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Linking progression of fibrotic lung remodeling and ultrastructural alterations of alveolar epithelial type II cells in the amiodarone mouse model

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The function of the lung critically depends on its structural integrity. Within the limited space of the chest the mammalian lung provides a large surface area with a very thin diffusion barrier for efficient gas exchange (61). An economical network of connective tissue elements including collagen and elastic fibers stabilizes the lung, allowing continuous volume changes during breathing without noticeable effort at the same time. Besides the connective tissue elements, the surfactant system of the lung is of utmost importance, since it stabilizes the interior surface area of the lung by reducing surface tension within the alveoli, particularly at low lung volumes (3). The alveolar epithelium consists of alveolar epithelial type I and type II (AE2) cells. AE2 cells, also termed the “defender of the alveolus” (10), are critically involved in epithelial regeneration and surfactant metabolism. Increasing evidence suggests that dysfunction of the AE2 cells is causally linked to interstitial lung diseases in humans and in animal models. Targeted injury of AE2 cells by diphtheria toxin has been shown to be sufficient to induce fibrotic remodeling in mice lungs (51). Furthermore, disturbances in the secretion pathway of lamellar bodies, the surfactant storage organelles of AE2 cells, are linked to the development of pulmonary fibrosis in mice mimicking features of the Hermansky-Pudlak syndrome-associated interstitial pneumonia (HPSIP) (17, 34). In these mice, obvious features of AEC2 cell stress include giant lamellar bodies, early lysosomal and late endoplasmic reticulum (ER) stress, and, finally, apoptosis of this cell type (34). Supporting this, increased susceptibility for the development of pulmonary fibrosis and abnormalities in the AE2 cells were reported in animal models of Hermansky-Pudlak syndrome (64). Hence, intracellular trafficking defects of surfactant components as such might contribute to a profibrotic milieu in the lung (64). On the same line, increased surface tension within alveolar air spaces, as observed in different models of lung fibrosis (19, 34), suggests impaired alveolar dynamics, a feature that is likely to be present in sporadic IPF as well (16, 46).

In familial IPF, mutations of the surfactant protein C (SP-C) gene lead to the accumulation of misfolded and misdirected SP-C, which in turn induced an unfolded protein response, ER stress, and, ultimately, apoptosis (5, 37, 39). Ultrastructural analyses of lung explants from patients with juvenile interstitial...
lung disease due to SP-C mutations revealed the existence of hypertrophic AE2 cells, often containing increased number of profiles of disorganized lamellar bodies (18). Similarly, spontaneous development of lung fibrosis in domestic cats, linked to the quantitative and qualitative alterations of lamellar bodies within hypertrophic AE2 cells, was reported, suggesting similarities between feline IPF and human IPF (62). IPF is one of the most common forms of diffuse parenchymal lung diseases. It is, however, associated with a very limited prognosis (43). Effective drug therapies completely stopping disease progression are currently not available, and lung transplantation remains the only therapeutic option (49). The current pathogenetic concept suggests that fibrotic pulmonary remodeling is a consequence of repetitive minor injuries of unknown origin of the alveolar epithelial lining with denudation of the basal lamina and a disturbed alveolar reepithelialization (13). The exact mechanisms leading to the ongoing injury of the alveolar epithelium have not yet been defined in IPF, but it has been hypothesized that premature senescence of AE2 cells, disturbances of the surfactant system, and mechanical stress are involved (6, 8). Recent studies focusing on AE2 cells in IPF and animal models of pulmonary fibrosis point towards a central role of this cell type in the disease pathogenesis, since impaired regeneration capacity in concert with increased rate of apoptosis (58) and ER and lysosomal stress is consistently observed (15, 26, 34). Such “cellular stress” signature of the AE2 cells found in familial cases with SP-C mutations is at least in part shared with sporadic IPF (26, 27). According to earlier ultrastructural observations, AE2 cells located at fibrotic septal walls accompanied by signs of acute injury showed marked abnormalities of lamellar bodies’ morphology in IPF (23). However, in areas with massive destruction and more progressed fibrotic remodeling like honey combing, AE2 cells were less often found and largely replaced by cuboidal cells, whose ultrastructure differed significantly from that of the AE2 cells (23). AE2 cells and myofibroblasts appear to be the most important constituents in IPF, at least in the initial phase of the disease, meaning that structural and molecular alterations of AE2 cells and the degree of fibrotic remodeling within septal walls are linked together. Hence, dysfunctional AE2 cells as morphologically characterized by hypertrophy (increase in cellular size), hyperplasia (increase in number), and abnormalities of the intracellular surfactant system might be involved in generating a profibrotic microenvironment in the lung (48).

