N-Glycosylation Augmentation of the Cystic Fibrosis Epithelium Improves *Pseudomonas aeruginosa* Clearance

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Chronic lung colonization with *Pseudomonas aeruginosa* is anticipated in cystic fibrosis (CF). Abnormal terminal glycosylation has been implicated as a candidate for this condition. We previously reported a down-regulation of mannose-6-phosphate isomerase (MPI) for core N-glycan production in the CFTR-defective human cell line (IB3). We found a 40% decrease in N-glycosylation of IB3 cells compared with CFTR-corrected human cell line (S9), along with a threefold-lower surface attachment of *P. aeruginosa* strain, PAO1. There was a twofold increase in intracellular bacteria in S9 cells compared with IB3 cells. After a 4-hour clearance period, intracellular bacteria in IB3 cells increased twofold. Comparatively, a twofold decrease in intracellular bacteria occurred in S9 cells. Gene augmentation in IB3 cells with hMPI or hCFTR reversed these IB3 deficiencies. Mannose-6-phosphate can be produced from external mannose independent of MPI, and correction in the IB3 clearance deficiencies was observed when cultured in mannose-rich medium. An *in vivo* model for *P. aeruginosa* colonization in the upper airways revealed an increased bacterial burden in the trachea and oropharynx of nontherapeutic CF mice compared with mice treated either with an intratracheal delivery adeno-associated viral vector 5 expressing murine MPI, or a hypermannose water diet. Finally, a modest lung inflammatory response was observed in CF mice, and was partially corrected by both treatments. Augmenting N-glycosylation to attenuate colonization of *P. aeruginosa* in CF airways reveals a new therapeutic avenue for a hallmark disease condition in CF.

Keywords: bacterial clearance; cystic fibrosis; gene therapy; N-glycosylation

The cystic fibrosis (CF) lung environment has a predilection for the opportunistic pathogen *Pseudomonas aeruginosa*. Most patients with CF develop a persistent colonization with *P. aeruginosa* (1, 2). This chronic lung infection causes an influx of cellular infiltrates that leads to airway obstruction and the destruction of lung tissue, and possibly respiratory failure (3–5). The life expectancy of patients with CF has increased thanks to treatments for these symptoms; still, a high incidence of premature death occurs. Therefore, there is a constant effort to develop novel therapies to improve the lives of infected patients with CF. CF lung deficiencies in bacterial clearance have been the focus of many efforts to clear microorganisms from airways (6, 7). Although the primary focus of chronic lung infection has been mucociliary dysfunction (7–10), other studies have revealed a breach in the innate immune response, abnormal

CLINICAL RELEVANCE

This work brings to light a potential novel therapeutic option for treating bacterial colonization in the airways of cystic fibrosis patients.

glycosylation, and deficiencies in bacterial ingestion and clearance by airway epithelial cells (2, 9, 11–14).

Abnormal glycosylation within the airways is a common CF phenotype (15–18). Unfortunately, the consequences of abnormal glycosylation are still unclear, and have hindered the development of a definitive explanation. Although several *P. aeruginosa* receptors have been targeted as candidates for increased *P. aeruginosa* in the CF airway, disparity and controversy has emerged. Investigators have demonstrated an increase in binding of *P. aeruginosa* to CF airway epithelium from higher levels of GalNAc β 1–4Gal asialo-GM1 bacterial receptors (12, 18–20). Alternatively, others have reported that the CFTR protein binds and coordinates ingestion of *P. aeruginosa* (11, 21–23). In addition, others have revealed a reduced activity of acid sphingomyelinase (ASMase) and abnormal glycosylation within lipid rafts contributing to the clearance deficiency (24, 25). Given the numerous, diverse observations, it remains plausible that the reason for this severe pathology is not a mutually exclusive event, and that other candidates have not been included.

