The Closer we Look the more we See? Quantitative Microscopic Analysis of the Pulmonary Surfactant System

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Key Words

Lung • Alveoli • Type II cells • Lamellar bodies • Surfactant • Stereology • Electron microscopy • Electron tomography

Abstract

The surfactant system of the lung has essential biophysical and immunomodulatory functions. Only at the electron microscopic level does surfactant reveal its morphological complexity - and beauty. Therefore, morphological tools are indispensable to characterize the surfactant system in health and disease. Stereology provides the gold standard for obtaining quantitative (morphometric) data in microscopy. The combination of microscopy and stereology allows for qualitative and quantitative analysis of the intraalveolar as well as the intracellular surfactant pool, both in its preserved microorganization and localization within the lung. Surfactant-producing alveolar epithelial type II cells can be counted and sampled for size estimation with physical disectors at a high magnification light microscopic level. The number of their surfactant storing lamellar bodies can be estimated using physical disectors at the electron microscopic level. Electron tomography allows for high resolution 3D visualization of lamellar body fusion pores. Intraalveolar surfactant subtypes

can be quantitated in situ, thus reflecting the functional state of the intraalveolar surfactant pool. By immunoelectron microscopy, surfactant protein distribution can be analyzed. These methods allow for a comprehensive quantitative analysis of surfactant (ultra-)structure. Here, we give an overview on the analysis of the normal and disordered surfactant system by electron microscopy and stereology.

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Pulmonary surfactant

Each day a human being inhales and exhales more than 10,000 liters of air. Within the lungs, the exchange surface for the diffusion of gases is distributed over about 300 to 500 million alveoli and is as large as 120 - 140 m² (nearly the size of a tennis court). At the same time, the thickness of the exchange barrier is only about 2 µm (50 times thinner than a sheet of air-mail stationery). This large and delicate surface has to be protected against collapse (i.e. atelectasis) as well as against invasion of pathogens. For both, biophysical and immunomodulatory, functions, the pulmonary surfactant system is principally responsible. Thus, the integrity of the pulmonary

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surfactant system is essential for normal lung function (for review, see [1-6]). Basically, surfactant helps to keep the large alveolar surface of the lung open, dry, and clean.

Surfactant consists of about 90% lipids and about 10% proteins. The protein fraction includes the surfactant-associated proteins SP-A, -B, -C, and -D. Only at the level of electron microscopy (EM) does surfactant reveal its morphological complexity (reviewed in [7]). Surfactant is synthesized, stored, secreted, and to a large extent recycled by type II alveolar epithelial cells [8]. Thus, an intracellular surfactant pool present in type II cells and an intraalveolar surfactant pool present at the surface of the fluid alveolar lining layer as well as within its hypophase can be distinguished (Fig. 1). Synthesis of surfactant material involves endoplasmic reticulum, at least partly Golgi complex, multivesicular bodies, and composite bodies (Fig. 2). The vast majority of intracellular surfactant material (all lipids and the hydrophobic SP-B and -C) is assembled in specific storage organelles, lamellar bodies, prior to secretion [9-11]. Intraalveolar surfactant consists of several subtypes. These include freshly secreted lamellar body-like forms, tubular myelin, the surface film, and small unilamellar vesicles. After secretion, lamellar body-like forms associate with SP-A and transform into lattice-like tubular myelin figures, which have been suggested to be precursors of the surface film although this has not been demonstrated directly. "Spent" surfactant components are found in the hypophase as small unilamellar vesicles that can be taken up either by type II cells for subsequent recycling or degradation or by alveolar macrophages for degradation. Therefore, intraalveolar surfactant subtypes that can be distinguished based on their morphology correspond to different functional stages in surfactant metabolism. In other words, the functional status of the surfactant system is directly reflected in intraalveolar surfactant subtype composition. This can either be assessed biochemically and biophysically using material obtained by bronchoalveolar lavage (BAL) (reviewed in [4, 12-14]) or morphologically by electron microscopy and stereology ([15]; see below).

