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In vivo glycosylation of MUC1 in airway epithelial cells

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The O-glycans that decorate mucin glycoproteins contribute to the biophysical and biochemical properties of these molecules and hence their function as a barrier and lubricant on epithelial surfaces. Alterations in mucin O-glycosylation in certain diseases may contribute to pathology. It is known that both the host cell type and the amino acid sequence of the mucin tandem repeat contribute to the O-glycosylation of a mucin molecule. We expressed an epitope-tagged MUC1 mucin cDNA construct in the airway cell line 16HBE14o- and the colon carcinoma cell line Caco2 and used Fast Atom Bombardment Mass Spectrometry to evaluate the contribution of the host cell to differences in O-glycosylation of a single mucin. Many of the glycans detected on the MUC1 mucin were common to both cell types, as would be predicted from biosynthetic constraints. However, MUC1 synthesized in the airway cell line showed comparatively low levels of sialylation but carried a range of oligo-*N-acetyllactosamine* structures that were not seen in the colon carcinoma cell line.
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Introduction

Mucous glycoproteins play a key role in the protection and lubrication of epithelial cells lining a number of organ systems including the airway, the pancreas and intestine. Human mucins are encoded by multiple genes all sharing a common motif, the tandem repeat which is evident at both nucleic acid and amino acid levels. The tandem repeat is rich in serine and threonine residues, which undergo significant O-glycosylation. This O-glycosylation is responsible for the biochemical and biophysical properties of mucins and so is a focus of studies to elucidate the biological properties of mucin molecules. Human mucins may be one of two main types, secreted mucins that are the classical high molecular weight glycoproteins released from intracellular granules and membrane-tethered mucins that are inserted into the cell membrane during their intracellular processing and are subsequently released from the cell surface. MUC1 was the first membrane-tethered mucin to be indentified and characterized from pancreas [1] and mammary gland [2] though it has a much broader expression pattern, including in other parts of the digestive system and in respiratory epithelium. Due to the possible involvement of membrane-tethered mucins in the pathology of diseases such as cystic fibrosis it is of importance to evaluate the differences in the biophysical properties of the mucins expressed in different types of normal epithelial cells, prior to embarking on studies of CF epithelial cells.

Elucidation of the O-glycosylation of individual mucins extracted from different cell types is difficult due to contamination with other molecules. To circumvent this problem an epitope-tagged MUC1 mucin (MUC1F) was generated and shown to produce O-glycosylated MUC1 when transfected into pancreatic duct and intestinal epithelial cells [3]. Presence of the FLAG epitope within the MUC1 protein enables immunopurification of this mucin for further biochemical and biophysical analysis [4–6]. MUC1F and derivatives of it have now been used to evaluate O-glycosylation of MUC1 in the airway epithelial cell line 16HBE14o- [7]. This transformed human bronchial epithelial cell line displays characteristic properties of differentiated epithelia such as tight junctions and cilia. Monolayers of the cell line generate transepithelial resistance [7] and express endogenous MUC1 mucin (unpublished observations). 16HBE14o- also demonstrates expression of CFTR and a regulated chloride conductance and has been used in a number of

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studies to evaluate airway epithelial function in CF [8–10]. In the work described in this paper, the O-glycosylation of MUC1F in 16HBE14o- has been compared to MUC1F expressed in the Caco2 colon carcinoma cell line and significant differences were observed. The airway cell line decorates MUC1F with oligo-*N-acetyllactosamine* (oligo-lacNAc) structures that are not seen in the colon carcinoma cell line but there is a relative paucity of sialylated structures. This novel use of a method that evaluates the glycosylation of a single mucin molecule in different cell types will have important applications.

Materials and methods

Generation of epitope-tagged MUC1F and MUC1F^2 constructs

The epitope-tagged (FLAG) MUC1 cDNA (MUC1F) was described previously [3]. Insertion of the FLAG sequence into MUC1F created two unique restriction enzyme sites, *Bgl*II and *Asp*I immediately adjacent to the epitope. The *Bgl*II site (at base 232 of MUC1F) was used to insert a second FLAG epitope immediately 3′ to first one. Briefly, a duplex of primers HS22 5′GAT CTT AGA TTA CAA GGA TGA CGA CGA CAA GGA 3′ and HS23 5′GAT CTC CTT GTC GTC GTC ATC CTT GTA ATC TAA 3′ encoding the FLAG epitope (DYKDDDDK) was ligated into the *Bgl*II site of MUC1F in pBluescript generating MUC1F^2. This was subsequently transferred to the pHβ-APrl-neo expression vector as a *Bam* HI fragment.

