Remodeling of alveolar septa after murine pneumonectomy

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Ysasi AB, Wagner WL, Bennett RD, Ackermann M, Valenzuela CD, Belle J, Tsuda A, Konerding MA, Mentzer SJ. Remodeling of alveolar septa after murine pneumonectomy. Am J Physiol Lung Cell Mol Physiol 308: L1237–L1244, 2015. First published April 10, 2015; doi:10.1152/ajplung.00042.2015.—In most mammals, removing one lung (pneumonectomy) results in the compensatory growth of the remaining lung. In mice, stereological observations have demonstrated an increase in the number of mature alveoli; however, anatomic evidence of the early phases of alveolar growth has remained elusive. To identify changes in the lung microstructure associated with neovalveolarization, we used tissue histology, electron microscopy, and synchrotron imaging to examine the configuration of the alveolar duct after murine pneumonectomy. Systematic histological examination of the cardiac lobe demonstrated no change in the relative frequency of dihedral angle components (Ends, Bends, and Junctions) (P > 0.05), but a significant decrease in the length of a subset of septal ends (“E”). Septal retraction, observed in 20–30% of the alveolar ducts, was maximal on day 3 after pneumonectomy (P < 0.01) and returned to baseline levels within 3 wk. Consistent with septal retraction, the postpneumonectomy alveolar duct diameter ratio (Dout:Din) was significantly lower 3 days after pneumonectomy compared to all controls except for the detergent-treated lung (P < 0.001). To identify clumped capillaries predicted by septal retraction, vascular casting, analyzed by both scanning electron microscopy and synchrotron imaging, demonstrated matted capillaries that were most prominent 3 days after pneumonectomy. Numerical simulations suggested that septal retraction could reflect increased surface tension within the alveolar duct, resulting in a new equilibrium at a higher total energy and lower surface area. The spatial and temporal association of these microstructural changes with postpneumonectomy lung growth suggests that these changes represent an early phase of alveolar duct remodeling.

IN MOST MAMMALS, REMOVING one lung (pneumonectomy) results in the compensatory growth of the remaining lung (5, 20). In mice, compensatory growth results in an increase in the number of alveoli to near-baseline levels in 3 wk; 74% of the new alveoli are formed within 6 days of surgery (9). The mechanism responsible for this rapid alveolar remodeling is unknown.

A potentially useful observation in postpneumonectomy compensation is that the lung growth is nonuniform. Of the four lobes in the murine right lung, most of the relative growth occurs in the cardiac lobe (also called the median, caval, or accessory lobe). Within the cardiac lobe, growth appears to be concentrated in discontinuous subpleural regions of the lobe (23). Perhaps relevant to the mechanism of compensatory growth, maximal deformation of the right lung is observed in discontinuous subpleural regions of the cardiac lobe (10, 11). When lung deformation is limited by plombage (18) or phrenic nerve transection (41), compensatory growth in the cardiac lobe is abrogated.

Since structural hierarchies channel mechanical forces from the macro to the micro level (24, 30), it is likely that macro-level deformation of the cardiac lobe is reflected in the configuration of lung parenchyma at the level of alveoli and alveolar septa. Given the small scale of lung microstructure, the techniques defining the structural configuration of alveoli and alveolar ducts have been largely limited to stereological analysis of histological sections and high-resolution imaging of fixed or casted lungs.

Previous stereological attempts to characterize the configuration of parenchyma at the level of alveoli and alveolar septa have focused on the stress-bearing components of the peripheral lung; namely, the connective tissue cables and septal membranes of the alveolar duct. Analogous to a parachute (6), the alveolar duct maintains its structure by tensions in its cables and membranes. In the normal lung, the cables and membranes (septa) can be characterized by the pattern of linear single septa (so-called “ends” or “E”), the linear junction of two septa (so-called “bends” or “B”), and the linear junction of three septa (so-called “junctions” or “J”) (6, 27, 28). In the postpneumonectomy lung, we anticipate that the altered tensions of the deformed lung will result in a change in the EBJ configuration of the alveolar duct; at minimum, we anticipate a change in the configuration of ducts located within the subpleural regions of the cardiac lobe.