Core N-glycosylation deficiency is an alternative candidate being explored. A previous study revealed a decrease in mannose-6-phosphate isomerase (MPI) gene expression in cultured CF epithelial cells (26). In addition, a carbohydrate-deficient transferrin increase, which results from a defect in N-glycosylation, has been revealed in patients with CF (27). Carbohydrate-deficient transferrin increase has also been reported in the human glycosylation disorder 1b (28), which is linked to a homozygous defect in the *MPI* gene. As an alternative to *MPI* gene augmentation, mannose-6-phosphate can be supplemented by direct phosphorylation of external mannose by hexokinase (29).

We decided to explore N-glycosylation on the surface of airway epithelial cells as a candidate for CF clearance abnormality. Although this study does not focus on a specific host receptor, the data presented in this article suggest that core N-glycosylation augmentation by MPI correction or hypermannose treatment improves bacterial attachment, ingestion, and clearance. *In vivo* studies were performed using a *Pseudomonas*-infected CF mouse model in which the susceptibility to bacterial colonization is CFTR dependent (30). This model was used to assess a potential clearance deficiency in CFTR-deficient mice, and to determine the feasibility of correction using *MPI* gene augmentation or a hypermannose diet.

MATERIALS AND METHODS

See the online supplemental for complete details.

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Cell Lines

IB3 cells originated from a patient with CF, and S9 cells are derived from IB3 cells with a CFTR correction.

IB3 Cell Treatment

Plasmid transfection used a proviral overexpression cassette (31) with hMPI cDNA or functional $\Delta 264$ hCFTR mini-gene previously developed (32), pTR2-CB-hMPI and pTR2-CB- $\Delta 264$ hCFTR. Control plasmid was human survival motor neuron (SMN) cDNA not reported as abnormally regulated (26), pTR2-CB-SMN. Mannose treatment medium was 50 nM, 500 nM, 5 μ M, 50 μ M, 500 μ M, or 5 mM mannose.

CFTR siRNA in S9 Cells

CFTR knockdown used a CFTR siRNA plasmid (33), pTR2-U6-CFTRsiRNA. siRNA sequence was 5-GGAUACAGACAGCGC CUGGdTdT-3 and 5-CCAGGCGCUGUCUGUAUCCdTdT-3, previously used by Singh and colleagues (34). The Ambion (Austin, TX, USA) siRNA control was used.

Bacteria

P. aeruginosa strain PAO1 and a PAO1-GFP fluorescent strain (35) were used *in vitro*. A mucoid *P. aeruginosa* strain (36) was used *in vivo*.

In Vitro Membrane Glycosylation Profiling

FITC-conjugated lectins, concanavalin A (ConA) and lens culinaris lectin (LcH) for N-glycosylation, and helix pomatia for N-acetylgalactosamine O-glycosylation, were used at 50 μ g/ml followed by FACS analysis.

In Vitro Bacterial Binding

Procedures have been described previously (23). Infection media were PAO1 or mucoid strain (1 hr at 37°C). Host cell lysis buffer (50 mM tris-HCl, 150 mM NaCl, and 1% NP-40; Sigma, St. Louis, MO) was added to release bacteria, which was collected, diluted, cultured, and then grown overnight and counted.

In Vitro Bacterial Ingestion

The PAO1-GFP strain was used to infect host cells followed by FACS. The initial steps mimic *in vitro* binding followed by a gentamicin treatment to kill extracellular bacteria.

In Vitro Bacterial Clearance and Host Cell Death

Clearance and host cell death analysis mimic ingestion analysis with additions. After gentamicin treatment, host cells were incubated for 4 hours (clearance period). After clearance, host cells were stained with 7AAD (BD Sciences, Franklin Lakes, NJ) for host cell death analysis. Dual-stain FACS analysis was performed.

FACS Analysis

FITC-conjugated lectins, PAO1-GFP fluorescence, and 7AAD analysis used a BD FACScalibur flow cytometer. For dual staining, GFP⁺ cells were gated and 7AAD staining was analyzed within GFP⁺ cells.