The associated proteins are considered to be the "smart molecules" within the surfactant system [16], being involved in surfactant structural assembly, function (both biophysics and immunomodulation), and homeostasis. The hydrophilic surfactant proteins SP-A and SP-D are members of the collectin subfamily of C-type lectins. Collectins, pattern-recognition proteins with extended collagen-like domains linked to C-type lectin domains, participate in the innate immune response by both binding a wide variety of glycoconjugates and modulating immune

Fig. 1. Schematic illustration of surfactant metabolism. Surfactant components are synthesized in alveolar epithelial type II cells via transcription, translation at the rough endoplasmic reticulum (rER), posttranslational processing in the Golgi apparatus (Golgi) and further processing and transport in multivesicular bodies (MVB). Some surfactant components might bypass the Golgi apparatus and MVB. Surfactant lipid and the hydrophobic proteins SP-B and -C are assembled in so-called composite bodies (CB) sharing structural characteristics of MVB and mature lamellar bodies (LB). Surfactant is stored in LB and secreted by exocytosis. SP-A and SP-D are secreted via an alternative route by direct exocytosis of small vesicles and/or MVB. After exocytosis into the aqueous hypophase (HY), lamellar bodies are unpacked and form tubular myelin (TM) figures, from which the surface film (SF) can be generated. Hypophase and surface film are termed the alveolar lining layer (ALL). Surfactant that is removed from the air-liquid interface is either reused or degraded. "Spent" surfactant has the morphological appearance of unilamellar vesicles (UV) and can be taken up and degraded by alveolar macrophages (AM), be cleared via the airways or be taken up by type II cells. There the surfactant components may be degraded, enter de novo synthesis or be transported to CB or LB for resecretion. The surface active „large aggregates“ (LA) fraction of intraalveolar surfactant obtained by bronchioalveolar lavage largely corresponds to TM whereas the inactive „small aggregates“ (SA) fraction largely corresponds to UV. Reproduced with permission from [159].

cell responses [6, 17-19].

The crucial role of the surfactant system for the maintenance of the functional integrity of the lung is clearly demonstrated by surfactant dysfunction disorders, which can be caused either at birth by developmental deficiency (due to lung immaturity or due to mutations affecting synthesis, assembly, secretion or uptake of surfactant components) or later by acquired dysfunction (due to damage of type II cells or inhibition/inactivation of intraalveolar surfactant). A primary deficiency of

surfactant in the immature lungs causes the respiratory distress syndrome of premature neonates (RDS) [20]. Impairment of an originally intact surfactant system is involved in the pathogenesis of a variety of other lung diseases, such as acute lung injury / acute respiratory distress syndrome (ARDS) as well as infectious, obstructive and interstitial lung diseases [4, 5, 13, 14].

Ultrastructural and stereological analysis of surfactant

Morphological tools are essential to characterize the surfactant system in health and disease. These include in particular EM-based methods and stereology. The high spatial resolution provided by EM is required to directly visualize and analyze surfactant. A variety of basic and advanced EM methods is available. Adequate fixation and processing methods (described in detail in [21-23]) have to be employed in order to preserve the phospholipid-rich surfactant material for transmission EM investigation and thus to obtain reliable qualitative and quantitative data on surfactant ultrastructure. The intracellular and intraalveolar surfactant subtypes can then be visualized by conventional transmission EM (Fig. 3).

No method of tissue fixation for EM yields a “perfect” representation of lung structure. While in general, conventional chemical fixation for EM (based on glutaraldehyde and osmium tetroxide) results in good overall preservation of cell and tissue ultrastructure, the selective nature of the chemical interactions taking place during fixation, processing and dehydration may induce artifacts [24]. Therefore, morphological characteristics and differences observed in biological EM samples need to be interpreted with care and knowledge of the preparation steps involved. This is of particular importance in EM studies of surfactant because, due to its high amounts of saturated phospholipids, surfactant material can be extracted to a considerable degree, thereby e.g. resulting in “empty” lamellar bodies. One way to prevent this is based on phospholipid stabilization by prolonged bloc-staining with uranyl acetate prior to dehydration [22, 23] (Fig. 2, 3). Theoretically, the ideal method for near in-vivo preservation of ultrastructure is physical fixation by rapid freezing. For EM, the cooling conditions necessary to avoid artifacts (ice crystal formation) are achieved by high pressure freezing of small (thickness = 200 μm) samples [25]. However, applied to the lung, high pressure freezing would lead to collapse of alveoli unless they are filled with an appropriate fluid prior to fixation.

