The surface-lining layer of airways in cystic fibrosis mice

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Running head: Lung lining layer in CF mice

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ABSTRACT

Lung disease is the major cause of death in individuals suffering from cystic fibrosis (CF), with abnormal lung-lining fluids occurring already in early infancy. However the precise aetiology of CF lung disease is still poorly understood. We investigated the structural components of the airway surface-lining layer in targeted $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ mutant mice and non-CF controls. Five lungs per animal group were fixed by intravascular triple perfusion. The ultrastructure of the surface-lining layer of large and small intrapulmonary conducting airways was systematically investigated according to a standard protocol in transmission and scanning electron micrographs. In both animal groups, the surface-lining layer consisted of an aqueous phase and an osmiophilic film of variable thickness at the air-fluid interface. The aqueous phase did usually extend less than 1 µm beyond the uppermost tips of the epithelial cells in both animal groups. The aqueous phase of the small airways was slightly more electron dense in $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ than in non-CF mice. Neither the ultrastructure of the surfactant film at the air-liquid interface nor the forms assumed by the osmiophilic structures associated with surfactant turnover in the aqueous layer differed significantly in $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ and non-CF mice. Hence, there were no signs of any ultrastructural abnormalities in the surface-lining layer of young adult $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ mice before infection with CF-related pathogens.
INTRODUCTION

Cystic fibrosis (CF) is a prevalent, lethal, autosomal recessive disorder, which is characterized by abnormal ion transport across epithelial membranes. This disturbance in ion transport is attributable to mutations within the transmembrane conductance regulator (CFTR) gene (39). Although many organs are implicated in CF, chronic lung disease is the foremost cause of morbidity and mortality. Albeit so, the precise aetiology of CF lung disease remains poorly understood. Clinically, it is characterized by inflammation and mucus obstruction of the airways and by chronic infection with a specific spectrum of bacteria [reviewed by Dinwiddie (7)].

The surface-lining layer of the airways in normal lungs consists of an aqueous phase and of a surfactant film at the air-fluid interface. The continuous aqueous phase lies adjacent to the airway epithelial cells and has a relatively low viscosity. Above this periciliary layer, a gel phase has been postulated to occur, whose thickness, continuity and very existence in different airway compartments and species are still disputed (12, 24, 33, 40). In small airways, the presence of a thick mucus layer has never been demonstrated (12, 15). However, all available evidence supports the existence of a continuous surfactant film at the air-fluid interface within the alveoli and central airways (3, 12, 15, 16, 19), which plays an important role in the displacement of particles towards the epithelium (13, 30). Whilst the function and homeostasis of the surfactant film lining the alveoli are well known, those of that in the airways have not been full elucidated (10, 25, 26).

As the dysfunction of the CFTR chloride channel in CF leads to abnormal surface liquids, many studies have been carried out to resolve the homeostasis of the volume and to determine the ion composition of the lung’s surface lining layer in health as well as in disease (e.g. 21, 22, 27, 34, 37). Recently, evidence has been furnished of a functional as well as a biochemical disturbance in the surfactant of patients with progressive CF [reviewed by Griese (18)].
Changes in the phospholipid and protein composition of this film may cause dramatic changes in the architecture of the surface-lining layer, which is the first barrier of defense against inhaled and deposited particles of biological or inorganic origin (11). But the ultrastructure of the lung-lining layer in CF is largely unknown and cannot be studied in humans.