Mouse Model

Cftr^{tm1Unc}-TgFABP-CFTR mice were used (37). The infection model was modified from a drinking water challenge previously developed (30). AAV vectors included AAV5-CB-GFP or AAV5-CB-mMPI. Vectors were delivered to the trachea (38) 2 weeks before infection. Hypermannose-treated mice were given 5 mg/ml mannose or glucose in drinking water for 4 weeks before infection.

Analysis of In Vivo Bacterial Load

Bacterial load was tracked by weekly oropharynx swabs. After diluting swabs, overnight growth occurred. Colonies were transferred to filter paper, and *P. aeruginosa* colonies were screened with Gaby-Hadley reagents (Sigma).

RESULTS

N-Glycosylation Deficiency in IB3 Cells

Profiling core surface N-glycosylation was analyzed to determine the effects of MPI transcript deficiency previously observed in IB3 cells. ConA and LcH were used to measure levels of N-glycosylation residues. Although ConA also binds α -mannosidic structure, it is widely used in measuring high-mannose, hybrid, and biantennary complex N-glycans, and is also used in N-glycan affinity chromatography (39–41). In addition, ConA is not known to measure O-linked glycans (42). ConA showed a 50% increase in S9 cells compared with IB3 cells, which was then reduced by CFTR siRNA treatment of S9 cells (Figures 1A and 1B). Similar results were revealed when using the LcH lectin instead of ConA, which attaches to the fucosylated core region of bi- and triantennary complex N-glycans (41, 42). This demonstrates that an N-glycosylation deficiency can be linked to a CFTR defect. Successful correction occurred by gene augmentation of hMPI in IB3 cells, achieving a 40% increase in N-glycosylation. The results were similar to CFTR correction. Growing IB3 cells in hypermannose media also significantly increased N-glycosylation. No significant difference in O-linked glycosylation between IB3 and S9 cells was observed using the helix pomatia lectin (data not shown) (41–43).

Figure 1. Percent difference of attached FITC-conjugated lectins, lens culinaris lectin (LcH) and concanavalin A (ConA) normalized to IB3 cells. IB3 treatments included pTR2-CB- $\Delta 264$ hCFTR, pTR2-CB-hMPI, pTR2-CB-SMN (control), or hypermannose media at 500- μ M concentration and S9 cells treated with pTR2-U6-CFTRsiRNA or siRNA negative control. (A) Data collected from FITC-ConA and (B) data collected using FITC-LcA/LcH. Statistical analysis was done using a two-tailed, paired *t* test (**P* < 0.05 analyzed against IB3; #*P* < 0.05 analyzed against S9). Data were collected from three separate trials.

Bacterial Ingestion Deficiency *In Vitro*

We observed a *cfr*-dependent bacterial attachment deficiency that was corrected with MPI gene augmentation and mannose treatment (see the online supplement), and assessed the consequences in bacterial ingestion. S9 cells showed twofold-higher intracellular PAO1-GFP when compared with IB3 cells. Furthermore, CFTR silencing in S9 cells reduced ingestion, with bacterial loads reflecting the results in IB3 cells (Figure 2A). This reduced bacterial ingestion phenotype in IB3 cells was complemented by gene augmentation using pTR2-CB-hMPI or pTR2-CB-Δ264 hCFTR, which achieved an 80% increase of intracellular bacteria (Figure 2A). In addition, mannose-rich medium resulted in a maximum of 30% increase at 5-μM concentration (Figure 2B).

Bacterial Clearance and Cell Death Deficiency

The ability of these IB3 cells to clear intracellular PAO1 adequately was tested. In IB3 cells, the amount of intracellular bacteria doubled after a 4-hour clearance period. Conversely, intracellular bacteria after the same clearance period was reduced to one-half in the S9 cells. CFTR gene knockdown in S9 cells reversed this clearance, which conferred an IB3-like phenotype. Furthermore, hMPI or hCFTR gene correction resulted in improved clearance (Figure 3A). From mannose treatment, intracellular PAO1-GFP at 5 μM mannose remained

unchanged after clearance. At all other mannose concentrations, except 50 nM and 500 mM, there was only a marginal increase compared with the twofold increase seen in untreated IB3 cells (Figure 3B).