Fig. 2. Transmission electron micrograph of an alveolar epithelial type II cell in the human lung. Fixation by airway instillation. Phospholipid stabilization by bloc-staining with uranyl acetate (for details of the protocol, see 22, 23). The biosynthetic pathway of surfactant involves multivesicular bodies (MVB) and composite bodies (CB). Finally, the phospholipid-rich surfactant material is stored in lamellar bodies (LB) prior to secretion onto the alveolar epithelial surface.

Fig. 3. Transmission electron micrograph of rat lung fixed by vascular perfusion. Both the intracellular surfactant pool (in lamellar bodies (LB) within type II cells) and the intraalveolar surfactant pool (here tubular myelin (TM) covering the alveolar epithelium) can be visualized.

Fig. 4. Comparison of lamellar body ultrastructure in the human (a) and rat (b) lung. Human lung lamellar bodies usually show a concentric arrangement of lamellae originating from a dense projection core (PC). Rodent lamellar bodies usually contain lamellae arranged as parallel stacks.

Moreover, this approach is, at least yet, not suitable for quantitative analysis of whole lungs by stereology (see below) because it precludes adequate sampling for which the whole (fixed) organ should be available. At current, this can only be achieved by chemical fixation under carefully controlled conditions, either by airway instillation or (in particular for intraalveolar surfactant) by vascular perfusion, both of which also fulfill the criteria of consistent reproducibility in space (homogeneity) and time (repeatability) [21]. Nevertheless, high pressure freezing-based cryopreparation methods hold great potential for qualitative EM studies of surfactant.

The presence of lamellar bodies is a characteristic feature of type II alveolar epithelial cells. In humans, a single type II cell contains about 200-500 lamellar bodies, whereas in mice one finds about 50-100 lamellar bodies per type II cell [26]. Notable species differences exist with respect to lamellar body ultrastructure. In the human lung, lamellar bodies are composed of concentrically arranged lamellae attached to a projection core which, depending on the section plane, is usually located at the periphery. The projection core contains densely stacked short membrane segments in a random orientation (Fig. 4a). In contrast, lamellar bodies of rodents do not contain a projection core, and their lamellae, again depending on the section plane, are usually arranged in parallel (Fig. 4b). Species differences in lamellar body ultrastructure have long been noted (e.g. [27-30]), but they are not always taken into consideration. However, these findings are mainly based on chemically fixed samples. Verification (or falsification) by cryo-electron microscopy of vitreous sections [31] therefore seems desirable. Interesting concepts regarding the formation of lamellar bodies with parallel or concentric arrangement of lamellae have been provided recently by Perez-Gil [32]. Remarkably, differences in the ultrastructural arrangement of lamellae

Fig. 5. Immunogold labeling for SP-B in the human lung. Fixation by airway instillation, freeze-substitution, and embedding in Lowicryl HM20 (for details of the protocol, see [22]). Within type II cells, SP-B is localized in the projection core (PC) of lamellar bodies. Reproduced with permission from [7].

are reflected by (and most likely caused by) a differential distribution of surfactant proteins, in particular SP-B [33] (Fig. 5). This finding emphasizes the importance of the combination of cryopreparation methods and colloidal gold immuno-EM for the investigation of surfactant protein localization, distribution and processing [33, 34].

