Function and Composition of Pulmonary Surfactant and Surfactant-Derived Fatty Acid Profiles Are Altered in Young Adults With Cystic Fibrosis*

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Study objectives: To determine whether chronic lung inflammation in young adult patients with cystic fibrosis (CF) alters the composition and function of surfactant and surfactant components in bronchoalveolar secretions.

Design: A prospective, descriptive study.

Setting: An adult CF center in a tertiary health-care center.

Participants: Thirteen normal volunteer (NV) subjects recruited via local advertising and 15 CF patients recruited from the CF center.

Interventions: None.

Measurements and results: We performed BAL and measured surfactant-associated protein A (SP-A) via enzyme-linked immunosorbent assay in BAL fluid (BALF), and quantitated total phospholipid, phospholipid subclass, and fatty acid subclass content of extracted BALF. We also determined the protein and phospholipid content, SP-A content, and functional characteristics of surfactant isolated from BALF via high-speed centrifugation. The phospholipid-to-protein ratio (milligram/milligram) of surfactant isolated by centrifugation (mean ± SEM) was 1.01 ± 0.07 for NV subjects and 2.62 ± 0.42 for CF patients (p = 0.0001). Minimal surface tension was < 1 dyne·cm⁻² in all samples from NV subjects, but 21.9 ± 0.73 dyne·cm⁻² for surfactant from CF patients. Immunoblotting of isolated surfactant revealed a marked decrease in SP-A for CF patients, compared to NV subjects. However, mean concentrations of SP-A in BALF that had not been subjected to high-speed centrifugation to isolate surfactant were not significantly different for CF patients (4.7 ± 0.8 µg/mL) vs NV subjects (4.6 ± 0.2 µg/mL). Additionally, phospholipid-to-protein ratios (0.32 ± 0.04 for NV subjects vs 0.10 ± 0.02 for CF patients; p < 0.0001) in extracted uncentrifuged BALF, and SP-A-to-protein ratios (microgram/milligram) in BALF were significantly depressed (74 ± 8 for NV subjects vs 16 ± 3 for CF patients; p < 0.0001). The phospholipid and fatty acid subclass profiles of extracted CF BALF vs NV BALF revealed a decreased mean phosphotidylcholine-to-sphingomyelin ratio (20.7 ± 10.0 vs 55.2 ± 8.7; p = 0.002), increased oleic acid content (12.1 ± 2.3 nmol/mL vs 3.2 ± 0.9 nmol/mL; p < 0.01), and increased arachidonic acid content (2.2 ± 0.5 nmol/mL vs 0.6 ± 0.3 nmol/mL; p < 0.05) for CF patients.

Conclusions: Altered phospholipid-to-protein ratios and phospholipid subclasses, altered surfactant-derived fatty acid profiles, high minimal surface tension, and decreased association of SP-A with lipid components of isolated surfactant indicate that surfactant components are considerably altered and dysfunctional in lower respiratory tract secretions of CF patients. Surfactant composition and function are altered in CF, and the pattern of phospholipid and surfactant-derived fatty acid subclass alterations in CF are characteristic of ongoing lung injury and may depress surfactant function.

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Key words: BAL; cystic fibrosis; fatty acid; phospholipid; pulmonary surfactant; surfactant protein A

Abbreviations: BALF = BAL fluid; CF = cystic fibrosis; ELISA = enzyme-linked immunosorbent assay; MST = minimal surface tension; NV = normal volunteer; PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecyl sulfate; SP = surfactant-associated protein; SP-A = surfactant-associated protein A; TTBS = 0.1% Tween-20/Tris-buffered saline solution
Lower respiratory tract secretions from patients with cystic fibrosis (CF) contain large numbers of neutrophils and free neutrophil-derived enzymes, which are associated with chronic endobronchial infection with *Pseudomonas aeruginosa* and other bacterial pathogens. Large numbers of BAL fluid (BALF) neutrophils and high concentrations of neutrophil elastase have been found in CF patients with stable, clinically mild lung disease, and lower respiratory tract inflammation is now also recognized in very young children with CF who have no evidence of active lower respiratory tract infection. Unfortunately, pulmonary dysfunction and airflow obstruction caused by chronic inflammatory lung injury progress despite nutritional interventions, measures to optimize secretion clearance, and antibacterial chemotherapy.

Pulmonary surfactant prevents airspace collapse by reducing surface contractile forces at low alveolar volumes at the air/liquid interface in alveoli, and it has been shown to prevent small airway collapse. Surfactant also possesses anti-inflammatory properties, facilitates mucus clearance, plays a role in prevention of pulmonary infection, and can scavenge extracellularly generated oxyradicals and enhance intracellular antioxidant enzyme content.