Amiodarone, a very potent antiarrhythmic drug, accumulates within lysosomes and related organelles such as lamellar bodies. Administration of this drug is associated with interstitial fibrosis without relevant inflammation in mice paralleled with surfactant accumulation, increased ER stress and lysosomal stress, and increased autophagy and apoptosis of AE2 cells (32, 33). We have recently demonstrated that LC3B, the autophagy marker protein, was preferentially localized at the limiting membrane and the interior of lamellar bodies both in healthy controls and after amiodarone challenge. Moreover, we observed classical autophagosomes, which are extremely rare in AE2 cells, in direct connection with the limiting membrane of the lamellar body after amiodarone challenge providing clear evidence that lamellar bodies and autophagosomes share the same source of membranes. In full support, knockdown of LC3B reduced amiodarone-induced accumulation of SP-C and apoptosis of alveolar epithelial cells (32). Hence, autophagy to a certain degree seems to be involved in cellular surfactant homeostasis and lamellar body formation in healthy AE2 cells while the excess of autophagy as indicated by an increase in LC3B and related increase in intracellular surfactant pool might induce dysfunction of AE2 cells and even cell death. Whether these autophagy-related disturbances of intracellular surfactant pool or whether an overall increase in autophagy would directly correlate with the severity of septal wall fibrosis is unclear. To answer this question, design-based stereology up to the electron microscopic level represents the method of choice. Design-based stereology allows sophisticated quantification of fibrotic remodeling in different compartments of the lung, e.g., within septal walls or within alveolar space, a feature that is not possible using other analytical methods. In the present study we analyzed the degree of septal wall fibrosis as a function of disturbances of the intracellular surfactant system. We tested the hypothesis whether ultrastructural alterations of AE2 cells including hypertrophy and hyperplasia as well as the alterations in the intracellular surfactant pool correlate with the severity of fibrosis in the amiodarone model of pulmonary fibrosis in mice by means of design-based stereology at the light and ultrastructural level. A high positive correlation between disturbances of the intracellular surfactant pool such as the volume of lamellar bodies on the one hand and the total amount of collagen fibrils within the septal wall tissue on the other hand could be found. These findings could be reproduced in sporadic human IPF diagnosed according to current criteria (43) at a qualitative level in areas with comparatively slight to moderate fibrosis.

MATERIALS AND METHODS

Animal models

The animal model of amiodarone-induced interstitial fibrosis has been described in detail elsewhere (33). Briefly, 20 male C57Bl/6 mice at the age of 8 wk received 0.8 mg/kg body wt amiodarone intraorally every 5th day. Animals were randomly divided into two groups. AD d7 and AD d14 were killed 7 and 14 days after first amiodarone treatment, respectively. Seven days after first amiodarone administration, collagen content as estimated by hydroxyproline level in our previous study was significantly increased and progressive till day 14 but remained stable thereafter up to day 28 (33). Age-, gender-, and body weight-matched C57Bl/6 mice served as controls. Both the University Animal Care Committee and the Federal Authorities for Animal Research of the Regierungspreisidium Giessen (Hessen, Germany) approved the study protocol.

Fixation, Sampling, and Processing

The quantitative morphometric methods used in this study are based on the official research policy statement of the American Thoracic Society/European Respiratory Society on quantitative assessment of lung structure (21). Five animals each in groups wild type and AD d7 as well as six animals in group AD d14 were included in this study. The perfusion fixation process was based on a previous protocol described by Vasilescu et al. (56). A multipurpose fixative was used containing 4% paraformaldehyde, 0.1% glutaraldehyde in 0.2 M HEPES buffer. The perfusion pressure was 30 cmH2O and the airway opening pressure during fixation was 13 cmH2O, respectively. Fixation was started after the pulmonary vascular bed was rinsed with 0.9% NaCl solution. The airway opening pressure was adjusted during expiratory limb after recruitment maneuver (30 cmH2O). After storage of the lungs for at least 24 h in fixative at a temperature of 4°C, the fluid displacement method was used to determine the total lung...
volume (45). Afterwards, a systematic uniform randomization was carried out (54). The whole lungs were embedded in 4% agar and cut in cranio-caudal direction in eight lung slices with a thickness of 2 mm each using a tissue slicer. The aim of the systematic uniform randomization is to give every part of the lung the same chance of being considered for the stereological assessment. Hence, the stereological parameters represent the entire lung. Tissue slices designated for light microscopy were osmicated, immersed in 4% aqueous uranyl acetate, dehydrated in an acetone solution with rising concentration (e.g., 70, 90, and 100%), and embedded in glycol methacrylate according to the manufacturer’s instructions (Technovit 8100; Heraeus Kulzer, Wehrheim, Germany). This protocol has been validated to minimize tissue deformation and therefore was appropriate for design-based stereology (47). The tissue slices assigned to electron microscopy had to undergo an additional unbiased sampling step. Areas from which a cube with an edge length about 1–2 mm should be taken from were randomly chosen by a point grid cast randomly on the slices. By means of this procedure, six to eight tissue blocks were obtained per lung. These small tissue blocks were postfixed in osmium tetroxide, stained en bloc in half-saturated aqueous uranyl acetate, dehydrated in a rising acetone series, and embedded in Epon. Six out of six to eight tissue blocks were taken by chance and further processed for stereological assessment.