Expression of epitope-tagged chimeric mucins

The Caco2 colon adenocarcinoma cell line [11] and 16HBE14o- airway epithelial cell lines [7] were cultured in Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 10% fetal bovine serum.

The MUC1F and MUC1F^2 constructs were transfected into Caco2 and 16HBE14o- cells by calcium phosphate precipitation [12] or Lipofectin (Life Technologies) and clones carrying integrated constructs were selected using G418 (Gibco BRL) at 600 μg/ml. Multiple clones were isolated for each cell line and evaluated for expression of the MUC1F mucin.

Preparation of cell lysates and immunopurification

Cell lysates were prepared from post-confluent flasks of cells with 10 μl/cm^2 of lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1% (w/v) Triton X-100, 0.02% (w/v) NaN_3). Lysates were processed as described previously [5] and used immediately for immunopurification with M2 antibody conjugated to agarose beads (Sigma). The conjugated agarose beads were pelleted by centrifugation at 200 g for 5 min, and rinsed twice with NET buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA) containing 1% (w/v) Triton X-100. For mass spectrometry (MS) studies the rinsed pellets were subsequently washed in NET buffer without Triton X-100. For western blot analysis immunoprecipitated mucin was eluted from the conjugated agarose beads with 500 μg/ml of FLAG peptide (Sigma) in NET buffer containing 1% (w/v) Triton X-100. The sample was heated to 80°C in sample loading buffer (2% (w/v) SDS, 62.5 mM Tris pH 6.8, 5% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue) for 5 min before loading on SDS-polyacrylamide gels. For MS, immunoprecipitated mucin was eluted as above but without Triton X-100 and the supernatant collected and freeze dried. (Yields of mucin were equivalent, as judged by western blots when material was eluted from the M2 beads with or without detergent.) MUC1F^2 prepared from Caco2 clone 400 was also concentrated and purified on a centricon YM-10 filter unit (Amicon).

Western blotting

Immunoprecipitated mucins were resolved using SDS-PAGE (3% stacking gel and 6% resolving gel or 3% stacking gel and 10% resolving gel) [13]. Proteins were electrophoretically transferred to Hybond-C Super membranes (Amersham Pharmacia Biotech Ltd., UK), and blocked in 5% (w/v) fat free dried skimmed milk (Marvel) in phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_3, 1.4 mM KH_2PO_3, pH 7.3) for 1 h. lmmunodetection with the M2 antibody (Sigma) was carried out as described previously [4,5]. Enhanced chemiluminescence reagents (ECL) and ECL sensitive film (Amersham Pharmacia Biotech Ltd., UK) were used for the final detection of antibodies.

Mass spectometry

Preparation of oligosaccharides

O-linked oligosaccharides were liberated from the immunoprecipitated mucins by reductive elimination (400 μl of 1 M $NaBH_4$ in 0.05 M NaOH at 45°C for 16 h) and desalted through a Dowex 50W-X8(H) column. Removal of traces of detergent was achieved by passing the desalted eluate through a Sep-Pak C_{18} column (Waters Corp.). Excess borates were removed by co-evaporation with 10% (v/v) acetic acid in methanol under a stream of nitrogen.

Chemical derivatisation for FAB-MS

Permethylation using the sodium hydroxide procedure was performed as described previously [14]. After derivatization the reactions were purified on Sep-Pak C_{18} [14].

FAB-MS analysis

FAB-MS spectra were acquired using a ZAB-2SE 2FPD mass spectrometer fitted with a caesium ion gun operated at 30 kV. Data acquisition and processing were performed using the VG Analytical Opus software. Solvents and matrices were as described previously [14].

Results

Expression of MUC1F and MUC1F^2 mucin in 16HBE14o- cells

The MUC1F construct was transfected into 16HBE14o- and Caco2 cells, clones exhibiting stable integration of the plasmid were selected by culture with G418 and then evaluated for expression of MUC1F. Though multiple Caco2 clones expressing MUC1F were isolated, as described previously [3,4] no 16HBE14o- clones expressing MUC1F were identified. Although a PCR screen with primers specific for the neomycin resistance gene confirmed the presence of the pHβ-APr1neo expression vector in 9 16HBE14o- clones (data not shown) none of these clones demonstrated M2-reactive material by western blot analysis of whole cell lysates. A further 22 G418-resistant 16HBE14o- clones carrying other MUC1F derived constructs (MUC1F(ΔTR), MUC1F/5ACTR and MUC1F/5BTR [5] also failed to express M2-reactive glycoprotein. These constructs are based on MUC1F but either lack the MUC1 tandem repeat (MUC1F(ΔTR] or have a portion of tandem repeat sequence from another mucin gene substituted for that of MUC1 (MUC1F/5ACTR and MUC1F/5BTR]. Several explanations might account for these observations, including low expression of the transgenes and/or masking of the M2 epitope.

