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CHANGES IN THE STRUCTURE AND FUNCTION OF ARTERIAL ELASTIC LAMELLAE AS A RESULT OF PULMONARY HYPERTENSION: STUDIES USING SCANNING ELECTRON MICROSCOPY, MASS SPECTROSCOPY, AND X-RAY DIFFRACTION

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INTRODUCTION

Pulmonary arterial hypertension (PAH) is a hemodynamic state characterized by a severe elevation in the mean pulmonary arterial pressure. Both idiopathic (primary) and congenital (secondary) forms of the disease impose increased hydraulic load on the right ventricle of the heart, resulting in cardiac remodeling and if left untreated, complete failure.

Right ventricular afterload in PAH arises from both distal vascular flow resistance and upstream arterial stiffening, the combination of which increases pulmonary vascular input impedance [1,2]. Much of the research on this disease has focused on downstream vascular tone, the corresponding pulmonary vascular resistance (PVR), and pharmaceutical methods of reducing PVR clinically. Less effort has been expended on elucidating the impact of compliance of the pulmonary arteries on right ventricle afterload. Specifically, the issue of whether the upstream pulmonary arteries have stiffened due to acute effects, such as changes in vascular tone caused by increased pressure, or due to chronic remodeling, i.e., changes in vascular structure, has not been completely addressed. A prior finite element study using a novel microstructure constitutive model of the pulmonary arteries provoked the hypothesis that structural protein cross-linking (specifically for elastin) may be a key method by which the pulmonary artery stiffens in PAH [3]. However, this has not been explicitly verified. Here, we explore the question of how the structure of the elastin lamellae changes with onset of PAH using scanning electron microscopy (SEM) and x-ray diffraction (XRD). Further, a desmosine-based mass spectroscopy method was used to quantify the percent cross-linking of elastin.

MATERIALS AND METHODS

Animal

PAH was induced in ET-B receptor deficient Long-Evans rats using hypoxia. Both control (non PAH) and experimental (PAH)

animals were studied. PAH animals were studied after 3-week exposure to hypoxia (simulating 17,000 feet). After sacrifice, a midline sternotomy was performed to expose the heart and lungs, and 1mL (1000 units) heparin was injected into the left ventricle of the heart. A small incision was made in the left atrium and 10mL phosphate-buffered saline (PBS) was pushed through the pulmonary circuit via pulmonary artery cannulation. The aorta was removed, followed by removal of the pulmonary artery from the left atrium to the left / right hilum of the lungs. The aorta and pulmonary artery were then prepared for light microscopy, SEM and XRD studies.

Elastin Scaffolds

Elastin scaffolds were prepared for use in both SEM imaging and elastin crosslink quantification. The scaffolds consist of pulmonary arteries in which the cells, collagen and other ECM components have been removed using a cyanogen bromide (CNBr) treatment [4]. Briefly, pulmonary artery samples were cut into 1 mm thick rings, treated with 50mg/mL CNBr in 70% formic acid (10mL / cm²) for 19h at 20°C with stirring. This was followed by 1h at 60°C and boiling for 5 min to inactivate the CNBr.

Light Microscopy

The pulmonary arteries of both normotensive and hypertensive rats were extracted and the trunk, right and left branches were sectioned and stained with hematoxylin / eosin (H&E) and Verhoff Van Giesen (VVG) stains to determine elastic lamellae morphology. A Nikon TE200 inverted biological microscope was used for imaging.

Scanning Electron Microscopy

Scanning electron microscopy was used to determine the overall morphology of the elastic lamellae. The protocol used has been described elsewhere [4]. Briefly, the SEM samples were fixed in Karnovsky's fixative (2.5% glutaraldehyde, 2% formaldehyde in

100mM cacodylic acid buffer, pH 7.4) followed by dehydration in a series of graded ethanol (50-100%). Critical drying was then used to remove the ethanol and gold was deposited on the sample prior to SEM analysis (Jeol JSM-6400 SEM).

X-Ray Diffraction

Both small-angle and wide-angle X-ray diffraction have been used for studies of soft-tissue components (in particular collagen and elastin). These methods can provide information on correlation distances from the molecular to the fiber level. For our studies, a simple test cell was built to allow collection of diffraction patterns of samples immersed in a saline solution. X-ray diffraction data was collected using a Bruker D8 Discover diffractometer with a 2-D detector (GADDS) and Cu K α radiation. The 2-D detector facilitates quick data acquisition and quantitative assessment of sample anisotropy.

