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Elastin Cables Define the Axial Connective Tissue System in the Murine Lung

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Abstract

The axial connective tissue system is a fiber continuum of the lung that maintains alveolar surface area during changes in lung volume. Although the molecular anatomy of the axial system remains undefined, the fiber continuum of the lung is central to contemporary models of lung micromechanics and alveolar regeneration. To provide a detailed molecular structure of the axial connective tissue system, we examined the extracellular matrix of murine lungs. The lungs were decellularized using a 24 hour detergent treatment protocol. Systematic evaluation of the decellularized lungs demonstrated no residual cellular debris; morphometry demonstrated a mean $39 \pm 7\%$ reduction in lung dimensions. Scanning electron microscopy (SEM) demonstrated an intact structural hierarchy within the decellularized lung. Light, fluorescence, and scanning electron microscopy of precision-cut lung slices demonstrated that alveolar duct structure was defined by a cable line element encased in basement membrane. The cable line element arose in the distal airways, passed through septal tips and inserted into neighboring blood vessels and visceral pleura. The ropelike appearance, collagenase resistance and anti-elastin immunostaining indicated that the cable was an elastin macromolecule. Our results indicate that the helical line element of the axial connective tissue system is composed of an elastin cable that not only defines the structure of the alveolar duct, but also integrates the axial connective tissue system into visceral pleura and peripheral blood vessels.

Introduction

Alveoli are air-filled cavities within the lung parenchyma that provide surface area for gas exchange. By volume, alveoli are the largest part of the lung. Alveoli do not function independently, but are integrated into an “ingenious” fiber continuum (Weibel, 2012). How the fiber continuum of the lung maintains alveolar surface area during changes in lung

volume is controversial (Frazer, 2012; Mitzner and Smaldone, 2012; Smaldone and Mitzner, 2012; Weibel, 2012); for some, it remains a “last frontier of gross anatomy” (Smaldone and Mitzner, 2012).

Early qualitative (Orsós, 1936) and later quantitative (Weibel and Gomez, 1962) microscopy studies have demonstrated that the lung has three different connective tissue systems; namely, 1) a strong peripheral system extending from the pleura, 2) a delicate fiber system supporting the septa, and 3) a fibrous connective tissue system extending from the airways into the acini. This latter system, referred to as the axial connective tissue system, has been imaged after caustic digestion (Carton et al., 1960; Carton and Dainauskas, 1964; Pierce and Ebert, 1965; Crissman, 1987), at different ages (Pierce and Ebert, 1965) and in a variety of disease states (Snider et al., 1962). The cumulative evidence suggests that the axial connective tissue network provides the fibrous support for alveolar duct; however, the precise anatomic role and molecular structure of the axial system remains uncertain.

Recent theoretical and practical developments have suggested the utility of revisiting the structure of the axial connective tissue system. First, the theoretical model advanced by Wilson and Bachofen provides a specific functional role for the axial fiber system (Wilson and Bachofen, 1982). In this model, the axial fibers or “line elements” form a helix that maintains alveolar structure by balancing the distortion created by alveolar surface tension (Bachofen and Wilson, 1997). The mechanical consequences of a disrupted axial fiber system have been implicated in disease states as varied as emphysema (Azcuy et al., 1962; Snider et al., 1962; Fukuda et al., 1989; Suki et al., 2012) and ventilator-associated lung injury (Dreyfuss and Saumon, 1998; Imanaka et al., 2001; Matthay et al., 2002; Gatto et al., 2004). Second, studies of post-pneumonectomy lung regeneration in humans and other mammals have suggested the importance of alveolar duct mechanics. In humans, functional MRI scans of the lung have demonstrated normalization of the lung microstructure in regions of greatest stretch (Butler et al., 2012). Similarly, neoalveolarization in mice has been localized to subpleural regions associated with the greatest deformation or stretch after pneumonectomy (Konerding et al., 2012; Filipovic et al., 2013; Filipovic et al., 2014). In both cases, lung growth has been spatially linked to the axial fiber system supporting the peripheral alveolar ducts.

In this report, we studied the axial connective tissue system of decellularized murine lungs. The decellularized lungs demonstrated a helical structure of the axial fibers. Enzymatic digestion indicated that the helical line element is composed of an elastin cable that not only defines the structure of the alveolar duct, but also integrates the axial connective tissue system into visceral pleura and blood vessels.