The biogenesis of lamellar bodies and the factors regulating the size and packing of the intracellular surfactant pool are only rudimentary understood [10]. Recent data from human patients and from mouse models with genetic alterations clearly demonstrate that disordered lamellar body biogenesis results in severe lung disease. In turn, these studies help understanding normal

lamellar body biogenesis by identifying key regulators. In principle, disorders of lamellar body biogenesis can be divided into underdevelopment (resulting in too few and/or too small lamellar bodies) or overdevelopment (resulting in too many and/or too large lamellar bodies). Examples for underdevelopment include SP-B deficiency [35-37], ABCA3 deficiency [38-41] and TRIC-B deficiency [42]. Overdevelopment is observed e.g. in SP-D deficiency ([43, 44]; see below), ABCG1 deficiency [45] and lysosomal diseases like Hermansky-Pudlak syndrome and Chediak-Higashi syndrome [46-50]. Besides the fact that lysosomal diseases result in lamellar body alterations, lysosomal enzymes (e.g. acid phosphatase, cathepsins) and lysosomal proteins (e.g. LAMP-1, CD63/LAMP-3) as well as an acidic internal pH have been detected in lamellar bodies. These findings led to their classification as lysosome-related organelles [10].

Further EM-based analytical tools include energy-filtering transmission EM for high resolution analysis of elemental composition, e.g. the detection of phosphorus in lamellar bodies [51, 52]. Besides detecting elements that are normally present in biological samples, this approach can also be applied in tracer studies [53]. A recent EM development holding great potential for surfactant analysis is electron tomography. With electron tomography, 3D stacks of “optical” slices with a z-resolution of about 2-5 nm are generated from tilt series recorded from thick (200-500 nm) sections [54-56]. This method is well suited for quantitative (stereological, see below) analysis of structures of a size comparable to, or smaller than, the thickness of a typical ultrathin (50-100 nm) section [57]. In particular, electron tomography has been demonstrated to be an ideal method for the 3D visualization and quantification of nanoparticles in the lung [23, 58] (Fig. 6).

Quantitative microscopy is essential in experimental morphology and pathology, including the phenotyping of genetically altered organisms. The gold standard for obtaining quantitative structural (morphometric) data at all microscopic levels is provided by state-of-the-art stereology (see [59, 60]). Stereology offers a toolkit of unbiased sampling rules (thus guaranteeing randomization of localization and spatial orientation of samples) and estimation tools based on geometric probes (sets of points, lines, planes, disectors) which allows for quantitative characterization of 3D objects on the basis of measurements made on 2D sections. By design, stereological methods make no assumptions on the size, shape, orientation or spatial distribution of the structure of interest. The objective is to estimate geometrical

Fig. 6. Three-dimensional rendering of multivesicular body (with its limiting membrane shown in black and membranes of internal small and large vesicles in red and blue) containing 15 nm gold nanoparticles (clustered group of small yellow particles in the middle), based on an electron tomography dataset. Vesicles outside the multivesicular body shown in green. Reproduced with permission from [58].

parameters that characterize the composition of a structure, using a few well-distributed samples from the whole. Typical parameters that can be estimated are volume (e.g. of alveolar septal tissue), surface area (e.g. of alveolar epithelium), length (e.g. of alveolar capillaries), number (e.g. of alveolar epithelial type II cells), mean size (e.g. of lamellar bodies), and barrier thickness (e.g. of blood-air barrier). Stereological methods are accurate, efficient, simple, and transparent. Thus, they provide data that are “real” (i.e. biologically meaningful total values related to a well-defined reference space), valid, reliable, generalizable, and comparable (e.g. by formal statistics).

The combination of EM and stereology allows for qualitative and quantitative analysis of the intraalveolar as well as the intracellular surfactant pool, both in its preserved microorganization and localization within the lung. In experimental studies, lungs can be analyzed by stereology in order to detect lung pathology and alterations in the surfactant system as well as treatment effects. The Euler number is an integer-valued connectivity measure basically defining the number of elements in a network [61]. In the lung, this approach can be used to obtain unbiased and direct estimates of the number of alveoli. Using physical disectors at the light microscopic

Table 1. Stereological parameters for surfactant analysis. Parameters characterizing alveolar epithelial type II cells, lamellar bodies, intra-alveolar surfactant and its subtypes, and surfactant proteins, are listed. Modified and extended after [26, 67].

