Quantitative Proteomics Reveals an Altered Cystic Fibrosis *In Vitro* Bronchial Epithelial Secretome

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Abstract

Alterations in epithelial secretions and mucociliary clearance contribute to chronic bacterial infection in cystic fibrosis (CF) lung disease, but whether CF lungs are unchanged in the absence of infection remains controversial. A proteomic comparison of airway secretions from subjects with CF and control subjects shows alterations in key biological processes, including immune response and proteolytic activity, but it is unclear if these are due to mutant CF transmembrane conductance regulator (CFTR) and/or chronic infection. We hypothesized that the CF lung apical secretome is altered under constitutive conditions in the absence of inflammatory cells and pathogens. To test this, we performed quantitative proteomics of *in vitro* apical secretions from air–liquid interface cultures of three life-extended CF ($\Delta F508/\Delta F508$) and three non-CF human bronchial epithelial cells after labeling of CF cells by stable isotope labeling with amino acids in cell culture. Mass spectrometry analysis identified and quantitated 666 proteins across samples, of which 70 exhibited differential enrichment or depletion in CF secretions (± 1.5 -fold change; $P < 0.05$). The key molecular functions were innate immunity (24%), cytoskeleton/extracellular matrix organization (24%), and protease/antiprotease activity (17%). Oxidative proteins and classical complement pathway proteins that are altered in CF secretions *in vivo* were not altered *in vitro*. Specific differentially increased proteins—

MUC5AC and MUC5B mucins, fibronectin, and matrix metalloproteinase-9—were validated by antibody-based assays. Overall, the *in vitro* CF secretome data are indicative of a constitutive airway epithelium with altered innate immunity, suggesting that downstream consequences of mutant CFTR set the stage for chronic inflammation and infection in CF airways.

Keywords: cystic fibrosis; bronchial epithelial secretions; quantitative proteomics; secretome; innate immunity

Clinical Relevance

This is the first report of quantitative proteomic analysis of cystic fibrosis (CF) and non-CF bronchial secretions in cell culture, which allowed for a highly quantitative and unbiased analysis of CF secretions in the absence of active infection and inflammation. Quantitative proteomics revealed altered immune and injury repair proteins in CF secretions, indicating that patients with CF may benefit from early treatment that prevents hyperinflammation and amplifies the host defense response before bacterial infection and chronic lung disease is established.

Cystic fibrosis (CF), the most common lethal autosomal recessive genetic disorder among the white population, is caused by mutations in the CF transmembrane

conductance regulator (*CFTR*) gene, with the most common mutation, $\Delta F508$ CFTR, occurring in 70% of patients with CF. CFTR, a member of the ATP-binding

cassette transporter superfamily and a cyclic adenosine monophosphate-dependent chloride channel and channel regulator in epithelial tissues (1), is also permeable

(Received in original form July 3, 2014; accepted in final form February 5, 2015)

This work was supported by a Children's National Board of Visitor's Grant (M.C.R.) and Cystic Fibrosis Foundation Rose 12PO, by Career Development Awards from 1K12 HL090020 and 5K12 HD001399–12 (D.K.P.), and by National Institutes of Health core grants 2R24HD050846–06 (National Center for Medical Rehabilitation Research) and UL1TR000075 (National Institutes of Health/National Center for Advancing Translational Sciences). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Center for Advancing Translational Sciences or the National Institutes of Health.

Author Contributions: Conception and design: M.C.R., J.R.P.-H., and K.J.B. Performing experiments: J.R.P.-H., K.J.B., D.K.P., A.T., and L.M.G. Analysis and interpretation: M.C.R., J.R.P.-H., K.J.B., D.K.P., A.T., L.M.G., and X.W. Drafting the manuscript for important intellectual content: M.C.R., J.R.P.-H., K.J.B., D.K.P., X.W.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 53, Iss 1, pp 22–32, Jul 2015

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Originally Published in Press as DOI: 10.1165/rcmb.2014-0256RC on February 18, 2015

Internet address: www.atsjournals.org

to iodide and thiocyanate (2), reduced glutathione (3), and bicarbonate (4). CFTR is expressed in many organs (5), but its functional loss in lung epithelial cells leads to pulmonary disease, which is characterized by inflammation, mucus obstruction, and chronic bacterial infection that accounts for CF morbidity and mortality (6, 7).

Defective functions of CFTR in lung epithelial cells predictably underlie the increased susceptibility of patients with CF to chronic airway infections (8). Inflammation is observed in the neonate lungs of humans (9, 10), pigs (11, 12), and ferrets (13) with CF, suggesting that early hyperinflammation contributes to the impaired responses of CF airways to bacterial infection. *In vivo*, significantly less sterile bronchoalveolar lavage fluid (BALF) is collected from the airways of newborn CF pigs and ferrets compared with their wild-type (wt) counterparts, indicating a defect in the ability to clear bacteria (14, 15). *Ex vivo*, airway surface liquid secretions from CF bronchial xenografts exhibit decreased bactericidal activity compared with non-CF secretions (16). *In vitro*, CF primary human bronchial epithelial (HBE) cells differentiated at air-liquid interface (ALI) produce secretions with decreased bactericidal activity in the absence of inflammatory or immune cells (17, 18).