Methods

Animals

Male mice, eight to ten week old wild type C57BL/6 (Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized as previously described (Gibney et al., 2011). The care of the animals was consistent with guidelines of the American Association for Accreditation of

Laboratory Animal Care (Bethesda, MD, USA) and approved by our Institutional Animal Care and Use Committee.

Decellularization protocol

The murine lungs were decellularized using a modification of a previously described 25 hour treatment protocol (Jensen et al., 2012). Briefly, heart-lung blocks were harvested with bicaval and aortic transection followed by tracheal and pulmonary artery cannulation. After serial 3cc flushes of 5% Penicillin-Streptomycin (Life Technologies, Carlsbad, CA, USA), 0.1% Triton-X-100 (Sigma-Aldrich, St. Louis, MO, USA) was instilled in the trachea followed by an 8 hour incubation at 27°C. The lungs were rinsed and a 2% sodium deoxycholate (Sigma-Aldrich, St. Louis, MO, USA) (w/v) solution was instilled into the trachea and pulmonary circulation. The lungs were incubated for an additional 14 hours at 4°C, followed by a 1 hour flush with a 1M NaCl solution (27°C). Finally, the lungs were treated with a 30ug/mL bovine pancreatic DNase (Sigma-Aldrich, St. Louis, MO, USA) solution for 1 hour at 27°C. Specimens were stored in phosphate buffered saline (Quality Biological, Gaithersburg, MD, USA) with 5% Penicillin-Streptomycin (Life Technologies, Carlsbad, CA, USA) at 4°C until further use.

Precision-cut lung slices

Agarose (Sigma-Aldrich, St. Louis, MO, USA) at 3% (w/v), warmed to 37°C, was infused into the trachea through a 20g Angiocath (BD Insyte, Sandy, Utah, USA), using the lowest pressure required to inflate the peripheral lung (typically 20 cm H₂O pressure) (Bennett et al., 2014). At total lung capacity, the trachea was clamped and the lung block 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/sec; oscillation frequency, 80–100 Hz; and oscillation amplitude, 0.6 mm.

Histochemical staining

Tissue sections were stained with a modification of a previously described method (Sweat et al., 1964). Briefly, the lung tissue the lung tissue was prepared in thin sections and stained with commercially available hematoxylin and eosin (H&E), Sirius red stain or elastic van Gieson (EvG) stain. The stained tissue sections were examined using standard, fluorescent and polarized light (Puchtler et al., 1973) illumination. Quantification was performed by independent observers blinded to the experimental condition.

Basement membrane digestion

To remove the basement membrane, the decellularized lungs were treated with 1% Trypsin-EDTA (PAA, Pasching, Austria) for 12 hours at 37°C. The specimens were treated with 1% Type 4 filtered collagenase (Worthington, Lakewood, USA) at 37°C for 7days 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.

Scanning electron microscopy (SEM)

After coating with 20–25 Å gold in argon atmosphere, the decellularized lungs were imaged using a Philips XL30 ESEM scanning electron microscope (Philips, Eindhoven, Netherlands) at 15Kev and 21µA. Stereopair images were obtained using a tilt angle difference of 6° on a eucentric sample holder using standardized computerization

Elastin staining

After decellularization, thin cut lung slices were incubated in 1mg/ml collagenase Type IV (Worthington, Lakewood, NJ, USA) for 7 days at 37°C on a vibrating plate. Collagenase media was changed every 30 minutes for the first 2 hours, then every hour for the next 2 hours, then every 24 hours thereafter. The collagenase treated lung was stained with 5-fold excess of rabbit polyclonal anti-elastin antibody (Biorbyt, Cambridge, UK). Both anti-elastin and control conditions were treated with a fluoresceinated goat polyclonal anti-rabbit antibody (Pierce Antibody Products, Rockford, IL, USA). Serial stained sections were examined by fluorescence grid confocal microscopy (Lee et al., 2009), processed in parallel and analyzed using MetaMorph 7.52 (Molecular Devices, Downingtown, PA) intensity applications as previously described (Gibney et al., 2012).

Results

Decellularized lung

To obtain cell-free matrix scaffolds, murine lungs were treated with a standard detergent protocol (see Methods). Systematic evaluation of the decellularized lung by light microscopy (H&E staining) and scanning electron microscopy (SEM) demonstrated no evidence of residual cellular elements (Figure 1A–C). Decellularization was associated with a $39\pm 7\%$ decrease in alveolar dimensions (Figure 1D). Despite the reduction in scale, SEM demonstrated an intact structural hierarchy from bronchi (Figure 2A) to alveolar ducts (Figure 2B–D).

