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A method for evaluating the murine pulmonary vasculature using micro-computed tomography

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ABSTRACT

Background: Significant mortality and morbidity are associated with alterations in the pulmonary vasculature. While techniques have been described for quantitative morphometry of whole-lung arterial trees in larger animals, no methods have been described in mice. We report a method for the quantitative assessment of murine pulmonary arterial vasculature using high-resolution computed tomography scanning.

Methods: Mice were harvested at 2 weeks, 4 weeks, and 3 months of age. The pulmonary artery vascular tree was pressure perfused to maximal dilation with a radio-opaque casting material with viscosity and pressure set to prevent capillary transit and venous filling. The lungs were fixed and scanned on a specimen computed tomography scanner at 8-μm resolution, and the vessels were segmented. Vessels were grouped into categories based on lumen diameter and branch generation.

Results: Robust high-resolution segmentation was achieved, permitting detailed quantitation of pulmonary vascular morphometrics. As expected, postnatal lung development was associated with progressive increase in small-vessel number and arterial branching complexity.

Conclusions: These methods for quantitative analysis of the pulmonary vasculature in postnatal and adult mice provide a useful tool for the evaluation of mouse models of disease that affect the pulmonary vasculature.

Introduction

The lung is a complex organ that is composed of airways, blood vessels, and parenchyma. Acquired diseases such as emphysema and bronchopulmonary dysplasia, and congenital diseases such as congenital diaphragmatic hernia, congenital heart disease, and primary pulmonary hypertension may have alterations in the pulmonary vasculature from

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vessel wall remodeling and reduction in vessel numbers due to vessel rarefaction or failed angiogenesis.[1-6] Pulmonary hypertension is a source of significant morbidity and mortality. The mechanisms are not fully understood, and treatments to prevent or reverse it are lacking.

Animal models have been used to investigate the histology, physiology, and molecular mechanisms of pulmonary hypertension and pulmonary vascular disease.[7-9] A major limitation in studying pulmonary hypertension and other pulmonary vascular disease is the lack of appropriate quantitative morphometric techniques to evaluate the entire intact pulmonary vascular tree. Current analysis of the pulmonary vasculature is restricted to histologic or stereologic techniques that require the use of random or systematic sampling from two-dimensional tissue sections to draw conclusions about three-dimensional (3D) structure.[10-15] Verified techniques such as point discrimination, line intercept count, and transection count use standardized methods such as a uniform grid to overlay on tissue section images and thus limit the amount of error associated with probing the structures of interest.[10,14,16-18] Such methods are limited by the assumption that each analyzed sample is sufficiently random yet simultaneously representative of the entire lung.[10] Hence, these methods do not account for the full complexity of the pulmonary vasculature. Furthermore, they do not allow whole-lung evaluation of branch-patterning, vessel generation, length, diameter, or volume of the pulmonary vasculature. Moreover, tissue sections alone may miss the true density of blood vessels or changes in vessel density and structure from one lobe to another.[15]

In recent years, 3D techniques to evaluate the pulmonary vasculature in small animal models have evolved to avoid the above limitations. Multidetector computed tomography (CT), magnetic resonance imaging, and micro-CT (μCT) have been used.[14,19-22] Micro-CT has a number of advantages. Images can be acquired *in vivo* or on postmortem tissue samples. Analysis may be combined with unbiased sampling procedures and can encompass the entire pulmonary vascular tree.[14,20,22] μCT can acquire images with resolution that is comparable to microscopy.[14] Recently, μCT was combined with arterial casting to evaluate pulmonary vascular development in the rat. Data were obtained and analyzed with proprietary software to provide automated measurements.[22] Besides the limitation of using noncommercially available software, few studies have applied these techniques to mice. Mouse models of pulmonary vascular disease are important for understanding the basis of pulmonary vascular disease. Thus, the purpose of this study was to develop a method for quantitative analysis of the total murine lung vascular tree using current techniques and commercially available software.

Materials and methods

Animals

Male C57BL/6-SVJ/129 hybrid mice from our existing colony were sacrificed at 2 weeks, 4 weeks, and 3 months (adult). Animal care and procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Lung perfusion and casting technique

Perfusion cannulas were constructed by inserting polyethylene-10 (PE-10) into polyethylene-50 (PE-50) tubing (Solomon Scientific, San Antonio, TX) in which a wire mandrel was inserted to maintain luminal patency during heating over a soldering iron tip to seal the tubing and form a trumpeted tip.

Mice were deeply anesthetized with isoflurane, heparinized, and lungs harvested either with or without measurement of right ventricular pressure. After removal of the anterior chest wall, the pulmonary artery was cannulated through the right ventricle, the left atrium incised, and the lungs perfused with 200 μL of phosphate-buffered saline containing sodium nitroprusside (10^{-4} M) to ensure maximum vessel dilation. The trachea was then cannulated with PE-10 tubing and inflated to 20 cm H_2O with 4% paraformaldehyde (Fig. 1A). Microfil (Flowtech Inc, Carver, MA), which was mixed in a ratio of 800 μL of Microfil, 100 μL of diluent, and 100 μL of casting agent to achieve a viscosity tailored to minimize capillary and venous transit, was slowly infused under a dissecting microscope. After the Microfil solidified, the trachea was ligated to maintain airway distension, and the lungs and/or trachea excised and fixed in paraformaldehyde for 24 hours.

Tissue imaging and analysis

Lungs were scanned on a MicroCT 40 (Scanco Medical, Wayne, PA). CT scans were obtained using the following settings: field of view (16 mm), energy or intensity (70 kVp, 114 μA, 8W), high resolution (i.e., 1000 projections), voxel size (8 μm), and an integration time (200 ms). This produced DICOM images at 8-μm resolution. Data (DICOM) were analyzed using Amira 5.5.0 software (FEI, Hillsboro, Oregon). A 3D reconstruction of the pulmonary arterial tree was achieved with multiple steps. Threshold segmentation identified the material of interest and was optimized by specimen. If bubbles were present within the arterial cast, a segmentation tool removed the bubbles with the command "Fill Holes." Images were processed with default settings using the "Remove Islands" and "Smoothing" segmentation functions. Connected components were attached to the Labelfield with a minimum size threshold of 100 pixels and a maximum size threshold of 0. "Connected components options" for "Labelfield" and "Spreadsheet" were selected as output styles, and "Preserve Exterior" was left unchecked. Next, material of interest was identified within the spreadsheet by identifying the largest nonexterior material available and noting the material designation (i.e., material X). Compute-arithmetic function was performed by designating the function to be performed on the material identified by the spreadsheet as A = X. A Castfield was generated from the result, and the output type selected is Labelfield. Using the Castfield generation, autoskeletonization was performed on the generated Labelfield (Fig. 1B).