Pulmonary surfactant, like lung matrix, represents a likely target for the chronic, neutrophil-dominated inflammation associated with CF. Neutrophil-derived lipases, oxidants, and proteases can biochemically alter surfactant in vitro. Activated neutrophils can directly orchestrate lipid peroxidation in dilinoleoylphosphatidylcholine vesicles, and this peroxidation injury is enhanced by pyrophosphate-chelated iron but attenuated by superoxide dismutase or catalase. Because unsaturated phosphatidylcholines comprise about 25% of phospholipids in surfactant, the potential for surfactant phospholipid peroxidation is considerable. In addition to damaging phospholipids, activated neutrophils can also inhibit de novo synthesis of phosphatidylcholine in primary cultures of rat type II alveolar cells, and superoxide dismutase or catalase is again protective. Activated neutrophils can also alter electrophoretic migration of surfactant-associated protein A (SP-A) and other markers of surfactant functional activity in vitro, and SP-A gene knockout mice are more susceptible to mucoid *P. aeruginosa* respiratory infection. Thus, activated neutrophils, which are abundant in the CF lower respiratory tract, may cause significant qualitative and quantitative alterations of both phospholipid and protein components of surfactant. Additionally, increased serum and endogenous proteins in lower respiratory tract secretions of the inflamed CF lung may inhibit surfactant function by altering the stoichiometric relationship between surfactant lipids and inhibitory proteins.

Because alteration of surfactant by neutrophil-derived mediators may depress mucociliary clearance, promote collapse of conducting airways and alveoli causing impaired gas exchange, and promote persistent infection, we performed bronchoscopy with BAL to obtain lower respiratory tract secretions and characterize biochemical and biophysical changes in surfactant from the lungs of young adult patients with CF. We examined the functional integrity of surfactant isolated via high-speed centrifugation by determining minimal surface tension (MST) on a pulsating bubble surfactometer (Electronetics; Amherst, NY). The relative amounts of protein and phospholipid components were quantified in BALF, methanol/dichloromethane-extracted BALF, and surfactant isolated by high-speed centrifugation. We also determined the SP-A content of BALF and isolated surfactant. Finally, because fatty acids are utilized for surfactant phospholipid synthesis, we determined phospholipid and surfactant-derived fatty acid subclass profiles in BALF.

### Materials and Methods

**Patient Population**

BALF was obtained from both normal volunteer (NV) subjects (n = 13) and patients with CF (n = 15). The 13 NV subjects, ranging in age from 19 to 24 years, were subjected to bronchoscopy and BAL. All NV subjects had unremarkable medical histories, normal results of physical examinations and spirometry, and no symptoms of an upper respiratory tract infection in the 4-week period prior to participation in the study. No study subjects had a history of tobacco smoking. All study protocols were approved by the University of Wisconsin Human Subjects Committee, and informed written consent was obtained from all subjects.

The 15 patients with CF were hospitalized at the University of Wisconsin Hospital for subacute exacerbations of their lung disease (n = 8), or were seen in the Adult Cystic Fibrosis Center...
Outpatient Clinic with stable chest symptoms and objective findings (n = 7). Patients with subacute respiratory exacerbations had two or more of the following: (1) increased cough and sputum production; (2) systemic symptoms and signs (malaise, anorexia, weight loss); (3) worsening infiltrates on chest radiographs; or (4) a ≥ 10% decline in FEV1 on spirometric testing. The diagnosis of CF was established by typical clinical manifestations of the disease, and confirmed by positive sweat tests in all patients with CF, who ranged in age from 17 to 42 years (mean age, 23.3 ± 1.8 years). All except one patient (who was chronically infected with *Staphylococcus aureus* only) were chronically infected with *P aeruginosa*.

**BAL**

NV subjects and CF patients were subjected to bronchoscopy and BAL as previously described.1 IV access was maintained throughout the procedure in CF patients, and oxygen was given as indicated with continuous oximetric monitoring. Atropine, 0.6 mg, or glycopyrrolate, 0.2 mg, was administered IV or IM, and midazolam was given IV or IM at the beginning of and during the procedure as needed. Upper airway and posterior pharynx anesthesia was obtained with 4% aerosolized lidocaine. The fiberoptic bronchoscope (Olympus BR 4B; Olympus Corporation of America; New Hyde Park, NY) was passed nasally unless anatomic problems necessitated passage via the oropharynx. Lidocaine (1%) was delivered via the bronchoscope onto the epiglottis and vocal cords prior to passage into the trachea, and 1 to 3 mL of lidocaine (1%) was administered to the tracheobronchial tree just prior to obtaining the wedge position. BAL was performed in all subjects by wedging the bronchoscope into a segmental bronchus of the right middle lobe or lingula. Four 60-mL aliquots of sterile 150 mM NaCl were injected and gently aspirated via hand-held syringe. Six aliquots of 40 mL were used in some CF patients to minimize the possibility of inducing hemorrhage. The percentage of BALF return was similar for both aliquot volumes. BALF was 2 centrifuged at 400 g for 10 min at 22°C. Aliquots of the resulting BAL supernatant fluid were immediately frozen at −70°C until assayed. Airspace cells were washed once in calcium- and magnesium-free Hank's balanced salt solution, and then resuspended in calcium- and magnesium-free Hank's balanced salt solution. A hemocytometer and a microcentrifuge were used to obtain total and differential cell counts. Neutrophil elastase and other proteins were assayed. Airspace cells were washed once in calcium- and magnesium-free Hank's balanced salt solution, and then resuspended and sonicated four times with 30-s bursts at 0°C, followed by ultracentrifugation with 2:1 (volume: volume) methanol: dichloromethane. The mass of phospholipid was calculated from the mass of phospholipid phosphorus, given that phosphorus comprises, on the average, 4% of the total mass of phospholipid.