Attempts to image the lung by conventional microCT technology have been limited by resolution (32). The alveolar diameter of the normal mouse alveolus is 60 μm (26), close to the resolution of microCT and nanoCT (3). In contrast, high-energy imaging, such as the synchrotron, provides less than 1-μm resolution (29, 31). In studying fixed or casted lungs, the synchrotron has been effectively used to produce 3D imaging of the lung microstructure (1, 21).

In this report, we used tissue histology and synchrotron imaging to examine the configuration of the alveolar duct after murine pneumonectomy. We found evidence that septal retraction, potentially related to an alteration in the force balance within the alveolar duct, participates in the remodeling associated with compensatory lung growth.

METHODS

Animals. Male mice, 8- to 10-wk-old, wild-type C57BL/6 (Jackson Laboratory, Bar Harbor, ME), were anesthetized as previously de-
scribed (14). The care of the animals was consistent with guidelines of the American Association for Accreditation of Laboratory Animal Care (Bethesda, MD) and was approved by our Institutional Animal Care and Use Committee.

**Decellularization protocol.** The murine lungs were decellularized by using a modification of a previously described 24-h treatment protocol (22). Briefly, heart-lung blocks were harvested with bicaval and aortic transection followed by tracheal and pulmonary artery cannulation with olive-tipped catheters. After serial 3-ml flushes of 5% penicillin-streptomycin (Life Technologies, Carlsbad, CA) in deionized water, tracheal instillation with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) was followed by incubation for 8 h at 27°C. The lungs were rinsed and a 2% sodium deoxycholate (Sigma-Aldrich) solution was instilled into the trachea and pulmonary circulation. The lungs were incubated for an additional 14 h at 4°C, followed by a flushing with a 1 M NaCl (Fisher Scientific, Waltham, MA) rinse solution (27°C, 1 h). Finally, lungs were treated with 30 μg/ml bovine pancreatic DNase (Sigma-Aldrich) solution for 1 h at 27°C. Specimens were stored in phosphate-buffered saline (Quality Biological, Gaithersburg, MD) with 5% penicillin-streptomycin (Life Technologies, Carlsbad, CA) at 4°C until further use.

**Anesthesia and pneumonectomy.** As previously described (41), animals were anesthetized with an intraperitoneal injection of ketamine 100 mg/kg (Fort Dodge Animal Health, Fort Dodge, IA) and xylazine 6 mg/kg (Phoenix Scientific, St. Joseph, MO). The animals were intubated under direct vision with a standard 20-gauge angiocatheter (BD Insyte, Sandy, UT) for the pneumonectomy procedure. The general anesthesia was performed by using a flexiVent rodent ventilator (SCIREQ, Montreal, QC, Canada). Ventilator rates of 200/min, tidal volume of 10 ml/kg with positive end-expiratory pressures of 3 cmH2O, and a pressure limit of 30 cmH2O were used. In all mice, the left lung was exposed with a fifth intercostal space thoracotomy and the hilum was ligated with a 5-0 silk tie (Ethicon, Somerville, NJ). A recruitment maneuver (termed TLC by SCIREQ) was performed while closing the thoracotomy. The animals were removed from the ventilator and extubated upon recovery of spontaneous ventilation. Subcutaneous buprenorphine 2.4 μg (Hospira, Lake Forest, IL) was administered twice daily for 48 h.

**Precision-cut lung slices.** Agarose (Sigma-Aldrich) at 3% (wt/vol), warmed to 37°C, was instilled into the trachea through a 20-gauge Angiocath (BD Insyte), at the lowest pressure necessary to inflate the peripheral lung (typically 20 cmH2O pressure) (4). At total lung capacity (TLC), the trachea was clamped and the lung block was placed in 4°C saline and allowed to harden. Sectioning was performed with the Leica VT1000 S vibrating blade microtome (Leica Biosystems, Nussloch, Germany) using stainless steel razor blades (Gillette, Boston, MA). The microtome was operated at the following adjustable settings: knife angle, 5–7°; sectioning speed, 0.05–0.2 mm/s; oscillation frequency, 80–100 Hz; and oscillation amplitude, 0.6 mm.