Stereological Analysis

Design-based stereology at the light microscopic level was conducted applying a computer-assisted stereology system (newCAST; Visiopharm, Horsholm, Denmark) equipped with a computer-controlled Ludl-stage to carry out a systematic uniform random area sampling. In our study, stereological parameters to appropriately characterize lung fibrosis in animal models were based on recently published recommendations (36, 40). Following a cascade sampling design, volume fractions (Vv) of structures of interest were determined within the given reference volume at different levels of magnification using a coherent point grid for point counting. As the magnification was increased within the given reference volume at different levels of magnification, volume fractions of alveolar air space (Vv(alv,par)), ductal air space (Vv(duc,par)), and septal wall tissue (Vv(sep,par)) as well as the surface density of the alveolar epithelium (SV(alvepi,par)), basal lamina (Vv(bl/sep)), collagen (Vv(col/sep)), interstitial cells (Vv(ic/sep)), endothelial cells (Vv(endo/sep)), and capillary lumen (Vv(cap/sep)) were used to determine the volume fractions of alveolar epithelium (Vv(alvepi/sep)), basal lamina (Vv(bl/sep)), collagen (Vv(col/sep)), interstitial cells (Vv(ic/sep)), endothelial cells (Vv(endo/sep)), and capillary lumen (Vv(cap/sep)). The arithmetic mean thickness of the blood-gas-barrier ([r(bg)]) was calculated as a volume-to-surface-ratio by point and intersection counting (60).

Function of Alveolar Surfactant

Function of alveolar surfactant was determined by means of established methods (33, 34). Mice were killed and subjected to bronchoalveolar lavage. Large surfactant aggregates were prepared by high speed centrifugation (48,000 × g, 1 h, 4°C) and adjusted to 2 mg/ml phospholipid content. After a 30-min incubation period at 37°C, samples were transferred to the disposable sample chamber, and adsorption was measured over an initial period of 12 s (γads = equilibrium surface tension). Next, pulsation was started by sinusoidally oscillating the bubble radius between 0.4 and 0.55 mm at a rate of 20 cycles/min. The pressure differences across the air/liquid interface were recorded continuously over 5 min and minimum surface tension (γmin = dynamic surface tension behavior under pulsation) was calculated using the Young-Laplace equation.

Ultrastructure of Human IPF Lung Explants

Detailed ultrastructural descriptions of AE2 cells in fibrosing lung diseases including IPF have been published in 1982 (23). As diagnostic criteria of the clinical entity IPF have changed within the last decades, we processed lung tissue of two IPF lung explants diagnosed according to current criteria (43) for transmission electron microscopic evaluation. These two patients with sporadic IPF were participating in the European IPF Registry/Biobank and gave informed consent for participation. As most parts of the lungs of IPF lung explants are completely destructed and characterized by honeycomb and cysts with very severe fibrosis, we focused on areas of the lung that were adjacent to such severe fibrotic areas as identified at macroscopic level but still contained air so that tissue did not descent in fixation solution. Tissue blocks with a diameter of 1–2 mm were sampled and immersion fixed in 1.5% glutaraldehyde, 1.5% paraformaldehyde in 0.15 M HEPES buffer for at least 24 h at 4°C. Afterwards, tissue was embedded in Epon as described above. The European IPF Registry and Biobank have been approved by the local ethic committee and data protection officers. Embedded tissue for electron microscopic evaluation from five healthy donor lungs was available from a previous study (41).
Statistics

Data were analyzed using one-way ANOVA with a Bonferroni correction for multiple comparisons. A value of $P < 0.05$ was considered significant. Correlation analyzes were carried out using Spearman-test and pooled data. Tests were performed using Prism GraphPad 5.0 software.

RESULTS

Qualitative Findings: Animal Models

Air spaces were homogenously inflated and septal walls were slim with open corner vessels in control animals, suggesting a successful perfusion fixation (Fig. 1). After amiodarone challenge, focal thickening of septal walls was observed (Fig. 1). In some regions with thickened septal walls alveolar ducts were enlarged, lacking alveoli (Fig. 1, asterisk), hinting at high surface tension and alveolar collapse (63) and collapse induration as a mechanism for a loss of ventilated alveoli (9, 31). Some areas demonstrated accumulation of intra-alveolar surfactant in alveolar space 7 days after first amiodarone challenge (Fig. 1, arrowhead), which was absent in the control group.

At the electron microscopic level traces of interstitial and alveolar edema were occasionally seen 7 days after challenge (Fig. 2). In addition, denudation of the basal lamina due to cell death (usually oncosis) of AE1 and AE2 cells was a typical finding (Fig. 2). Interstitial edema was present 7 days after amiodarone within the thick side of the septal wall, where, as opposed to the thin side, stabilizing connective tissue elements like collagen fibrils (Fig. 2, asterisk) are found. At day 14, edema was not present anymore. Instead large bundles of collagen fibrils could be observed within the alveolar septal wall tissue often in close neighborhood to enlarged profiles of AE2 cells, which were completely filled with enlarged and numerous lamellar bodies (Figs. 2 and 3). In addition, the thickness of the basal lamina appeared to have increased. Some AE2 cells could be located within thickened septal wall tissue, indicating that they had lost their usual spatial orientation.

Fig. 1. Amiodarone and septal wall thickening. Representative light microscopic images of toluidine blue stained sections: enlarged alveolar ducts (asterisk) with thickened septal walls deprived of their alveoli were seen in AD d7 and AD d14 (killed 7 and 14 days after 1st amiodarone treatment, respectively). Adjacent alveoli appeared to be enlarged compared with the control group. In some alveoli surfactant material could be observed after amiodarone treatment but not in untreated controls (arrowhead).