With the advent of mouse models for CF in 1992 (8, 36), it became possible to study the mechanisms underlying lung disease in association with this disorder. The $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ mouse, generated by insertional mutagenesis (8), expresses a low percentage of wild-type $Cftr$ m-RNA owing to aberrant splicing (9). This lower-than-normal level of CFTR appears to be responsible for the only mild intestinal disturbances manifested by this phenotype and consequently for its improved survival rates, which permits an investigation of the pulmonary aberrations. A similar phenotype is manifested in human compound heterozygotes, who are also predicted to partially retain normal CFTR function (32). Although pancreatic function is improved in these patients, they can still develop severe lung disease. Physiological studies with $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ mutant mice have revealed the cAMP-dependent chloride secretion of lung epithelial cells to be reduced, as is the case in CF-patients (8, 35). Furthermore, these mice develop a more severe form of lung disease than do their non-CF littermates (6), which is pathogen-specific, there being no overt clinical signs of pulmonary dysfunction at birth and prior to bacterial exposure (8). Moreover, the pertinent volumes and surface areas of the airway and alveolar compartments, as well as the thickness of the air-blood barrier are the same in young adult $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ and non-CF mice before infection with CF-relevant pathogens (14). However, Zahm et al. (41) have demonstrated mucociliary clearance, but not ciliary beat frequency, to be impaired in the trachea, of untreated $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ mice. The authors accounted for their observations by a postulated increase in mucus production. Other investigators have reported the phospholipid pool size to be increased in the bronchoalveolar lavage of uninfected
Cftr<sup>tm1HGU</sup>/Cftr<sup>tm1HGU</sup> mice (5), a defective metabolism of primary phospholipids having been forwarded as a possible explanation for these findings. It would thus appear that structural changes in the surface-lining layer of Cftr<sup>tm1HGU</sup>/Cftr<sup>tm1HGU</sup> mice antecede infection with CF-relevant pathogens. But so far, the architecture of the surface-lining layer in mice, and especially in CF-mice, has received very little attention.

In the present study, we compared the ultrastructures of the surface-lining layer of the intrapulmonary conducting airways in untreated young adult Cftr<sup>tm1HGU</sup>/Cftr<sup>tm1HGU</sup> and non-CF mice.
MATERIALS AND METHODS

Animals. \(Cftr^{tm1HG-U}/Cftr^{tm1HG-U}\) transgenic mice with an MF1/129 background (n=5), as well as the non-CF controls (3 wild-type mice and 2 mice heterozygous for \(Cftr\)) were obtained from Dr. Julia Dorin in Edinburgh (8). They all bore coded earmarks which were deciphered only after the raw data had been collected. The ages (111 to 144 days) and body weights (25 to 42 g) of \(Cftr^{tm1HG-U}/Cftr^{tm1HG-U}\) and control mice did not differ significantly from each other. The mice were housed and maintained under standard conditions of animal husbandry. All experimental procedures were performed in accordance with the Swiss Federal Act on Animal Protection and the Swiss Animal Protection Ordinance, and approved by the Cantonal Veterinary Department Bern, Switzerland (permit no. BE106/97).

Anaesthesia and preparation for lung fixation. Mice were anesthetized by an intraperitoneal injection first of ketamine hydrochloride (Ketalar®, Parke-Davis, Walther-Lambert AG, Baar, Switzerland) and then of pentobarbital (Grogg Chemie AG, Bern, Switzerland), each at a dose of 50 mg/kg of body weight. Under these conditions, mice are deeply anesthetized and in analgesia, but still able to breathe spontaneously. Intravascular blood coagulation was inhibited by an intraperitoneal injection of heparin [250 IU/animal (Liquemine®, Hofmann-La Roche AG, Reinach, Switzerland)]. A cannula was inserted into a tracheostoma and tightly affixed in readiness for manual ventilation after the formation of a pneumothorax.

Lung fixation. In order to preserve the airways and alveoli in an air-filled state, the lungs were perfusion fixed \(in situ\). After performing a thoracotomy and removing the ventral portion of the chest wall, a cannula was introduced through the right ventricle into the pulmonary artery and held firmly in place with surgical gut. The left atrial auricle was then opened and the circulating blood flushed out with a plasma substitute (polygeline, Haemaccel®, Behring AG, Marburg, Germany). Three slow inflation-deflation cycles were then set in train, which
inflated the lungs to total capacity [TLC (the 25-cm level of a water column)] and prevented atelectasis. The lungs were maintained at 60% TLC by reducing the inflation pressure to 5 cm of water (on the deflation limb), and the intravascular perfusion of fixatives was then begun. The three sequentially applied fixative solutions, 2.5% glutaraldehyde, 1.0% osmium tetroxide and 0.5% uranyl acetate (1, 20) each contained 3% dextran (T-70; Grogg Chemie AG, Bern, Switzerland) to maintain the oncotic pressure. This triple perfusion fixation has been shown in small rodents to preserve the surface-lining layers of intrapulmonary conducting airways and alveoli, including the phospholipids of the surfactant film at the air-fluid interface (12, 15, 16).