**Determination of Surfactant Functional Activity**

Surfactant functional activity was assessed utilizing a pulsating bubble surfactometer (Electronics) with a pulsatot rate of 20/min in an environment of 37°C and 100% relative humidity.27 MST and time to MST were determined on surfactant suspended in normal saline solution at approximately 1 mg/mL phospholipid concentration. This concentration, although higher than that likely present in alveolar lining fluid, was used to avoid concentration effects.

**Extraction and Determination of BALF Phospholipid Content**

Phospholipid concentration in BALF aliquots that were not subjected to high-speed centrifugation was determined by adapting methods published by Chen et al28 and Ames and Dubin.29 One milliliter of BALF was extracted with 1.25 mL of CCl4, followed by vortexing, addition of 2.5 mL of methanol with vortexing, addition of another 1.25 mL of CCl4 with vortexing, followed by addition of 1.25 mL of H2O with vortexing. Aliquots of the lower organic layer (0.5 mL) were obtained (extracted BAL) and evaporated to dryness under nitrogen at 37°C, as were 0.5-mL aliquots of phosphorus standards (Na2HPO4, J.T. Baker Chemical; Phillipsburg, NJ). Thirty microliters of 10% Mg(NO3)2 was then added, followed by evaporation of the solvent at 45°C over 15 min with subsequent conversion to ash over 10 min. Pyrophosphates were hydrolyzed by adding 0.3 mL of 0.5 mol/L HCl and heating in a boiling water bath for 15 min while protected from light, and 0.7 mL of 10% ascorbic acid (one part) combined with 0.42% ammonium molybdate in one normal H2SO4 (six parts) was then added, followed by heating at 45°C for 20 min. After cooling to room temperature, absorbance was read at 520 nm using a DU-650 spectrophotometer (Becton Instruments; Fullerton, CA). Total protein was determined for unextracted BALF via a modified Lowry assay for microtiter plates as previously described.1

**Determination of SP-A Content of BALF**

The amount of SP-A in samples of BALF was determined via an enzyme-linked immunosorbent assay (ELISA) technique using a rabbit anti-human SP-A polyclonal antibody as previously described by Moya et al.30 Standards were made with purified SP-A obtained from alveolar proteinosis material after the method reported by Akorn and Mendelson.31 Briefly, lamellar bodies obtained by ultracentrifugation were resuspended and sonicated 4 times with 30-s bursts at 0°C, followed by vortexing for 15 min and centrifugation at 12,000 g for 15 min at 0°C. The supernatant was recovered and calcium was added to achieve a final concentration of 10 mM. This mixture was...
incubated on ice for 15 min and centrifuged again at 12,000g for 15 min at 0°C. The resulting pellet was resuspended in water, and its purity was determined via sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and silver staining.

An aliquot of the lavage fluid with a final protein concentration of 100 µg/mL was used for the ELISA. Duplicates of three serial dilutions of each sample were used, and the amount of protein from the samples to be loaded onto 96-microwell plates (Nunc ImmunoPlate II Polysorp; Intermountain Science; Kaysville, UT) was varied if necessary to obtain an SP-A reading on the linear aspect of the curve. Following preadsorption with serum to remove antibodies against other serum proteins, the rabbit polyclonal antibody was incubated with the samples overnight at 4°C in 96-well plates. Following overnight incubation, the free antibody was transferred into a similar 96-microwell plate previously coated with purified SP-A. Antibody binding was allowed to proceed at room temperature for 30 min. Unbound antibody was removed by a series of washes with phosphate-buffered saline-Tween 20 buffer (Sigma Chemical; St. Louis, MO). Finally, peroxidase-conjugated goat anti-rabbit IgG antibody (ICN Biomedicals; Costa Mesa, CA) was added and allowed to bind for 2 h at room temperature. After washing the plate with PBS-Tween 20 buffer, ABTS (2,2'-azinobis-[3-ethylbenzothiazoline]-6-sulfonic acid; Sigma Chemical) was pipetted into the wells after addition of hydrogen peroxide. The colorimetric reaction was stopped by addition of 5% SDS, and the plate was read at 450 nm. The lowest concentration of SP-A detectable by this method was approximately 0.2 ng/µg of total protein.

**Determination of SP-A Content of Isolated Surfactant**

SDS-PAGE was performed using a minigel electrophoresis apparatus (Mini-protein II; Bio-Rad Laboratories; Hercules, CA). Equal total protein loads were delivered onto individual wells. Electrophoresis was performed initially at 45 V and increased to 100 V once the dye front traversed the stacking gel, and the gel was electrophoresed until the dye front reached the bottom of the gel. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane. Unbound sites on the nitrocellulose were blocked with powdered nonfat milk. The secondary antibody (rabbit IgG anti-human SP-A, kindly provided by Dr. Jeffrey Whitsett, Cincinnati, OH) after washing, the secondary antibody (goat anti-rabbit IgG peroxidase conjugate) was applied. Color was developed with 4-chloro-1-napthol and hydrogen peroxide (oxidized products form purple precipitates). Molecular weight was ascertained from accompanying bands of known standards.