**Histology and morphometry.** Tissue sections were stained with a modification of a previously described method (33). Briefly, the lung tissue was prepared in thin sections and stained with commercially available Sirius red and Verhoeff-van Gieson (VVG) stain. Serial histological sections of the entire cardiac lobe were obtained, stained, and imaged (7). Images were coded and observers were blinded to experimental condition. Dihedral angle components were identified as previously described (27).

**Basement membrane digestion.** To remove the basement membrane, the decellularized lungs were treated with 1% trypsin-EDTA (PAA, Pasching, Austria) for 12 h at 37°C. The specimens were treated with 1% Type 4 filtered collagenase (Worthington, Lakewood, IL) at 37°C for 7 days with frequent enzyme changes. The specimens were later fixed with 2.5% glutaraldehyde and 1% buffered osmium, dehydrated in an intermediate ascending acetone range and a final critical-point drying process.

**Detergent lavage.** The lavage procedure was a modification of a previously described detergent treatment protocol (2). After administration of anesthesia (ketamine 100 mg/kg, xylazine 0.6 mg/kg), the mouse was intubated with an 18- to 20-gauge Angiocath (BD Insyte) and placed on the flexiVent (SCIREQ) for determination of TLC. TLC was determined by the mean of five TLC maneuvers. Two hundred units of heparin were then administered intraperitoneally. The mouse was temporarily removed from the ventilator and 0.01% Triton X-100 (in PBS) was instilled into the lung via the endotracheal tube at a volume equal to 70% TLC. Detergent solution was aspirated from the endotracheal tube and the lavage was repeated two additional times. A small volume (6% TLC) of Triton X-100 solution was then instilled and left in the lungs and the mouse was placed back on the ventilator.

**Vascular corrosion casting.** After general anesthesia, systemically heparinized (2,000 U/kg ip) mice underwent thoracotomy and the pulmonary artery was cannulated through the right ventricle with an olive-tipped cannula and retained in place with a 5-0 silk tie (Ethicon, Somerville, NJ). A recruitment maneuver (termed TLC by SCIREQ) was performed while closing the thoracotomy. The animals were removed from the ventilator and extubated upon recovery of spontaneous ventilation. Subcutaneous buprenorphine 2.4 μg (Hospira, Lake Forest, IL) was administered twice daily for 48 h.

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**SEM.** After standard-protocol gold coating in argon atmosphere, the decellularized lungs were imaged by using a Philips XL30 ESEM scanning electron microscope (SEM) (Philips, Eindhoven, Nether-
lands) at 15 keV and 21 μA. Stereopair images were obtained by using a tilt angle difference of 6° on an eucentric sample holder and standardized computerization to confirm observations in planar images.

Synchrotron radiation X-ray tomographic microscopy. Corrosion-casted lung samples were imaged at the TOMCAT beamline of the Swiss Light Source at the Paul Scherrer Institute. A slit system tailored the monochromatic X-ray light to a profile of ~1.4 mm². The X-rays that penetrated the sample were converted to visible light by a thin scintillator screen (Crismatec Saint-Gobain, Nemours, France). After magnification by a diffraction-limited microscope, the projection images were digitalized by a high-resolution charge-coupled device camera (Photonic Science, East Sussex, UK). The corrosion

Fig. 2. Histology of the cardiac lobe in control (A and C) and postpneumonectomy day 3 mice (B and D). The cellular lung in control (A) and postpneumonectomy day 3 (B) cardiac lobe were stained with Sirius red. Normal septa were thin and projected into the alveolar duct (A, ellipses). Postpneumonectomy day 3 sections frequently demonstrated “plump” septa that appeared to be retracted away from the duct lumen (B, arrows). C and D: lungs of control and postpneumonectomy day 3 mice were decellularized and the cardiac lobe stained with Verhoeff-van Gieson stain. The dilated alveolar ducts demonstrated areas of condensed matrix (D, arrows), suggesting both cellular and matrix alterations after pneumonectomy. Bar = 100 μm.

