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Terminal glycosylation of cystic fibrosis airway epithelial cells

Andrew D. Rhim, Vaishali A. Kothari, Paul J. Park, Andrew E. Mulberg, Mary Catherine Glick and Thomas F. Scanlin*

Department of Pediatrics and The Cystic Fibrosis Center, University of Pennsylvania School of Medicine, and The Children's Hospital of Philadelphia, Philadelphia, PA, 19104-4318 USA

Cystic fibrosis (CF) has a characteristic glycosylation phenotype usually expressed as a decreased ratio of sialic acid to fucose. The glycosylation phenotype was found in CF/T1 airway epithelial cells (ΔF508/ΔF508). When these cells were transfected and were expressing high amounts of wtCFTR, as detected by Western blot analysis and *in situ* hybridization, the cell membrane glycoconjugates had an increased sialic acid content and decreased fucosyl residues in α1,3/4 linkage to antennary *N*-acetyl glucosamine (Fucα1,3/4GlcNAc). After the expression of wtCFTR decreased, the amount of sialic acid and Fucα1,3/4GlcNAc returned to levels shown by the parent CF cells. Sialic acid was measured by chemical analysis and Fucα1,3/4GlcNAc was detected with a specific α1,3/4 fucosidase. CF and non-CF airway cells in primary culture also had a similar reciprocal relationship between fucosylation and sialylation. It is possible that the glycosylation phenotype is involved in the pathogenesis of CF lung disease by facilitating bacterial colonization and leukocyte recruitment.

Keywords: **glycosylation, cystic fibrosis, airway epithelial cells, α1,3fucose, sialic acid, CFTR, CFTR transfection**

Abbreviations: **CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; wt, wild type; Fucα1,3/4GlcNAc, fucosyl residues linked α1,3 or α1,4 to antennary *N*-acetyl glucosamine**.

Introduction

Cystic fibrosis (CF) is the most common lethal genetic disease of Caucasians. More than a decade ago, the gene CFTR (cystic fibrosis transmembrane regulator) was cloned and shown to be an apical chloride channel of epithelial cells [1]. More than 800 mutations have been described but 49% of the patients are homozygous ΔF508. However, identification of the gene and characterization of the gene product have not led to a reduction of the morbidity and mortality, which are mainly due to progressive lung disease. A phenotypic consequence of mutations in the CFTR gene is the altered oligosaccharide composition of both secreted and membrane glycoproteins. Originally described as an increase in fucosylation and a decrease in the sialylation of secreted glycoproteins [2], this altered glycosylation has been confirmed in both mucins and membrane glycoproteins (reviewed in 3). In studies performed on the surface glycoconjugates of CF and non-CF fibroblasts, it was demonstrated by ^{1}H-NMR spectroscopy that the key features of the oligosaccharides unique to CF had increased Fuc linked α1,3 to antennary GlcNAc, increased Fuc linked α1,6 to core GlcNAc, and decreased terminal NeuAc [4].

Specific information regarding the details of oligosaccharide structures at the surface of airway epithelial cells is lacking, however some information is available from binding studies of a variety of pathogenic organisms to airway epithelial cells [5,6]. Both *Pseudomonas aeruginosa* and *Haemophilus influenzae* have fucose binding proteins [7,8]. *Pseudomonas* [9] and *Haemophilus* [10] also bind to asialo GM1 and a number of other oligosaccharides [11].

Colonization and infection of the airways with specific bacteria, accompanied by a neutrophilic inflammatory response, have been recognized as hallmarks of CF lung disease [12]. Indeed, it has been demonstrated that leukocytes may infiltrate the lung prior to bacterial colonization [13–15]. Thus, the increased numbers of leukocytes and the ability of the bacteria to colonize the CF airways may be independently linked to the altered function of CFTR. Direct evidence

* To whom correspondence should be addressed: Thomas F. Scanlin, MD, The Children's Hospital of Philadelphia, Abramson Pediatric Research Center, Cystic Fibrosis Center, 3516 Civic Center Blvd., Rm 402, Philadelphia, PA 19104-4318; Tel.: +215-590-3608; Fax: +215-590-4298; E-mail: scanlin@email.chop.edu

linking mutated CFTR function to the presence of *Pseudomonas aeruginosa* was provided by Zar *et al*. [16], who demonstrated an increased binding of *P. aeruginosa* to primary cultures of CF ciliated nasal epithelial cells, with the highest percentage binding to cells which were homozygous for ΔF508. This was confirmed by two reports [17,18]. Davies *et al*. [17] showed that the increased binding of *Pseudomonas* to CF airway epithelial cells could be reversed by transfection with wtCFTR.