A major host response to airway bacterial challenge is apical secretion of proteins (19). Alterations of protein components or levels in CF lung epithelial secretions may be a downstream consequence of mutations in the *CFTR* gene, resulting in a failure of CF lungs to appropriately respond to infection and inflammation. Proteomic analyses of lung mucus and BALF from patients with CF and patients without CF show alterations in levels of proteins related to defense response and immunity, the complement pathway, cellular proliferation and adhesion, wound repair, stress response, apoptosis, proteolysis, and decreased surfactant protein in CF secretions (20, 21). However, secretions from patients with CF typically contain inflammatory cells and bacteria, so alterations in some protein levels may be more related to inflammation and infection than to mutant CFTR.

HBE cells differentiated at ALI mimic native airway epithelial structure and function (22, 23). This model system, which is free of pathogens and inflammatory cells, is useful for studying the role of the airway

epithelium in lung biology, especially in relation to CF pathophysiology (17, 18, 24, 25), and to evaluate the responses of CF epithelium to corrector drugs (26). The composition of proteins secreted by normal differentiated HBE cells is similar to that found in induced mucus of healthy individuals (27). We hypothesized that mutant CFTR results in altered protein levels in the CF airway epithelial secretome under constitutive conditions at ALI in the absence of infection and inflammatory cells. To test this, we used stable isotope labeling with amino acids in cell culture (SILAC), a highly accurate and quantitative mass spectrometry (MS)-based proteomics technique (28), and three CF ($\Delta F508/\Delta F508$) and three non-CF life-extended HBE cell lines that could be passaged several times (29) to ensure >98% amino acid incorporation for quantitative secretome analyses.

Materials and Methods

Procurement of CF and Non-CF Cell Lines and Primary Cells

Life-extended HBE cell lines, three non-CF (UNCN1T, UNCN2T, and UNCN3T) and three CF (UNCCF1T, UNCCF2T, and UNCCF3T), were a generous gift from Scott H. Randell (University of North Carolina) and have been previously described (29). They express CFTR, and the non-CF cells exhibit cyclic adenosine monophosphate-induced chloride current upon forskolin stimulation (29). Immunoprecipitation and immunoblot techniques (30) showed that wt and $\Delta F508$ CFTR were expressed in life-extended cells grown in our lab (data not shown). Normal primary HBE cells for proteomic comparison studies were purchased from Lonza (Walkersville, MD).

Metabolic Labeling of Life-Extended CF and Non-CF HBE Cells

Before seeding at ALI, passage 12 UNCCF cells were labeled by SILAC for two passages (28). Cells were proliferated in bronchial epithelial growth medium (Lonza) containing “heavy” (“labeled”) $^{13}\text{C}_6$ -Arg (2 mM) and $^{13}\text{C}_6$, $^{15}\text{N}_2$ -Lys (0.2 mM) (Cambridge Isotopes, Andover, MA). The non-CF UNC cell lines were proliferated in bronchial epithelial growth medium containing the abundant “light” (“unlabeled”) $^{12}\text{C}_6$ -Arg (2 mM) and

$^{12}\text{C}_6$, $^{14}\text{N}_2$ -Lys (0.2 mM) (Sigma-Aldrich, St. Louis, MO). Incorporation of heavy amino acid isotopes in secreted proteins was verified by MS to be >98%.

Establishment of ALI Cell Cultures

SILAC-labeled passage 14 UNCCF and unlabeled, in the presence of heavy or light media, UNC non-CF cells were differentiated to an *in vitro* epithelium as described in the online supplement.

Collection of ALI Apical Secretions

Secretions were collected as described by Kesimer and colleagues (27). Apical surfaces were incubated with 1 ml $1\times$ PBS twice for 30 minutes each, and washes were pooled for each cell line. Secretions were subjected to centrifugation ($300\times g$ for 5 min at 4°C); supernatants were transferred to clean tubes and stored at -80°C .

Sample Preparation and Quantitative Secretome Analysis

Samples were processed according to well-established protocols as described in the online supplement.

Identification of Altered Proteins in CF Secretions

Proteins with altered levels in CF secretions were determined by filtering quantitated proteins from five sets of labeled CF/unlabeled non-CF (N) peptides combinations as described in the online supplement using the following parameters: at least two unique peptides, a difference in the $\log_2$ ratio of CF/N greater than ± 0.58 (i.e., a ± 1.5 -fold change), and statistical significance ($P < 0.05$) in three out of five sets. For each sample combination, a significance of ± 1.5 -fold change for a protein was determined with a one-sample parametric *t* test that took into account normalized-scan count and standard deviation.

Protein Classification

Protein annotations were acquired using the keyword and gene ontology terms in the Uniprot Protein Knowledgebase (<http://www.uniprot.org>). Proteins quantified in SILAC-labeled CF and non-CF secretions were uploaded to Panther Database (31) (<http://www.pantherdb.org>) for categorization of biological processes. Secreted proteins were identified by Secretome P (32) and Signal P (33) databases and comparison to proteins

identified in “The Human Secretome Atlas Initiative” (34).