**Tissue sampling and processing for microscopic analysis.** The lungs were cut into 2-mm-thick slices running perpendicular to the longitudinal axis. Every second slice (with a random start) was processed for transmission electron microscopy [TEM (Fig. 1)]. The other slices were destined for scanning electron microscopy (SEM) or other purposes. Each of the Epon-embedded slices to be used for TEM were sub-sampled, one sub-slice being taken from the apical region and one from the hilum of the left lobe, and one sub-slice from the basal region and one from the hilum of the right lobe. 10-µm thick sections were then cut parallel to the sub-slice face using a Reichert Jung microtome (2050 Supercut; Leica, Glatbrugg, Switzerland). Airway-containing regions were sampled according to their size with the aid of a WILD M10 stereomicroscope (Leica). The tissue blocks corresponding to the identified regions were sawed out of the Epon sub-slices and cut into semi-thin (1- to 2-µm-thick) and ultrathin (60- to 80-nm-thick) sections. Semi-thin sections, stained with Toluidine Blue, were used for a general evaluation of the fine pulmonary structures in the light microscope. Ultrathin sections were transferred to uncoated 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined in a Philips 300 transmission electron microscope (Philips AG, Zürich, Switzerland) operating at 60 kV. The location of the first micrograph
was selected at random. If the lining layer was not preserved at this position, the adjacent optical field was chosen. One micrograph was taken at low magnification (final magnification \( \times 9,300 \)) to evaluate the histology of the airway wall. A series of four micrographs, embracing adjacent regions of the airway surface, was then taken at a higher magnification (final magnification \( \times 29,700 \)). An additional micrograph was taken at a final magnification of \( \times 115,500 \) to resolve details such as osmiophilic film layer-number.

For SEM, two 2-mm-thick slices, one from the left and one from the right lung, were sampled from each mouse, dehydrated in ethanol, critical-point-dried and sputter-coated with platinum. They were examined in a Philips XL 30-FEG scanning electron microscope (Philips) operating at 10 kV.

**TEM analysis.** A total of 150 micrographs from each animal group were analyzed according to a standard protocol for the presence of specified structural elements of the airway wall and the surface-lining layer (Tables 1 and 2). The epithelium, the muscle layer and the connective tissue were evaluated on the low-magnification micrographs, whereas the surface-lining layer was evaluated only on the higher-magnification ones. The aqueous layer adjacent to the epithelial surface, within which the cilia and microvilli of the epithelial cells and the surface macrophages are embedded, was evaluated for its uniformity ("regular" or "irregular" distribution of its components) and electron density ("opaque" or "transparent"). Osmiophilic structures within the surface-lining layer, most of which are linked with the surfactant system, were assessed predominantly by their shape (film-forming, lamellar structures, tubular myelin structures, vesicular forms or membrane fragments), but also sometimes by their location (at the air-fluid interface or within the aqueous phase).

**SEM analysis.** TEM permits a detailed ultrastructural analysis of the surface-lining layer, but the area scored, though representative, is necessarily small. SEM was therefore used to view and investigate the surface-lining layer from above over larger areas.
Statistical analysis. Data were compared using the two-sided Mann-Whitney (Wilcoxon) rank-sum test, the level of significance for differences being set at $p < 0.05$. Firstly, the group mean values for each parameter were compared. Secondly, the group mean values for each parameter in large and small airways were compared. And thirdly, the mean values for large and small airways within the same animal group were compared. Within the control group, data pertaining to the two mice heterozygous for $Cftr$ were then excluded and the statistical analysis was repeated.
RESULTS

*Fine pulmonary structure and the airway wall.* The fine pulmonary structure appeared to be normal in both animal groups (Table 1, Figs. 3 and 4). The epithelium of the large intrapulmonary conducting airways was columnar to cuboidal, depending on the distance from the extrapulmonary airways, and low cuboidal in the small ones. The epithelium consisted mainly of ciliated and non-ciliated (Clara) cells in both animal groups, with ciliated cells being scored more frequently in the larger than in the smaller airways, and vice versa for the non-ciliated ones. The connective tissue layer tended to be thicker in \( Ctfr^{tm1HGU}/Ctfr^{tm1HGU} \) than in non-CF mice, but the difference did not attain statistical significance. According to definition, there were more layers of muscle cells in large than in small airways. But one macrophage was recorded; it was lodged in the aqueous phase of a small airway in a \( Ctfr^{tm1HGU}/Ctfr^{tm1HGU} \) mouse. Neither granulocytes nor lymphocytes were observed within either the lung-lining layer or any grade of airway wall.