It is reported here that CF airway epithelial cells have the glycosylation phenotype of decreased sialylation and increased fucosylation. Most importantly, this phenotype is shown to be reversed by expression of wtCFTR. These results provide an additional molecular link between mutations in CFTR and the pathogenesis of CF lung disease.

Materials and Methods

Materials

All cells were cultured in serum-free media from Clonetics, San Diego, CA on Falcon flasks (Becton Dickenson Laboratory, Bedford, MA). Trypsin (3× crystalized) was from Worthington Biochemical Corporation, Lake Wood, NJ. Thiobarbituric acid was from Acros Organics, Pittsburgh, PA. Sephadex G25 and G10 were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Dowex 1 × 8 (200–400 mesh) was purchased from Sigma Chemicals, St. Louis, MO. L-[5-6-^{3}H]Fucose (60 Ci/mmol) was from NEN Boston, MA.

Cell culture and harvest

Tracheal epithelial cells from a patient homozygous for the ΔF508 mutation were immortalized with SV40 T antigen and termed CF/T1. CF/T1 cells were transfected with wild type (wt) CFTR cDNA and showed functional correction of the chloride channel [19]. Both the parent CF/T1 cells and the transfected cells, LCFSN, were obtained from Dr. J.R. Yankaskas, University of North Carolina. LCFSN cells are referred to here as CF/T1 (wt) cells since they were transfected with wtCFTR. CF/T1 (wt) and CF/T1 cells were cultured as described [19]. Another cell line of CF airway epithelial cells, CF/T43, was obtained from the nasal epithelia of a patient homozygous for the ΔF508 mutation. These cells were immortalized with the SV40 T antigen [20] and obtained from Dr. J.R. Yankaskas. Normal airway epithelial cells, BEAS-2B, were immortalized by an adeno/SV40 virus and obtained from Dr. J.F. Lechner, NIH [21]. BEAS-2B and CF/T43 cells were cultured as described previously [22]. All cell lines were passaged once per week at 1×10^6 cells per 25 cm^2 flask.

CF/T1 and CF/T1 (wt) cells harvested between 32 and 60 passages in culture were analyzed. In total, three series were analyzed. A series was defined as a sequence of weekly passages from the original CF/T1 (wt) or CF/T1 cells. Four to eight cultures in each series were examined. After approximately 40 passages in culture, CF/T1 cells transfected with wtCFTR cDNA (CF/T1 (wt) cells) exhibited a significant cell division-dependent loss of expression of wtCFTR and reverted to the initial CF phenotype as shown by *in situ* hybridization and western blot analysis. The loss of the expression of wtCFTR with passage in culture exhibited a logarithmic decrease as described under *Statistical Methods*.

Airway cells in primary culture were obtained from nasal polyps of CF patients after surgery. CF and non-CF bronchial epithelial cells were obtained from lung transplants according to the Institutional Review Board of The Children's Hospital. The tissue was treated as previously described [23].

When radioactive glycopeptides were prepared, the cells were metabolically labeled (5 μCi per 25 cm^2 flask) with L-[5,6-^{3}H]fucose for 18 h prior to harvest. The cells were harvested on day 7 with controlled trypsination releasing the membrane glycopeptides while maintaining the viability of the cells [4,24]. Aliquots were taken for cell counts and protein determinations using bovine serum albumin as a standard [25]. The cell pellets and ^{3}H-glycoproteins were lyophilized and stored at −40 °C until used.

