 h). Membranes were blocked with 5% Marvel in Tris-buffered saline containing Tween-20 (TBS-t) for 15–20 h at 4°C prior to incubation with a C595 mouse monoclonal antibody in the same buffer for 15–20 h at 4°C. Immunocomplexes were detected with a horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody (Dako, Glostrup, Denmark) for 2 h, prior to detection with enhanced chemiluminesence detection reagents and developed on Hyperfilm (GE Healthcare).

Hydrazinolysis of MUC1 O-glycans

MUC1 was dialyzed against 0.1% trifluoroacetic acid (TFA) at 4°C for over 36 h in a microdialysis block and lyophilized. Hydrazinolysis was performed as in Patel et al. (1993) and Merry et al. (2002). Briefly, lyophilized MUC1 was cryogenically dried for 24 h and *O*-glycans were released by incubation at 60°C for 6 h with anhydrous hydrazine. Excess hydrazine was removed by evaporation and the released *O*-linked glycans were re-*N*-acetylated by incubation with acetic anhydride in a saturated solution of sodium bicarbonate; sodium salts were removed using Dowex AG50 X12(H+) 200–400 mesh (Bio-Rad Laboratories). *O*-Glycans were separated from peptide components by descending paper chromatography on a pre-washed Whatmann 3 MM chromatography paper in butanol:ethanol:water (8:2:1 v/v) for 48 h. Glycans were recovered from their chromatography paper origin by 4 × 1.5 mL washes and concentrated using a rotary evaporator.

2-AB labeling of MUC1 O-glycans

2-AB labeling was performed as in Bigge et al. (1995), using the Ludger Tag™ 2-AB kit (Ludger Ltd, Oxford, UK). 2-AB-labeled glycans were separated from excess fluorophore by ascending paper chromatography with acetonitrile (Royle et al. 2006). Glycans were eluted from their paper origin with water.

Normal Phase-HPLC of MUC1 2-AB-labeled O-glycans

2-AB-labeled glycans were separated on a 2695 Alliance Separation Module, fitted with a temperature control module and a 474 fluorescence detector (Waters, MA). Fluorescence was measured at 420 nm with excitation at 330 nm. Separation was performed as in Royle et al. (2002) using a TSK Amide-80 250 × 4.6 mm column (Anachem, Luton, UK).

Exoglycosidase digestion of 2-AB-labeled O-glycans

2-AB-labeled glycans were subject to a range of exoglycosidase digestions followed by NP-HPLC. Digestions were preformed in a 50 mM sodium acetate buffer, pH 5.5, for 16 h at 37°C. Exoglycosidases used were *Arthrobacter ureafaciens* sialidase (ABS EC 3.2.1.18), 1–2 units/mL; *Streptococcus pneumonia* sialidase recombinant from *Escherichia coli* (NAN1 EC 3.2.1.18), 1 unit/mL; bovine kidney α-fucosidase (BKF EC 3.2.1.51) 1 unit/mL; almond meal α-fucosidase (AMF EC 3.2.1.111), 3 milliunits/mL; bovine testes β-galactosidase (BTG EC 3.2.1.23), 2 units/mL; Jack bean β-*N*-acetyl-hexosaminidase (JBH EC 3.2.1.30), 10 milliunits/mL, purchased from Prozyme (San Leandro, CA).

Weak anion exchange-HPLC fractionation of MUC1 2-AB-labeled O-glycans

The fluorescently labeled glycans were separated on a 2695 Alliance Separation Module, fitted with a temperature control module and a 474 fluorescence detector (Waters). Fluorescence was measured at 420 nm with excitation at 330 nm. Separation was performed as in Royle et al. (2002) using a Vydac 301VHP575 7.5 × 50 mm column. Both the undigested and ABS-digested *O*-glycans of MUC1 were subject to WAX-HPLC and fractions were collected and subjected to further analysis by NP-HPLC.

Glycan analysis by liquid chromatography-electrospray ionization-mass spectrometry

Glycans were analyzed using an LC Packings Ultimate HPLC equipped with a Famos autosampler (Dionex Ltd, Leeds, UK) interfaced with a Q-Tof Ultima Global mass spectrometer (Waters-Micromass, Manchester, UK). Chromatographic separation was achieved using a 2 × 250 mm microbore NP-HPLC TSK gel Amide-80 column (Hichrom, Beskshire, UK) with the same gradient and solvents as used with the standard NP-HPLC but at a lower flow rate of 40 μL/min (Royle et al. 2002). The mass spectrometer was operated in positive ion mode with 3 kV capillary voltage; RF lens 60; source temp 100°C; desolvation temp 150°C; cone gas flow 50 L/h; and desolvation gas flow 450 L/h.