To evaluate the possibility that the M2 was being masked by O-glycosylation or other processing specific to the 16HBE14o- cell line a MUC1F^2 construct was generated that contained tandem FLAG epitopes (DYKDDDDK) at the same site as the single epitope in MUC1F. MUC1F^2 was transfected into 16HBE14o- and Caco2 cells and 7 16HBE14o- and 6 Caco2 clones exhibiting stable integration of MUC1F^2 were selected by culture with G418. Western blot analysis of whole cell lysates using M2 antibody detected the presence of MUC1F^2 in three of the Caco2 clones (Figure 1A, H400, H402, H403). Two of the 16HBE14o- clones (Figure 1B H503, H504) demonstrated a high level of MUC1F^2 expression with multiple glycoforms between 75 and >250 kDa and, as with MUC1F expressed in Caco2 the pattern of M2-reactive glycoforms was specific to the clone isolated [3,4].

O-glycosylation of MUC1F^2 mucin in 16HBE14o- cells

O-linked oligosaccharides from the immunoprecipitated mucins were prepared from clones H503 and H504 and analysed as described previously [5].

The FAB-MS spectra of the permethylated products of reductive elimination from H503 and H504 are shown in Figure 2A and B respectively, and assignments of ions are given in Table 1. Only peaks that correspond to known sugar structures have m/z assignments in Figure 2. Material was purified from each cell line on two separate occasions and the data shown to be reproducible with the exception of some variations in the abundance of sialylated glycans, for example m/z 895 ($NeuAc_1Hex_1HexNAc_1$) and 1344 ($NeuAc_1Hex_2HexNAc_2$)

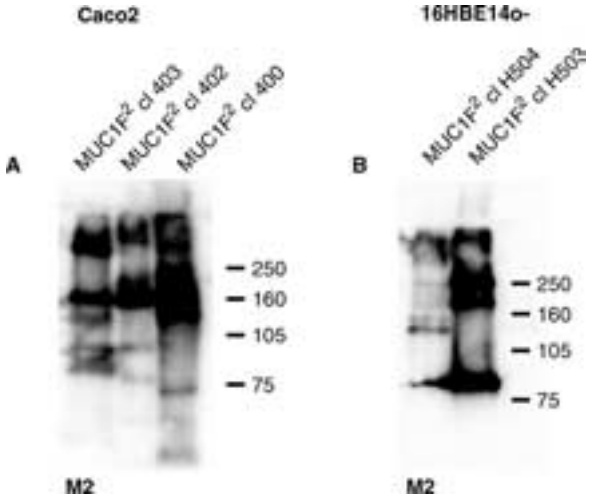

Figure 1. Detection of MUC1F² in (A) Caco2 cells (clones 400, 402 and 403) and (B) 16HBE14o- cells (clones H503 and H504). Western blot analysis of MUC1F² mucins in whole cell lysates separated by SDS-PAGE. Detection was by M2 anti-FLAG antibody and ECL. Migration of full range rainbow protein molecular weight markers (Amersham) is indicated in kDa.

were of low abundance in two of the preparations. Also, because of the very low abundance of structures with masses above m/z 2000, detection of signals at m/z 2056 ([$Fuc_1Hex_4HexNAc_4$]-ol) and 2331 ([$Hex_5HexNAc_5$]-ol] (Figure 2B] was dependent on sample quality.

The oligosaccharide compositions in Table 1 suggest the presence of two families of O-glycans on the MUC1F^2 mucin. Members of the first family have compositions of $Hex_1HexNAc_1$-ol and $NeuAc_1Hex_1HexNAc_1$-ol which are consistent with type 1 core structures. Members of the second family of pseudomolecular ions have compositions $Hex_1HexNAc_2$-ol, $Hex_2HexNAc_2$-ol, $Fuc_1Hex_2HexNAc_2$-ol,

Table 1. Assignment of pseudomolecular ions observed in FAB-MS spectra of permethylated O-glycans from MUC1F² in 16HBE14o- cells