*Aqueous phase.* A periciliary aqueous layer was observed in 95-100% of all micrographs (Table 2, Fig. 2). In general, its constituent material tended to be more uniformly ("regularly") and less irregularly distributed, as well as more electron dense ("opaque") and less transparent in the larger than in the smaller airways of both groups. However, this airway-size-related difference reached statistical significance \( p < 0.05 \) only in the \( Ctfr^{tm1HGU}/Ctfr^{tm1HGU} \) group of mice. A comparison of the electron density of the material lining just the small airways in each animal group, revealed a greater, though non-significant, opacity in the \( Ctfr^{tm1HGU}/Ctfr^{tm1HGU} \) mice.

The aqueous phase did not usually extend much beyond the uppermost tips of the epithelial cells (< 1 \( \mu m \)). A mucus layer lying beneath the osmiophilic surfactant film at the air-fluid interface, which therefore had to be extremely thin, could not be unambiguously distinguished from the rest of the aqueous phase on TEM micrographs in either group.
Epiphase. A second aqueous phase, overlying the first and delimited on both sides by an osmiophilic film, was encountered three times (Table 2, Figs. 2 and 5C). In one instance (large airway of a \(Cftr^{tm1HGU}/Cftr^{tm1HGU}\) mouse), the epiphase consisted of electron dense, diffusely-distributed floccular material; in the second [large airway of a non-CF (heterozygous) mouse], it contained multi-lamellar structures and vesicles; and in the third [small airway of a non-CF (wild-type) mouse], it was composed exclusively of vesicular structures.

Osmiophilic structures. Osmiophilic elements associated with the surface-lining layer, and which are mostly linked with the surfactant system, were found in 88-99% of all micrographs (Table 2, Fig. 2). Osmiophilic surfactant films, located at the air-fluid interface (Figs. 3, 4 and 5), were observed in 75-88% of all micrographs. Bilayered and multilayered surfactant films were found in the smaller as well as in the larger airways of both animal groups. Films tended to be more diffuse in \(Cftr^{tm1HGU}/Cftr^{tm1HGU}\) than in non-CF mice (non-significant), especially in the large airways.

Multilamellar (Fig. 5A) and tubular-myelin structures (Fig. 5B) were encountered more often in the smaller than in the larger airways of both animal groups. Extracellular vesicles (Figs. 3 and 4) occurred at the lowest frequency in the large airways of \(Cftr^{tm1HGU}/Cftr^{tm1HGU}\) mice. Membrane fragments were more often recorded in the smaller than in the larger airways of both animal groups. Overall, such fragments were more frequently encountered in the aqueous layer of \(Cftr^{tm1HGU}/Cftr^{tm1HGU}\) mice. The differences, however, did not attain statistical significance.

When data pertaining to the two control mice that were heterozygous for \(Cftr\) were excluded from the analysis, the statistical significances of the aforementioned differences respecting osmiophilic structures remained unchanged.
Surface-lining layer by SEM. The analysis of nine large and seven small airways in $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ mice and of ten large and seven small airways in non-CF ones confirmed the TEM findings. The surface-lining layer covered the cilia and microvilli of epithelial cells and tended to be more compact in the larger than in the smaller airways of both animal groups (non-significant) (Fig. 6). The thickness of the surface-lining layer beyond the uppermost tips of the epithelial cells, which included the non-SEM-resolvable surfactant film at the air-fluid interface, was constant at $<1 \mu m$. 
DISCUSSION

In the present study, we have demonstrated the surface-lining layer of intrapulmonary conducting airways in \( \textit{Cftr}^{\text{tm1HGU}}/\textit{Cftr}^{\text{tm1HGU}} \) and the non-CF mice to consist of a continuous aqueous phase containing various surfactant-related osmiophilic structures (vesicles, tubular myelin, multilamellar structures and membrane fragments) and an osmiophilic surfactant film of variable thickness at the air-fluid interface. The surface-lining layer did usually extend less than 1 \( \mu \text{m} \) beyond the uppermost tips of the epithelial cells in both animal groups.