In vitro host cell death as a method for clearance was analyzed in the IB3 cells. There was a twofold increase in cell death using S9 cells compared with IB3 cells, and S9 CFTR siRNA reduced the cell death response after the clearance period. This IB3 deficiency was corrected by hMPI or hCFTR gene transfer, resulting in a 50% increase in cell death (Figure 3C). Culturing IB3 cells in mannose-rich medium revealed variable correction of the cell death defect, which peaked at 50 and 500 μM (Figure 3D).

Tracking Bacterial Load in CFTR-Deficient Mouse Airways

A mucoid strain isolated from a patient with CF (36) was used to infect the airways of CF mice. The aggregation property of mucoid strains can potentially improve the chances of establishing colonization (44). The persistence of this bacterial infection was tracked during the infection period and for 6 weeks afterward. In both nontherapeutic groups (AAV5-GFP or hyperglucose) of CF mice, the bacterial infection in the upper airway persisted for the length of the study. Comparatively, in treated mice (AAV5-MPI or hypermannose), an acute infection occurred for the first 2 weeks, but then dissipated to nearly undetectable levels (Figures 4A and 4B).

Tracking the Health of Mice after Bacterial Exposure

The systemic effects of the bacterial infection in the *Cfr^{tm1Unc}*-TgFABP-CFTR mice were tracked by monitoring the weight of the mice. In all groups (treated and controls), there was an acute weight loss during infection. Although all groups showed a significant improvement in weight lost during the 6 weeks after the infection, only the treatment groups (AAV5-MPI and hypermannose) had an overall increase in weight. Conversely, the nontherapeutic mice still had significant weight loss at the end of the study (Figures 4C and 4D).

Measuring Bacterial Load in Trachea and Lungs

Bacterial load was measured in trachea homogenates and in lung homogenates. There was a twofold increase in bacterial load in the trachea from the nontherapeutic CF mice (AAV5-GFP or hyperglucose) compared with their respective treatment groups (AAV5-mMPI or hypermannose) (Figure 5). In addition, 70% of the lung homogenates from nontherapeutic mice had detectable levels of *P. aeruginosa* compared with 30% in treated mice (data not shown).

Lung Pathology

Persistent bacterial infection in the CF lung results in chronic inflammation. The observations depicted in Figure 6 demonstrate an increase in multifocal, moderate inflammation, with more cases of bronchiectasis in nontherapeutic mice compared with the treatment groups. In addition, 50% of the treated mice presented with no lung inflammation compared with only 20% in the nontherapeutic mice (Table 1).

DISCUSSION

Previous studies have been successful in improving bacterial clearance in the CF airway, yet it is still unclear why *P. aeruginosa* plagues the CF airway. In addition, efforts to pinpoint the glycosylation phenotypes linked to deficient clearance of this pathogen have not yielded a clear understanding of this condition.

Figure 2. Difference in percentage of green fluorescent protein (GFP) fluorescence of host cells due to internalized GFP plus PAO1 strain shown as a percentage change compared with untreated IB3 cells. GFP fluorescence from ingested PAO1-GFP was detected by FACS analysis. (A) IB3 cells were treated with pTR2-CB-Δ264 hCFTR, pTR2-CB-hMPI, or pTR2-CB-SMN (control), and S9 cells were treated with pTR2-U6-CFTRsiRNA or siRNA negative control. (B) IB3 cells were treated with the indicated amount of mannose. Statistical analysis was done using a two-tailed, paired *t* test (**P* < 0.05 analyzed against IB3; #*P* < 0.05 analyzed against S9). Data were collected from three separate trials.