Acknowledgements

The authors would like to acknowledge Brian Matthews (Oxford Glycobiology Institute, University of Oxford) for expert hydrazinolysis release and Tony Hitch and Stuart Jones (Clinical Chemistry, Nottingham City Hospital) for CA15.3 analysis.

Conflict of interest statement

John Robertson is a shareholder and director of Oncimmune. Andrea Murray and Sarah Storr are employees of Oncimmune. Caroline Chapman consults for Oncimmune.

Abbreviations

ABC, advanced breast cancer; 2-AB, 2-aminobenzamide; ELISA, enzyme-linked immunosorbant assays; GU, glucose units; HPLC, high performance liquid chromatography; LC-ESI-MS, liquid chromatography-electrospray ionization mass spectrometry; N-CAM, neural cell adhesion molecule; NP, normal phase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TF, Thomsen–Friedenreich antigen; VNTR, variable number of tandem repeats; WAX, weak anion exchange.

References

Baruch A, Hartmann M, Yoeli M, Adereth Y, Greenstein S, Stadler Y, Skornik Y, Zaretsky J, Smorodinsky NI, Keydar I, et al. 1999. The breast cancer associated MUC1 gene generates both a receptor and its cognate binding protein. *Cancer Res*. 59:1552–1561.

Bigge JC, Patel TP, Bruce JA, Goulding PN, Charles SM, Parekh RB. 1995. Nonselective and efficient fluorescent labeling of glycans using 2 aminobenzamide and anthranilic acid. *Anal Biochem* 230:229–238.

Boshell M, Lalani EN, Pemberton L, Burchell J, Gendler S, Taylor-Papadimitriou J. 1992. The product of the human MUC1 gene when secreted by mouse cells transfected with the full length cDNA lacks the cytoplasmic tail. *Biochem Biophys Res Commun*. 185:1–8.

Engelmann K, Kinlough CL, Müller S, Razawi H, Baldus SE, Hughey RP, Hanisch F-G. 2005. Transmembrane and secreted MUC1 probes show trafficking dependent changes in *O*-glycan core profiles. *Glycobiology*. 15:1111–1124.

Fujimoto I, Bruses JL, Rutishauser U. 2001. Regulation of cell adhesion by poly-sialic acid. *J Biol Chem*. 276:31745–31757.

Fukuda M, Lauffenburger M, Sasaki H, Rogers ME, Dell A. 1987. Structures of novel sialylated *O*-linked oligosaccharides isolated from human erythrocyte glycophorins. *J Biol Chem*. 262:11952–11957.

Gendler S, Lancaster CA, Taylor-Papadimitriou J, Duhig T, Peat N, Burchell J, Pemberton L, Lalani E, Wilson D. 1990. Molecular cloning and expression of human tumour-associated polymorphic epithelial mucin. *J Biol Chem*. 265:15286–15293.

Heyderman E, Steele K, Ormerod MG. 1979. A new antigen on the epithelial membrane: Its immunoperoxidase localisation in normal and neoplastic tissue. *J Clin Pathol*. 32:35–39.

Hilkens J and Buijs F. 1988. Biosynthesis of MAM-6, an epithelial sialomucin. *J Biol Chem*. 263:4215–4222.

Hoffman S, Sorkin BC, White PC, Brackenbury R, Mailhammer R, Rutishauser U, Cunningham BA, Edelman GM. 1982. Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. *J Biol Chem*. 257:7720–7729.

Julian JA and Carson DD. 2002. Formation of MUC1 metabolic complex is conserved in tumour derived and normal epithelial cells. *Biochem Biophys Res Commun*. 293:1183–1190.

Karsten U, Serttas N, Paulsen H, Danielczyk A, Goletz S. 2004. Binding patterns of DTR-specific antibodies reveal a glycosylation-conditioned tumour-specific epitope of the epithelial mucin (MUC1). *Glycobiology*. 14:681–692.

Levitin F, Stern O, Weiss M, Gil-Henn C, Ziv R, Prokocimer Z, Smorodinsky NI, Rubinstein DB, Wreschner DH. 2005. The MUC1 SEA module is a self-cleaving domain. *J Biol Chem*. 280:33374–33386.

Ligtenberg MJL, Kruijshaar L, Buijs F, Meijer MV, Litvinov SL, Hilkens J. 1992. Cell-associated episialin is a complex containing two proteins derived from a common precursor. *J Biol Chem*. 267:6171–6177.

Lloyd KO, Burchell J, Kudryashov V, Yin BWT, Taylor-Papadimitriou J. 1996. Comparison of *O*-linked carbohydrate chains in MUC1 mucin from normal breast epithelial cell lines and breast carcinoma cell lines – demonstration of simpler and fewer glycan chains in tumour cells. *J Biol Chem*. 271:33325–33334.