Fig. 2. Amiodarone and injury of the blood-gas barrier. Representative electron microscopic images of septal wall morphology: basal lamina was denuded in AD d7 as a consequence of epithelial cell injury (arrowhead). The endothelium was unaffected. In AD d14 bundles of collagen fibrils (asterisk) were increased and observed in thickened septal walls underneath enlarged alveolar epithelial type II (AE2) cells completely filled with lamellar bodies (lb). Alv, alveolar air space; bl, basal lamina; endo, endothelium; caplumen, capillary lumen; IC, interstitial cell; ed, interstitial edema.
regarding the contact to alveolar air space, e.g., as a consequence of alveolar collapse (Fig. 3). As published previously by our group (33), more and larger profiles of AE2 cells containing high amounts of lamellar bodies suggested hyperplasia and hypertrophy of AE2 cells in this animal model (Fig. 3).

Human IPF Lung Explants and Healthy Donor Lungs

Alveolar epithelium was injured as shown by fragmentation (Fig. 4, arrow) and denudation of the basal lamina in areas with slight septal wall thickening (Fig. 4, arrowhead). In direct neighborhood to areas of denuded basal lamina, AE2 cells were enlarged and filled with abundant lamellar bodies (Figs. 4 and 5). Some of the AE2 cells were lifted off the basal lamina and showed signs of cell death like swelling and vacuolization (Fig. 4, DC) (35). Septal walls were thickened as a consequence of an increase in components of the extracellular matrix (ECM) including collagen fibrils (Figs. 4 and 5, asterisk). A prominent finding was the marked thickening of the basal lamina in these regions. Enlarged and numerous AE2 cells were present that contained larger and above all more lamellar bodies compared with the healthy controls. Within interstitial tissue, profiles of AE2 cells occurred. Microvilli-like structures separated these AE2 cells from a surrounding basal lamina. As microvilli are usually observed at the luminal and not at the basolateral surface, this observation might be a consequence of alveolar collapse within the space between the AE2 cell and the denuded basal lamina representing the residual alveolar lumen. Alternatively, the polarization of the cell got lost and this might be considered a morphological correspondent of epithelial-mesenchymal transition.

Quantitative Findings: Amiodarone Model of Pulmonary Fibrosis

Stereological data related to general lung architecture and AE2 cells including intracellular surfactant pool and composition of the septal wall tissue are summarized in Tables 1–3 and Figs. 6–9.

General lung architecture and surfactant function. Amiodarone induced a significant increase in total volume of septal wall tissue per lung [V(sep,lung); Table 1]. While the total volume of alveolar air spaces [V(alvair,lung)] remained virtually unchanged, a marked increase in the total volume of ductal air space [V(ductair,lung)] could be found in AD d7 being in line with the qualitative finding of enlargement of alveolar ducts. In turn, the surface area of alveolar epithelium per unit

![Fig. 3. Amiodarone and ultrastructure of AE2 cells. Representative electron microscopic images showing ultrastructure of AE2 cells and the surfactant system: more and larger profiles of AE2 cells were observed in AD d7 and AD d14 (arrows) in particular in areas with thickened septal walls. These enlarged AE2 cells contained more and larger profiles of lamellar bodies.](image-url)
parenchyma \([S(V(alvepi/par))\] was significantly decreased in this group but was nearly normalized in AD d14. Alveolar number per lung \([N(alv,lung)\] showed a trend towards lower values in both amiodarone groups (Fig. 6A) while septal walls were significantly thickened (Fig. 6B). A significant negative correlation of pooled data between septal wall thickness and alveolar number supports the concept of collapse induration where alveolar collapse leads to septal wall thickening (Fig. 6C). In addition, the remaining alveoli were characterized by a larger number-weighted mean volume \([V_N(alv))\]. This finding supports the notion that alveolar collapse and collapse induration, e.g., due to high surface tension and at later time points fibroproliferation, led to thickening of septal walls around enlarged alveolar ducts. In line with this speculation, we observed remnants of the basal lamina within thickened septal walls 7 and 14 days after first amiodarone challenge (Fig. 6D–F). Remnants of the basal lamina have been suggested by Katzenstein and Myers (38) to result from collapse induration, which is likely to contribute to the loss of lung capacity in fibrosing lung diseases (31). In this context, we performed measurements to characterize the function of bronchoalveolar lavage-derived intra-alveolar surfactant by means of a pulsating bubble surfactometer. Seven days after first amiodarone challenge we observed a significant increase in minimum surface tension compared with control animals (Fig. 7). The surfactant dysfunction was associated with a decline in alveolar number and the occurrence of collapse induration at the ultrastructural level. Fourteen days after first amiodarone challenge, the function of the intra-alveolar surfactant had partly recovered although the alveolar number remained stable. This suggests that collapse induration is an irreversible process.

