Lung surfactant in a cystic fibrosis animal model: increased alveolar phospholipid pool size without altered composition and surface tension function in cftr<sup>m1HGU/m1HGU</sup> mice

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Abstract

**Background** – Progressive pulmonary dysfunction is a characteristic symptom of cystic fibrosis (CF) and is associated with functional impairment and biochemical alterations of surfactant phospholipids in the airways. However, the fundamental question of whether surfactant alterations in the CF lung are secondary to the pulmonary damage or are present before initiation of chronic infection and inflammation has yet to be resolved in patients with cystic fibrosis but can now be addressed in CF mice that exhibit the basic defect in the airways. A study was therefore undertaken to investigate the pool sizes, composition, and function of lung surfactant in the non-infected cftr<sup>m1HGU/m1HGU</sup> mouse.

**Methods** – The amount and composition of phospholipid classes and phosphatidylincholine molecular species were determined in bronchoalveolar lavage (BAL) fluid and lavaged lungs by high performance liquid chromatography (HPLC). Surfactant protein A (SP-A) levels in BAL fluid were determined by ELISA and surfactant for functional measurements was isolated from BAL fluid by differential ultracentrifugation. Equilibrium and minimal surface tension of surfactant was assessed by the pulsating bubble surfactometer technique. MF1, BALB/c, C57/BL6, and C3H/He mice served as controls.

**Results** – BAL fluid of cftr<sup>m1HGU/m1HGU</sup> mice contained 1.02 (95% confidence interval (CI) 0.89 to 1.16) µmol phospholipid and 259 (239 to 279) ng SP-A. BAL fluid of MF1, BALB/c, C57/BL6, and C3H/He mice contained 0.69 (0.63 to 0.75), 0.50 (0.42 to 0.57), 0.52 (0.40 to 0.64), and 0.45 (0.27 to 0.63) µmol phospholipid, respectively. After correction for the different body weights of mouse strains, phospholipid levels in BAL fluid of cftr<sup>m1HGU/m1HGU</sup> mice were increased by 64 (52 to 76)%, 60 (39 to 89)%, 72 (45 to 113)%, and 92 (49 to 163)%, respectively, compared with controls. The amount of SP-A in BAL fluid and the composition of phospholipid as well as phosphatidylincholine molecular species in BAL fluid and lung tissue was unchanged in cftr<sup>m1HGU/m1HGU</sup> mice compared with controls. The increase in phospholipid levels in BAL fluid of cftr<sup>m1HGU/m1HGU</sup> mice resulted from an increased fraction of large aggregates which exhibited normal surface tension function.

**Conclusion** – In cftr<sup>m1HGU/m1HGU</sup> mice surfactant homeostasis is perturbed by an increased phospholipid pool in the alveolar compartment.

Keywords: cystic fibrosis, surfactant pool size, cftr<sup>m1HGU/m1HGU</sup> mouse.

Cystic fibrosis is an inherited disease with a defect in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This gene codes for an integrated plasma membrane protein which is responsible for cAMP regulated chloride transport through the plasma membrane. Mucus viscosity is increased in bronchial secretions from patients with cystic fibrosis. Additionally, airway surfactant from patients with cystic fibrosis chronically infected with *Pseudomonas aeruginosa* shows impaired function and altered phospholipid composition, possibly due to enzymatic destruction and contamination of surfactant secondary to infection. However, a primary alteration of surfactant metabolism in cystic fibrosis may additionally exist since the CFTR gene is expressed in Clara cells and type II pneumocytes. Type II pneumocytes synthesise and secrete all the phospholipid and apoprotein components of surfactant, while Clara cells release surfactant apoproteins SP-A, SP-B, and SP-D into the airways. Surfactant phospholipids comprise about 80% phosphatidylcholine (PC), with dipalmitoyl phosphatidylcholine (PC16:0/16:0) being the most important molecular species, and about 10% phosphatidylglycerol (PG). Phospholipid homeostasis may be generally altered in cystic fibrosis since the rate of choline incorporation into PC is increased in isolated blood platelets and fibroblasts from patients with cystic fibrosis. It is possible that defective CFTR gene expression may altersurfactant phospholipid homeostasis before colonisation of the lungs with opportunistic bacteria. Since the concentration of surfactant phospholipids and the ratio between individual components, particularly between SP-A and phospholipids, can influence the anti-
body-independent defence functions of surfactant, altered phospholipid homeostasis may consequently affect pulmonary integrity. To investigate any such primary alterations in surfactant homeostasis we analysed the concentration of surfactant phospholipids and SP-A in lungs of cftr<sup>m1HGU/m1HGU</sup> mice. We further investigated in this mouse model the composition of surfactant phospholipid classes as well as phosphatidylycholine molecular species and, finally, the surface tension function of lung surfactant in comparison with healthy controls. In cftr<sup>m1HGU/m1HGU</sup> mice a cftr gene defect was generated by insertional mutagenesis into exon 10 of the cftr gene in which aberrant skipping results in the synthesis of 5–10% wild type transcript and therefore relatively mild symptoms of cftr deficiency. It was our aim to determine whether there was any primary deviation in pulmonary phospholipids of cftr<sup>m1HGU/m1HGU</sup> mice in the absence of chronic pulmonary infection.