Axial system. Microscopic analysis of precision-cut decellularized lung slices confirmed the classic observations of Orsos (Orsós, 1936). Examination of 200µm lung slices demonstrated the presence of helical “line elements” within the alveolar ducts (Figure 3). Axial histologic sections of alveolar ducts stained with Sirius red demonstrated characteristic collagen birefringence (red/orange) as well as elastin intrinsic fluorescence (green) in regions of the duct corresponding to the line element (Figure 4A, arrows). SEM demonstrated a discrete cable with the characteristic “twisted ropelike” appearance of elastin (Gotte et al., 1974); the cable was encased in basement membrane (Figure 4C–D).

Axial connections

Light and fluorescence microscopy suggested that the alveolar duct cables inserted into the visceral pleura (Figure 5AB). To identify the interconnections of the cable network, SEM was used to trace the cable line elements from their origins in the proximal airway to their point of insertion. At the pleural surface, the alveolar duct cables inserted into the pleural connective tissue system (Figure 5C). The cable line elements also demonstrated connections with blood vessels paralleling the alveolar duct (Figure 5D).

Elastin cable

The microstructure of the line element was investigated using the enzymatic digestion of the encasing basement membrane. Prolonged collagenase digestion (7 days) removed the basement membrane and revealed the typical “twisted ropelike” (Gotte et al., 1974) appearance of elastin (Figure 6A–C). The post-digestion morphologic appearance of the elastin cable was similar to the pre-digestion appearance (see Figure 4B–D for comparison), suggesting collagenase resistance. Anti-elastin monoclonal antibody staining provided specific evidence for a dominant elastin component of the cable structure (Figure 6DE, inset).

Discussion

In this report, we extended classic morphologic studies to provide a specific structural definition of the axial connective tissue system of the lung. Using decellularized murine lung, we demonstrated a helical “line element” defining the internal circumference of the alveolar duct. The line element was composed of a basement membrane sheath surrounding a cable-like structure that arose in the proximal airways, passed through septal tips, and inserted into blood vessels and visceral pleura. SEM morphology, collagenase resistance and anti-elastin immunostaining indicated that the cable was an elastin macromolecule. Further, the discrete cable-like structure of the elastin line element suggested an important functional role for the cable in maintaining alveolar duct structure.

The cable line element described here is an important extension of the original description of the axial system by Orsos (Orsos, 1907; Orsós, 1936). In his classic paper, Orsos used light microscopy of thick lung sections to describe a “respiratory elastic scaffold” that extended from the respiratory bronchioles to alveolar rings (Orsós, 1936). This scaffold included larger bundles with few interconnections. Gil and Weibel also described the “thick bundles of connective tissue...which surround the alveolar mouth,” but primarily focused on the fiber arrangement in the alveolar wall (Gil and Weibel, 1969, 1972; Gil and Reiss, 1973; Gil et al., 1979; Gil and Martinez-Hernandez, 1984). In both cases, the elastic scaffold and the thick bundles correspond to the elastin line element described here.

The cable identified in this report is both similar and dissimilar to the supramolecular elastin in other tissues. Similar to elastin structures elsewhere, the elastin line element demonstrated collagenase resistance (Senior et al., 1991) and a twisted ropelike appearance (Gotte et al., 1974; Ronchetti et al., 1998). The cable’s structure was unusual because its ropelike appearance was observed in cables approaching 1 microns rather than much smaller 50nm fibers found in other tissues (Yu et al., 2007; Gasiorowski et al., 2013). Further, supramolecular elastin in most tissues is an amorphous mass of fibers (Mithieux and Weiss, 2005). Since the mechanical properties of the elastin polymer are believed to be a consequence of the entropic (disorganized) structure (Vrhovski and Weiss, 1998), the cable identified here not only had a distinctive structure, but likely had unique mechanical properties as well.

The cable line element and its basement membrane sheath highlight the structural and functional importance of the lung extracellular matrix. Elastin is secreted from smooth

muscle cells and fibroblasts as a soluble monomer (tropoelastin) that must be crosslinked into a functional polymer (Kagan and Sullivan, 1982). The supramolecular assembly of elastin involves a scaffolding of fibrillin-rich microfibrils within the extracellular matrix (Muiznieks and Keeley, 2013); the distribution and composition of these molecular scaffolds likely determine the supramolecular assembly of the elastin cables observed here. Given the structure and function of the cable line element, we speculate that elastogenesis in the lung is intimately tied to both myofibroblast activity and the mechanical forces associated with ventilation.