**AE2 cells and intracellular surfactant.** Compared with the control group, amiodarone induces a progressive increase in the number-weighted mean volume of AE2 cells \([V_N(AE2))\] from day 7 to 14 (Fig. 8A; Table 2). The total number of AE2 cells per lung \([N(AE2,lung))\] also increased at day 7 but remained stable thereafter (Fig. 8B). The hypertrophy of AE2 cells (increase in cellular volume) could partly be explained by an progressive increase in the total volume of lamellar bodies per cell \([V(lb,AE2))\; Fig. 8C]. As demonstrated by the volume-weighted mean volume, the lamellar bodies as such increased in size after amiodarone challenge (Fig. 8D).

**Composition of septal wall tissue.** Although alveolar collapse as such could at least partly explain the increase in septal wall thickness after amiodarone challenge, this is not the case regarding the marked increase in the total volume of alveolar septal walls per lung (Table 3). Hence, we analyzed the composition of septal walls at the ultrastructural level by dividing alveolar septal walls into different components: alveolar epithelium, basal lamina, collagen fibrils, amorphous ECM, interstitial cells, endothelium, and capillary lumen. A slight increase in the total volume of the alveolar epithelium...
[\text{V(alvepi,sep)}] was observed, which could most likely be attributed to hyperplasia and hypertrophy of AE2 cells and also to the swelling of alveolar epithelial type I cells. The total volume of endothelial cells [\text{V(endo,sep)}] and capillary lumen [\text{V(caplumen,sep)}], however, remained more or less stable after amiodarone challenge. Unlike the volume of capillaries within septal walls, the surface area of endothelium [\text{S(endo,sep)}] demonstrated a significant increase in AD d7, hinting towards angiogenesis, as a consequence of septal wall remodeling induced by amiodarone.

The most important factor leading to an increase in the volume of the septal walls could be assigned to the components of the interstitial tissue. The amorphous ECM [\text{V(aECM,sep)}] and collagen fibrils [\text{V(coll,sep)}] demonstrated a progressive increase after first amiodarone challenge and their volume increased by the factor 3 to 4 (Fig. 9A). The total volume of interstitial cells [\text{V(IC,sep)}] demonstrated an initial increase till day 7 by the factor 3 compared with the control group but then decreased again till day 14 after first amiodarone challenge. In essence, the described remodeling process led to an increase in the arithmetic mean thickness of the blood-gas barrier [H9270(bgb)] due to deposition of connective tissue elements within the alveolar septal walls (Fig. 9B).

Correlation analysis. A progressive remodeling, characterized by an increase in the volume of collagen fibrils until day 14 after amiodarone, could be observed. These findings were in line with the increase in the hydroxyproline level after the first amiodarone challenge as a typical marker used to assess the severity of pulmonary fibrosis (33). A correlation between the total volume of collagen fibrils within the alveolar septal wall (ultrastructural level) and the hydroxyproline level (biochemical level) could also be observed in the bleomycin model of pulmonary fibrosis (12). Hence, in the present study, we used the volume of collagen fibrils within the septal wall as an important severity marker of pulmonary fibrosis. High positive correlations between the volume of collagen fibrils within septal walls on the one hand and hypertrophy of AE2 cells, total volume of lamellar bodies per AE2 cell, and the volume-weighted mean size of lamellar bodies on the other hand could be established (Fig. 10, A–C). The same was the case taking the volume of amorphous ECM into consideration (data not shown).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>AD d7</th>
<th>AD d14</th>
<th>P Value</th>
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<tr>
<td>V(lung), cm³</td>
<td>0.52 (0.07)</td>
<td>0.93 (0.07)*</td>
<td>0.72 (0.20)</td>
<td>&lt;0.01</td>
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<tr>
<td>V(des,lung), mm³</td>
<td>/</td>
<td>34.3 (23.6)</td>
<td>27.9 (7.3)</td>
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<tr>
<td>N(alv,lung), 10⁶</td>
<td>3.09 (0.06)</td>
<td>2.22 (0.83)</td>
<td>2.23 (0.56)</td>
<td>0.08</td>
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<tr>
<td>V(Nalv/par), 1/cm³</td>
<td>6.33 (0.02)</td>
<td>2.72 (0.83)*</td>
<td>3.69 (1.03)†</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>S(alvepi,lung), cm²</td>
<td>209 (37.1)</td>
<td>210 (35.5)</td>
<td>245 (28.1)</td>
<td>NS</td>
</tr>
<tr>
<td>S(alvepi/par), cm²/cm³</td>
<td>431 (50.9)</td>
<td>273 (21.7)*</td>
<td>424 (82.6)‡</td>
<td>&lt;0.01</td>
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<tr>
<td>V(alvair,lung), cm³</td>
<td>0.29 (0.06)</td>
<td>0.37 (0.09)</td>
<td>0.31 (0.08)</td>
<td>NS</td>
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<tr>
<td>V(ductair,lung), cm³</td>
<td>0.12 (0.03)</td>
<td>0.33 (0.06)*</td>
<td>0.17 (0.07)‡</td>
<td>&lt;0.01</td>
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<tr>
<td>V(sep,lung), mm³</td>
<td>75.0 (8.7)</td>
<td>146.7 (16.8)*</td>
<td>128.5 (21.6)†</td>
<td>&lt;0.01</td>
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<td>t(sep), µm</td>
<td>7.32 (1.35)</td>
<td>14.5 (4.0)*</td>
<td>10.4 (0.9)‡</td>
<td>&lt;0.01</td>
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<tr>
<td>Lm, µm</td>
<td>78.2 (13.1)</td>
<td>118.6 (6.0)*</td>
<td>76.7 (18.3)†</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are means (SD). AD d7 and AD d14, killed 7 and 14 days after 1st amiodarone treatment, respectively; V, volume; N: number; Nv, numerical density; vN, number-weighted mean volume; S, surface area; Ss, surface area density; t, arithmetic mean thickness; des, destructed lung parenchyma; alv, alveoli; par, lung parenchyma; alvepi, alveolar epithelium; alvair, alveolar air spaces; ductair, ductal airspaces; sep, septal wall tissue; Lm, mean linear intercept length. One-way ANOVA and Bonferroni test for statistical significance: *control vs. AD d7; †control vs. AD d14; ‡AD d7 vs. AD d14.
shown). Altogether, alterations in intracellular surfactant pool and the severity of the pulmonary fibrosis are tightly correlated in this model.