Macao B, Johansson DGA, Hansson GC, Härd T. 2006. Autoproteolysis coupled to protein folding in the SEA domain of the membrane bound MUC1 mucin. *Nat Struct Mol Biol*. 13:71–76.

Martersteck C, Kedersha NL, Drapp DA, Tsui TG, Colley KJ. 1996. Unique α2,8-polysialylated glycoproteins in breast cancer and leukemia cells. *Glycobiology*. 6:289–301.

Merry AH, Neville DCA, Royle L, Matthews B, Harvey DJ, Dwek RA, Rudd PM. 2002. Recovery of intact 2-aminobenzamide labeled *O*-glycans released from glycoproteins by hydrazinolysis. *Anal Biochem*. 304:91–99.

Müller S, Goletz S, Packer N, Gooley A, Lawson AM, Hanisch F-G. 1997. Localisation of O-glycosylation sites on glycopeptides fragments from lactation-associated MUC1. *J Biol Chem*. 272:24780–24793.

Müller S, Alving K, Peter-Katalinic J, Zachara N, Gooley AA, Hanisch F-G. (1999). High density O-glycosylation on tandem repeat peptide from secretory MUC1 of T47D breast cancer cells. *J Biol Chem*. 274:18165–18172.

Müller S and Hanisch F-G. 2002. Recombinant MUC1 probe authentically reflects cell-specific O-glycosylation profiles of endogenous breast cancer mucin. *J Biol Chem*. 277:26103–26112.

Patel T, Bruce J, Merry A, Bigge C, Wormald M, Jaques A, Parekh R. 1993. Use of hydrazine to release in intact and unreduced form both *N*- and *O*-linked oligosaccharides from glycoproteins. *Biochemistry*. 32:679–93.

Robertson JFR, Jaeger W, Syzmendera JJ, Selby C, Coleman R, Howell A, Winstanley J, Jonssen PE, Bombardieri E, Sainsbury JRC, et al. 1999. The objective measurement of remission and progression in metastatic breast cancer by use of serum tumor markers. *Eur J Cancer*. 35:47–53.

Royle L, Mattu TS, Hart E, Langridge JI, Merry AH, Murphy N, Harvey DJ, Dwek RA, Rudd PM. 2002. An analytical and structural database provides a strategy for sequencing *O*-glycans from microgram quantities of glycoproteins. *Anal Biochem*. 304:91–99.

Royle L, Radcliffe CM, Dwek RA, Rudd PM. 2006. Detailed structural analysis of *N*-glycans released from glycoproteins in SDS–PAGE gel bands using HPLC in combination with exoglycosidase assay digestions. *Methods Mol Biol*. 347:125–144.

Smorodinsky N, Weiss M, Hartmann ML, Baruch A, Harness E, Yaakobovitz M, Keydar I, Wreschner DH. 1996. Detection of a secreted MUC1/SEC protein by MUC1 isoform specific monoclonal antibodies. *Biochem Biophys Res Commun*. 228:115–121.

Wing DR, Garner B, Hunnam V, Reinkensmeier G, Andersson U, Harvey DJ, Dwek RA, Platt FM, Butters TD. 2001. High-performance liquid chromatography analysis of ganglioside carbohydrates at the picomole level after ceramide glycanase digestion and fluorescent labeling with 2-aminobenzamide. *Anal Biochem*. 298:207–17.

Wreschner DH, Hareuveni M, Tsarfaty I, Smorodinsky N, Horev J, Zaretsky J, Kotkes P, Weiss M, Lathe R, Dion A, et al. 1990. Human epithelial tumour antigen cDNA sequences. Differential splicing may generate multiple protein forms. *Eur J Biochem*. 189:463–73.

Yabe U, Sato C, Matsuda T, Kitajima K. 2003. Polysialic acid in human milk. *J Biol Chem*. 278:13875–13880.

Yasukawa Z, Sato C, Sano K, Ogawa H, Kitajima K. 2006. Identification of disialic acid containing glycoproteins in mouse serum: A novel moidification of immunoglobulin light chains, vitronectin and plasminogen. *Glycobiology*. 16:651–665.

Yu LG, Andrews N, Zhao Q, McKean D, Williams JF, Connor LJ, Gerasimenko OV, Hilkens J, Hirabayashi J, Kasai K, et al. 2007. Galectin-3 interaction with Thomsen–Fridenreich disaccharide on cancer associated MUC1 causes increased cancer cell endothelial adhesion. *J Biol Chem*. 282:773–781.

Zuber C, Lackie PM, Catterall WA, Roth J. 1992. Poly-sialic acid is associated with sodium channels and the neural cell adhesion molecule N-CAM in adult rat brain. *J Biol Chem*. 267:9965–9971.