Elastin Crosslink Quantification

Elastin is a matrix composed of tropoelastin protein molecules bound to one another through desmosine and isodesmosine crosslinks. The protocol to quantify elastin cross-linking has been described elsewhere [5]. Briefly, the elastin scaffold of the pulmonary artery is cut into pieces and 1 nmol PE-Cys / mg lung and 0.5-1 mL of water are added. The tissue is then hydrolyzed with an equal volume of 12N HCl and reacted for 48h at 110°C. The hydrochloride is then dried in a rotary evaporator and hydrolysates are dissolved in ultrapure water. The hydrolysate is then loaded into a HPLC-Mass Spectrometer for crosslink quantification. Dry weights of lyophilized elastin scaffolds are also taken to determine the crosslink density.

RESULTS

The following results are representative of the preliminary studies conducted on normotensive rats. Light micrographs of the pulmonary artery trunk are shown in Figure 1. Tissue sections were stained with either H&E or VVG. For H&E cell nuclei stain dark purple, for VVG cell nuclei and elastin stain black, collagen fibers stain red and muscle stains greenish yellow. An SEM image of the intimal and medial layers of an elastin scaffold is shown in Figure 2. The image clearly shows the directionality and overall morphology of the elastic lamellae. A typical X-ray diffraction pattern seen for elastin scaffolds is shown in Figure 3. Few changes were seen in diffraction patterns between pure elastin and whole artery samples. Analysis of the diffraction data to recover orientation distribution function for elastin is currently ongoing.

CONCLUSIONS

We show that a variety of diagnostic methods can be applied to explore structural changes at the protein and extra cellular matrix level for the pulmonary artery. Ongoing effort is aimed at completing and inter-relating SEM, XRD, and cross-linking results for normotensive and PAH arteries.

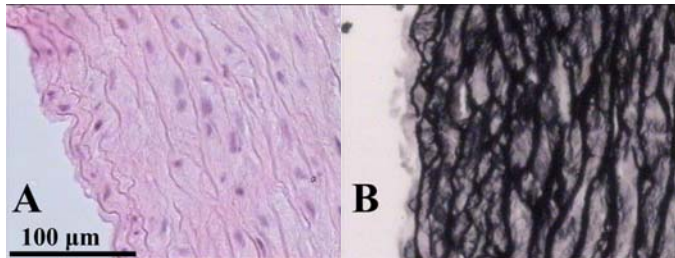


Figure 1. Representative H&E (A) and VVG (B) staining of pulmonary arteries, 400x magnification.

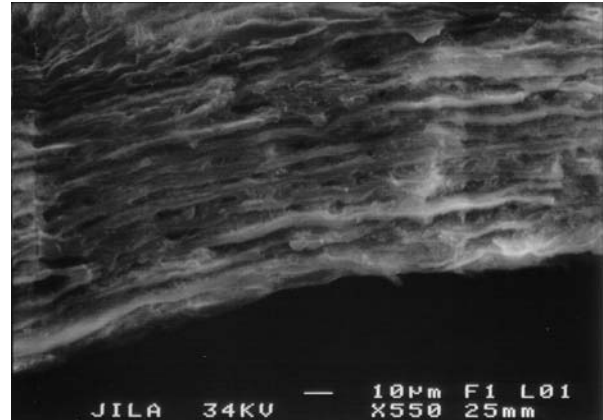


Figure 2. SEM image of artery. 550x magnification

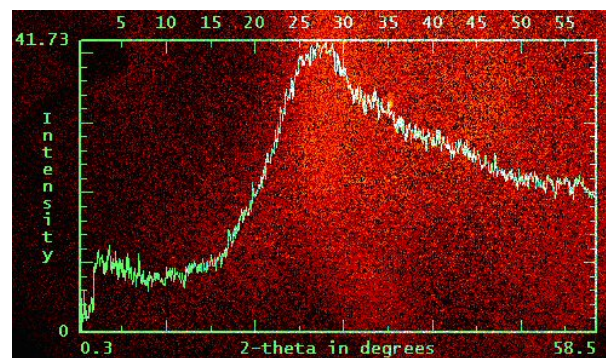


Figure 3. X-ray diffraction pattern of elastin scaffold

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