Figure 3. Change of intracellular PAO1-GFP from host cells after a 4-hour clearance period compared with initial internalized bacterial levels after infection, ingestion, and gentamicin treatment to kill extracellular PAO1-GFP, and the difference in host cell death from treated and untreated host cells after the same clearance period. (A and C) IB3 cells were treated with pTR2-CB-Δ264 hCFTR, pTR2-CB-hMPI, or pTR2-CB-SMN (control), and S9 cells were treated with pTR2-U6-CFTRsiRNA or siRNA negative control. (B and D) IB3 cells were cultured in indicated concentrations of mannose rich media. Statistical analysis was done using a two-tailed, paired *t* test (**P* < 0.05 analyzed against IB3; #*P* < 0.05 analyzed against S9). Data were collected from three separate trials.

This study did not focus on a specific host receptor. Still, efforts to identify a host receptor, which may be altered in CF, have been made. Although some have reported the bacterial receptor asialo-GM1 glycosylation residue as a contributor to the increased *P. aeruginosa* (12, 19), disparate evidence has suggested otherwise (45). Moreover, this would not explain how *P. aeruginosa* supplants *Staphylococcus aureus* in patients with CF given that *S. aureus* has shown

higher affinity to asialo-GM1 (46). The CFTR protein has also emerged as a potential receptor for *P. aeruginosa* binding directly to the airway epithelial cells (11, 21, 47) and subsequent internalization. Our ability to enhance bacterial clearance in IB3 cells independent of *cftr* correction with MPI gene augmentation or a mannose treatment would suggest that this corrective effect does not include attachment to the CFTR protein.

Figure 4. Total mucoid *Pseudomonas aeruginosa* CFU collected from weekly oropharynx culture swabs from *cftr* mice that were infected for 2 weeks with the mucoid strain via drinking water and treated with (A) AAV5-CB-mMPI viral vector, with AAV5-CB-GFP as control (*n* = 6) or (B) hypermannose diet of 5 mg/ml in drinking water with hyperglucose diet as control (*n* = 4). Weekly percentage changes in weight compared with Week 0 weight of infected mice treated with (C) AAV5-CB-mMPI, with AAV5-CB-GFP as control (*n* = 6), or (D) hypermannose diet of 5 mg/ml in drinking water with hyperglucose diet as control (*n* = 4). *P* < 0.05 using one-way ANOVA for repeat sampling between groups.

Figure 5. Cultured CFU from trachea homogenates of *cfr* mice infected with mucoid strain and treated with (A) AAV5-CB-mMPI viral vector, with AAV5-CB-GFP as a nontherapeutic control ($n = 6$) or (B) hypermannose diet of 5 mg/ml in drinking water, with hyperglucose diet as a non-therapeutic control ($n = 4$). Statistical analysis was done using a two-tailed, paired *t* test.

Yu and colleagues (24) showed that reduced ASMase activity in IB3 cells decreased internalized *P. aeruginosa*. In addition, IB3 cells presented with a decreased apoptosis response. This deficiency in bacterial clearance by internalization and host cell death can be corrected, not only by CFTR correction, but also by treatment with bacterial sphingomyelinase from *S. aureus*. ASMase deficiency is associated with a severe defect in functional ASMase. Site-directed mutagenesis of ASMase demonstrated that the development of mature ASMase protein was

hindered by the removal of N-glycosylation sites (48). Taken together, these studies suggest that N-glycosylation abnormalities on ASMase in CF epithelial cells deficient in MPI expression could contribute to the bacterial clearance deficiency.

Our *in vitro* data has revealed that there is a strong correlation between the N-glycosylation deficiency in IB3 cells and improvement in bacterial ingestion and clearance by host cellular death. These IB3 deficiencies in bacterial binding, bacterial ingestion, bacterial clearance, and host cellular death can be partially corrected by treatment with pTR2-CB-MPI, or by growing CFTR-deficient host cells in mannose-rich media, both of which increased the levels of N-glycosylation residues on the surface of IB3 cells. It should be mentioned that this response was not strain specific, because the mucoid strain showed similar results to the PAO1 strain (data not shown).