The demonstration of a helical line element within the alveolar duct is particularly relevant to mechanical models of lung microstructure. Wilson and Bachofen proposed a model based on the geometric relation between the surface- and force-bearing elements of the lung alveolar duct (Wilson and Bachofen, 1982). The model predicts that abnormally high surface tension results in alveolar flattening, septal retraction and a widened alveolar duct. In contrast, low alveolar surface tension results in increased alveolar surface area, septal lengthening and a narrowed alveolar duct. Because of the cable line element, the alveolar walls—containing relatively delicate septal fibers and alveolar capillaries—are protected from normal mechanical stresses (under 80% total lung capacity) as they maintain a constant alveolar surface area (Bachofen et al., 1982). The structure of the cable observed here provides anatomic evidence of this force-bearing line element and support for the Wilson model.

Finally, the static structural images in this report can only imply the functional consequences of a helical line element. Future dynamic imaging may provide a test of the helical structure of the cable: does the cable function as a line element that integrates alveolar septal tips into a continuous helix, or merely links alveolar entrance rings that function as independent “hoops”? Dynamic imaging may also provide insights into the mechanical properties of this elastic structure. These studies will have important implications for our understanding of alveolar duct structure and function.

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Abbreviations

2D	2-dimensional
3D	3-dimensional
AD	alveolar duct
BV	blood vessel
EvG	elastic van Gieson
P	pleura
H&E	hematoxylin & eosin

IP	intraperitoneal
LM	light microscopy
SD	standard deviation
SEM	scanning electron microscopy

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Figure 1.

Decellularized murine lung. A–B) H&E staining of thin sectioned decellularized lungs (A, bar=500μm; B, bar=100μm). No residual cellular debris is evident. C) Scanning electron microscopy (SEM) of the decellularized lung demonstrating the residual extracellular matrix (bar=100μm). D) Comparison of calibrated alveolar diameters in control (cellular) and decellularized lungs (DC) examined by light microscopy (LM) and SEM. Box plot demonstrates the 1st and 3rd quartiles, upper and lower limits ("whiskers"), and median (red cross).

Figure 2.

The decellularized murine cardiac lobe. After decellularization, the cardiac lobe was imaged by scanning electron microscopy. Alveolar ducts (AD) are shown (A, bar = 200um; B, bar = 50um; C, bar = 20um; D, bar = 50um).

Figure 3. Alveolar ducts in 200um precision-cut lung slices examined by light microscopy (A,B) and scanning electron microscopy (SEM)(C,D). Helical line elements were apparent when imaged along the long axis of the alveolar duct (C–D, bar=10um).

Figure 4.

Microscopy of the cable line element of the axial connective tissue system. A) Fluorescence microscopy of thin tissue sections stained with Sirius red demonstrating red/orange collagen birefringence as well as green elastin staining at septal tips (white arrows)(bar=100um). B–D) SEM of the line element demonstrated a central cable encased in basement membrane (B–C, bar=10um; D, bar=2um).

Figure 5. Microscopy of the lung demonstrating the connections of the axial connective tissue system. A) EvG staining of a cellular lung, with characteristic black elastin staining (arrows), demonstrating multiple attachments to the visceral pleura (bar=80um). B) Fluorescence microscopy of thin tissue sections stained with Sirius red revealing red/orange collagen birefringence as well as green elastin staining at septal tips (white arrows) and at the pleural insertion (ellipse). C) SEM demonstrating visceral pleural (P) insertion of parallel cables (dotted lines). D) The cable line element of the alveolar duct (AD) also inserts into juxtaposed blood vessels (BV)(bar=20um).

Figure 6.

Macromolecular elastin cable network. A–C) Scanning electron microscopy (SEM) of decellularized lung after 7 days of collagenase digestion. Note the frequent helical cable pattern and the “twisted rope-like” appearance of the cable (arrows)(A, bar=50um;B–C, bar=20um). Anti-elastin immunofluorescence (D) and control (E) staining of serial sections of the collagenase-treated decellularized murine lung (bar=120um). Control sections were treated with fluorescein-labeled detection antibody alone. Despite the expected elastin autofluorescence, the relative fluorescence intensity (RFI) of the anti-elastin antibody staining was consistently twice the control fluorescence (inset; E=anti-elastin treatment; C=control with secondary antibody alone). Mean $\pm$ 1 S.D.