Fig. 3. Morphometry of the alveolar duct in the murine cardiac lobe. Serial sections of the cardiac lobe were obtained in various conditions. A: width of the alveolar duct was expressed as a ratio between outer diameter (distance between outer walls, D_out) and inner diameter of the duct (distance between septal tips, D_in). B: E length was measured as the greatest length along the axis of the septa. These parameters were compared in cardiac lobes from nonsurgical controls, sham thoracotomy mice, detergent-treated mice, postpneumonectomy day 3 (POD 3) mice, areas of the cardiac lobe remote from known growth areas (remote) and postpneumonectomy day 22 (POD 22) mice. Error bars = 1 SD. POD 3 duct diameters were indistinguishable from detergent-treated mice (P > 0.05), but significantly different from the other controls (**P < 0.001). N = 3–5 mice per condition.
casts were scanned without binning with an optical magnification, resulting in voxels with an isometric side length of 650 nm; 1,001 projections were obtained for each scan along with dark and flat field images at an integration time of 150 ms each. Flat-field corrected sinograms were rearranged from the data online by using cluster five 12-core 2.67 GHz Intel Xeon nodes with 48 GB RAM each. An optimized regreidding reconstruction algorithm was used (17, 25). For reconstructing and visualizing the regions of interest, we used a 16-node Linux PC (Pentium 4, 2.8 GHz with 512 MB RAM) and optimized filtered back-projections. A global thresholding approach was used for volume rendering. Mean and median filtered and binarized (by Otsu’s method) images were obtained by use of MAVI-Modular Algorithms for Volume Images (2014) version 1.5.1, developed in the image analysis department of Fraunhofer ITWM, Germany.

**Quadratic energy difference model.** The application of the quadratic energy difference model was based on published work (39). Here, our assumptions included a small relevant change in surface area. Surface tension ($\gamma$) estimates in air ($\gamma_A = 16$ dyn/cm) and after detergent treatment ($\gamma_D = 42$ dyn/cm) were based on published data (2, 15). Energy curves were calculated based on a lung volume of 80% TLC.

**Statistical analysis.** Data analysis was performed by using XLstat (Addinsoft, New York, NY) add-in for Microsoft Excel. Results are reported as means ± one standard deviation unless otherwise noted. Statistical significance is determined by ANOVA with a P value of 0.05. Pairwise comparisons were performed as needed by Tukey’s test.

**RESULTS**

**Septal retraction.** To identify the histological features of lung regeneration, we evaluated postpneumonectomy histology sections for tissue density (planar projection) and dihedral angle (EBJ) frequency. Measurements of tissue density, expressed as a percent of total cross-sectional area, demonstrated significant variability but an apparent increase on day 3 after pneumonectomy (Fig. 1A). The internal configuration of the lung, as reflected by relative frequency of dihedral angle components (EBJ), did not change postpneumonectomy (Fig. 1B, $P > 0.05$). Although the frequency of septal ends (E) was unchanged, systematic histological analysis of the subpleural cardiac lobe 3 days after pneumonectomy demonstrated that a subset of septal ends were shorter and thicker (Fig. 2B, arrows). Decellularized day 3 postpneumonectomy lungs demonstrated apparent retraction of the septal tip elastin cable (Wagner W, Bennett RD, Ackermann M, Ysasi AB, Belle JM, Valenzuela CD, Pabst AM, Tsuda A, Konerding MA, Mentzer SJ, unpublished observations) (Fig. 2D, arrows). On day 3, the septa appeared retracted but there was no significant change in planar surface area of the cellularized lung (Sirius red stain: control $22.1 \pm 11.3\%$ and pneumonectomy $26.6 \pm 11.1\%$) or the decellularized extracellular matrix (VVG stain: control $39.9 \pm 10.8\%$ and pneumonectomy $39.2 \pm 21.2\%$) ($P > 0.05$).