### Methods

#### MATERIALS

Specific pathogen-free cftr<sup>m1HGU/m1HGU</sup> mice supplied from Edinburgh as well as Ztm:MF1, BALB/cZtm, C57/BL6Ztm, and C3H/HeZtm mice were bred at our local animal house. cftr<sup>m1HGU/m1HGU</sup> mice originated from five different breeding lines. Mice were kept in a flexible film isolator under specific pathogen-free conditions. The hygienic status was tested monthly according to Kunstyr and the mice were free from murine pathogens. The temperature within the isolator was maintained at 20–24°C with 40–50% relative humidity. Animals were fed an irradiated (5 Mrad) standard diet (Altromin 1314) and autoclaved water (134°C, 50 minutes) was given ad libitum. Mice subjected to analysis were 5–7 weeks old and the numbers of experiments were not based on any pre-study power calculations. High performance liquid chromatography (HPLC) grade solvents were supplied by Baker (Deventer, Holland). All other solvents and chemicals were of analytical grade and from various commercial sources.

#### HISTOLOGICAL EXAMINATION

For histological examination cftr<sup>m1HGU/m1HGU</sup> mice (n = 6, 6–10 weeks old) and MF1 mice (n = 6) were sacrificed by cervical dislocation and bled by incision of the abdominal aorta. The thorax was opened and, after perfusing the lung with 1 ml cold phosphate buffered saline via the right ventricle, the trachea was cannulated with a small catheter. The lung was then filled with 1 ml OCT embedding medium (Miles Inc, Elkhart, Indiana, USA) diluted 1:4 with 4% buffered formalin and further immersed in 4% buffered formalin for fixation. Paraffin-embedded sections (5 μm) were prepared and stained with haematoxylin and eosin.

#### PHOSPHOLIPID EXTRACTION

For phospholipid quantitation in cell-free BAL fluid two aliquots of 300 μl were placed into phosphate-free glass tubes, the volumes adjusted to 1 ml with 154 mM saline, and this material was extracted with chloroform/methanol according to the method of Bligh and Dyer. The chloroform extract was dried under a stream of nitrogen and the glass tube directly used for phospholipid quantitation. To quantify phospholipids in subfractions of BAL fluid aliquots of P60000 and S60000 were extracted with chloroform/methanol as outlined above. Lavaged lungs were extracted according to Folch et al, the extract adjusted to 10 ml with chloroform/methanol 9:1 (v/v) and two aliquots of 100 μl used for phospholipid quantitation. Phospholipid phosphorus was determined by the method of Bartlett after digesting the organic compounds at 190°C for 35 minutes in the presence of 500 μl 70% perchloric acid (w/v) and 200 μl 30% hydrogen peroxide (w/v). The amount of phospholipid in total samples was calculated from phospholipid phosphorus in the measured sample aliquots and the original sample volume (BAL fluid, P60000, S60000) or extract volume (total lung extract).

#### ANALYSIS OF PHOSPHOLIPID CLASSES

The distribution of total phospholipid classes in lipid extracts was determined by normal phase HPLC as described previously. In brief, aliquots containing 80–100 nmol phospholipid dissolved in chloroform/methanol (1:4 v/v) were resolved isocratically on an aminopropyl silica column (Nucleosil NH<sub>2</sub>, 5 mm, Schambeck, Germany) per kg body weight prior to sacrifice. This standard procedure was used since it does not cause respiratory depression during anaesthesia. The trachea was cannulated with a small catheter, the abdomen opened, and the animal bled by dissecting the abdominal aorta. The thorax was then opened, the lungs ventilated once with 1 ml of ambient air, and the lung perfused with 154 mM saline at room temperature at a pressure of 15 cm H<sub>2</sub>O via the right ventricle. The lung was then flushed five times via the tracheal catheter with 1 ml 154 mM saline and the pooled bronchoalveolar lavage (BAL) fluid was centrifuged for 10 minutes at 150 and 4°C to remove cells. The total recovery of BAL fluid was 4.5 ml and did not differ between the experimental groups. BAL fluid and lavaged lungs were frozen at −80°C until further analysis.