Validation of Secreted Proteins by Antibody-Based Techniques

Specific proteins were validated by Western blot or ELISA using independent sets of secretions from CF and non-CF life-extended or primary HBE cell cultures (described in Table E3 in the online supplement).

Results

Comparison of Secretomes from Normal Differentiated HBE Primary Cells and Cell Lines

To assess the validity of using life-extended HBE cell lines to identify differentially secreted proteins, we compared secretions from primary normal differentiated HBE cells (35) with secretions from life-extended non-CF differentiated HBE cell lines (both cohorts analyzed in the Children’s National Proteomic Core Facility) regarding categorization of proteins contributing to biological processes (Figure E1). Percentages are based on the number of proteins/total proteins identified that contribute to specific categories (out of 100%) for each cohort; many proteins appear in more than one category. The biological processes with the highest number of proteins were metabolic, cellular, cell communication, transport, and immune system. There was a $\leq 3.3\%$ difference for all categories, with the exception of metabolic processes, for which the life-extended secreted proteins showed a 6.2% enrichment, demonstrating that the biological processes of proteins secreted by primary non-CF HBE cells and by life-extended non-CF HBE cell lines were similar but not identical. This type of comparison has previously shown that the protein composition of *in vitro* ALI secretions and induced sputum are similar, with no more than a 6% difference observed in proteins contributing to each biological process (27).

CF Life-Extended HBE Cell Lines for Accurate Quantitative Proteomics

A quantitative comparison of secreted proteins requires complete isotope labeling of cells, which typically requires a minimum of two to three passages (36). Because primary HBE cells can generally be passaged only three times before becoming

senescent (22, 23), life-extended CF and non-CF HBE cells (29) were used in conjunction with SILAC. Secretions from CF HBE cells were shown by MS to be fully labeled ($>98\%$) with $^{13}\text{C}_6$ -arginine and $^{13}\text{C}_6$, $^{15}\text{N}_2$ -lysine after passing twice on plastic, followed by plating on collagen-coated Transwell inserts with 2 days of proliferation (submerged media) and 3 to 4 weeks of differentiation at ALI in the presence of media with heavy isotopes (data not shown).

Characterization of Proteins in ALI Apical Secretions from Normal and CF HBE Cell Lines

MS analysis confidently identified and quantitated 666 proteins with at least two unique peptides across five combinations of secretions from life-extended CF and non-CF HBE ALI cultures (Day 19, CF3T:N1T; Day 21, CF2T:N1T; Day 21, CF3T:N3T; Day 27 CF2T:N1T; Day 27 CF3T:N3T). N2T secretions were not included in the proteomic data analysis as the N2T secretome profile more closely resembled CF secretomes than non-CF secretomes. The N2T non-CF donor was an 18-pack-year smoker. N2T cells exhibit decreased CFTR expression and function (29), and cigarette smoke decreases CFTR expression/function and increases mucus dehydration (37, 38).

Subcellular localization analysis classified 59.5% of the apical proteins as secreted (32, 33); 5.9% of proteins were previously identified in HBE exosomes (39). Twenty-eight percent of the apical proteins were classified as cellular (Figure E2A). Because recent publications show that numerous proteins previously not categorized as secreted are consistently

present in *in vivo* and *in vitro* secretions (34, 40), we compared our list of cellular proteins classified by current data bases with those published in *The Human Secretome Atlas Initiative* (34), resulting in 55% of the cellular proteins being reclassified as secreted and bringing the total percentage of secreted proteins to 81.7% (Figure E2B).

Many of the most abundant proteins in UNC life-extended CF and non-CF HBE ALI apical secretions (Table E1) were also among the most abundant proteins in normal primary HBE ALI secretions (27, 35). A scatter plot of the fold change of CF (labeled peptides)/non-CF (N; unlabeled peptides) ($\log_2 \text{CF/N}$) showed that the relative expression of most proteins was unchanged (ratio of $\leq \pm 1.5$ or $\log_2 \leq \pm 0.58$) (Figure 1).

Quantitative Proteomic Comparison of CF versus Non-CF *In Vitro* HBE ALI Apical Secretions

Of the 666 identified and quantitated proteins, 70 were significant ($P < 0.05$) with ± 1.5 -fold change, 56 were increased, and 14 were decreased in CF compared with non-CF apical secretions (Table E2). Molecular function categorization by Uniprot of the 70 differentially abundant proteins in CF versus non-CF apical secretions showed the major functions to be innate immunity (24%), protease/antiprotease activity (17%), cytoskeleton structure (14%), and extracellular matrix (ECM) organization (10%), as well as energy metabolism (12%) and ion-dependent activity (10%). Many proteins appear in more than one category (Figure 2).

Figure 1. Relative protein expression. Scatter plot of the ratio of labeled cystic fibrosis (CF)/unlabeled non-CF (N) peptides ($\log_2 \text{CF/N}$) for each of the 666 proteins quantified in CF and non-CF air-liquid interface (ALI) apical secretions. The ratio of labeled (CF) to unlabeled (non-CF) peptides for each protein was determined ($\log_2 \text{CF/N}$). Although CF/N was unchanged for most proteins, 70 proteins had a $\log_2 \text{CF/N}$ of ± 0.58 (± 1.5 -fold change), with a P value of < 0.05 .