Signal (m/z)	*Assignment [M + Na]$^+$*
534	($Hex_1HexNAc_1$)-ol
779	($Hex_1HexNAc_2$)-ol
895	($NeuAc_1Hex_1HexNAc_1$)-ol
983	($Hex_2HexNAc_2$)-ol
1157	($Fuc_1Hex_2HexNAc_2$)-ol
1228	($Hex_2HexNAc_3$)-ol
1344	($NeuAc_1Hex_2HexNAc_2$)-ol
1432	($Hex_3HexNAc_3$)-ol
1606	($Fuc_1Hex_3HexNAc_3$)-ol
1677	($Hex_3HexNAc_4$)-ol
1882	($Hex_4HexNAc_4$)-ol
2056	($Fuc_1Hex_4HexNAc_4$)-ol
2331	($Hex_5HexNAc_5$)-ol

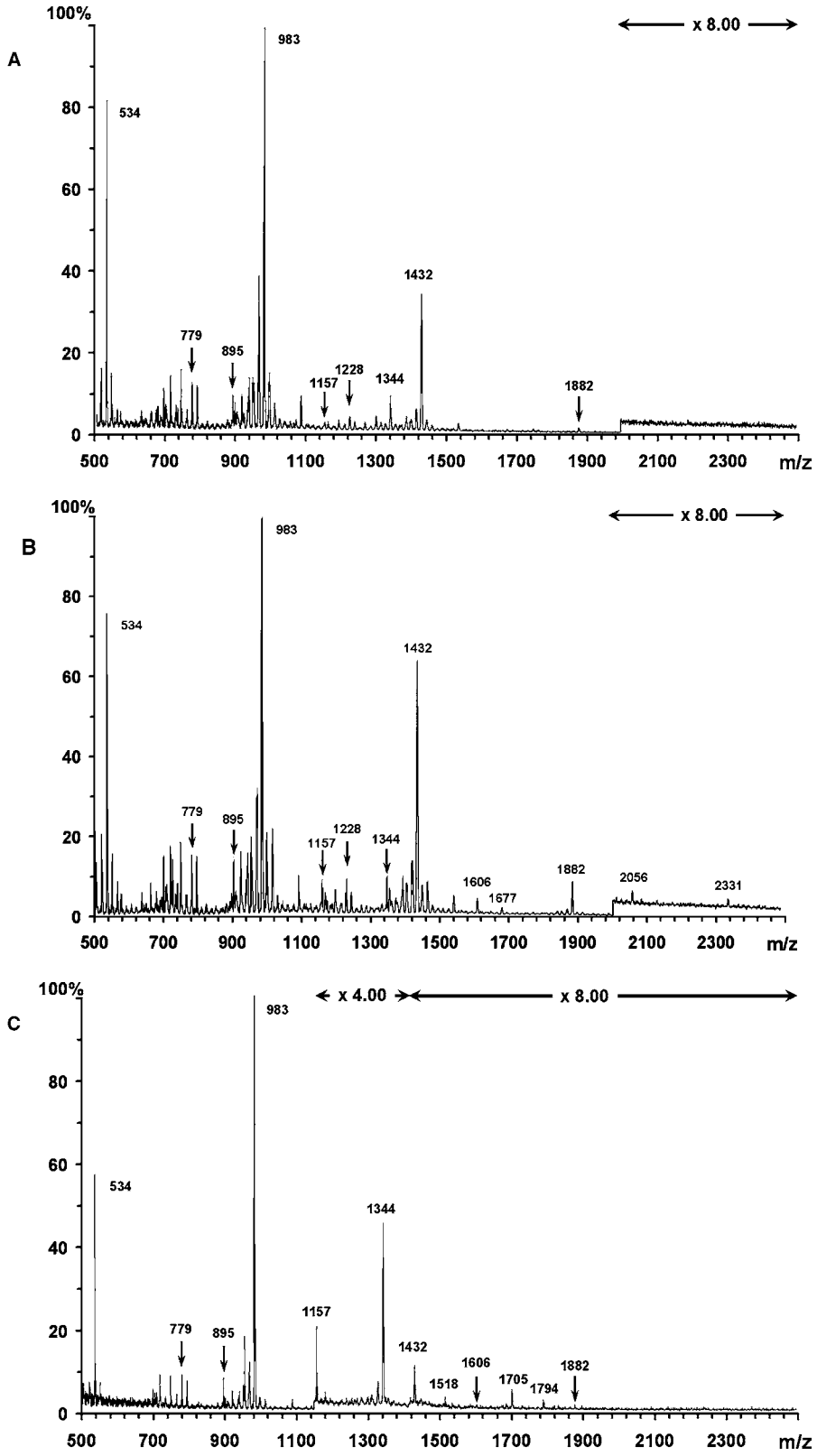

Figure 2. FAB-MS of permethylated O-glycans from MUC1F² in 16HBE14o- (A) clone H503, (B) clone H504 and (C) MUC1F² in Caco2, clone 400. Peaks with assigned m/z values correspond to the O-glycan structures shown in Tables 1 and 2. The signals at m/z 1089 and 1538 are matrix artefacts and the minor clusters flanking each of the major signals are due to derivatisation artefacts. Magnified parts of the spectrum are shown above each panel.