### Harvesting of Bronchoalveolar Lavage Fluid and Lavaged Lung Tissue

Animals were anaesthetised with an intraperitoneal injection of 100 mg ketamine hydrochloride (Ketanest, WDT Corporation, Garbsen, Germany) and 4 mg xylazine hydrochloride (Rompun, Bayer AG, Leverkusen, Germany) per kg body weight prior to sacrifice. This standard procedure was used since it does not cause respiratory depression during anaesthesia. The trachea was cannulated with a small catheter, the abdomen opened, and the animal bled by dissecting the abdominal aorta. The thorax was then opened, the lungs ventilated once with 1 ml of ambient air, and the lung perfused with 154 mM saline at room temperature at a pressure of 15 cm H<sub>2</sub>O via the right ventricle. The lung was then flushed five times via the tracheal catheter with 1 ml 154 mM saline and the pooled bronchoalveolar lavage (BAL) fluid was centrifuged for 10 minutes at 150 and 4°C to remove cells. The total recovery of BAL fluid was 4.5 ml and did not differ between the experimental groups. BAL fluid and lavaged lungs were frozen at −80°C until further analysis.

### Refining Bronchoalveolar Lavage Fluid and Lavaged Lung Tissue

Animals were anaesthetised with an intraperitoneal injection of 100 mg ketamine hydrochloride (Ketanest, WDT Corporation, Garbsen, Germany) and 4 mg xylazine hydrochloride (Rompun, Bayer AG, Leverkusen, Germany) per kg body weight prior to sacrifice. This standard procedure was used since it does not cause respiratory depression during anaesthesia. The trachea was cannulated with a small catheter, the abdomen opened, and the animal bled by dissecting the abdominal aorta. The thorax was then opened, the lungs ventilated once with 1 ml of ambient air, and the lung perfused with 154 mM saline at room temperature at a pressure of 15 cm H<sub>2</sub>O via the right ventricle. The lung was then flushed five times via the tracheal catheter with 1 ml 154 mM saline and the pooled bronchoalveolar lavage (BAL) fluid was centrifuged for 10 minutes at 150 and 4°C to remove cells. The total recovery of BAL fluid was 4.5 ml and did not differ between the experimental groups. BAL fluid and lavaged lungs were frozen at −80°C until further analysis.
acid (1460:500:30:0.3 v/v/v/v), adjusted to pH 6.3 by titration with NH₄OH (25% v/v in water). Eluted phospholipids were quantitated by fluorescence (excitation wavelength 340 nm, emission wavelength 440 nm) after post-column formation of mixed micelles in the presence of 1,6-diphenyl-1,3,5-hexatriene (DPH).

ANALYSIS OF PHOSPHATIDYLCHOLINE

Molecular species

The composition of individual molecular species of phosphatidylcholine (PC) was determined as previously described by Postle.²⁹ A PC fraction was isolated from the total lipid extract on a 100 mg Varian Bondelut NH₄ disposable cartridge (Jones Chromatography, Hengoed, Mid Glamorgan, UK). PC molecular species were then resolved on a Spherisorb ODS II column (Shambeck SFD, Bad Honnorf, Germany) of internal diameter 250 × 4.6 mm at 50°C using a mobile phase of methanol/water (92.5:7.5 v/v) containing 40 mM choline chloride at a flow rate of 1 ml/min. Eluted PC molecular species were quantified by post-column fluorescence derivative formation as outlined above.

DETERMINATION OF TOTAL PROTEIN AND SP-A

Total protein was determined according to the method of Lowry et al.²⁹ SP-A was determined by an ELISA technique using polyclonal anti-human SP-A antibodies which cross-reacted with murine SP-A.²¹ Briefly, polyclonal anti-human antibodies were prepared in chickens and New Zealand rabbits by repeated injection of approximately 1 mg purified human SP-A in Freund’s complete adjuvant. The chicken and rabbit antisera were then used as the first and second antibodies in a capture ELISA. The microtitre plates (Limbro, ICN Biomedicals Ltd, High Wycombe, Bucks, UK) were coated with chicken anti-human SP-A IgY (10 μg/ml in 35 mM Na₂CO₃, pH 9.6) at 4°C overnight and the non-specific binding sites were blocked with TBS-NTC (50 mM Tris, 50 mM NaCl, 2 mM CaCl₂, 0.05% (v/v) Tween-20, 0.05% (w/v) NaNO₂, pH 7.4) containing 1 mg/ml bovine serum albumin for one hour at 37°C. Purified human SP-A (10–1000 ng/ml) to be used as standards and BAL fluid samples were duplicated in four serial dilutions in TBS-NTC buffer. The final amounts of SP-A were calculated using these four dilutions. After extensive washes of the wells with TBS-NTC buffer, the samples and standards (100 μl) were added to each well and incubated for three hours at 37°C. After a series of washes with buffer the plates were then incubated with biotinylated rabbit anti-human IgG (50 μg/ml, 100 μl/well) for two hours at 37°C. Finally, streptavidin-alkaline phosphatase conjugate (Sigma Chemical Co. Ltd, Poole, Dorset, UK) diluted 1:10000 in TBS-NTC containing 1 mg/ml BSA was added and allowed to bind for one hour at room temperature. After washing the plates with TBS-NTC buffer, p-nitrophenyl phosphate (Sigma) was used as a substrate for the phosphatase reaction and incubated for 30 minutes at 37°C. The reaction was stopped by addition of 1 N NaOH and the plate was read at 405 nm. Correlation coefficients of the standard curve were 0.90–0.95. Sample dilutions were adjusted to within the linear range. Total amounts of SP-A in the BAL fluid were calculated from the apparent SP-A concentrations per ml BAL fluid.