The *in vivo* methods were complicated by the absence of robust colonization in CF mice. Infecting *Cfr^{Δm1Unc}-TgFABP*-CFTR mice with bacteria-tainted water established only a modest upper airway infection. This *cfr*-dependent upper airway colonization was achieved through the use of a *P. aeruginosa* mucoid strain; therefore, we are unable to suggest that this infection can be achieved using alternative strains. Still, it should be mentioned that Coleman and colleagues (30) demonstrated a range of success using different *P. aeruginosa* strains with mucoid and nonmucoid characteristics. CFTR gene transfer was precluded from the *in vivo* studies, because the overall goal of the study was to demonstrate that N-glycan augmentation improves bacterial clearance independent of CFTR correction.

Unfortunately, the results from the mouse model study were not as profound as those from the *in vitro* study. Nevertheless, a significant increase in bacterial load occurred in the upper airway of untreated mice, along with significant weight loss compared with MPI gene augmentation or the noninvasive high-mannose diet. To date, no CFTR-deficient mouse model has been ideal for developing a chronic infection. Therefore, any potential therapeutic benefit that can be achieved from proposed treatments will unlikely be dramatic unless a more severe CFTR-defective mouse phenotype can be established.

Finally, we cannot completely rule out the possibility that the mannose receptor is involved in the *in vivo* corrective response. Mannose receptors contain an N-glycan-rich domain (49), and efforts to correct N-glycosylation deficiency can potentially influence the innate immune response to the *P. aeruginosa* challenge. Typically, rAAV transduction of monocytes *in vivo* is

Figure 6. Images of inflammatory conditions revealed by hematoxylin and eosin staining of lung sections from *cfr* mice infected for 2 weeks with the mucoid strain through the drinking water. Shown are cases, in nontherapeutic mice, of (A) bronchiectasis (star) and (B and C) multifocal, moderate inflammation (within circle [B]) and, in therapeutic mice, (D) healthy airways and (E and F) noninflammatory conditions.

TABLE 1. INFLAMMATION SCORES FROM LUNG SECTIONS OF INFECTED WHITSETT MICE

	AAV5-mMPI Treated (%)	Mannose Treated (%)	AAV5-GFP Control (%)	Glucose Control (%)
Bronchiectasis	17	0	33	25
None (0)	50	50	17	25
Mild focal (1)	34	25	33	25
Moderate multifocal (2)	17	25	50	50
Severe multifocal (3)	0	0	0	0
Necrotic (4)	0	0	0	0

Definition of abbreviations: AAV, adeno-associated virus; GFP, green fluorescent protein; MPI, mannose-6-phosphate isomerase.

Data from a pathology report submitted by a trained pathologist who ranked the severity of inflammation from lung sections with H&E staining in a blinded fashion.

negligible, except for AAV5. Therefore, AAV5 MPI gene augmentation for improved gene transfer to the lung epithelium could potentially impact mannose receptors on monocytes.

A potential role of MPI expression in bacterial clearance has been demonstrated. Nonetheless, efforts to pinpoint why MPI down-regulation occurs in IB3 cells, as well as investigating the effects on mucin secretions from IB3 cells via an air-liquid interface cell culture study, need to be explored. Even in the absence of such answers, MPI gene augmentation and a non-invasive hypermannose diet showed significant improvement in bacterial clearance and reduced levels of detrimental lung inflammation. Hypermannose diet is already used as a clinical treatment for the glycosylation disorder 1b caused by a homozygous mutation of MPI (29), and the data presented from this study demonstrate that N-glycan augmentation has therapeutic potential for the bacterial clearance deficiency in CF.

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