Figure 2. Classification of altered proteins in CF secretions by molecular function. The list of 70 proteins with altered levels in CF secretions (Table E2) was uploaded to Uniprot.org for classification of molecular function. The predominant molecular functions of differentially enriched or depleted proteins in CF apical secretions are innate immunity (24%), protease/antiprotease activity (17%), cytoskeleton structure (14%), energy metabolism (12%), extracellular matrix (ECM) organization (10%), and ion-dependent activity (10%).

The 20 proteins classified as innate immunity proteins (including those in the complement pathway), proteinases, antiproteinases and ECM proteins that exhibited altered abundance are listed in Table 1. Mucins (MUC5AC, MUC5B, and MUC16), fibronectin, and calcium-binding proteins (S100A2 and S100P) were increased in CF secretions, whereas the antimicrobial protein BPIFA1/PLUNC, uteroglobin-related protein 2 (UGRP2), and complement pathway proteins (clusterin, complement factor I and complement C4A/B) were decreased. Thirteen proteinases or proteinase inhibitors exhibited altered abundance. Eight of the nine proteinases and three of the four proteinase inhibitors had increased levels in CF secretions, suggesting a protease/antiprotease imbalance in the endogenous CF secretome. In addition, six ECM proteins (fibronectin; perlecan; galectin-3; and laminin subunits $\alpha 3$, $\beta 3$, and $\gamma 2$) were increased in CF secretions. Taken together, the proteomics data indicate an imbalance in the CF secretome, under constitutive conditions, of innate immunity proteins, proteases/antiproteases, and ECM proteins.

Validation of Proteins with Altered Abundance in CF Secretions

A subset of innate immunity and ECM proteins and proteinases was evaluated using antibody-based assays. Secretions from (1) independent sets of differentiated

life-extended CF ($n = 3$) and non-CF ($n = 3$) cells and (2) primary CF ($n = 4$) and non-CF cells ($n = 3$ or 4) at Days 27 to 29 ALI were evaluated. For primary cell secretions, a summary of the days at ALI on which secretions were collected, samples used for specific immunoassays, and the smoking status of donors are presented in Table E3.

Secretory Mucins. We first determined whether increased levels of mature MUC5AC and MUC5B mucins would be observed in secretions from independent sets of CF and non-CF life-extended cells using Western blot analyses. Densitometry analysis showed a 2.5-fold increase of MUC5AC mucin in the three CF secretions relative to N1T and N3T. This increase was not statistically significant due to high levels of MUC5AC in secretions from the non-CF smoker N2T (Figure 3A). In contrast, densitometry analysis of MUC5B mucin showed a significant 3-fold increase in CF life-extended secretions ($P = 0.03$) (Figure 3B).

To test whether the increased mucin levels in the CF secretome resulted from higher transcript levels in the life-extended CF cells, the mRNA abundance of MUC5AC and of MUC5B was determined by real-time PCR. Data showed that MUC5AC and MUC5B mRNA levels were statistically increased 3.0- and 2.3-fold in CF cell lines compared with N1T and N3T (Figure 3C), consistent with the secretome and immunochemical data.

Immunoblot analysis of apical secretions from primary CF and non-CF HBE cultures at Days 27 to 29 ALI showed MUC5AC to be significantly increased in four out of four samples from individuals with CF compared with samples from three individuals without CF ($P = 0.03$) (Figure 4A). MUC5B levels were increased in apical secretions from three out of four primary CF HBE ALI cultures, compared with secretions from three primary non-CF HBE ALI cultures (Figure 4B). The increase was not significant, suggesting variability of MUC5B expression in individuals with CF. Secretions collected from primary cells at Day 21 did not exhibit differential mucin protein levels (data not shown).

Fibronectin and matrix

metalloprotease-9. Fibronectin and matrix metalloprotease (MMP)-9 were evaluated as examples of ECM proteins and proteases, respectively. ELISA analysis of fibronectin levels in apical secretions from life-extended ALI cultures demonstrated a statistically significant ($P = 0.04$) 3-fold increase in CF secretions, with fibronectin levels averaging 99 ± 24 ng/ml in CF secretions and 35 ± 29 ng/ml in non-CF secretions (Figure 5A). Fibronectin levels from primary ALI cultures also demonstrated a statistically significant ($P = 0.01$) 4-fold increase in CF secretions, with fibronectin levels averaging 70 ± 31 ng/ml in CF secretions and 17 ± 5 ng/ml in non-CF secretions (Figure 5B).

ELISA analysis of MMP-9 levels in apical secretions from life-extended HBE ALI cultures demonstrated a trend toward increased MMP-9 in CF secretions (Figure 6A), which was not significant due to high levels of MMP-9 secretions from the non-CF smoker N2T cell line. Analysis of MMP-9 in apical secretions from primary HBE ALI cultures also demonstrated a trend toward increased levels in CF secretions (Figure 6B); this was not significant, likely reflecting the inherent variability of protein expression in primary cells from different individuals (26) and/or the smoking history of non-CF individuals (Table E3).