\textit{Lung fixation in situ:} In the present study, the ultrastructure of the surface-lining layer of intrapulmonary conducting airways was investigated for the first time in the lungs of mice that had been fixed by the sequential intravascular perfusion of glutaraldehyde, osmium tetroxide and uranyl acetate. This triple perfusion-fixation technique has been previously shown in small laboratory animals to preserve the surface structures of alveoli and intrapulmonary conducting airways, including the surfactant film at the air-fluid interface (1, 12, 15, 16). Chemical fixation followed by dehydration is still the only technique available for imaging whole lungs in an air-filled state.

\textit{The aqueous phase.} The aqueous phase, especially that of the small airways, tended to be more electron dense in \( \textit{Cftr}^{\text{tm1HGU}}/\textit{Cftr}^{\text{tm1HGU}} \) than in control mice. This appearance might be suggestive of a higher concentration of proteins or other constituents, which would support the hypothesis that surface fluids are underhydrated in CF. However, water depletion of the surface fluids in CF leads to a reduction in the volume of the periciliary phase (37), and there was no evidence of this in the present study.

The aqueous phase of the airways in both animal groups was located between the epithelium and the surfactant film at the air-fluid interface and covered the uppermost extremities (cilia and microvilli) of the epithelial cells. The surface-lining layer has been observed to be thinnest over protruding Clara cells and in positions where macrophages
overlie the epithelial surface; it was thickest in ciliated zones (Figs 3 and 4). The combined height of an epithelial cell (including cilia and/or microvilli) and the surface-lining layer appears to remain constant for a given lung compartment so as to furnish a regular (non-undulating) surface which continues into the alveoli (3, 16). Recently, Bachofen and Schürch (2) have demonstrated that the surface tension exerted by the surfactant film at the air-fluid interface influences the underlying structures in the airways as well as in the alveoli.

Predictably, the surface-lining layer did usually not extend much beyond the uppermost tips of the epithelial cells (< 1 µm) in the intrapulmonary conducting airways of both animal groups (12, 15). A second aqueous phase (epiphase) overlying the first and separated from it by osmiophilic-film structures, which might represent "mucus islands" or "streets" (38), was observed only rarely in the present study. These findings accord with the absence of a substantial mucus layer in the small airway compartments. Although Zahm et al. (41) have reported an accumulation of mucus in their $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ mice, this occurred within the proximal trachea, were goblet cells and submucosal glands are admittedly scarce but nevertheless present.

The air-fluid interface. Preservation of the extremely fragile surfactant film at the air-fluid interface is problematic, and the chemical fixation and dehydration techniques used in the present study are known to induce its artificial rupturing or detachment from the air-fluid interface (16). But such artifacts were readily identified as blunt-ending structures in the transmission electron microscope and as fissures in the scanning electron microscope, and they did not interfere with the analysis.

In the present study, surfactant films of variable thickness were found in the airways of both animal groups. Multilayered surfactant films have been previously observed using the captive-bubble surfactometer, and have been interpreted as a "surface-associated surfactant reservoir" (31). Diffuse films, which were more often observed in $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ mice,
may be generated by tangential cutting of bilayered or multilayered surfactant films. This being the case, it is not clear why they were encountered more frequently in Cftr<sup>tm1HGU</sup>/Cftr<sup>tm1HGU</sup> than in non-CF mice. However, if all of these diffuse films represented tangentially-cut multilayers, then the airways of Cftr<sup>tm1HGU</sup>/Cftr<sup>tm1HGU</sup> mice, especially the large ones, would have thicker surfactant films than those of non-CF controls.

The phospholipid pool size of fluid derived from bronchoalveolar lavages has been reported to be larger in Cftr<sup>tm1HGU</sup>/Cftr<sup>tm1HGU</sup> than in non-CF mice, which indicates that the surfactant pool size is larger. The authors interpreted this finding in terms of a decreased re-uptake of phospholipids by the CF epithelium (4, 5).