Sialic acid analysis

The large membrane glycoproteins were hydrated with 2 ml of water and freed of salts and free [^{3}H]fucose on Sephadex G-25 columns (2.5 × 35.6 cm) in water and lyophilized. The material from 1×10^7 cells, which was excluded from the column, was resuspended in 1 ml of 0.1 N H_2SO_4. After hydrolysis at 80 °C for 60 min, the total suspension was poured over a column (0.5 × 7.0 cm) of Dowex 1 × 8. The column was washed with 2 ml of water, and eluted with 1 ml of 0.05 M sodium acetate, 0.4 ml (×7) of 0.6 M sodium acetate, and 0.4 ml (×2) of 1 M sodium acetate. Aliquots (0.2 ml) were analyzed for sialic acid with thiobarbituric acid [26].

Fucα1,3/4GlcNAc detection

The twice desalted ^{3}H-membrane glycopeptides from 6.2×10^7 cells were lyophilized and resuspended with water (50 cpm/μl) and treated with α1,3/4 fucosidase from almonds as previously described [27]. The enzyme-treated glycopeptides as well as a control of non enzyme-treated glycopeptides were incubated for 18 h at 37 °C. [^{3}H]fucose released from the fucosidase-treated glycopeptides was detected by scintillation counting after gel filtration on a column (2.5 × 35.6 cm) of Sephadex G-10. [^{3}H]Fucose released by the enzyme was reported as fucosyl residues in α1,3/4 linkage to branch *N*-acetyl glucosamine (Fucα1,3/4GlcNAc).

In situ Hybridization

CFTR mRNA was detected by *in situ* hybridization as previously described using a digoxygenin-labeled RNA probe specific for CFTR exon 14 [28]. Cells (1×10^5) were grown for 24 h on coverslips and fixed with 4.4% paraformaldehyde. After hybridization for 18 h, the cells were developed

by antibody/alkaline phosphatase reagents [29] and photographed.

ImageQuant v.2.1 for Macintosh was used to quantify the results of *in situ* hybridization experiments. Total staining data was obtained for both experiments employing the wtCFTR mRNA probe in sense and antisense orientations. Total staining obtained with the wtCFTR probe in the sense orientation was subtracted from antisense experiments to obtain the value, total relative staining, which was correlated with the relative amount of wtCFTR mRNA in the respective cells.

Western blot analysis

Western blot analysis for the detection of CFTR was performed as described [22] using antibody pAb3145 to CFTR [28]. Cells (5×10^5; 50 μg of protein) were dissolved in sample buffer [22] and loaded onto each lane. CFTR was detected by enhanced chemiluminescence reagents and subsequently analyzed by Image Quant Analysis v.2.1 for Macintosh. Relative protein quantities were calculated by the detection of pixel volumes of the scanned film. The area of the individual lanes in which these analyses took place was held constant. Background pixel volume, obtained from an area of no protein, was subtracted to normalize across lanes.

Statistical methods

Two-tailed, heterodescic student's t-tests were performed on all data as specified with all p-values less than 0.05 were considered significant. Logarithmic regression analysis was performed on the western blot of wtCFTR protein to characterize the relationship between the amount of wtCFTR protein in the cells and the number of passages in culture of the cells. The data fit the model [wtCFTR protein = −11.75*log (passage/30340.9)] with an absolute r of 0.99.

Exponential regression analysis was performed on the specified data to further characterize the relationship among the variables. The number of passages in culture of the cells analyzed was taken to be the independent variable, x. The dependent variable, y, was taken to be the amount of sialic acid or Fucα1,3/4GlcNAc found on the corresponding cells. The generated values characterized the rate of change of the quantity of cell surface sialic acid or Fucα1,3/4GlcNAc according to an increasing number of passages in culture and was termed the exponential decay constant in sialic acid experiments or exponential growth constant in fucose experiments.

Results

Decrease in sialic acid as the expression of wtCFTR decreased

Using the immortalized CF cell line (CF/T1) and these cells transfected with wtCFTR (CF/T1) (wt) cells) as described in *Materials and Methods*, it was shown that after transfection, the sialic acid content of the membrane glycoconjugates was increased by 80% (Figure 1). When CF/T1 (wt) cells expressed significantly lower levels of wtCFTR (Figures 2 and 3), the sialic acid content of the membrane glycoconjugates decreased (Figure 1). The sialic acid content of the membrane glycoconjugates of CF/T1 (parent) cells (Figure 1) was similar to that of CF/T1 (wt) cells, which were expressing nominal amounts of wtCFTR.