Complement. For the complement studies, we evaluated C3 activation because total complement C3 levels were unchanged in SILAC/MS analysis of CF versus non-CF apical secretions, whereas levels of C3 regulators were altered (Table 1). Complement factor B, a key activator of C3 in the alternate complement pathway, was increased, whereas complement factor I and clusterin (inhibitors of the complement

Table 1. Altered Innate Immunity, Protease/Antiprotease, and Extracellular Matrix Proteins in Cystic Fibrosis Secretions*

Molecular Function or Protein Name	Uniprot ID	Fold-Change (CF/N)	SEM	Log ₂ CF/N	Total Peptides
Innate Immunity					
Mucin-16	Q8WXI7	8.91	1.07	3.16	239
Mucin-5AC	P98088	8.14	7.19	3.03	202
Carcinoembryonic antigen-related cell adhesion molecule 5	P06731	5.07	2.89	2.34	48
Histone H4	P62805	4.82	1.94	2.27	52
Serum amyloid A	P02735	4.43	2.35	2.15	37
Mucin-5B	Q9HC84	4.35	2.56	2.12	2,090
Protein S100-P	P25815	4.02	0.65	2.01	63
Fibronectin	P02751	3.82	2.45	1.93	386
Protein S100-A2	P29034	3.46	2.69	1.79	31
Histone H2B	O60814	3.27	2.02	1.71	41
Galectin-3	P17931	2.25	0.32	1.17	69
Heat shock cognate 71-kD protein	P11142	1.94	0.49	0.95	108
Heat shock 70-kD protein 1	P08107	1.84	0.28	0.88	44
Protein Plunc	Q9NP55	0.58	0.06	−0.79	1,216
Uteroglobin-related protein 2	Q96QR1	0.35	0.27	−1.5	49
Prolactin-inducible protein	P12273	0.31	0.04	−1.71	103
Complement pathway					
Complement factor B	P00751	1.83	0.33	0.87	1,709
Complement factor I	P05156	0.48	0.12	−1.06	73
Complement C4-A/B	P0C0L4	0.27	0.3	−1.88	81
Clusterin	P10909	0.27	0.22	−1.92	206
Extracellular matrix organization					
Fibronectin	P02751	3.82	2.45	1.93	386
Laminin subunit β-3	Q13751	3.82	1.66	1.93	228
Laminin subunit α-3	Q16787	3.74	1.65	1.9	192
Perlecan	P98160	3.15	1.47	1.65	256
Laminin subunit γ-2	Q13753	2.66	0.98	1.41	142
Galectin-3	P17931	2.25	0.32	1.17	69
Gelsolin	P06396	0.55	0.05	−0.86	1,367
Fibulin-2	P98095	0.37	0.2	−1.43	65
Proteases					
Tubulointerstitial nephritis antigen-like	Q9GZM7	3.58	0.08	1.84	27
Matrix metalloproteinase-9	P14780	3.18	0.83	1.67	32
Cathepsin B	P07858	2.54	0.35	1.34	122
Calpain small subunit 1	P04632	2.07	0.95	1.05	48
Complement factor B	P00751	1.83	0.33	0.87	1,709
Cathepsin D	P07339	1.79	0.18	0.84	406
Gelsolin	P06396	0.55	0.05	−0.86	1,367
Serine proteases					
Kallikrein-11	Q9UBX7	2.49	0.21	1.31	39
Kallikrein-10	O43240	1.96	0.51	0.97	37
Serine protease inhibitors					
Serpin B3	P29508	4.42	2.08	2.15	503
Serpin B4	P48594	3	3.07	1.58	219
Plasminogen activator inhibitor 1	P05121	2.4	0.43	1.26	27
Serpin B5	P36952	0.4	0.25	−1.32	63

Definition of abbreviation: CF/N, ratio of labeled cystic fibrosis to unlabeled non-CF peptides.

*All altered proteins are significant ($P < 0.05$).

pathway) were decreased. When C3 is activated, it is cleaved into smaller subunits that allow conformational rearrangement to an active conformation (41). Our global proteomic strategy could not distinguish between intact and fragmented C3 because all detected C3 peptides were averaged. Immunoblots of C3 in life-extended CF and non-CF ALI apical secretions showed intact C3 in N1T and N3T secretions and depletion of intact C3 in all three CF cell lines and the

N2T smoker cell line (Figure 7A), which likely reflects proteolysis/activation of C3. Immunoblots of C3 in apical secretions from six CF and six non-CF primary ALI cultures did not show intact C3 in either cohort. However, an increased abundance of low-molecular-weight complement C3 fragments was observed in CF compared with non-CF apical secretions, especially fragments of the expected sizes (e.g., C3 dg, C3 d, and C3Cα chain fragment 1) (Figure 7B). Differences in

high- versus low-molecular-weight C3 bands in secretions from life-extended and primary cultures may reflect differences in handling and storage (e.g., protease inhibitors were added to life-extended secretions but not to primary secretions collected at UNC). C3 d fragments, which are used to demonstrate activation of C3 (42), were observed in CF secretions, and C3 dg was observed in both CF and non-CF secretions. Overall, analysis of C3 showed increased proteolysis and