DISCUSSION

The present study is the first stereological characterization of the amiodarone model of pulmonary fibrosis in mice. Moreover, for the first time, a quantitative link between alterations of AE2 cells and the degree of the deposition of collagen fibrils within the septum wall as a marker of severity of fibrosis could be established in an animal model. At a qualitative level these findings were reproducible in human IPF samples, where, in areas with thickened septal walls due to excessive ECM deposition but more or less maintained alveolar architecture and signs of lung injury (denuded basal lamina), AE2 cells appeared to be hypertrophic containing more and enlarged lamellar bodies. In humans amiodarone has been reported to induce a broad spectrum of pulmonary diseases ranging from acute respiratory distress syndrome to pulmonary fibrosis (7). In the current study, repetitive intratracheal instillation of amiodarone was used to mimic repetitive alveolar epithelial injury, a key characteristic of IPF. As demonstrated earlier by our group, amiodarone induces lysosomal stress (including autophagy) and endoplasmic reticulum stress and results in autophagy-dependent apoptosis of AE2 cells (32–34). Moreover, electron microscopic tomography convincingly demonstrated that autophagosomes and lamellar bodies share the same source of membranes and that LC3B preferably localizes to the limiting membrane or the interior of lamellar bodies. It is hence reasonable to state that autophagy manifests the formation of increased and enlarged lamellar bodies in this animal model of pulmonary fibrosis (32). Therefore, the mouse model of repetitive intratracheal instillation of amiodarone was exploited to establish a correlation between disturbances of the intracellular surfactant system as a severity marker of epithelial stress defined by ultrastructural criteria and the degree of septal wall fibrosis. Therefore, we tried to translate our ultrastructural findings from the animal model to human IPF samples. AE2 cells are critical for maintaining healthy alveolar homeostasis, which is also reflected by the term “defender of the alveolus”

Fig. 6. Collapse induration. Total number of alveoli per lung (A) and arithmetic mean thickness of septal walls (B). Septal wall thickness and total number of alveoli demonstrated a negative correlation (C), meaning that alveolar collapse and collapse induration could explain at least partly the loss of alveoli in this model. D–F: at the ultrastructural level, basal laminae could be observed within thickened septal walls (arrowhead). Alvepi, alveolar epithelial cell. Individual data and mean are given.

Fig. 7. Amiodarone and the function of alveolar surfactant. Seven days after first amiodarone challenge, minimum surface tension \( \gamma_{\text{min}} \) of bronchoalveolar lavage-derived alveolar surfactant is significantly increased and recovers somewhat on day 14. Data were obtained at a concentration of 2 mg phospholipids/ml.
Besides their critical role in surfactant homeostasis which is inevitable to stabilize alveolar micro architecture during respiratory cycle, the role of AE2 cells as self-renewing stem cells of the alveolar epithelial lining is well accepted (57). Moreover, direct cell contacts with surrounding cells including fibroblasts located underneath the basal lamina (50) suggest a direct epithelial-mesenchymal communication, which is expanded by autocrine and paracrine mediators including, for instance, growth factors or inflammatory factors secreted by AE2 cells. These factors are known to regulate epithelial-mesenchymal cross talk and in turn influence alveolar micro architecture including turnover of components of the ECM under physiological and pathological conditions (20, 25, 30). In addition, in vitro studies suggested that AE2 cells, in concert with interstitial cells, are involved in the production of components of the ECM including collagen type I (11, 25) and these extracurricular functions have to be regulated very tightly in order not to interfere with the regular pulmonary functions as gas exchanger. Hence, dysfunctional AE2 cells have been suggested as key factors in generating a profibrotic microenvironment in the lung in pulmonary fibrosis (48). Recent studies linked ER stress of AE2 cells due to excessive accumulation of misfolded surfactant proteins to AE2 cell dysfunction and, as a final consequence, apoptosis in familial (37, 39) as well as sporadic IPF (26, 27). In HPSIP, AE2 cells demonstrate a massive defect in secretion of intracellular surfactant components leading to an accumulation within the cell (17). This has been proven to be a potent trigger for lysosomal and ER stress, resulting in apoptosis (34), inflammation (2), and fibrotic remodeling (64). Hence, altered surfactant homeostasis by AE2 cells might be involved in generating a profibrotic environment in the lung. In the amiodarone model, increased autophagy has been shown to be a critical mechanism for accumulation of SP-C and cell death (32). In addition, qual-