Parameter	Description	Method
Type II cells		
N(typell, lung)	total number of type II cells per lung	physical disector LM
$v_N(\text{typell})$	number-weighted mean volume of type II cells	nucleator or rotator
Lamellar bodies		
V(lb, typell)	total volume of lamellar bodies per type II cell	point counting
V(lb, lung)	total volume of lamellar bodies per lung	$V(\text{lb, typell}) \cdot N(\text{typell, lung})$
N(lb, typell)	total number of lamellar bodies per type II cell	physical disector EM
$v_N(\text{lb})$	number-weighted mean volume of lamellar bodies	$V(\text{lb, typell})/N(\text{lb, typell})$
$v_V(\text{lb})$	volume-weighted mean volume of lamellar bodies	point-sampled intercepts
	size distribution of lamellar bodies	$v_V = v_N(1 + CV^2(v))$
Intraalveolar surfactant		
V(alvsurf, lung)	total volume of intraalveolar surfactant per lung	point counting
- $V_V(\text{lb}/\text{alvsurf})$	-volume fraction of lamellar body-like forms	
- $V_V(\text{tm}/\text{alvsurf})$	-volume fraction of tubular myelin	
- $V_V(\text{mv}/\text{alvsurf})$	-volume fraction of multilamellar vesicles	
- $V_V(\text{uv}/\text{alvsurf})$	-volume fraction of unilamellar vesicles	
Surfactant proteins	surfactant protein distribution	relative labeling index

level, the method is practically reduced to counting alveolar openings [62-64]. Surfactant-producing alveolar type II cells can be counted and sampled for local size estimation with physical disectors at a high magnification light microscopic level on semithin sections. The number of their surfactant storing lamellar bodies can be estimated using physical disectors at the EM level, thus providing a morphological measure of the intracellular surfactant pool [44]. These parameters therefore allow to distinguish between type II cell hyperplasia and hypertrophy and to dissect the ways type II cells handle the formation of lamellar bodies quantitatively - essential information in studies addressing type II cell alterations or lamellar body biogenesis. Electron tomography combined with stereology [57] allows for high resolution (2-5 nm) 3D visualization and quantification of lamellar body fusion pores. By quantitative immunoelectron microscopy [58], surfactant protein distribution can be analyzed with the relative labeling index [34]. Intraalveolar surfactant subtype composition - and therefore function - can be estimated by point or intersection counting [65, 66]. These design-based stereological methods allow for a comprehensive quantitative lung phenotype and surfactant (ultra-)structure analysis (reviewed in [26, 67-69]; see also Table 1). Two case studies shall illustrate their application.

Phenotype analysis of lung collectin deficient mice

The surfactant-associated proteins SP-A and SP-D belong to the family of collectins (C-type lectins with

collagen-like domains). Both the SP-A and SP-D protein monomers consist of four subunits: an N-terminal region, a collagen-like region, a neck region, and a C-terminal carbohydrate recognition domain. Three monomers form a homotrimer, and either six homotrimers form a bouquet-like octadecamer (SP-A) or four homotrimers usually form an x-like dodecamer (SP-D). SP-A and SP-D are multifunctional proteins involved in surfactant subtype assembly and stability as well as in immunomodulation (binding and opsonization of pathogens, interaction with immune cells, regulation of mediator production, clearance of apoptotic cells, direct antimicrobial function) [6, 17-19]. SP-D and, to a somewhat lesser degree, SP-A mRNA and protein have also been localized outside the lung [70-76], indicating a general role in mucosal immunity. However, there are still some controversies and inconsistent findings regarding extrapulmonary sites of SP-A and SP-D expression and localization, most likely due to possible sampling artifacts, differences in detection methods and open questions regarding the "normality" of human tissue samples obtained at autopsy or during surgery.