Figure 3. Validation of increased levels of MUC5AC and MUC5B mucins and mRNA in life-extended CF and non-CF human bronchial epithelial ALI cultures. (A and B) Secretions (10 μ g) from life-extended UNCCF1–3T ($n = 3$) and UNCN1–N3T ($n = 3$) ALI cultures at Day 21 were separated by electrophoresis on 1% agarose gels, transferred to polyvinylidene fluoride, and probed with anti-MUC5AC antibody (A). Blots were then stripped and reprobed with anti-MUC5B antibody (B) as described in MATERIALS AND METHODS. In each panel, blots are shown on the *left* and densitometry graphs on the *right*, with fold changes (sample with lightest density = 1). MUC5B mucin showed a significant 3-fold increase in CF life-extended secretions ($*P = 0.03$). (C) UNC CF1–3T, N1T, and N2T life-extended cell lines were differentiated at ALI for 21 days. RNA was isolated, and mucin and actin mRNA levels were quantified by real-time PCR as described in the online supplement. Fold changes were determined by comparing MUC5AC and MUC5B mRNA levels with β -actin mRNA levels. Data are representative of $n = 2$ for the non-CF and $n = 3$ for the CF cell lines repeated twice in duplicate for MUC5AC data and once in duplicate for MUC5B data in each cell line. Statistical analysis was performed by Student's t test compared with control. $*P < 0.03$; $**P < 0.005$.

activation of C3 in CF apical secretions from life-extended and primary ALI cultures in the absence of infection and inflammatory cells.

Discussion

This study, to the best of our knowledge, is the first quantitative comparison of *in vitro* apically secreted proteins by CF and non-CF lung epithelium. By using life-extended HBE cells (29) and a SILAC/liquid chromatography/MS/MS approach (28), we obtained an unbiased quantitative comparison of proteins constitutively present in CF and non-CF lung epithelial secretions. Seventy proteins exhibited a significant ($P < 0.05$) fold change (± 1.5), demonstrating that CF lung epithelium exhibits a unique apical secretome in the absence of immune and inflammatory cells. The structural/molecular functions of proteins with differential abundance (many with multiple known functions) were categorized predominantly as innate immunity, proteases/protease inhibitors, and cytoskeleton/ECM organization. These functions are activated *in vivo* in the airway epithelium after injury to support epithelial migration and spreading and secretion of MMPs to aid in ECM remodeling (43, 44). Our data show that an intrinsic inflammatory state constitutively exists in CF lung epithelial secretions *in vitro* and support the proinflammatory state previously demonstrated *ex vivo* using xenografts from CF and non-CF fetal tracheal tissues (45). Additionally, the UNCCF1T cell line exhibits significantly slower migration (46).

Some of the altered biological processes identified in our *in vitro* CF secretions were also identified in BALF from adults with CF and control subjects analyzed by shotgun proteomics and gene ontology (21). Analysis of BALF and sputum, especially from patients with CF (20, 21), is convoluted by contributions of immune cells and other nonepithelial cell types as well as bacteria and serum exudate. Thus, the finding that wound healing and repair, defense response and immunity, and proteolysis are key processes in CF apical secretions both *in vivo* (21) and *in vitro* (this study) implicates these processes in early and late pathogenesis of CF lung disease. In contrast, the lack of differential abundance in the CF secretome of oxidative stress proteins that predominate in CF

Figure 4. Validation of increased MUC5AC and MUC5B mucins in secretions from 4-week primary CF and non-CF human bronchial epithelial ALI cultures. Secretions (30 μ g) from individual primary non-CF ($n=3$) (lanes 1–3) and CF ($n=4$) (lanes 4–7) ALI cultures were separated by electrophoresis on 1% agarose gels, transferred to polyvinylidene fluoride, and probed with anti-MUC5AC antibody (A). Blots were then stripped and reprobed with anti-MUC5B antibody (B). MUC5AC was significantly increased in four out of four CF samples compared with three individuals without CF. $^*P=0.03$. In each panel, blots are shown on the left and densitometry graphs on the right, with fold changes (sample with lightest density = 1).

BALF (21) and in sputum and serum from patients with CF (47) indicates that oxidative responses are important in the chronic CF lung disease. The discussion below focuses on specific proteins that exhibited altered abundance in CF secretions in the three predominant

categories and were validated in independent sets of secretions from life-extended and/or primary HBE CF and non-CF cells. Although the validation studies overall support the proteomics data, there are challenges in using secretions from a small sample size to analyze clinical

Figure 5. ELISA validation of increased fibronectin in independent sets of secretions from 4-week life-extended (A) and primary (B) CF and non-CF human bronchial epithelial (HBE) ALI cultures. Secretions (equal amounts of protein) were analyzed in duplicate for fibronectin by ELISA and demonstrated a statistically significant 3-fold increase in CF ($n=3$) compared with non-CF ($n=3$) apical secretions from life-extended HBE ALI cultures ($^*P=0.02$) (A) and a statistically significant 4-fold increase in CF ($n=4$) compared with non-CF ($n=4$) apical secretions from primary HBE ALI cultures ($^*P=0.008$) (B). Gray bars: concentration in ng/mL; error bars indicate $\pm$ SD.

significance due to the inherent variability of protein expression in primary cells from different individuals (26).