**Determination of Phospholipid Subclasses in Extracted BALF**

Five milliliters of BALF was added to 5 mL of methanol and 10 mL of dichloromethane in screw-top, Teflon-lined glass centrifuge tubes. After vigorous agitation for 5 min, the tubes were centrifuged and the lower layer was withdrawn via a stainless steel needle and syringe. The extract was evaporated to dryness under a stream of pure nitrogen with heating to 35°C. The extract was then dissolved in 1 mL of methanol/dichloromethane (1:2) and transferred via syringe to sealed autosampler vials for determination of phospholipid subclasses by high-pressure liquid chromatography. Phospholipids were analyzed using a normal-phase, Econosphere, Silica, 5-µm column (Alltech Applied Science; Deerfield, IL) eluted with a gradient of water in acetonitrile. The initial eluent was 2.5% water in acetonitrile for 4 min, followed by a slightly concave gradient reaching 25% water at 12 min. At 15 min, the gradient was returned to 2.5% water for 5 min before injection of the next sample. Two pumps (Gilson Medical Electronics; Middleton, WI) were controlled by a Dynamax program (Rainin Instrument; Woburn, MA), which also controlled an autosampler (Shimadzu Sil-9A; Shimadzu Scientific; Kyoto, Japan), which injected 50 µL of sample or standard. Phospholipid peaks were detected via a Rainin UV-1 absorbance detector (Rainin Instrument) set at 205 nm. Some chromatograms were also detected via an evaporative light-scattering detector (Varex ELSD, Alltech Associates; Deerfield, IL). The Dynamax program recorded chromatograms and integrated areas under peaks. Phospholipid standards were purchased from Sigma Chemical, and standards were run between every five to six test samples.

**Detection of Nitrotyrosine in SP-A via Western Blot Analysis**

Surfactant isolated from CF BALF as above via high-speed centrifugation was diluted in sample buffer to a final concentration of 62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.925% bromophenol blue. Following heating for 4 min in a boiling water bath, samples were fractionated via SDS-PAGE (10% polyacrylamide) and subsequently transferred to nitrocellulose sheets by blotting at 100 V for 2 h in an ice-water bath using Tris buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, and 0.05% SDS, pH 8.2. Nitrocellulose blots were then blocked for 2 h at room temperature in a solution containing 5% nonfat milk; Tris-buffered saline solution containing 10 mM sodium azide and 0.1% Tween-20. Blots were then washed with 0.1% Tween-20/Tris-buffered saline solution (TTBS) and incubated with primary antibody (anti-nitrotyrosine; Upstate Biotechnology; Lake Placid, NY) diluted in TTBS for 1 h at room temperature. Blots were washed with TTBS and then incubated with secondary antibody (goat anti-rabbit IgG HRP conjugate; Upstate Biotechnology) in TTBS for 1 h at room temperature. Blots were again washed and secondary antibody detected via chemiluminescence (ECL; Amersham; Arlington Heights, IL) and exposure of blots to radiographic film.
Results

Clinical and BALF analysis data for the CF patients vs NV subjects are given in Table 1. NV subjects had no BAL neutrophil influx and did not have measurable activities of myeloperoxidase or neutrophil elastase in BALF (data not shown). All patients with CF had large numbers of neutrophils and considerable levels of myeloperoxidase and neutrophil elastase activity in BALF, similar to values reported previously.2

The surfactant fraction (isolated surfactant) containing heavy surfactant (lamellar bodies isolated by centrifugation at 40,000g for 30 min, subsequent washing on a 0.7-mol/L sucrose cushion, and reisolation by centrifuging at 40,000g for 30 min) contained SP-A and relatively little albumin or other protein bands by PAGE for both NV subjects and CF patients. The volume of the surfactant pellets (as subjectively assessed) retrieved from the BALF of CF patients was, as a rule, much smaller than that isolated from the BALF of NV subjects. However, adequate amounts of surfactant were recovered from each individual to perform most assays. Supernatant remaining after the initial BALF centrifugation (40,000g for 30 min) was centrifuged overnight at 100,000g, and was found to contain little phospholipid (<10% of the phospholipid content of the heavy fraction).

The ratio of phospholipid-to-protein (milligram/milligram) for isolated surfactant (isolated via high-speed centrifugation) was 2.62 ± 0.42 for surfactant from 15 individual CF patients and 1.01 ± 0.07 for surfactant from 13 NV subjects (p = 0.0001 by independent t test; Fig 1). This ratio appeared to be increased mainly due to relatively decreased protein (174 ± 66 µg/mL for CF patients vs 338 ± 76 µg/mL of resuspended surfactant for NV subjects) in the isolated surfactant. Mean phospholipid concentrations were 364 ± 99 µg/mL for CF patients vs 363 ± 96 µg/mL of resuspended surfactant for NV subjects. When BAL was performed a second time on five of the CF patients following treatment with a 2-week course of IV antipseudomonal antibiotics for subacute exacerbations of their lung disease, no significant change in the phospholipid-to-protein ratio was observed for surfactant obtained prior to treatment (2.40 ± 0.73) vs that obtained following antibiotic therapy (2.50 ± 0.86).