$Hex_2HexNAc_3$-ol, $NeuAc_1Hex_2HexNAc_2$-ol, $Hex_3HexNAc_3$-ol, $Fuc_1Hex_3HexNAc_3$-ol, $Hex_3HexNAc_4$-ol, $Hex_4HexNAc_4$-ol, $Fuc_1Hex_4HexNAc_4$-ol and $Hex_5HexNAc_5$-ol, corresponding to other core structures. The predominant structures in this family are $Hex_{2-4}HexNAc_{2-4}$-ol (m/z 983, 1432 and 1882, respectively], whose compositions are consistent with the presence of oligo-N-acetylactosamine (oligolacNAc] antennae. Minor, mono-fucosylated variants of each of these structures are also present, giving the signals at m/z 1157, 1606 and 2056.

Comparison of O-glycosylation of MUC1F mucin in 16HBE14o- and Caco2 cells

The assignment of pseudomolecular ions observed in FAB-MS spectra of permethylated glycans from $MUC1F^2$ in Caco2 are shown in Figure 2C and Table 2. A comparison of with $MUC1F^2$ in 16HBE14o- shows that, though many of the structures are common to both cell types, as would be expected due to biosynthetic constraints (that is, within the limitations of available substrates and glycoprotein processing enzymes) there are some clear differences. The most striking of these is the abundance of the pseudomolecular ion at m/z 1432 in the 16HBE14o- $MUC1F^2$ spectrum that corresponds to the structure $Hex_3HexNAc_3$-ol. This structure was at comparatively low levels in $MUC1F^2$ synthesized in Caco2 as was the $Hex_4HexNAc_4$-ol structure detected as m/z 1882, which was also seen in both 16HBE14o- clones. These glycans have compositions consistent with the presence of oligolacNAc antennae. The $Fuc_1Hex_4HexNAc_4$-ol structure and $Hex_5HexNAc_5$-ol structures detected respectively as m/z 2056 and m/z 2331 were also not detected on $MUC1F^2$ synthesized in Caco2. These data suggest that the glycosyltransferases generating the oligolacNAc structures are more active in the airway cell line. Another difference between the spectra for $MUC1F^2$ synthesized in 16HBE14o- and Caco2 cells is the paucity of sialylated structures seen in the clones H503 and H504. Though both cell lines have an abundant peak at m/z 983 ($Hex_2HexNAc_2$-ol], only the monosialylated derivative at 1344 is evident at low abundance in clones H503 and H504. In contrast in the Caco2-derived MUC1F, mono- and disialyl variations of the m/z 983 composition occur at m/z 1344 and m/z 1705, as well as a fucosylated non- and mono-sialyl variation at m/z 1157 and m/z 1518 respectively. This suggests that either the 16HBE14o- cells have low activity of sialyltransferases and/or they express a sialidase.

Table 2. Assignment of pseudomolecular ions observed in FAB-MS spectra of permethylated O-glycans from MUC1F in Caco2 cells

Signal (m/z)	*Assignment $[M + Na]^+$*
534	$(Hex_1HexNAc_1)$-ol
708	$(Fuc_1Hex_1HexNAc_1)$-ol
779	$(Hex_1HexNAc_2)$-ol
895	$(NeuAc_1Hex_1HexNAc_1)$-ol
983	$(Hex_2HexNAc_2)$-ol
1157	$(Fuc_1Hex_2HexNAc_2)$-ol
1344	$(NeuAc_1Hex_2HexNAc_2)$-ol
1432	$(Hex_3HexNAc_3)$-ol
1518	$(NeuAc_1Fuc_1Hex_2HexNAc_2)$-ol
1606	$(Fuc_1Hex_3HexNAc_3)$-ol
1705	$(NeuAc_2Hex_2HexNAc_2)$-ol
1794	$(NeuAc_1Hex_3HexNAc_3)$-ol
1882	$(NeuAc_1Hex_3HexNAc_3)$-ol

Discussion

The O-glycosylation of the same glycoprotein expressed in different cells is known to show variation that is in part dependent on the glycosyltransferases expressed in each cell type. This complicates the analysis of defects in O-glycosylation that may be associated with a number of human diseases.