Innate Immunity: Mucins

Secretory mucins are large, highly O-glycosylated, and inherently difficult to quantify (48). Immunochemical analysis of MUC5AC and MUC5B mucins secreted by primary and life-extended cells validated the markedly increased levels in CF secretions identified by SILAC/MS/MS analysis, which quantified multiple MUC5AC and MUC5B peptides in bands that spanned agarose gels. This further supports the concept that the protein backbones of MUC5AC and MUC5B are spontaneously cleaved in a consistent manner that represents overall abundance of the mature mucins and can be reliably quantified by our MS/MS approach (35, 49). Although it is generally accepted that MUC5AC and MUC5B mucins are increased in CF secretions (48, 50), it has been thought that this primarily reflects the inflammatory milieu of CF airways (5, 51). However, our data show that, in the absence of inflammatory and bacterial cells, MUC5AC and MUC5B mRNA levels are increased in life-extended CF cell lines and that MUC5AC and MUC5B mucins are present at higher levels in CF secretions from primary differentiated cell lines and cells, suggesting that the increased levels of secretory mucins may be a direct consequence of the lack of functional CFTR in goblet cells. However, the expression of CFTR in specific lung epithelial cells is controversial, and detection may be antibody dependent. CFTR has been localized to serous cells in submucosal glands (52, 53) and to goblet cells (52), with weaker staining in ciliated cells. In contrast, CFTR has been localized to the apical membrane of ciliated cells, with weaker or undetectable staining in submucosal glands (54). The controversy with CFTR expression in *ex vivo* tissue is also evident with *in vitro* models. One study shows that CFTR is expressed in ciliated, but not goblet, cells in primary differentiated HBE epithelium (54), whereas more recent studies (55) demonstrate CFTR expression in goblet cells both in differentiated primary HBE cells and in CFTR-expressing Calu-3 cells (56). Although CFTR localization has not been investigated in the life-extended HBE cells, both CF and non-CF cells differentiate to a pseudostratified

Figure 6. ELISA validation of increased matrix metalloproteinase (MMP)-9 in independent sets of secretions from 3-week life-extended and primary CF and non-CF HBE ALI cultures. Secretions (equal amounts of protein) were analyzed in duplicate for MMP-9 by ELISA and demonstrated increased levels in CF secretions from life-extended (A) and primary (B) ALI cultures, but the increased levels were not significant. Gray bars: concentration in ng/ml; error bars indicate $\pm$ SD.

epithelium with abundant goblet and fewer ciliated cells, and the non-CF cells express functional CFTR (29).

Innate Immunity: Complement

The complement system is a significant component of the host defense/innate immune response. Complement dysregulation in CF lungs has been

indicated by a semiquantitative shotgun proteomics analysis of CF and non-CF BALF from humans (21) and ferrets (13) exhibiting altered levels or an absence of several complement proteins. In this *in vitro* study, C3, the primary component of the complement pathway, was the most abundant protein in the secretome, clearly demonstrating that C3 is apically secreted

by lung epithelial cells. C3 levels were unchanged between CF and non-CF *in vitro* secretions, in contrast to BALF from humans (21) and ferrets (13) with CF, where C3 is significantly decreased or increased, respectively. Although our global proteomic strategy could not distinguish between intact and fragmented C3 levels, immunochemical analysis of *in vitro* secretions showed increased proteolysis/activation of C3 in both life-extended and primary CF cells compared with non-CF cells, suggesting that the CF lung microenvironment facilitates activation of C3 *in vivo*, resulting in overall decreased levels of total C3.

ECM Organization and Protease/Antiprotease Activity

Analyses of CF secretions in the absence of immune cells and bacteria support the concept of an inherent inflammatory state in CF epithelium that contributes to repair/remodeling (44). Several proteases and protease inhibitors were quantitatively increased in CF apical secretions, including serine proteases and SERPINs, whereas the protease gelsolin and the antiprotease Serpin B5 exhibited decreased levels. Because unregulated proteases can damage respiratory epithelium and impair ciliary function (57), this continues the theme of a state of repair induced by inflammation (44), as previously demonstrated in UNCCF1T cells (46).

MMP-9 belongs to a family of MMP zinc- and calcium-dependent proteolytic enzymes that regulate ECM turnover, especially during tissue repair after injury (58). MMP-9, which was increased approximately 3-fold in our CF secretome and showed a trend toward increased MMP-9 in CF *in vitro* primary secretions, is the specific isoform present at high levels in the BALF of patients with CF *in vivo* (58). MMP-9 is present in CF BALF but is not detected in non-CF BALF (21). Tissue inhibitor of metalloproteinases 1 (TIMP-1), which inhibits MMP-9 and other MMPs, is increased in CF BALF (21). TIMP-1 levels were unchanged in the CF and non-CF *in vitro* secretomes, suggesting a protease/antiprotease imbalance in the *in vitro* epithelial model that may not exist in CF chronic lung disease. Increased MMP-9 in the airways, if uninhibited, has the potential to degrade ECM and epithelial tight junctions, inhibiting repair and contributing to the pathogenesis of chronic airway diseases.