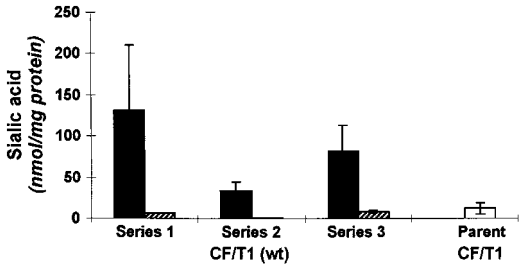

Figure 1. Summary of the sialic acid content in three series of CF/T1 (wt) and all CF/T1 cells. Mean total sialic acid values from CF/T1 (wt) cells expressing (■) high, or (▨) low levels of wtCFTR protein, and (□) CF/T1 parent cells. When cells expressing high levels of wtCFTR were compared to cells with low levels of wtCFTR expression for series 2 and 3, the *p* values were <0.025 and <0.05, respectively. Cells expressing high levels of wtCFTR for series 1, series 2, and series 3, when compared to CF/T1 cells, had *p* values equal to <0.012, <0.012, and <0.004, respectively. Bars and lines represent the mean and standard deviation, respectively.

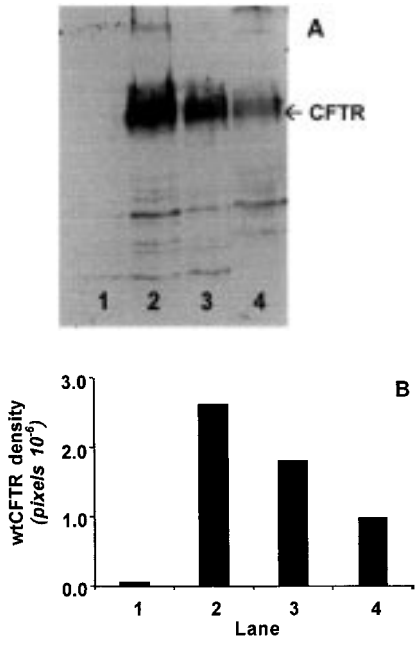

Figure 2. The expression of wtCFTR protein in CF/T1 (wt) cells. (A) Western blot analysis with antibody pAb3145 to wtCFTR protein in CF/T1 (wt) cells. (lane 1) parent CF/T1; CF/T1 (wt) cells in noted passages (P): (lane 2) P33; (lane 3) P36; (lane 4) P41. The cells (5×10^5; 50 μg of protein) were solubilized and loaded onto each lane. (B) Density of CFTR protein is represented as pixels in each of the gel lanes. See *Materials and Methods*.

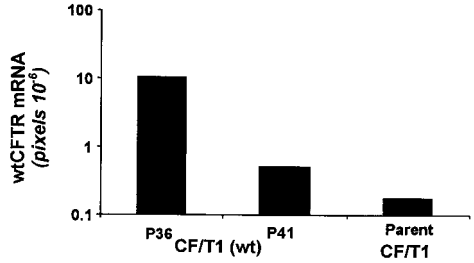

Figure 3. The expression of wtCFTR mRNA transcripts in CF/T1 (wt) cells. *In situ* hybridization using a probe to exon 14 of human CFTR in antisense and sense orientations, was developed with antibody-alkaline phosphatase reagents. The relative expression was quantified by ImageQuant for the specific staining of CF/T1 (wt): P36; and P41; and CF/T1 parent as described in *Materials and Methods*.

In order to further characterize the significant loss of wtCFTR at higher passages, the cells were passaged in culture until this could no longer be reliably detected by Western analysis, usually after passage 40. Thus, three series of cells growing from passages 32 to 50 were examined, and in each case (Figure 1), the sialic acid content of the membrane glycoconjugates was significantly reduced after wtCFTR had declined in CF/T1 (wt) cells. wtCFTR was followed by western blot analysis with antibody pAb3145 and by *in situ* hybridization using an Exon 14 probe to CFTR. Examples are shown in Figures 2 and 3.