MST as determined with a pulsating bubble surfactometer was <1 dynes·cm⁻¹ in all samples from NV subjects (n = 13) but 21.9 ± 0.73 dynes·cm⁻¹ for surfactant from CF patients (n = 12). The length of time required to attain MST was not significantly different between the two subject groups (4.0 ± 2.6 min for CF surfactant and 3.4 ± 2.3 min for NV surfactant). Because the relative protein content of isolated CF surfactant tended to be lower than that of NV surfactant, it is relatively unlikely that exogenous protein accounted for the abnormal surface-active characteristics of isolated surfactant.

PAGE with immunoblotting was performed on isolated surfactant from seven CF patients and revealed a marked decrease in SP-A, as compared to surfactant isolated from NV subjects when identical protein loads were applied to the gels (Fig 2). Gel scanning generally revealed a twofold to fourfold reduction in SP-A in isolated CF surfactant, and in two instances, virtually no SP-A could be identified.

Table 1—Clinical Data and BAL Characteristics*

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NV Subjects</th>
<th>CF Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age range, yr</td>
<td>21 ± 1</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>FEV₁, % predicted</td>
<td>103 ± 8</td>
<td>54 ± 5</td>
</tr>
<tr>
<td>Volume return, %</td>
<td>67 ± 2</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>Cells × 10³/mL BALF</td>
<td>90 ± 9</td>
<td>1,290 ± 190</td>
</tr>
<tr>
<td>% macrophage</td>
<td>91 ± 2</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>% neutrophil</td>
<td>1.2 ± 0.1</td>
<td>51 ± 7</td>
</tr>
<tr>
<td>% lymphocyte</td>
<td>7 ± 2</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>% eosinophil</td>
<td>0.4 ± 0.2</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Total protein, µg/mL</td>
<td>69 ± 6</td>
<td>356 ± 67</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SEM.
SDS PAGE utilizing silver or Commassie blue staining showed a similar decrease in the protein band corresponding to SP-A (approximately 35 kd).

In contrast to SP-A content of isolated surfactant, mean SP-A concentrations (SP-A per milliliter of BALF) as determined via ELISA in BALF that had not been subjected to high-speed centrifugation were not significantly different between CF patients and NV subjects, although the ratio of SP-A to total protein in crude BAL was significantly depressed for CF patients when compared to NV subjects (Table 2). However, it should be noted that SP-A levels in BALF of patients with CF ranged from undetectable to values within or above the range obtained for NV subjects. Western blots of isolated surfactant stained with antibodies against nitrotyrosine did not demonstrate the presence of nitrotyrosine residues on SP-A isolated from either CF patients or NV subjects (data not shown).

The phospholipid content of BALF extracted with 2:1 dichloromethane:methanol (in contrast to surfactant isolated via high-speed centrifugation) was higher for CF patients than NV subjects, but the difference was not statistically significant (Table 2). However, the mean value for phospholipid-to-total protein ratio of extracted BALF was significantly diminished. Similarly, phospholipid subclass content as determined via high-pressure liquid chromatography was generally increased for extracted BALF from CF patients, compared to NV subjects (Table 3), and the profile of alterations in relative percentages of phospholipid subclasses for CF BALF showed diminished mean percentages of phosphatidylglycerol and phosphatidylcholine but increased phosphatidylethanolamine, phosphatidyl-inositol, and sphingomyelin. Although, these changes were only statistically significant for phosphatidylglycerol and sphingomyelin, the mean phosphatidylcholine to sphingomyelin ratio was significantly depressed for CF patients vs NV subjects (20.7 ± 10.0 vs 55.2 ± 8.7; p = 0.002).

Surfactant-derived fatty acid profiles in extracted BALF from a subset of individuals as determined by gas chromatography/mass spectrometry were altered for CF patients, as compared to NV subjects (Table 4). Importantly, the fatty acid profile for extracted BALF from both CF patients and NV subjects differed considerably from that for purified polymorphonuclear leukocytes or monocytes (data not shown), suggesting that WBC membrane contamination did not present a major problem in the interpretation of results. Although arachidonic acid was detected in both CF and NV BALF, we could not detect docosahexanoic acid (22:6) in BALF from NV subjects or CF patients. However, we did not analyze free fatty acid profiles in BALF.