In alveolar epithelial type II cells, both proteins are mainly secreted by a lamellar body-independent pathway via small vesicles, thus their secretion is not directly connected with the regulated secretion of lamellar body content (i.e. lipid, SP-B and SP-C). Interestingly, intraalveolar SP-A is mainly bound to surfactant phospholipids whereas the majority of SP-D, which only binds to the minor surfactant lipid phosphatidylinositol, is not. In the alveolus, SP-A binds to the outer membranes of freshly secreted lamellar bodies, thereby initiating their transformation to tubular myelin with SP-A localized in

Fig. 7. Immunogold labeling for SP-A in the human lung. Within the alveolar lumen, SP-A associates with the peripheral membranes of freshly secreted lamellar body-like forms (LBL). A strong signal for SP-A is found in the lattice of tubular myelin (TM).

the corners of the lattice [34, 77] (Fig. 7). Although the functions of tubular myelin are still not understood, one might speculate that it provides a matrix for SP-A, resulting in a differential distribution of the two lung collectins in the alveolus, with one (SP-A) lipid-bound in a “spider-web” microdomain and the other (SP-D) free in the aqueous hypophase. However, findings from *in vitro* [78] and *in vivo* [79, 80] studies suggest that SP-D can influence intraalveolar surfactant ultrastructure, in particular by promoting conversion to and reuptake of unilamellar vesicles.

The clinical relevance of low or even absent SP-D levels has been demonstrated in several studies analyzing BAL material [81-87]. By this approach, decreased intraalveolar SP-D levels have e.g. been reported in smokers (developing COPD), in cystic fibrosis patients (correlating with the degree of inflammation), in ARDS patients (correlating with a worse clinical outcome) and in children with recurrent bronchitis (correlating with a higher incidence of pneumonias and a worse clinical outcome).

SP-D gene targeted mice exhibit a surprisingly complex phenotype [43, 88-91]. This includes accumulation of intraalveolar surfactant material (lipoproteinosis), alterations of type II cells and alveolar macrophages as well as emphysematous alterations associated with in-

Fig. 8. Electron micrographs from lungs of wildtype (a, c, e) and lung collectin (SP-A/SP-D) double deficient mice (b, d, f) demonstrating qualitative findings for parenchymal architecture (a, b), type II cells (c, d), and alveolar macrophages (e, f). Parenchymal architecture shows enlarged airspaces, thickened alveolar septae, intraalveolar surfactant accumulation, occurrence of enlarged foamy alveolar macrophages (AM), and alveolar epithelial type II cell (AECII) hyperplasia in SP-A/SP-D deficient mice (b). Type II cells of SP-A/SP-D deficient mice occasionally show giant lamellar bodies (gLB) (d). Alveolar macrophages of SP-A/SP-D deficient mice take up giant lamellar body-like forms (gLBL) from the alveolar space and are filled with surfactant material (f).

creased levels of matrix metalloproteinases and reactive oxygen and nitrogen species, suggesting an ongoing inflammatory process leading to destructive lung remodeling.

Stereological lung analysis of mice either deficient in SP-D alone [44] or in both SP-A and SP-D [92] has been performed. Mice double deficient in SP-A and SP-D reveal fewer and larger alveoli (consistent with emphysema-like pathology), alveolar type II cell