Increase in Fucα1,3/4GlcNAc as the expression of wtCFTR decreased

At the same time that sialic acid was elevated, Fucα1,3/4GlcNAc was decreased in the membrane glycoconjugates of CF/T1 (wt) cells by 66%. When wtCFTR decreased (Figures 2 and 3), Fucα1,3/4GlcNAc was increased. This change in glycosylation of the membrane glycoproteins was examined in three series of cells ranging from passage 32 to 50 (Figure 4). As wtCFTR expression decreased in CF/T1 (wt) cells, the increase in Fucα1,3/4GlcNAc on glycoproteins correlated with the percentage of Fucα1,3/4GlcNAc released by the enzyme from the parent cell glycoconjugates (Figure 4).

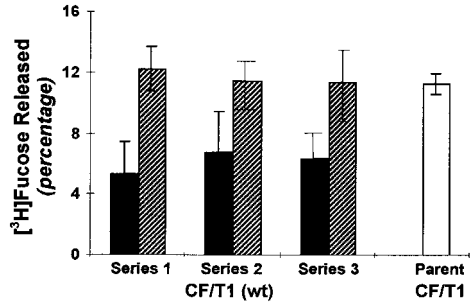

Figure 4. The presence of Fucα1,3/4GlcNAc in CF/T1 (wt) cells and the parent CF/T1 cells. Three series of CF/T1 (wt) cells were examined and each series summarized into cells expressing (■) high or (▨) low levels of wtCFTR, and (□) CF/T1 parent cells. When cells expressing high levels of wtCFTR were compared to cells with low wtCFTR expression for series 1, the p value was <0.022. Cells expressing high levels of wtCFTR for series 1 and 2, when compared to CF/T1 cells, had p values of <0.0009 and <0.001, respectively.

[^{3}H]fucose was released by α-L-fucosidase specific for fucosyl residues in α1,3/4 linkage to branch GlcNAc. The fact that the increased fucosylation was not an artifact of growth conditions was shown by the consistency of the parent CF/T1 cells when compared to CF/T1 (wt) cells, which were grown over a similar series of passages (Figure 5). CF/T1 cells maintained expression of Fucα1,3/4GlcNAc of $12.1 \pm 0.68\%$ (Figure 5B) whereas CF/T1 (wt) cells (Figure 5A) varied from 4.6% to 14.4%, reflecting the expression of wtCFTR.

The data given in Figures 1 and 4 as well as the 1st order decay constants of sialic acid (Figure 6A) and Fucα1,3/4GlcNAc (Figure 6B), lend solid support that there is a correlation between sialic acid content and wtCFTR expression as well as Fucα1,3/4GlcNAc content and wtCFTR expression in this cell system.

Ratio of NeuAc : Fucα1,3/4GlcNAc

The ratio of NeuAc : Fucα1,3/4GlcNAc was calculated in CF/T1 cells (parent), and CF/T1 (wt) cells expressing high or low levels of wtCFTR. As reflected by the results given in Figures 1 and 4, when CF/T1 (wt) cells were expressing high levels of wtCFTR, a ratio of 10.6 ± 4.8 was obtained. When the expression of wtCFTR cells decreased, the ratio dropped to 1.23 ± 0.3, which was similar to that of the parent cells, 0.45 ± 0.3 (Figure 7). This data represented the analyses performed from all three series.

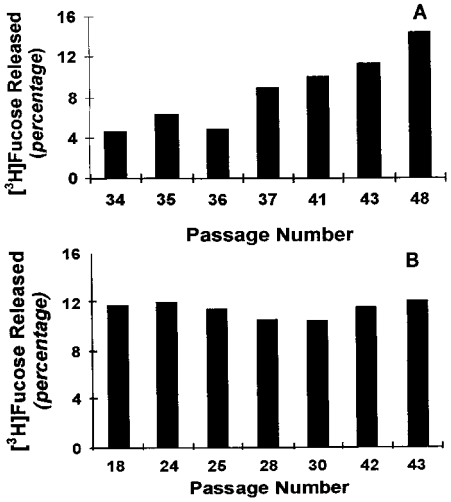

Figure 5. The presence of Fucα1,3/4GlcNAc in CF/T1 (wt) and parent CF/T1 cells. (A) One series of sequential passages of CF/T1 (wt) cells compared to (B) CF/T1 cells analyzed in sequential passages. Note consistency of (B) when compared to (A).