Table 2. Alveolar type II cells and intracellular surfactant

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>AD d7</th>
<th>AD d14</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N(AE2,lung), 10⁶</td>
<td>2.76 (0.30)</td>
<td>4.63 (0.90)</td>
<td>4.48 (2.59)</td>
<td>NS</td>
</tr>
<tr>
<td>Nv(AE2/par), 1/cm³</td>
<td>5.54 (0.49)</td>
<td>5.74 (0.81)</td>
<td>6.93 (3.39)</td>
<td>NS</td>
</tr>
<tr>
<td>v(AE2), μm³</td>
<td>321 (33)</td>
<td>522 (25)*</td>
<td>648 (40)†‡</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>V(lb,AE2), μm³</td>
<td>58.3 (11.0)</td>
<td>101.7 (21.6)*</td>
<td>146.9 (17.3)*‡</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>V(lb,lung), mm³</td>
<td>0.16 (0.04)</td>
<td>0.46 (0.04)</td>
<td>0.69 (0.47)*‡</td>
<td>0.03</td>
</tr>
<tr>
<td>v(lb), μm³</td>
<td>0.95 (0.19)</td>
<td>1.63 (0.59)</td>
<td>1.80 (0.54)*‡</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Values are means (SD). AE2, alveolar type II cells; lb, intracellular surfactant; Vv, volume fraction; v, volume-weighted mean volume. One-way ANOVA and Bonferroni test for statistical significance: *control vs. AD d7; †control vs. AD d14; ‡AD d7 vs. AD d14.

Table 3. Composition of septal wall tissue

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>AD d7</th>
<th>AD d14</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>V(alvepi,sep), mm³</td>
<td>18.3 (2.9)</td>
<td>35.6 (7.20)*</td>
<td>31.6 (6.6)†</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>V(BL,sep), mm³</td>
<td>3.55 (0.94)</td>
<td>9.02 (2.18)*</td>
<td>6.85 (1.83)†</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>V(aECM,sep), mm³</td>
<td>7.48 (2.14)</td>
<td>27.3 (7.6)*</td>
<td>28.0 (11.6)*</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>V(col,sep), mm³</td>
<td>0.73 (0.29)</td>
<td>2.15 (0.52)*</td>
<td>3.06 (1.01)†</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>V(IC,sep), mm³</td>
<td>10.8 (3.4)</td>
<td>32.9 (13.9)*</td>
<td>23.3 (3.6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>V(endo,sep), mm³</td>
<td>15.1 (3.2)</td>
<td>21.1 (2.6)*</td>
<td>17.2 (2.7)</td>
<td>0.02</td>
</tr>
<tr>
<td>V(caplumen,sep), mm³</td>
<td>19.1 (6.4)</td>
<td>18.5 (5.6)</td>
<td>17.6 (3.1)</td>
<td>NS</td>
</tr>
<tr>
<td>S(endo,sep), cm²</td>
<td>620 (138)</td>
<td>978 (141)*</td>
<td>716 (123)‡</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>V(inter,sep), mm³</td>
<td>22.5 (3.1)</td>
<td>71.5 (21.8)*</td>
<td>61.2 (16.0)*</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are means (SD). Alvepi, alveolar epithelium; BL, basal lamina; aECM, amorphous extracellular matrix; col, collagen fibrils; IC, interstitial cells; endo, endothelium; caplumen, capillary lumen; inter, interstitial tissue; bgb, blood-gas barrier. One-way ANOVA and Bonferroni test for statistical significance: *control vs. AD d7; †control vs. AD d14; ‡AD d7 vs. AD d14.
tative ultrastructural findings suggested an increase of intracellular surfactant pool size as a consequence of amiodarone treatment. Lamellar bodies are part of the lysosomal pathway and diverse mechanisms have been identified or suggested regarding the biogenesis of this organelle (42, 59). From a structural point of view autophagy has been convincingly linked to the intracellular surfactant pool, partly revealing its link to the degree of interstitial fibrosis. The volume of intracellular surfactant, defined by morphological criteria as the volume of lamellar bodies, could be an appropriate parameter of cellular stress and AE2 cell dysfunction inducing autophagy in the amiodarone model of pulmonary fibrosis.

In the present study, we quantified alterations of the intracellular surfactant pool as well as the structural remodeling process within the septal wall tissue using design-based stereology at the light and electron microscopic level. We found a progressive hypertrophy of AE2 cells within the first 14 days after amiodarone challenge, accompanied by a dramatic increase in the total volume of lamellar bodies per AE2 cell. The later could be attributed to an increase in the mean size of lamellar bodies. These changes regarding the intracellular surfactant pool were escorted by a thickening of the septal walls as indicated by an increase in the arithmetic mean thickness of septal walls, a loss of ventilated alveoli, and an increase in interstitial tissue elements within septal walls. In particular, the total volume of ECM and above all collagen fibrils as defined by ultrastructural criteria demonstrated a progressive increase till day 14. In IPF, alveolar collapse has been discussed to explain the observed decrease in alveolar surface area and volume of air space per lung (9). These pathological findings, however, could partly be reproduced by the amiodarone model of lung fibrosis in the present study and are most likely a consequence of an impaired intra-alveolar surfactant function, a feature this mouse model shares with human IPF as well as with the bleomycin model (16, 31).