Few attempts have been made to use a combination of molecular biological and classic biochemical approaches to evaluate O-glycosylation of mucin glycoproteins. This is partly due to the difficulties of working with these molecules that are encoded by large genes that contain tandemly repeated sequence motifs. We used an epitope-tagged MUC1 mucin molecule [3] to evaluate the O-glycosylation of MUC1 in an airway and a colon carcinoma cell line, by FAB-MS. The procedure was sufficiently sensitive to detect consistent differences in the sugar structures that are added to the MUC1 mucin in these two cell lines. This approach will enable further evaluation of mucin O-glycosylation in different cell lineages. It will also allow detection of reproducible differences in O-glycosylation of mucins in cell lines carrying human disease-associated mutations and matched pairs of lines that have the mutation corrected by introduction of a normal transgene. This could prove valuable in the elucidating possible abnormalities in mucin O-glycosylation in diseases such as cystic fibrosis, which is associated with failure to clear thickened mucus secretion from several organ systems.

Comparison of the O-glycosylation of epitope-tagged MUC1 synthesized in the human airway line 16HBE14o- and the colon carcinoma cell line Caco2 is of interest as both these cell lines express endogenous MUC1 [15] and unpublished). Given the known biosynthetic constraints on O-glycosylation it is not surprising that many of the glycans seen on MUC1 are common to both cell types. For example the major peaks on the FAB-MS spectra are at m/z 534 ($Hex_1HexNAc_1$-ol) and m/z 983, ($Hex_2HexNAc_2$-ol) in both cell lines. Cancer cell lines are known to produce glycans with markedly altered glycosylation and an abundance of short structures in comparison to the normal epithelium from which they were derived. For example the normal colonic epithelium shows characteristic core 3-based glycosylation associated with UDP-GlcNAc/GalNAc β3-Nacetylglucosaminyl-transferase (reviewed in [16]. However as this enzyme is absent from colon carcinoma cells these show alternative core-structures including core 2, core 5 and core 6. The 16HBE14o- cell line was derived from normal human bronchial epithelium by transformation with SV40 largeT and it maintains many of the differentiation markers of the

normal epithelium. Hence, mucin production by 16HBE14o- is more likely to reflect processing in the normal epithelium.

The most noticeable difference between the carbohydrate structures detected on $MUC1F^2$ produced in the airway and colon lines is the abundance of the $Hex_3HexNAc_3$-ol structure (m/z 1432) in the 16HBE14o- $MUC1F^2$ spectrum, a structure that was present at low levels on $MUC1F^2$ synthesized in Caco2. $MUC1F^2$ isolated from Caco2 cells also showed extremely low levels of the $Hex_4HexNAc_4$-ol structure (m/z 1882) that was detected in both 16HBE14o- clones and lacked the $Fuc_1Hex_4HexNAc_4$-ol (m/z 2056) and $Hex_5HexNAc_5$-ol (m/z 2331) structures that were detected in clone H504 on one occasion. These data suggest that the glycosyltransferases generating the oligolacNAc structures are more active in the airway cell line. The other major difference between the spectra for $MUC1F^2$ synthesized in 16HBE14o- and Caco2 cells is the low abundance of sialylated structures in the clones H503 and H504. Though both cell lines have a major peak corresponding to the precursor $Hex_2HexNAc_2$-ol structure (m/z 983), only low levels of the monosialylated derivative at m/z 1344 are evident in contrast to the mono- and disialyl (m/z 1705) and fucosylated non- (m/z 1157) and mono-(m/z 1518) sialyl variations seen in Caco2. This suggests that either the 16HBE14o- cells have a low activity of sialyltransferases and/or they express a sialidase, possibilities that warrant further evaluation.

The 16HBE14o- cell line is frequently used as a model of the airway epithelium to study bacterial interaction with host cells [10]. Since some bacteria are known to exploit the mucin layer on the epithelium in pathogenesis [17–21], the characterization of the O-glycans on membrane-tethered MUC1 mucin expressed in 16HBE14o- cells may be particularly relevant to these studies. These data also illustrate the power of this mucin expression system to evaluate cell-type specific and disease-associated changes in mucin O-glycosylation.

Acknowledgments

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