**Alveolar duct configuration.** To characterize the effect of septal retraction on alveolar duct microstructure, we measured several morphometric parameters of the alveolar duct. Because of the heterogeneity of septal retraction, the most dilated quartile of alveolar ducts were measured in a variety of conditions including no-surgery controls, sham thoracotomy controls, detergent-treated lungs, regions of lung remote to growth areas, and postpneumonectomy lungs on days 3 and 22 after surgery. Consistent with septal retraction, 1) the postpneumonectomy alveolar duct diameter ratio ($D_{out}:D_{in}$) was significantly lower than in all controls except for the detergent-treated lung ($P < 0.001$), and 2) the septa were generally wider in both postpneumonectomy and detergent-treated conditions (Fig. 3B, $P < 0.05$). When analyzed over 22 days, there was a significant decrease in the duct diameter ratio and septal length on day 3 postpneumonectomy (Fig. 4, A–C, $P < 0.05$). There was no change in other duct parameters (e.g., B angle, Fig. 4D).

![Fig. 4. Morphometry of the cardiac lobe alveolar ducts between pneumonectomy (day 0) and postpneumonectomy day 22. Serial sections of the cardiac lobe were obtained in various conditions. A: when all of the alveolar ducts were evaluated, variability was noted on day 3 after pneumonectomy ($^*P < 0.05$). B: when the duct measurements at all time points were combined and the most dilated 20% plotted as a function of time after pneumonectomy, most of the diluted ducts were identified on day 3 after pneumonectomy. C: similarly, septal length (E) decreased on day 3 after pneumonectomy ($^*P < 0.05$). The difference in septal length E was also significant between day 3 and day 12 after pneumonectomy ($^{**}P < 0.01$). D: in contrast, other structural features, such as B angle, were unchanged ($^*P > 0.05$). N = 3–6 mice per time point.](http://ajplung.physiology.org/DownloadedFrom/10.1152/ajplung.00042.2015)
Vascular evidence of septal retraction. As the primary gas-exchange surface in the lung, normal alveolar septa are characterized by a dense capillary plexus. Although septal retraction may prevent capillary perfusion and the identification of clumped capillaries in histological sections, vascular casting in the postpneumonectomy lungs should demonstrate clumped or matted capillaries. Consistent with this prediction, corrosion casts and SEM demonstrated regions of the peripheral cardiac lobe with dense capillaries (Fig. 5). Similarly, synchrotron imaging of subpleural regions of the cardiac lobe in casted postpneumonectomy lungs demonstrated ducts with retracted septa and areas of dense capillary matting (Fig. 6).

Increased surface tension. The morphometric similarity of postpneumonectomy day 3 and detergent-treated alveolar ducts...
suggested that septal retraction could be the result of a significant increase in alveolar surface tension after pneumonectomy. The micromechanical implications of increased surface tension can be interpreted by using the energy relationship described by Wilson (39). In the equilibrium configuration, the elastic energy of the normal lung is at a minimum (Fig. 7, red dot). With an increase in surface tension, analogous to detergent treatment or possibly postpneumonectomy changes, the configuration of the lung reaches a new equilibrium at a higher total energy and lower surface area (Fig. 7, blue dot). Wilson has speculated that the reduction in surface area caused by increased surface tension may lead to alveolar walls that “buckle and fold” (38).

**DISCUSSION**

In this report, we demonstrated subtle morphological changes in a subset of alveolar ducts postpneumonectomy. The changes in alveolar duct microstructure included septal retraction and widening of the alveolar duct. A prediction of septal retraction, namely, clumps of capillaries at the base of the septa, were demonstrated by SEM and synchrotron imaging.

The degree of septal retraction was consistent with an alteration in the balance between surface and tissue forces. The spatial and temporal association of these microstructural changes with postpneumonectomy lung growth suggests that these changes represent an early phase of alveolar duct remodeling.

An underappreciated observation in postpneumonectomy lung growth is the absence of obvious histological evidence of regeneration; that is, neovalveolization in peripheral regions of the cardiac lobe does not demonstrate the cellular aggregation associated with wound healing (7). Consistent with a subtle histological process, morphometry of the cardiac lobe demonstrated only a modest increase in tissue density on day 3 after pneumonectomy. In contrast, a more reliable finding was the retraction of alveolar septa and a heterogeneous widening of alveolar ducts. We estimate that 20–30% of alveolar ducts in the cardiac lobe demonstrated retracted septa on day 3 after pneumonectomy. These results are consistent with previous findings of noncontiguous regions of alveolar angiogenesis in the subpleural cardiac lobe (1, 23).