A former study from 1982 analyzed changes of epithelial cells at the ultrastructural level in human pulmonary fibrosis including IPF (23). As opposed to healthy lung tissue, AE2 cells found in areas of thickened septal walls with slight fibrotic changes were characterized by the occurrence of giant lamellar bodies. In the present study, we also analyzed areas of thickened septal walls from two IPF patients diagnosed according to the current criteria (43). We could confirm the qualitative findings by Kawanami et al. (23) and found larger and more profiles of AE2 cells filled with abundant and larger lamellar bodies in close neighborhood to bundles of collagen fibrils and denudation of the basal lamina. These observations indicated that AE2 cell changes and interstitial remodeling are colocalized with areas of ongoing epithelial injury. Interestingly, immunohistochemical staining in areas of human IPF lungs that appeared at light microscopic level more or less normal demonstrated an increased, dot-like staining for LC3B while in areas with severe fibrosis (e.g., fibroblastic foci) LC3B was reduced, meaning that in areas with severe fibrosis and fibroblast foci autophagy seems to be insufficient while in other areas autophagy could be increased (1). In human IPF these biochemical markers of autophagy seem to be paralleled by the ultrastructural presentation of alterations of intracellular surfactant where in severely affected areas lamellar bodies are rare, disrupted, and small while in other regions, e.g., giant

Fig. 9. Collagen content and blood-gas barrier. Total volume of collagen fibrils within septal walls per lung [V(col,sep); A] and the arithmetic mean thickness of the blood-gas barrier [θ(bgb); B]. Individual data and mean are given.

Fig. 10. AE2 cells and severity of fibrosis. Correlation analyses between the total volume of collagen within the septal walls and the number-weighted mean volume of AE2 cells (A) and total volume of lamellar bodies per AE2 cell (B) and volume-weighted mean volume of lamellar bodies (C).
lamellar bodies can be observed. However, severe fibrosis such as honeycombing and fibroblast foci is not present in the amiodarone model.

Intratracheal delivery of amiodarone might not truly mimic the drug delivery to the lung as it occurs clinically with systemic amiodarone therapy. Our animal model, however, exhibits histopathological alterations including patchy interstitial fibrosis, foamy AE2 cells, lung phospholipidosis, and AE2 apoptosis that resemble clinical amiodarone induced pulmonary toxicity. Of note, we recently reported severe surfactant accumulation and association of lamellar bodies with autophagosomal structures in amiodarone-treated mice. Hence, under the assumption that the increased intracellular surfactant pool is an indicator of cellular stress including autophagy with excessive accumulation of surfactant proteins and phospholipids, which might in turn be involved in driving fibrotic remodeling, we were able to establish a high positive correlation between the increase in the intracellular surfactant pool and the amount of collagen fibrils within septal wall tissue in the amiodarone model. Since both interstitial and epithelial cells are responsible for the turnover of components of the ECM, we also quantified the volume of interstitial cells within the septal walls. A correlation between the volume of interstitial cells and the degree of fibrosis was not found. These data clearly demonstrate that the alterations of AE2 cells and the septal wall fibrosis are linked together, meaning that they are either induced by the same factors or even causally linked, e.g., the excessive burden/stress due to increased surfactant pool provokes the generation of a more profibrotic environment by the AE2 cells, as seen in HPSIP.

In the amiodarone model, the total volume of destructed lung parenchyma was limited and amounted to <4% of total lung volume, which is less as has been found in the bleomycin model (>10%) (31). The bleomycin model is much more complex including interstitial fibrosis within septal walls, extensive intra-alveolar fibrosis, and alveolar collapse (28, 29, 31). In the amiodarone model, the most prominent findings were thickened septal walls due to interstitial fibrosis and less intra-alveolar fibrosis or collapse induration.

Conclusion

Amiodarone challenge induces a progressive interstitial remodeling with considerable increase in components of ECM especially collagen fibrils within the septal walls. Similar alterations regarding the different ultrastructural components have also been demonstrated to occur in IPF lungs. The progressive increase in collagen fibrils could be tightly correlated to disturbances of the intracellular surfactant system suggesting that both pathological alterations are mediated by the same factors or that there is a causal relationship. In other words accumulation of intracellular surfactant potentially linked to autophagy-related dysfunction of AE2 cells and cell death after amiodarone challenge is clearly correlated with the degree of septal wall fibrosis. Qualitative findings observed in thickened septal walls with slight to moderate fibrotic remodeling from patients with IPF suggest that hypertrophy of AE2 cells and an increase in the intracellular surfactant pool could also be linked to a fibrotic remodeling process in humans at least in the early stages of the disease process.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