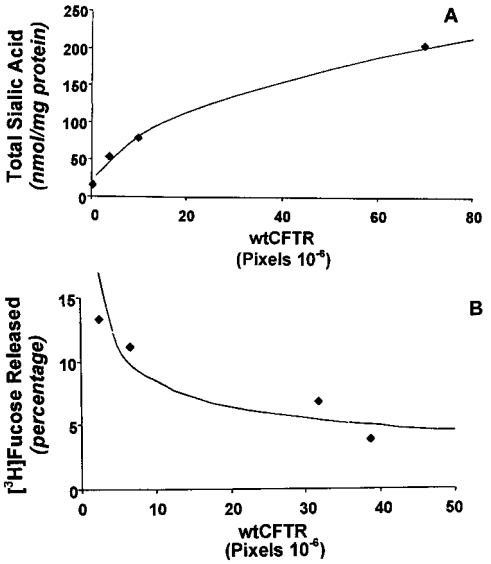

Figure 6. The dependence of total sialic acid or Fucα1,3/4GlcNAc on the expression of wtCFTR protein characterized as an exponential equation. The line represents the exponential equation generated relating (A) total sialic acid or (B) Fucα1,3/4GlcNAc with wtCFTR protein content. (A) Closed squares indicate actual data points for series CF/T1 (wt) cells at passages 59, 46, 41, and 31 from left to right, (B) Closed squares indicate actual data points for series CF/T1 (wt) cells at passage 48, 43, 35, and 34 from left to right.

Table 1. Sialic acid and Fucα1,3/4GlcNAc content in immortalized and primary airway cell glycoconjugates

Airway cells	*Sialic Acid (nmol/mg protein)*	*Fucose (% released)*
BEAS-2B	7.64 ± 4.01^{a} (3)	3.28 ± 0.03^{b} (3)
CF/T43	2.34 ± 0.322^{a} (4)	27.45 ± 1.63^{b} (2)
Non-CF (primary)	37.45 ± 1.45^{c} (2)	2.75 ± 0.35^{d} (2)
CF (primary)	16.42 ± 2.00^{c} (3)	18.45 ± 0.42^{d} (2)

(a) $p < 0.021$; (b) $p < 0.013$; (c) $p < 0.00064$; (d) $p < 0.0009$. Numbers in parentheses are the number of experiments performed.

Fucosylation and sialylation of CF/T43 cells and non-CF cells, BEAS-2B

To determine how general the reciprocal relationship of sialic acid to Fucα1,3/4GlcNAc was in other airway epithelial cell lines, the sialic acid and Fucα1,3/4GlcNAc content of CF/T43 cells and BEAS-2B cells were examined. Table 1 shows that the sialic acid content of the membrane glycoconjugates of non-CF cells is 3.2 times higher than that of the CF cells. At the same time, Fucα1,3/4GlcNAc found on the CF membrane glycoproteins was 8.4-fold higher than normal membrane glycoproteins. Both of these analyses were complimentary to those of the CF/T1 cell system (Figure 7). These cells also served as a control for the overexpression of wtCFTR in the CF/T1 (wt) cells.

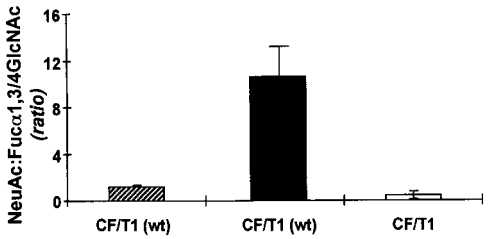

Figure 7. Ratio of NeuAc to Fucα1,3/4GlcNAc in CF/T1 cells. (□) CF/T1 cells (parent), and CF/T1 cells transfected with wtCFTR cDNA expressing (■) high or (▨) low levels of wtCFTR were analyzed for total sialic acid (NeuAc) content and Fucα1,3/4GlcNAc. The difference in the ratio between high and low levels of wtCFTR expression in CF/T1 (wt) cells was significant with a p value of <0.04. CF/T1 (wt) cells with high levels of wtCFTR compared to CF/T1 (parent) cells had a p value equal to 0.01.