Consistent with the increased phospholipid and phosphatidylcholine in CF BALF extract (mostly surfactant lipid), absolute concentration of palmitic acid (the major fatty acid component of surfactant) was also increased significantly. In addition, the other striking finding was the marked increase in CF BALF phospholipid-derived monounsaturated oleic acid, both in absolute terms and percentage. Surprisingly, perhaps, the polyunsaturated fatty acids, linoleic acid and arachadonic acid, appeared to be higher in absolute terms in CF patients, as compared to extracted BALF from NV subjects. No differences in monohydroxylinoleic acid/native linoleic acid ratio were observed between groups. Accordingly, no direct evidence of enhanced lipid peroxidation or related

![Figure 2](https://publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21949/)

**Table 2—** SP-A and Total Phospholipid in BALF*

<table>
<thead>
<tr>
<th>Variables</th>
<th>NV Subjects</th>
<th>CF Patients</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-A, µg/mL BALF</td>
<td>4.63 ± 0.24</td>
<td>4.37 ± 0.83</td>
<td>NS</td>
</tr>
<tr>
<td>Protein, µg/mL BALF</td>
<td>69 ± 6</td>
<td>356 ± 67</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PL, µg/mL BALF</td>
<td>19.7 ± 1.3</td>
<td>29.2 ± 5.3</td>
<td>NS</td>
</tr>
<tr>
<td>µg SP-A/µg PL</td>
<td>0.25 ± 0.02</td>
<td>0.31 ± 0.14</td>
<td>NS</td>
</tr>
<tr>
<td>µg SP-A/mg protein</td>
<td>74.5 ± 8.1</td>
<td>16.1 ± 2.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PL/TP ratio (µg/µg)</td>
<td>0.32 ± 0.04</td>
<td>0.10 ± 0.02</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SEM. Total phospholipid determined on CCl₄-extracted BALF. PL = phospholipid; TP = total phospholipid.
consumption of polyunsaturated fatty acids were observed in the CF group.

Discussion

We found that surfactant isolated from the BALF of patients with CF via high-speed centrifugation was chemically and physically altered, compared to surfactant from NV subjects. The total protein measurement and the total SP-A content of isolated surfactant from patients with CF were relatively decreased, compared to isolated surfactant from NV subjects. Additionally, the surface activity of surfactant from CF BALF was markedly depressed, and BALF phospholipid and surfactant-derived fatty acid profiles were altered in CF patients. Our findings of increased phospholipid-to-protein ratios of surfactant isolated from CF BALF via high-speed centrifugation coupled with impaired surfactant function indicate that the phospholipid and protein composition and dynamics of surfactant are altered in CF.

SP-A, the most abundant surfactant-associated protein (SP), is necessary for the reconstitution of tubular myelin in vitro, and acts synergistically with SP-B and SP-C to enhance adsorption and spreading of phospholipid at the air/liquid interface of the alveoli. SP-A also appears to modulate the uptake, recycling, and secretion of phospholipids by type II cells, although recent studies in SP-A-deficient mice suggest that surfactant function is nearly normal despite absence of SP-A. Additionally, SP-A has also been implicated in enhancing bacterial killing by alveolar macrophages, preventing infection with Pseudomonas aeruginosa, and in preventing contaminating serum proteins from altering the surface activity of the surfactant monolayer. SP-A can be cleaved under inflammatory conditions, and its ability to assume its hexameric quaternary structure via self-association, mediate lipid aggregation, and bind mannose is altered by oxidant stress or tyrosine nitration. Thus, diminished amounts of SP-A due to diminished production or degradation and/or altered SP-A function due to structural changes may have important consequences for lung function, homeostasis, and susceptibility to infection in CF patients.

When SP-A levels were determined in BALF that had not been subjected to high-speed centrifugation to isolate surfactant, we found that SP-A levels varied considerably among individual CF patients. However, the mean concentration of SP-A in BALF from CF patients did not differ from that of NV subjects, suggesting that association of SP-A with lipid components of surfactant from CF BALF may be altered, such that less SP-A can be isolated from surfactant separated from BALF via high-speed centrifugation. Additionally, SP-A production by alveolar type II cells may be depressed for some individuals with CF who had very low SP-A levels in BALF.

Numerous investigators have reported abnormalities in fatty acid and phospholipid profiles, MST, or SPs of lower respiratory tract secretions from patients with CF. Gilljam et al and Griese et al found that isolated surfactant from bronchial washings or BALF displayed a diminished ability to achieve MST in a bubble surfactometer. However, Hull et al did not observe a difference in MST between CF vs non-CF “controls” subjected to bronchoscopy for evaluation of stridor. We found a distinctly clear-cut difference between older patients with CF vs NV subjects for BALF obtained in an identical BAL protocol used for individuals from both subject groups (CF and NV). The subjects studied by Hull et al were, however, very young, and may have had little inflammatory change in their lungs to render surfactant dysfunctional.

Table 3—Phospholipid Subclass Composition of Extracted BALF

<table>
<thead>
<tr>
<th>Phospholipid Class</th>
<th>NV Subjects (n = 10)</th>
<th>CF Patients (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mL BALF</td>
<td>%</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>0.9 ± 0.1</td>
<td>6.8 ± 1.2</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>3.1 ± 0.3</td>
<td>21.0 ± 2.6</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>0.8 ± 0.1</td>
<td>5.7 ± 0.8</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>9.9 ± 1.1</td>
<td>64.8 ± 3.1</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>0.3 ± 0.1</td>
<td>1.7 ± 0.5</td>
</tr>
</tbody>
</table>

aData are presented as mean ± SEM.