hyperplasia and hypertrophy and an increased volume of lamellar bodies per type II cell and per lung, due to an increased number of lamellar bodies per cell (Fig. 8). Despite the occasional occurrence of giant lamellar bodies (Fig. 8d), their mean size is unchanged, however variation in size is increased [92]. This phenotype is basically due to SP-D deficiency alone since similar alterations are seen in SP-D single deficient mice [44]. The only obvious ultrastructural alteration present in SP-A single deficient mice under unchallenged conditions is the absence of tubular myelin [93]. However, surfactant-specific functions of SP-A may become relevant under stress [94, 95] because high levels of SP-A enhance the resistance of surfactant to protein inhibition [96, 97] whereas SP-A deficiency results in a higher susceptibility to protein inhibition [98]. Moreover, the seemingly normal surfactant reuptake by type II cells in SP-A deficient mice is only achieved by compensatory upregulation of other (non-SP-A-mediated) endocytic mechanisms that cannot be further enhanced by secretagogues, suggesting that the system operates at its maximum capacity already at baseline conditions [99].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been implicated as a possible mediator of the alterations seen in SP-D deficient mice. GM-CSF is an important regulator of the alveolar epithelium, surfactant homeostasis, and lung host defense [100, 101]. Alveolar epithelial type II cells and alveolar macrophages express GM-CSF and both subunits of the GM-CSF receptor. GM-CSF is required for the expression of the transcription factor PU.1, which in turn is required for most of the differentiated functions of alveolar macrophages, including the production of reactive oxygen species and the catabolism of surfactant [102]. Consequently, mice deficient in GM-CSF develop alterations reminiscent of pulmonary alveolar proteinosis [103], a disease that, in its acquired form, is caused by auto-antibodies against GM-CSF [101]. Additional deletion of GM-CSF in SP-D deficient mice leads to pronounced emphysema but less pronounced alterations of type II cells and lamellar bodies, indicating that these structural abnormalities are differentially influenced by GM-CSF. These results suggest that GM-CSF, either directly or indirectly, regulates surfactant metabolism in and proliferation of type II cells, but is not required for the development of emphysema in SP-D-deficient mice [44].

One obvious attempt to correct the phenotype of SP-D deficient mice is to substitute SP-D [104]. Indeed, intranasal treatment of SP-D deficient mice with a recombinant fragment of human SP-D (rfhSP-D) con-

taining a short stalk of the collagen-like domain is able to largely prevent the emphysema-like alterations as well as those of type II cells and lamellar bodies [105]. However, treatment with rfhSP-D without the short stalk of the collagen-like domain fails to do so [106], suggesting that this part of the protein, most likely by assembling three monomer fragments to form a homotrimer, is essential for its proper biological function. The therapeutic potential of rfhSP-D containing the short stalk therefore deserves further evaluation.

Surfactant in lung ischemia/reperfusion injury

In clinical lung transplantation, primary graft dysfunction (PGD) is a major problem in the early post-transplant period, leading to increased short- and long-term morbidity and mortality [107-109]. The main reason for the development of PGD is ischemia/reperfusion (I/R) injury. I/R injury manifests as acute lung injury/ARDS, a syndrome of acute and persistent lung inflammation characterized by intraalveolar edema formation, arterial hypoxia and pulmonary hypertension [110].

Alterations in surfactant composition and function are associated with I/R associated acute lung injury in the course of experimental and clinical lung transplantation (reviewed in [13, 110]). In particular, major surfactant phospholipids are decreased as are levels of SP-A and SP-B, both of which are important for formation and stabilization of surface active surfactant forms (tubular myelin). The reduced biophysical function of surfactant (increase in minimum surface tension) is reflected by an increase in the small-to-large-aggregate ratio, corresponding to a relative increase in inactive surfactant forms [111-116].

These data are based on the biochemical and biophysical analysis of surfactant material obtained by BAL. However, BAL can only harvest the intraalveolar surfactant pool, and any topographical information is lost. Moreover, BAL and surfactant isolation require strictly controlled conditions to avoid errors and to make results comparable [117], especially when intraalveolar edema fluid complicates analysis [118]. An alternative approach, at least in experimental studies, is morphological surfactant analysis by EM and stereology [15]. Compared to BAL, it has the advantage of allowing for analysis of the intraalveolar and intracellular surfactant pool in situ, with preserved localization and microstructure. Due to the correlation of intraalveolar surfactant subtype structure

Fig. 9. Perfusion-fixed rat lung samples from control (a) and ischemia/reperfusion injury (b) group. In the control group, a freshly secreted lamellar body-like form (LBL) on top of the alveolar epithelium and tubular myelin (TM) underneath the surface film (SF) are present in the alveolar lumen (a). In the ischemia/reperfusion injury group, alveolar lumina are filled with protein-rich edema fluid (b). On one side of the alveolar septum, swellings and fragmentations of the alveolar epithelium (thin extensions of type I cells) are present, leading to partial denudations of the alveolar epithelial basal lamina (arrows). Surfactant is present mainly as unilamellar vesicles (UV). Reproduced with permission from [65].

and function (Fig. 1), the small-to-large-aggregate ratio known from BAL studies can be measured in lung tissue samples as the ratio of unilamellar vesicles to tubular myelin.