**Osmiophilic structures in the aqueous layer.** Osmiophilic elements related to surfactant turnover, such as multilamellar structures, tubular myelin or vesicles, are commonly encountered in the aqueous layer of alveoli [for review, see Goerke (17)]. But we found them also in the intrapulmonary conducting airways of both Cftr<sup>tm1HGU</sup>/Cftr<sup>tm1HGU</sup> and non-CF mice, and more frequently in smaller than in larger ones. The occurrence of these structures in the intrapulmonary conducting airways of small rodents has been previously reported (12, 15). Vesicular structures are involved in surfactant film formation and their location at the air-fluid interface has been demonstrated by Schürch et al. (31). In the present study, vesicles were less frequently encountered at the air-fluid interface in the large conducting airways of Cftr<sup>tm1HGU</sup>/Cftr<sup>tm1HGU</sup> than in non-CF mice. These vesicles have also been suggested to represent superfluous surfactant drained from the alveoli (23). However, if such were the case, then they would have been expected to occur more frequently in Cftr<sup>tm1HGU</sup>/Cftr<sup>tm1HGU</sup> mice, since it is these animals that have the larger surfactant pool size (4, 5).

**The airway wall.** Non-ciliated cells were abundant in the intrapulmonary conducting airways of both animal groups. These cells correspond to the different subtypes of Clara cells, which represent the prevailing secretory cell type in mice (28, 29). There was no evidence of
the epithelial metaplasia that has been observed in \( \text{Cftr}^{\text{tm1HGU}}/\text{Cftr}^{\text{tm1HGU}} \) mice after repeated exposure to CF-relevant pathogens (6).

Zahm et al. (41) have reported a significant increase in the number of inflammatory cells within the lamina propria of the tracheal mucosa of untreated \( \text{Cftr}^{\text{tm1HGU}}/\text{Cftr}^{\text{tm1HGU}} \) mice. Although the lamina propria tended to be thicker (non-significant) in \( \text{Cftr}^{\text{tm1HGU}}/\text{Cftr}^{\text{tm1HGU}} \) than in non-CF mice, we observed no inflammatory cells within the mucosa of the intrapulmonary airways.

In summary, the present study demonstrates the existence of no major ultrastructural differences in the surface-lining layer of intrapulmonary conducting airways between untreated young adult \( \text{Cftr}^{\text{tm1HGU}}/\text{Cftr}^{\text{tm1HGU}} \) and non-CF mice. These observations are relevant to the pathogenesis of human CF, firstly because the slightly “leaky” \( \text{Cftr}^{\text{tm1HGU}}/\text{Cftr}^{\text{tm1HGU}} \) mouse mutant is deemed to serve as a model for the human compound heterozygotes, and secondly because CF is believed to begin in the small-airway compartment.
Acknowledgements

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REFERENCES


Table 1. *Histology of the airway wall*

<table>
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<tr>
<th>Structure analyzed</th>
<th>Non-CF</th>
<th>Non-CF</th>
<th>Cftr&lt;sup&gt;tm1HGU&lt;/sup&gt; / Cftr&lt;sup&gt;tm1HGU&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>small airs.</td>
<td>large airs.</td>
<td>small airs.</td>
</tr>
<tr>
<td>Cuboidal cells, single layer*</td>
<td>95</td>
<td>80</td>
<td>95</td>
</tr>
<tr>
<td>Ciliated cells†</td>
<td>50</td>
<td>76</td>
<td>57</td>
</tr>
<tr>
<td>Non-ciliated cells‡</td>
<td>95</td>
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<td>99</td>
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<tr>
<td>Macrophages‡</td>
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<td>0</td>
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</tr>
<tr>
<td>Connective tissue*</td>
<td>70</td>
<td>60</td>
<td>85</td>
</tr>
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Numbers represent the percentages of micrographs in which a particular entity was observed. Airways were sized according to their diameter and wall histology. *Structures recorded on low-magnification micrographs (small airways: 20 micrographs; large airways: 10 micrographs). †Structures recorded on low-as well as on high-magnification micrographs (small airways: 100 micrographs; large airways: 50 micrographs).
Table 2. *Ultrastructure of the airway surface by TEM*