Fucosylation and sialylation of CF and non-CF airway cells in primary culture

Airway cells from CF patients or normal donors at the time of lung transplantation were grown in primary culture. The sialic acid and Fucα1,3/4GlcNAc content of the membrane glycopeptides of these cells was similar to the results of other CF or non-CF cells (Table 1). That is, the membrane glycoconjugates of the airway cells from the non-CF source had 2.3 times more sialic acid than the CF cells. At the same time, the primary CF cells had 6.7 times more Fucα1,3/4GlcNAc than normal epithelial cells. Although the study of the primary cells represents only a few analyses, they were consistent with those of the immortalized cell lines. The fact that the CF and non-CF airway cells in primary culture reflect the ratios of NeuAc:Fucα1,3/4GlcNAc of the immortalized cells provided further evidence of the altered glycosylation phenotype in CF.

Discussion

Airway epithelial cells derived from a CF patient homozygous for the most common mutation in CFTR (ΔF508) were used to examine the glycosylation phenotype of decreased sialylation and increased fucosylation. Previously, CF fibroblasts [4] and other CF material [3] were shown to have alterations in terminal glycosylation. The direct relationship to the expression of wtCFTR was shown by transfection of CF airway cells with wtCFTR. After correction of the Cl^- channel by transfection, the sialic acid content was increased by 80% (Figure 1) and Fucα1,3/4GlcNAc was decreased by 66% (Figure 4). Subsequently, when wtCFTR expression decreased significantly in the cell population, the sialic acid content and Fucα1,3/4GlcNAc returned to the levels of the non-transfected CF/T1 (parent) cells. The resultant cell division-dependent decline in wtCFTR expression was tightly correlated with the decrease of sialic acid and with the increase in Fucα1,3/4GlcNAc content as shown in Figures 6A and 6B.

To further confirm these results, another CF airway cell line (homozygous ΔF508) was examined and compared to a non-CF airway cell line. Again, the reciprocal relationship of NeuAc to Fucα1,3/4GlcNAc was noted. Most importantly, airway cells in primary culture showed a similar reciprocal relationship of fucosylation and sialylation (Table 1).

While others have shown that sialic acid is decreased in CF airway cells using indirect methods [3], the first direct chemical measurement of total sialic acid content in membrane glycoconjugates of CF airway cells is presented here. Since the original observation that sialylation was necessary to maintain circulating hepatic glycoproteins [30], there have been many reports of the importance of sialylation in cell interactions [31]. For example, the lack of sialylation can cause glycoproteins to become a toxin or a bacterial receptor. The binding of pathogenic bacteria such as *H. influenzae* to CF cells but not to non-CF cells may be a direct functional application of the potential role of fucosylation and sialylation in the pathology of CF [32].

It is believed that glycosyltransferases are localized in the Golgi in an organized manner which is representative of oligosaccharide biosynthesis. Thus ΔF508 CFTR may play a role in faulty compartmentalization of terminal glycosyltransferases resulting in the glycosylation phenotype observed in CF cells. Brefeldin A is known to disrupt the *cis*, *medial*, and *trans* Golgi causing an intercollation with the ER [33]. When CF airway cells were treated with Brefeldin A, the binding of the membrane glycoproteins to lentil lectin, a fucose binding lectin, was increased significantly [34] suggesting that a change in compartmentalization influenced fucosylation. Additional events [35–38] may influence the structure of the oligosaccharides of a cell membrane; however regardless of the mechanism, the link between CFTR mutations (ΔF508 in particular) and the CF glycosylation phenotype is clear. While speculative at present, it is possible that increased fucosyl residues and decreased sialyl residues, could facilitate bacterial colonization and neutrophil adherence in the CF airway. Confirmation of these hypotheses will provide the rationale to attempt blockade therapy with glycomimetics before bacterial colonization and inflammation are established in the CF airway.

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