In principle, I/R injury induced surfactant alterations can occur due to damage of alveolar epithelial type II cells (thereby affecting the intracellular pool) or due to biophysical/biochemical mechanisms leading to inhibition/inactivation of secreted surfactant (thereby affecting the intraalveolar pool) [5]. Known mechanisms in acute lung injury/ARDS include peroxidation of surfactant lipids and nitration of surfactant proteins by reactive oxygen and nitrogen species [119-122], phospholipid hydrolysis by phospholipases [123, 124], cell death of type II cells [125-127], and proteolytic damage to SP-A by neutrophil elastase [128, 129]. In addition, plasma proteins entering the alveolar space during edema formation inhibit surface film formation by competitive adsorption and inactivate surfactant components [130-133].

EM-based stereological analysis of intraalveolar surfactant in a rat lung model of I/R injury reveals a decrease in active subtypes (tubular myelin) and an increase in inactive subtypes (unilamellar vesicles) (Fig. 9). These alterations correlate with postischemic lung function and they can be found in edematous as well as in non-edematous lung regions which indicates that their occurrence is independent from the presence of intraalveolar edema. Thus, they are rather a cause than a mere result of edema formation [65, 66]. An increased alveolar surface tension results in an increased pressure gradient from the capillary to the alveolar lumen, and thus in fluid fluxes across the blood-air barrier into alveoli. Components of the edema fluid, in particular plasma proteins, then further inactivate surfactant. This results in a vicious cycle of surfactant

dysfunction and edema formation.

Experimental (e.g. [134-138]) and clinical [139-142] studies demonstrate that exogenous surfactant therapy successfully supplements the disturbed endogenous surfactant system, thereby attenuating I/R injury and improving lung preservation and graft function [14]. Compared to other forms of acute lung injury/ARDS, the potential advantage of exogenous surfactant therapy of the donor in lung transplantation is that surfactant can be given prophylactically, i.e. before inhibition and inactivation manifest, so that PGD can be prevented [14].

EM-based stereological studies show that early (i.e. preischemic donor lung) treatment with an exogenous surfactant preparation prevents atelectasis formation, intraalveolar edema formation and air-blood barrier damage and stabilizes intraalveolar surfactant composition [143-145] and may therefore become a therapeutic option to optimize lung preservation during clinical lung transplantation.

Future improvements in exogenous surfactant therapy may be achieved by making exogenous surfactant preparations more resistant against inactivation or by improving their anti-inflammatory properties, e.g. by supplementing additives [146-154]. In general, surfactant-protective strategies should not only target the intraalveolar but also the intracellular pool (i.e. type II cells and their lamellar bodies). EM and stereology will be important analytical tools in experimental studies testing such therapeutic approaches.

Conclusions

It is morphology which links a genetic blueprint to the functions of an organism. In that sense, surfactant

ultrastructure reflects genetic alterations (surfactant dysfunction mutations) as well as the functional status of the surfactant system. Compared to other microscopic techniques, EM stands out because it provides an “open view” at high resolution on cells and tissues by visualizing the complete structural context. By this, EM is also open for unexpected findings. To go beyond simple description of structure, a quantitative, i.e. stereological, approach is necessary to generate data that allow for statistically valid comparisons between groups, e.g. to detect differences between mutations or for correlation with physiological or biochemical data. Developments in cryopreparation methods (high pressure freezing, freeze substitution, ultrathin cryosectioning), in immunoelectron microscopy (colloidal gold immunolabelling), in 3D EM (electron tomography), and in quantitative microscopy (design-

based stereology) now provide a basis for the analysis of the molecular topography of biological systems [55, 56, 59, 155-158]. This armamentarium is available to characterize the lung and its surfactant system in health and disease. We can therefore indeed expect to see more if we look more closely - and more thoroughly.

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