PREPARATION OF SURFACTANT FROM BAL FLUID

Surfactant was prepared from cell-free BAL fluid by centrifugation at 60 000 g and 4°C for one hour.²² The 60 000 g supernatant (S60000) was harvested and frozen at −80°C for further lipid analysis. The 60 000 g pellet (P60000) was resuspended in 60 μl 154 mM saline supplemented with 1.5 mM calcium chloride and the phospholipid concentration determined from an aliquot and then adjusted to the desired concentration for functional analysis (see below).

FUNCTIONAL SURFACTANT ANALYSIS

For functional analysis P60000 was adjusted to 1.33 μmol phospholipid/ml with 154 mM saline/1.5 mM calcium chloride. Equilibrium (γequil) and minimal (γmin) surface tensions were then determined using a pulsating bubble surfactometer (Electronics Co, Amherst, New York, USA).²³ Briefly, a bubble was created in a surfactant suspension at 37°C and γequil was determined as surface tension 10 seconds after formation of the bubble. The bubble was then pulsed for five minutes at a frequency of 20 oscillations per min between a minimal bubble radius of 0.4 mm and a maximal radius of 0.55 mm. The pressure across the bubble was measured by a pressure transducer. γequil and γmin were calculated using the LaPlace equation.²⁴

DATA ANALYSIS

Data were expressed as means with 95% confidence intervals (CI) in parentheses. Statistical analyses were performed by one factor analysis of variance (ANOVA) and the two tailed Student’s t test using commercial software (GraphPad InStat Version 1.1, San Diego, USA). Statistical values were corrected for multiple comparisons using the Bonferroni method and statistical values of regression coefficients were calculated using the same software.

Results

HISTOLOGICAL EXAMINATION

The morphology of cfr⁻¹HGU⁻¹HGU mice showed a regular structure of lung tissue (fig 1). No signs of inflammation as leucocytic infiltration or interstitial oedema were found in any of the animals examined. Comparison of the number and distribution of Clara cells and type II alveolar epithelial cells revealed no detectable differences between MFI and cfr⁻¹HGU⁻¹HGU mice.
Figure 1  Histological photographs of cftr<sup>m1HGU/m1HGU</sup> and control mouse lungs. Microphotographs were produced from haematoxylin and eosin stained sections of lung tissue (5 μm) from a cftr<sup>m1HGU/m1HGU</sup> mouse (a, b) and from an MF1 control mouse (c, d). The morphology in both lungs revealed no histopathological findings such as leucocytic infiltration or altered numbers of Clara and type II alveolar epithelial cells. The morphology was similar in all examined animals. * = alveolar duct; ↓ = Clara cell; ▲ = type II alveolar epithelial cell. Bar represents 50 μm.

In both controls and cftr<sup>m1HGU/m1HGU</sup> mice there were significant positive correlations between the amounts of phospholipid in BAL fluid or lavaged lung tissue and the body weight of the animals (fig 2). We therefore corrected the amount of phospholipid in BAL fluid for the body weight of the individual mouse strains contributing to the amount of phospholipid in the BAL fluid, we calculated the correlation between body weight and the amount of phospholipid in BAL fluid and lavaged lung tissue.

In BAL fluid from cftr<sup>m1HGU/m1HGU</sup> mice the amount of phospholipid was 1.02 (95% CI 0.89 to 1.16) μmol/total BAL fluid but it was significantly lower in controls (table 1). In BAL fluid from BALB/c, C57BL/6, and C3H/He mice the concentrations of phospholipid were 0.50 (0.42 to 0.57), 0.52 (0.40 to 0.64), and 0.45 (0.27 to 0.63) μmol/total BAL fluid, respectively, and in BAL fluid from MF1 mice it was between the values of these controls and cftr<sup>m1HGU/m1HGU</sup> mice (0.69 (0.63 to 0.75) μmol/total BAL fluid). Assuming that different body weights of the individual mouse strains contributed to the amount of phospholipid in the BAL fluid, we calculated the correlation between body weight and the amount of phospholipid in BAL fluid and lavaged lung tissue.
Table 1 Amounts of phospholipid (PL) and apparent surfactant protein A (SP-A) in BAL fluid and of phospholipid in lavaged lung tissue of cftrm1HGU/m1HGU mice versus individual healthy control strains