<table>
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<tr>
<th>Structure analyzed</th>
<th>non-CF</th>
<th></th>
<th>Cftr&lt;sup&gt;tm1HGU&lt;/sup&gt;/Cftr&lt;sup&gt;tm1HGU&lt;/sup&gt;</th>
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<td></td>
<td>small airways</td>
<td>large airways</td>
<td>small airways</td>
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<td>n*=40</td>
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<tr>
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<tr>
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<td>28</td>
<td>35</td>
</tr>
<tr>
<td>Grouped</td>
<td>13</td>
<td>3</td>
<td>14</td>
</tr>
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Numbers represent the percentages of micrographs in which a particular entity was observed. Airways were sized according to their diameter and wall thickness. *n=number of micrographs analyzed. †Statistically significant difference between large and small airways within the same animal group, p < 0.05. #The epiphase is a second aqueous layer, overlying the first and separated from it by an osmiophilic film.
FIGURE LEGENDS

Fig. 1. Tissue sampling. The left (LL) and right (RL) lungs were embedded in agar and cut into 2-mm-thick slices, in a direction perpendicularly to the longitudinal axis. Every second slice (*) was embedded in Epon. Two slices from each lung lobe were sub-sampled from the embedded material. Airways were sized and sampled on 10-µm-thick sections with the aid of a stereomicroscope. Tissue blocks containing the identified regions were sawed out of the Epon sub-slices and cut into semi-thin and ultra-thin sections for the structural analysis.

Fig. 2. Data from the ultrastructural analysis of the surface-lining layer of intrapulmonary conducting airways in $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ (n=5) and non-CF mice (n=5). Percentage mean values (together with the standard deviation) are represented for each structure analyzed. $A$: small airways; $B$: large airways; $C$: pooled data for large and small airways.

Fig. 3. Transmission electron micrographs of large intrapulmonary conducting airways. $A, B$: $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ mouse. $C, D$: non-CF mouse. $A, C$: overview of the airway wall. The epithelium contains both ciliated (CI) and non-ciliated (NC) cells and multiple layers of muscle cells (M). Details of the boxed regions are represented at higher magnification in ($B$) and ($D$). $B, D$: the periciliary aqueous layer (AL) contains multiple vesicles (V) and is covered by an osmiophilic surfactant film of variable thickness (double arrows). Note that the periciliary aqueous phase is thinner over the protruding Clara cells and thicker over the ciliated cells. Bars = 5 µm ($A, C$) and 0.5 µm ($B, D$).

Fig. 4. Transmission electron micrographs of small intrapulmonary conducting airways. $A, B$: $Cftr^{tm1HGU}/Cftr^{tm1HGU}$ mouse. $C, D$: non-CF mouse. $A, C$: overview of the airway wall. The epithelium contains both ciliated (CI) and non-ciliated (NC) cells. Details of the boxed
regions are represented at higher magnification in (B) and (D). B, D: the periciliary aqueous layer (AL) contains multiple vesicles (V) and is covered by a surfactant film (double arrow), which follows closely the upper-extremities of the cilia and microvilli. Note that the periciliary aqueous phase is extremely thin over the protruding Clara cells. Bars = 5 µm (A, C) or 0.5 µm (B, D).

Fig 5. Transmission electron micrographs of osmiophilic structures in the aqueous layer (AL), which is situated between the epithelium (EP) and the surfactant film at the air-fluid interface (double arrows). In (A) a multilamellar structure (ML) is illustrated and in (B) a tubular myelin structure (TM). In (C), a second aqueous layer (epiphase) containing vesicles (V) is illustrated. This overlies the periciliary phase and is separated from it by an osmiophilic film (solid arrow) of comparable thickness to that at the air-fluid interface (double arrow). Bars = 0.5 µm.

Fig. 6. Low- (A, C) and high- (B, D: boxed regions in A and C, respectively) magnification scanning electron micrographs of the surfaces of large intrapulmonary conducting airways. A, B: *Cftr*^tm1HGU//Cftr*^tm1HGU* mouse. C, D: non-CF mouse. The surface-lining layers (SLL) extend less than 1 µm beyond the uppermost endings of the epithelial cells. The surfactant films at the air-fluid interfaces cannot be resolved. A = alveoli; EP = epithelium; CI = cilia; CT = connective tissue; and M = muscle layer of the airway wall. Bars = 5 µm (A, C) and 1 µm (B, D).
Figure 1
Figure 3

Surface-lining layer of airways in cf mice
Figure 4
Figure 5

Surface-lining layer of airways in cf mice

21/08/03
Figure 6

Surface-lining layer of airways in cf mice