Our results highlight two neglected features of lung geometry. First, alveoli are not simple spheres, but rather complex polyhedral structures (13). The alveolar walls are quasi-planar, reflecting the force balance between neighboring alveoli. These straight septa correspond to the septal ends E observed in our study. Second, alveoli are not simple balloons that “collapse” with increased surface tension, but rather subunits of an alveolar duct structure that are minimized with increased surface tension. The effect of increased surface tension in alveolar ducts is “retraction” of the alveolar septa (2); this shortening of the straight alveolar septa minimizes the surface area of both neighboring alveoli. The degree of septal retraction depends on the balance between tissue and surface forces (38, 40).

The mechanism responsible for septal retraction appears to be increased surface tension. The cause of the increased surface tension is suggested by the spatial location of the dilated alveolar ducts; that is, the widened alveolar ducts were spatially coincident with areas of maximal postpneumonectomy lobar deformation (10, 11). One possibility is that parenchymal deformation triggered processes that contributed to the inactivation of alveolar surfactant (19). Another possibility is that parenchymal deformation had a direct effect on alveolar type II cells, perhaps exhausting surfactant production (16, 34) or even triggering type II cell apoptosis (8, 12). Notably, it is also possible that septal retraction reflects the active remodeling of the axial connective tissue cables that balance surface tension in the alveolar duct (37). Currently, there are no relevant data on type II cell function or remodeling of the connective tissue system of the alveolar duct after pneumonectomy.

Our assessment of microstructure primarily relied on assessment of the alveolar duct using modified stereological measures of EBJ. EBJ measurements are useful because the ends, bends, and junctions of the alveolar duct reflect fundamental features of alveolar structure (6, 27, 28). The interaction between the membrane and cable systems of the lung produce linear features that can be identified and quantified in two-dimensional tissue sections. We assumed that the frequency of EBJ features was relatively constant over the 3 wk of lung remodeling because the fundamental structure of the alveolar duct was preserved; the alteration of the duct was primarily observed in the length of septal membranes. The consequence of septal retraction was an increase in internal alveolar duct diameter (decrease in the \( D_{in}/D_{out} \) ratio).

Notably, we applied the morphometric measurements to targeted areas of the lung; namely, in subpleural regions of the cardiac lobe. There was no attempt at random sampling, but rather comprehensive serial sampling of growth areas of the
cardiac lobe. EBJ measurements were performed on samples obtained with controlled lung volumes, fixation techniques, and image processing. The reproducibility of our findings was insured by internal comparisons within the cardiac lobe; that is, at every time point after pneumonectomy, there were readily identifiable ducts without septal retraction. The preserved alveolar ducts provided a useful comparison to assess potential technical bias.

Our initial work used a variety of fixation techniques including vascular and airway fixation. An unexpected finding was the effective preservation of retracted septa with airway fixation. Our initial concern for fluid-associated artifact, specifically, the loss of surface tension and normalization of alveolar sepsa, was not observed. In fact, the septal retraction with airway fixation was indistinguishable from the retraction observed with vascular fixation. Septal retraction appears to reflect a fundamental change in duct microstructure, not a superficial change easily altered by fixation techniques.

The similar morphological appearance of the postpneumonectomy and detergent-treated alveolar septa suggested that increased surface tension was responsible for the retracted septa. Since surface tension cannot be measured in the subset of alveolar ducts undergoing septal retraction, we relied on a numerical model, Wilson’s quadratic energy difference model (39), to interpret the mechanical consequences of altered surface forces. Although the model is limited in its ability to describe large changes in surface area (39), the model is useful in describing the energy implications of increased surface tension. We interpret the higher total energy and decreased surface area described by the model as a transitional state in lung regeneration; that is, septal retraction appears to be an early step in the process of new alveolar growth. We suspect that repartitioning of the alveolar duct will increase surface area to near-baseline levels (35). This process likely involves the normalization the lung energy, perhaps through the increased production of surfactant and/or remodeling of the axial connective tissue system. Future work will need to address these possibilities.

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

AUTHOR CONTRIBUTIONS

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