<table>
<thead>
<tr>
<th></th>
<th>cftrm1HGU/m1HGU</th>
<th>MF1</th>
<th>BALB/c</th>
<th>C57BL/6</th>
<th>C3H/He</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PL (nmol/BAL fluid)</strong></td>
<td>(n = 25)</td>
<td>(n = 11)</td>
<td>(n = 13)</td>
<td>(n = 8)</td>
<td></td>
</tr>
<tr>
<td>1.02 (0.89 to 1.16)</td>
<td>0.69 (0.63 to 0.75)**</td>
<td>0.50 (0.42 to 0.57)**</td>
<td>0.52 (0.40 to 0.64)**</td>
<td>0.45 (0.27 to 0.63)**</td>
<td></td>
</tr>
<tr>
<td>4.04 (3.86 to 4.22)</td>
<td>4.78 (4.54 to 5.02)**</td>
<td>3.32 (3.16 to 3.47)**</td>
<td>3.97 (3.78 to 4.16)</td>
<td>3.63 (3.54 to 3.73)</td>
<td></td>
</tr>
<tr>
<td><strong>PL (nmol/lung)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23.3 (22.0 to 24.7)</td>
<td>26.0 (24.8 to 27.2)</td>
<td>18.5 (17.2 to 19.9)</td>
<td>20.6 (18.3 to 22.9)</td>
<td>19.9 (18.1 to 21.7)</td>
<td></td>
</tr>
<tr>
<td><strong>PL (% of total lung PL)</strong></td>
<td>43.4 (39.0 to 47.9)</td>
<td>26.5 (24.7 to 28.5)**</td>
<td>27.1 (23.0 to 31.2)**</td>
<td>25.2 (20.4 to 30.0)**</td>
<td>22.6 (16.5 to 29.1)**</td>
</tr>
<tr>
<td><strong>SP-A (ng/total BAL fluid)</strong></td>
<td>175 (162 to 185)</td>
<td>197 (185 to 209)</td>
<td>195 (170 to 220)</td>
<td>195 (170 to 220)</td>
<td></td>
</tr>
<tr>
<td><strong>Protein (mg/total BAL fluid)</strong></td>
<td>19.9 (18.0 to 21.8)</td>
<td>12.6 (11.8 to 13.4)</td>
<td>19.9 (18.1 to 21.7)</td>
<td>19.9 (18.1 to 21.7)</td>
<td></td>
</tr>
<tr>
<td>*∗∗∗ p&lt;0.001 versus controls</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Data are means and 95% confidence intervals in brackets of the indicated numbers of experiments (n).

Table 2 Phospholipid composition of bronchoalveolar lavage (BAL) fluid and lung tissue in cftrm1HGU/m1HGU mice versus MF1 and other control (BALB/c; C57BL/6; C3H/He) mice

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>cftrm1HGU/m1HGU (n = 13)</th>
<th>MF1   (n = 6)</th>
<th>Other controls (n = 21)**</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BAL fluid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>84.2 (81.2 to 87.1)</td>
<td>83.3 (81.8 to 84.8)</td>
<td>85.3 (83.8 to 86.8)</td>
</tr>
<tr>
<td>PG</td>
<td>11.1 (9.0 to 13.3)</td>
<td>12.0 (10.6 to 13.4)</td>
<td>10.4 (7.6 to 12.0)</td>
</tr>
<tr>
<td>SPH</td>
<td>1.1 (0.8 to 1.4)</td>
<td>0.8 (0.4 to 1.1)</td>
<td>1.1 (0.5 to 1.5)</td>
</tr>
<tr>
<td>PE</td>
<td>2.1 (1.1 to 2.4)</td>
<td>3.2 (2.1 to 4.5)</td>
<td>1.1 (0.5 to 1.5)</td>
</tr>
<tr>
<td>PS</td>
<td>1.6 (1.2 to 2.0)</td>
<td>0.7 (0.4 to 1.1)</td>
<td>2.1 (1.2 to 2.7)</td>
</tr>
<tr>
<td><strong>Lung tissue</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>51.3 (49.8 to 52.9)</td>
<td>50.9 (48.7 to 51.3)</td>
<td>49.4 (48.3 to 50.3)</td>
</tr>
<tr>
<td>PG</td>
<td>4.2 (3.1 to 5.4)</td>
<td>4.2 (3.1 to 5.4)</td>
<td>3.4 (2.6 to 4.2)</td>
</tr>
<tr>
<td>SPH</td>
<td>8.2 (7.9 to 8.5)</td>
<td>8.4 (8.0 to 8.7)</td>
<td>8.3 (8.0 to 8.6)</td>
</tr>
<tr>
<td>PE</td>
<td>34.1 (31.9 to 36.2)</td>
<td>36.2 (34.1 to 38.5)</td>
<td>36.7 (35.5 to 37.9)</td>
</tr>
<tr>
<td>PS</td>
<td>2.2 (1.8 to 2.6)</td>
<td>1.2 (1.0 to 1.4)</td>
<td>2.2 (1.8 to 2.7)</td>
</tr>
</tbody>
</table>