Figure 7. Western blot analysis of complement C3 in secretions from life-extended and primary CF and non-CF HBE ALI cultures. Western analysis of secretions (20 μ g protein) from (A) an independent set of 3-week life-extended UNCCF1-3T ($n=3$) and UNCCF1-3T ($n=3$) ALI cultures at Day 21 and (B) individual 3- to 4-week primary non-CF ($n=6$) (lanes 1–6) and CF ($n=6$) (lanes 7–12) ALI cultures from Days 21 to 33. Identification of complement fragments was based on molecular weight (Uniprot.org).

A variety of proteins traditionally associated with the ECM also exhibited increased levels in SILAC analysis of CF secretions. In general, ECM proteins serve as an anchor/scaffold for cell proliferation and differentiation, and their increased levels in CF secretions contribute to the theme of inherent and ongoing repair in CF airway epithelium (44, 46, 59). SILAC/MS/MS demonstrated that fibronectin, an ECM protein typically secreted basally in airway epithelium, was one of the most increased and abundant proteins in CF apical secretions, and ELISA validated a significant increase in fibronectin abundance in CF apical secretions from both life-extended and primary ALI cultures. Normal adult lung tissue constitutively expresses low amounts of cellular fibronectin compared with fetal and injured adult lung (60). Fibronectin expression by HBE cells is increased by exposure to proinflammatory mediators *in vitro* (61), and the increased fibronectin levels observed in CF epithelium *in vitro* may be indicative of an inflammatory state. Altered fibronectin production has also been implicated in airway remodeling and compromised lung repair in asthma (62).

Concluding Comments

The absence of functional CFTR in lung epithelium is characterized by NF- κ B activation, epithelial cell injury, and ineffective repair, which predictably would be exacerbated by chronic inflammation and infection (8). That CFTR is a negative regulator of NF- κ B with inherent anti-inflammatory properties is an emerging concept (8, 63–65), although it is still controversial (66). Our secretome data support the concept of increased

endogenous NF- κ B hyper-activity in CF HBE cells at ALI: as many of the 20 most highly differentially abundant secreted proteins are up-regulated by NF- κ B. These include MUC5AC (67, 68), MUC5B (69), fibronectin (70), complement C3 (71), MMP-9 (72), calpain small subunit 1 (73), cathepsin B (74), and plasminogen activator inhibitor 1 (75). In addition, the innate immune mediators S100P and serum amyloid A, which were also among the highly differentially abundant proteins in CF secretions, activate NF- κ B (76, 77). Although the mechanism(s) whereby CFTR regulates NF- κ B activity have not been established, the lack of functional CFTR in CF lung cells may alter lung responses early in life, as reported for human (9, 10), pig (11, 12), and ferret (13, 14) neonates with CF, and affect how CF individuals become susceptible to chronic infection (8).

This quantitative proteomics approach has limitations. Life-extended, rather than primary, HBE cell lines (29) were used to isotope-label cells for quantitative proteomic analysis. The number of non-CF samples was reduced from three to two in proteomic analyses because data analysis showed that the N2T secretome profile more closely resembled the CF secretomes than the non-CF secretomes, in line with reports that smoking reduces CFTR expression (37, 38). Proteomics data were validated in secretions from three or four primary CF or non-CF donor cells, but further studies with larger cohorts would be useful due to the inherent variability of protein expression in primary cells from different individuals (26). Regardless, this study establishes that the human CF secretome is unique in the absence of active infection and demonstrates altered

abundance of immunity and epithelial repair proteins in constitutive CF apical secretions. Additionally, it suggests that oxidative stress proteins, which are present at increased levels in CF BALF and sputum (21, 46), do not exhibit differential abundance in CF secretions at homeostasis, suggesting they are not part of the constitutive epithelial cellular response to the lack of functional CFTR but rather markers of chronic CF lung disease. Further use of quantitative proteomics to investigate the response of CF cells to bacterial infection regarding their secretome may provide insights as to how mutant CFTR leads to altered host responses in CF lungs. A better understanding of perturbations in the CF lung secretome could revolutionize the way clinicians treat CF lung disease by allowing treatment to focus on prevention of hyperinflammation and amplifying the host defense response of patients with CF, especially young children, before bacterial infection and chronic lung disease is established. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Scott H. Randell, Ph.D., and Leslie Fulcher (University of North Carolina) for the generous gifts of the life-extended UNCCF and non-CF cell lines and of apical secretions from primary CF and non-CF HBE ALI cultures; Mehmet Kesimer, Ph.D. (University of North Carolina) for the generous gift of the LUM5-1 MUC5AC antibody; Asha S. Payne, M.D. and Diego Preciado, M.D., Ph.D., for providing us with fibronectin and MMP-9 ELISA kits; Thomas Peters-Hall for programming a Ruby script to filter protein data; Heather Gordish-Dressman, Ph.D., for statistical analysis; and Kanneboyina Nagaraju, Ph.D., for immunology and complement expertise.

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