†p < 0.05.
Girod et al,50 Hull et al,49 and Griese et al47 all observed an increase in total phospholipid for CF vs control groups, but this increase was only significant for the sputa samples evaluated by Girod et al,50 and may reflect a high content of cellular debris in expectorated secretions as opposed to sampling of more distal bronchoalveolar areas. Alteration of lipid subclasses with a relative depression of the phosphatidylcholine fraction was also observed.46–48 However, a recent investigation52 in children with CF showed only negligible changes in phospholipid subclasses in BAL when analyzed via electrospray ionization mass spectrometry. Phospholipid content of extracted BALF was elevated for subjects with CF compared to NV in our study, but did not differ significantly between the NV and CF groups. Altered lipid subclasses in CF surfactant may, in part, account for the increased adhesiveness of mucus for epithelial mucosa and depression of mucus clearance50 or conversion of P aeruginosa to mucoid strains that produce a mucoexopolysaccharide capsule.48 We found that phospholipid subclasses were altered to a pattern likely to increase rigidity of mucus, as reported by Girod et al,50

An exciting, recently described finding in CF gene knockout mice is the linkage of pathologic changes in affected organs to tissue-specific alterations in fatty acid metabolism that can be reversed with docosahexaenoic acid administration.53 This and other investigations54–56 suggest that abnormalities in fatty acid metabolism are closely linked to the genetic defect in CF and can potentially be treated via measures other than gene therapy. We found that the surfactant-derived fatty acid profile of extracted BALF from CF patients also differed from that of NV subjects; a significant increase in phospholipid-associated oleic acid reminiscent of a pattern seen in serum-free fatty acids in ARDS57 was observed, and arachidonic acid content was increased fourfold. Our investigation was unable to muster evidence for extensive surfactant phospholipid peroxidation, but the presence of increased amounts of oleic acid may inhibit the ability of surfactant to reach low surface tension, as has been observed both with oscillating bubble surfactometry and in situ.58 Docosahexaenoic acid could not be detected in extracted BALF from either NV or CF subjects. However, docosahexaenoic acid is very readily degraded or may negligibly associate with surfactant lipids. Methodologic problems may have prevented its detection relative to other, more abundant fatty acid, or it may be detectable in BALF-free fatty acids, which we did not characterize in this study.

When SPs (SP-A and SP-B) were measured by Hull et al49 and Griese et al,47 conflicting results were obtained. Hull et al49 found an increase in SP-A and a trend toward diminished SP-B levels, whereas Griese et al47 found decreased SP-A and a nonsignificant increase in SP-B. Of note is the fact that total BALF protein for CF patients in the study by Griese et al47 was identical to that of the control subject group, despite the intense inflammation in CF airways that would be expected to elevate protein content. Postle et al52 found the median value for SP-A to be significantly depressed in BALF from children with CF. Our findings demonstrated diminished recovery of SP-A in surfactant isolated via high-speed centrifugation, but did not demonstrate a significant difference in mean SP-A concentrations of BALF not subjected to high-speed centrifugation (determined via ELISA) for CF patients vs NV subjects, suggesting that production of SP-A may be maintained for many individuals, but that association of SP-A with surfactant phospholipids is altered, possibly due to oxidative or other structural alterations of SP-A in the inflamed CF airspaces.

Because oxidants appear to play a potentially important role in the pathophysiology of CF lung disease, and extracellular nitric oxide synthase has been identified in areas of inflamed human lung,59

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<th>Table 4—BALF Fatty Acid Profiles*</th>
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<tr>
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<tr>
<td>Fatty Acids</td>
</tr>
<tr>
<td>Myristic (14:0)</td>
</tr>
<tr>
<td>Palmitic (16:0)</td>
</tr>
<tr>
<td>Palmitoleic (16:1)</td>
</tr>
<tr>
<td>Stearic (18:0)</td>
</tr>
<tr>
<td>Oleic (18:1)</td>
</tr>
<tr>
<td>Linoleic (18:2)</td>
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<tr>
<td>Arachidonic (20:4)</td>
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*Data are presented as mean ± SEM.
†p < 0.05.
‡p < 0.01.
we speculated that damage to SP-A by oxyradicals and/or nitroradicals in vitro, which has been demonstrated in vitro,15 may alter the physicochemical properties of SP-A and association of SP-A with lipid components of surfactant. However, despite the observation that oxyradicals such as peroxynitrite can alter SP-A by nitrosylating tyrosine residues,15 we could not detect nitrotyrosine residues on SP-A immunoblots from CF BALF. Nonetheless, other chemical modifications that were not examined such as chlorination, oxidation, or carbohydrate cleavage may render SP-A dysfunctional or alter its Ig affinity. Additionally, nitrated SP-A may not associate with lipids, lipid components may prevent SP-A nitration, or nitrated SP-A may have been lost during preparation of samples for subsequent assay. Another explanation for our inability to identify nitrosylated SP-A may be a lack of inducible nitric oxide synthase and nitric oxide production as recently described for CF respiratory epithelium.60,61