Table 3 Molecular species of phosphatidylcholine (PC) in bronchoalveolar lavage (BAL) fluid and lung tissue in cftrm1HGU/m1HGU versus MF1 and other control (BALB/c; C57BL/6; C3H/He) mice

<table>
<thead>
<tr>
<th>PC species</th>
<th>cftrm1HGU/m1HGU (n = 9)</th>
<th>MF1   (n = 14)</th>
<th>Other controls (n = 17)**</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BAL fluid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.3 (4.9 to 5.8)</td>
<td>5.7 (5.3 to 6.1)</td>
<td>4.7 (3.9 to 5.4)</td>
<td></td>
</tr>
<tr>
<td>57.9 (56.4 to 59.4)</td>
<td>58.3 (57.3 to 59.9)</td>
<td>58.3 (56.0 to 60.7)</td>
<td></td>
</tr>
<tr>
<td>19.8 (18.8 to 20.9)</td>
<td>17.9 (16.9 to 18.9)</td>
<td>13.5 (10.3 to 16.8)*</td>
<td></td>
</tr>
<tr>
<td>6.3 (5.6 to 6.9)</td>
<td>6.9 (6.5 to 7.2)</td>
<td>9.0 (8.0 to 9.9)**</td>
<td></td>
</tr>
<tr>
<td>0.4 (0.2 to 0.6)</td>
<td>0.2 (0.1 to 0.3)</td>
<td>1.2 (0.4 to 2.0)</td>
<td></td>
</tr>
<tr>
<td>1.1 (0.9 to 1.2)</td>
<td>1.2 (0.6 to 2.6)</td>
<td>1.4 (1.1 to 1.7)</td>
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</tr>
<tr>
<td>0.4 (0.2 to 0.6)</td>
<td>0.2 (0.1 to 0.3)</td>
<td>0.8 (0.5 to 1.2)</td>
<td></td>
</tr>
<tr>
<td>0.4 (0.2 to 0.5)</td>
<td>0.3 (0.1 to 0.9)</td>
<td>0.5 (0.3 to 0.7)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>3.9 (2.9 to 4.9)</td>
<td>3.7 (3.3 to 4.1)</td>
<td>3.4 (2.7 to 4.3)</td>
</tr>
<tr>
<td><strong>Lung tissue</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.6 (3.0 to 4.1)</td>
<td>4.0 (3.5 to 4.5)</td>
<td>2.9 (2.6 to 3.1)</td>
<td></td>
</tr>
<tr>
<td>34.8 (33.2 to 36.3)</td>
<td>35.6 (34.8 to 36.3)</td>
<td>35.2 (32.1 to 34.3)</td>
<td></td>
</tr>
<tr>
<td>10.8 (9.9 to 11.8)</td>
<td>8.4 (7.8 to 9.1)**</td>
<td>7.8 (6.5 to 9.0)**</td>
<td></td>
</tr>
<tr>
<td>11.1 (10.2 to 12.0)</td>
<td>10.2 (9.3 to 11.2)</td>
<td>11.6 (11.1 to 12.2)</td>
<td></td>
</tr>
<tr>
<td>3.6 (3.5 to 3.8)</td>
<td>3.3 (2.9 to 3.6)</td>
<td>4.0 (3.7 to 4.3)</td>
<td></td>
</tr>
<tr>
<td>4.3 (4.0 to 4.6)</td>
<td>4.0 (3.6 to 4.4)</td>
<td>4.9 (4.6 to 5.2)</td>
<td></td>
</tr>
<tr>
<td>3.8 (3.5 to 4.4)</td>
<td>3.8 (3.3 to 4.2)</td>
<td>4.0 (3.7 to 4.5)</td>
<td></td>
</tr>
<tr>
<td>3.4 (3.1 to 3.7)</td>
<td>4.9 (4.4 to 5.4)</td>
<td>3.2 (3.0 to 3.5)</td>
<td></td>
</tr>
<tr>
<td>1.4 (1.2 to 1.6)</td>
<td>2.7 (1.4 to 3.9)</td>
<td>1.6 (1.4 to 1.7)</td>
<td></td>
</tr>
</tbody>
</table>

In lavaged lung tissues from cftrm1HGU/m1HGU mice the amount of phospholipid was 4.04 (3.86 to 4.22) nmol/whole lung and 175 (166 to 185) nmol/whole lavaged lung/g body weight. While the absolute values per lavaged lung displayed some significant differences between cftrm1HGU/m1HGU mice and two of the control strains — namely, MF1 and BALB/c — there were no significant differences after correcting these values for body weight (table 1). Consequently, BAL fluid from cftrm1HGU/m1HGU mice contained 19.9 (18.0 to 21.8) mol% of total lung phospholipid (BAL fluid + lavaged lung), while in MF1, BALB/c, C57BL/6, and C3H/He mice the BAL fluid contained only 12.6 (11.8 to 13.4) mol%, 13.0 (11.2 to 14.7) mol%, 11.3 (9.2 to 13.5) mol%, and 11.0 (7.5 to 14.5) mol%, respectively (table 1). The apparent concentration of SP-A, a major component of surfactant, was therefore 64 (52 to 76)% of SP-A in MF1, BALB/c, C57BL/6, and C3H/He mice.
were those of sphingomyelin (SPH) or phosphatidylethanolamine (PE) increased in either BAL fluid or lung tissue of \textit{cftr}^\text{m1HGU/m1HGU} mice. Because there were no differences in the phospholipid compositions of BALB/c, C57BL/6 and C3H/He mice, these data were combined and are shown as “other controls”. Since the key molecule of surface tension function of lung surfactant is PC16:0/16:0, and since alterations in the concentrations of individual molecular species of PC are a more sensitive indicator of pulmonary surfactant alterations than the determination of PC fatty acids by gas chromatography,\textsuperscript{19} we subsequently investigated these PC molecular species in both BAL fluid and lavaged lung tissue. The BAL fluid and lavaged lung tissue of \textit{cftr}^\text{m1HGU/m1HGU} mice predominantly contained PC16:0/16:0 (table 3), comprising 57.9 (56.4 to 59.4) mol\% and 34.8 (33.2 to 36.3) mol\% of total PC, respectively. These values were identical to those from control mice. Other typical components of surfactant—which for example, palmito-lyrystoyl-PC (PC16:0/14:0) and palmitoyl-palmitoleoyl-PC (PC16:0/16:1)—were also without major changes in \textit{cftr}^\text{m1HGU/m1HGU} mice compared with controls. The highly unsaturated PC species palmito-lylarchidonoyl-PC (PC16:0/20:4), stearo-lylarchidonoyl-PC (PC18:0/20:4), and palmito-lydocosahexa- enoyl-PC (PC16:0/22:6) were not detected in significant amounts in BAL fluid but made up 12–14\% in lung tissue. Again, there were no major differences between \textit{cftr}^\text{m1HGU/m1HGU} and control mice.

**SURFACE TENSION FUNCTION OF SURFACTANT IN BAL FLUID**

The relative concentration of active surfactant isolated by differential ultracentrifugation at 60 000g (P60000) compared with the phospholipids in the supernatant (S60000) of the BAL fluid is shown in fig 3. In BAL fluid from \textit{cftr}^\text{m1HGU/m1HGU} mice the amount of phospholipid in P60000 was significantly higher than in controls. To evaluate whether the surface tension function of the P60000 was different from that of controls we measured the surface tension function of this material. Equilibrium surface tension (\(\gamma_{\text{equil}}\)) after 10 seconds of static adsorption of P60000 from \textit{cftr}^\text{m1HGU/m1HGU}, MF1, and other control mice was 24.7 (19.1 to 30.3) mN/m, 25.0 (24.2 to 25.9) mN/m, and 28.6 (25.5 to 31.6) mN/m, respectively (table 4). Dynamic measurement of minimal surface tension (\(\gamma_{\text{min}}\)) showed that P60000 from \textit{cftr}^\text{m1HGU/m1HGU} mice reached values below 5 mN/m as quickly as surfactant from the control mice (table 4).

**COMPOSITION OF PHOSPHOLIPID CLASSES AND PHOSPHATIDYLCHOLINE (PC) MOLECULAR SPECIES**

Investigation of phospholipid compositional changes in BAL fluid and lung tissue of \textit{cftr}^\text{m1HGU/m1HGU} mice was in the first instance addressed by HPLC analysis of phospholipid classes. As shown in table 2 there were no differences in the relative composition of phospholipid classes in BAL fluid and lung tissue of \textit{cftr}^\text{m1HGU/m1HGU} and control mice. Importantly, the contributions of PC and phosphatidyglycerol (PG) were not decreased, nor surfactant and important for its interactions with type II alveolar cells, phospholipids, bacteria and alveolar macrophages, was 259 (239 to 279) ng/total BAL fluid of \textit{cftr}^\text{m1HGU/m1HGU} mice which was identical to the concentrations in BAL fluid of control mice (table 1). Due to the increase in the concentration of phospholipids in the BAL fluid, the ratio of SP-A to phospholipid was decreased by 33–45\% from 0.40 (0.34 to 0.46), 0.47 (0.35 to 0.58), 0.42 (0.36 to 0.53), and 0.49 (0.33 to 0.63), respectively, in the individual control strains to 0.27 (0.23 to 0.31) in \textit{cftr}^\text{m1HGU/m1HGU} mice (table 1).