Surprisingly, the degree of abnormality in surface tension we observed for pulmonary surfactant from patients with CF approaches that of infants with respiratory distress syndrome23 or adults with ARDS.62 However, the technique used (pulsating bubble surfactometry) is not necessarily the best for determining MST.63 and the rapid decline in MST may reflect limitations of this method in measuring the ability of surfactant to lower surface tension. Nonetheless, the differences in MST between NV subjects and CF patients were striking. It appears unlikely that altered or deficient SP-A is playing a major role in the higher surface tension in CF, as SP-A knockout mice display only subtle changes in surfactant function.64 It is more likely that altered lipid composition (eg, increased oleic acid, increased sphingomyelin, decreased phosphatidylcholine) or altered SP-B or SP-C is responsible for the increased MST in CF patients.64

This alteration in surface tension may promote airway as well as alveolar collapse, but these CF patients were not in acute respiratory failure with extensive atelectasis as observed in infants. Infants and small children have a decreased ability to clear secretions, a very compliant chest wall, horizontally positioned ribs (and therefore no bucket handle effect to expand the thoracic cavity), smaller airways, decreased numbers of true alveoli, and decreased collateral ventilation.65 Such factors may cause infants to be more susceptible to gross atelectasis than older patients with CF, even when surfactant function is severely impaired. Additionally, although MST of airway secretions from infants with respiratory distress syndrome is abnormally high, surfactant isolated from these secretions by ultracentrifugation had low surface tension.23 Because nonsedimentable proteins and amino acids from epithelial lining fluid of infants with respiratory distress syndrome increase the surface tension of exogenous surfactant,23 an increase in serum proteins in epithelial lining fluid of such patients likely accounts for the difference in the surface tension-lowering capacity of ultracentrifuged vs nonultracentrifuged airway secretions. Indeed, the addition of SP-A to abnormal airway secretions from neonates with respiratory distress syndrome improves surface activity.66

All studies that have analyzed surfactant composition and function in CF have had suboptimal controls for comparison or have sampled sputum or bronchial secretions without truly obtaining and examining BALF. The nature and method of sampling respiratory tract secretions, inadequate “normal” comparison groups, methodologic differences, and differing age distributions of subject groups may account for the somewhat conflicting results among previously published studies. However, all suggest that surfactant proteins or phospholipid components of lower respiratory tract secretions are altered substantially in CF patients and that surfactant function is likely impaired. In contrast to other studies of surfactant structure and function in CF, our investigation sampled bronchoalveolar secretions via BAL and demonstrated alterations in phospholipid-to-protein ratios (in isolated surfactant and in extracted BALF), SP-A, MST, phospholipid, and surfactant-derived fatty acid profiles for subjects with CF as compared to NV subjects.

Although surfactant in CF may be dysfunctional as a consequence of proteolytic, lipolytic, or oxidative degradation by inflammatory mediators present in lower respiratory tract secretions, type II cell dysfunction due to inflammatory mediators or a primary defect related to the presence of an abnormal CF transmembrane conductance regulator protein may also contribute to surfactant abnormalities. Administration of exogenous surfactant can reverse neonatal respiratory distress syndrome,67 can significantly improve gas exchange and lung mechanics in lung-injured animals,68 and may suppress pulmonary inflammation.11 Prevention of surfactant damage by employing anti-inflammatory tactics or replacement of surfactant itself (eg, during episodes of respiratory exacerbations of CF) may benefit patients with CF by improving lung function via enhanced airway patency and secretion clearance, decreasing the rate of lung destruction over time, and/or attenuating lower respiratory tract infection. Although a pilot study with nebulized bovine surfactant in patients with CF did not demonstrate short-term improvement in pulmonary function,69 a multicenter trial of aerosolized surfactant demonstrated improved pulmonary function and ciliary sputum transportabi-
The role of surfactant replacement therapies for patients with CF deserves additional investigation and may prove to be beneficial in certain situations such as acute respiratory failure.

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REFERENCES

39. van Iwaarden F, Welmans B, Verhoef J, et al. Pulmonary surfactant protein A enhances the host-defense mechanism of...
52 Postle AD, Mander A, Reid KB, et al. Deficient hydrophilic
51 Sahu S, Lynn WS. Lipid composition of airway secretions
50 Girod S, Galabert C, Lecuire A, et al. Phospholipid compo-
48 Krieg DP, Bass JA, Mattingly SJ. Phosphorylcholine stimu-
47 Griese M, Birrer P, Mattingly SJ. Phosphorylcholine stimu-
luates capsule formation of phosphate-limited mucoid Pseudo-
46 Gilljam H, Andersson O, Ellin A, et al. Composition and
45 Haddad IY, Zhu S, Ischiropoulos H, et al. Nitration of
44 Haddad IY, Zhu S, Ischiropoulos H, et al. Nitration of
43 Oosting RS, van Greevenbroek MM, Verhoef J, et al. Struc-
42 Stuart GR, Sim RB, Malhotra R. Characterization of radioio-
41 Liau DF, Yin NX, Huang J, et al. Effects of human polymor-
40 Baker CS, Evans TW, Randle BJ, et al. Damage to surfactant-
specific protein in acute respiratory distress syndrome. Lancet 1990; 335:1232–1237
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