**Discussion**

Cystic fibrosis is an inherited disease with a defect in the \textit{CFTR} gene resulting in the expression of a defective \textit{CAMP}-dependent chloride channel.\textsuperscript{12} Previous reports of impairment to the biophysical properties of airway secretions, as well as lipid extracts of such secretions in patients with cystic fibrosis as-
Lung surfactant in a cystic fibrosis animal model

Increased phospholipid concentration together with a decreased SP-A to phospholipid ratio in the alveolar space. Decreased clearance of surfactant along the conductive airways due to the impaired bronchopulmonary clearance in CFTR deficiency is unlikely to play a major role since this is typically a minor route for surfactant clearance. Recent findings in isolated cells from patients with cystic fibrosis, however, support the concept that a primary alteration of phospholipid metabolism may contribute to this increased surfactant phospholipid pool in the alveolus. In isolated fibroblasts and platelets from patients with cystic fibrosis phospholipid synthesis was increased, but such cells do not secrete major amounts of phospholipid. However, despite the tempting explanations outlined above, other explanations for an increase in surfactant phospholipids in the alveolar space should be mentioned. The cftr gene transcript regulates the cAMP-dependent Cl− current and thereby the transport of other ions and of water through cells. Cftr expression in type II alveolar epithelial cells could indirectly alter the homeostasis of the watery alveolar hypophase and of cAMP, thereby influencing surfactant phospholipid homeostasis.

The alterations in cftrHGU/mHGU mice described in this study are different from those previously described in chronically infected airways of human subjects. While in studies on airway secretions from patients with cystic fibrosis alterations in phospholipid classes, fatty acid composition of phospholipids, and biophysical properties (adsorption velocity) were described, we did not detect such changes in BAL fluid from cftrHGU/mHGU mice without infection. Similarly, no impairment of surface tension function of the surfactant isolated from the BAL fluid of cftrHGU/mHGU mice was detected. We therefore conclude that such alterations are due to chronic inflammation and leucocyte infiltration of cystic fibrosis lungs, secondary to colonisation with opportunistic bacteria. Our data support the concept that any alteration in the phospholipid composition is due to chronic infection and inflammation rather than an effect of CFTR mutations.

While phospholipids were increased in the BAL fluid, the amount of SP-A remained unchanged. This unexpected finding is in agreement with a recent study by Doyle et al who showed that phospholipid and SP-A secretion into the alveolus are independently regulated.

While the mechanisms of such changes in cftrHGU/mHGU mice are still uncertain and await further investigation, the potential consequences for pulmonary defence of an increased amount of phospholipid and a decreased ratio of SP-A to phospholipid are more obvious. Surfactant contributes to pulmonary integrity as a first line of defence. Phospholipid inhibits the activity of SP-A to opsonise bacteria and depresses the respiratory burst of alveolar macrophages upon challenge. Increased phospholipid concentrations together with a decreased SP-A to phospholipid ratio in cftrHGU/mHGU mice, and possibly in patients with cystic fibrosis, may...
impair the resistance of the lungs against bacteria. Since human SP-A was used as a standard in the immunoassay, the values obtained for the mouse SP-A are not absolute and are only for use for the comparison of SP-A levels within the different strains of mice examined in this study. Nevertheless, our results are consistent with recent findings on BAL fluid from patients with cystic fibrosis which showed an increased concentration of PC, while the ratio between SP-A and phospholipid was even more decreased in the BAL fluid of patients with cystic fibrosis than in that of cfrmHGU mice. However, while the data from human BAL fluid originate from lungs that already show symptoms of chronic impairment and, possibly, proteolytic degradation of SP-A due to inflammation, the cfrmHGU mice used in this study were kept under specific pathogen-free conditions. Histological examination of lungs confirmed the absence of leucocytic infiltration or other visible alteration of lung structure and cellular distribution, and the absence of increases in membrane phospholipids such as PE or S phospholipid to SP-A was increased in the BAL fluid of patients with cystic fibrosis compared with cfrmHGU mice may in part be due to secondary degradation of SP-A caused by chronic inflammation of the lung.

We conclude that, in cfrmHGU mice without chronic inflammatory lung disease, the composition of phospholipid classes and phosphatidylcholine molecular species, the concentration of SP-A, and the surface tension function of surfactant are all unchanged compared with control mice. However, the total amount of phospholipid and the ratio of phospholipid to SP-A was increased in the BAL fluid, possibly as a result of altered surfactant functions.

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Lung surfactant in a cystic fibrosis animal model: increased alveolar phospholipid pool size without altered composition and surface tension function in cftrm1HGU/m1HGU mice.

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