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Comparative biology of decellularized lung matrix: Implications of species mismatch in regenerative medicine

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ABSTRACT

Lung engineering is a promising technology, relying on re-seeding of either human or xenographic decellularized matrices with patient-derived pulmonary cells. Little is known about the species-specificity of decellularization in various models of lung regeneration, or if species dependent cell-matrix interactions exist within these systems. Therefore decellularized scaffolds were produced from rat, pig, primate and human lungs, and assessed by measuring residual DNA, mechanical properties, and key matrix proteins (collagen, elastin, glycosaminoglycans). To study intrinsic matrix biologic cues, human endothelial cells were seeded onto acellular slices and analyzed for markers of cell health and inflammation. Despite similar levels of collagen after decellularization, human and primate lungs were stiffer, contained more elastin, and retained fewer glycosaminoglycans than pig or rat lung scaffolds. Human endothelial cells seeded onto human and primate lung tissue demonstrated less expression of vascular cell adhesion molecule and activation of nuclear factor-κB compared to those seeded onto rodent or porcine tissue. Adhesion of endothelial cells was markedly enhanced on human and primate tissues. Our work suggests that species-dependent biologic cues intrinsic to lung extracellular matrix could have profound effects on attempts at lung regeneration.

1. Introduction

For patients with end-stage lung disease, lung transplantation can be the only definitive therapy. However, this procedure is accompanied by high morbidity and mortality, is limited by a shortage of suitable donor lungs, and requires life-long immunosuppression [1]. Thus, many investigators are looking to lung regeneration as a possible solution to these limitations. The advent and subsequent improvement of decellularization/recellularization protocols has provided a theoretical means for the creation of patient-specific artificial lungs that could address the current donor tissue shortage while potentially alleviating the need for immunosuppressants [2–5].

In order to develop scaffolds for the construction of bioengineered lungs, donated tissues are decellularized using a series of detergents to remove donor DNA and cellular debris. The resulting decellularized scaffolds are not simply inert "organ templates"; rather these systems impart regulatory signals that actively govern fundamental cellular processes such as proliferation and migration [6], induction of metastatic or fibrotic activity [7], response to growth factors [8], and also provide information regulating appropriate location of specific cell constituents within the matrix [9,10]. These extracellular matrix (ECM) regulatory

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signals are transmitted via biological cues such as ECM composition and organization, mechanical cues such as matrix stiffness or stretch, and through architectural features such as surface topography [11–13]. This "biophysical language" is especially important for cells that undergo mechanical loading (e.g., during breathing), reside in interstitial spaces, or are being reintroduced into decellularized tissue scaffold for the purposes of organ regeneration [14]. By providing cells with a suitable scaffold that has retained the appropriate matrix signals, it is more likely that cells reseeded into decellularized scaffolds will have a more functional outcome.

Xenographic lung tissue from rodent, porcine and primate sources are often considered as a model of human tissue, although these systems have innate differences in matrix composition and architecture (Supplemental Table 1). Porcine lungs are also regarded as an ideal donor source for xenotransplantation, and considerable amounts of financial resources have been dedicated to building porcine bioengineered lungs for transplantation into human recipients [4,15–18]. Despite recent advancements in eliminating critical xenographic cellular contribution(s) to organ failure, the survival of primate recipients of porcine lungs is less than 5 days [19–21]. Therefore, it is likely that in addition to cellular differences in species, there are other factors in xenographic tissues that could lead to transplantation failure. Surprisingly, there are few studies dedicated to examining how the innate differences in animal-derived lung ECM impact human cells. Characterizing the species-dependent impact of the decellularization process is a necessary step towards understanding what models are appropriate for informing the construction of human-derived bioengineered lungs. In this work, we first seek to characterize the species-specific responses to decellularization in relevant models of lung regeneration. Secondly we determine whether human cells exhibit species-dependent cell-matrix interactions within these systems.

2. Materials and methods

2.1. Rat, pig, primate, and human lung tissue

Yale University Institutional Animal Care and Use Committee approved all experimental work performed on rat, porcine, and primate animals in this study. All animal care complied with the Guide for the Care and Use of Laboratory Animals. This study utilized lungs from 12 old Sprague Dawley rats (3–6 months old); 6 Yorkshire pigs (18–25 kg); 4 African Green Vervet monkeys (5–8 years old); and 2 Humans (65–68 years old). All animals (rat, pig and primate) were euthanized via intraperitoneal injection of 150 mg/kg sodium pentobarbital (Sigma). All lungs were intravenously pretreated with 500 U/kg heparin (Sigma-Aldrich). Following euthanasia, the lungs were perfused via the right ventricle with PBS containing 50 U/mL heparin and 1 mg/mL sodium nitroprusside (Fluka). For rat and primate lungs, tracheal and pulmonary artery cannulae were inserted and sutured into place to provide access for perfusion and decellularization. For pig lungs, the accessory lobe was dissected away and the lobar bronchus and artery were cannulated. The accessory lobe, which is the smallest of the seven lobes in the pig lung, makes for a convenient model system for decellularization studies due to its size [22–24].

The native human whole lungs were acquired *en bloc* from brain-dead organ donors during transplant organ recovery through a research protocol with Gift of Life Michigan (Ann Arbor, MI). The University of Michigan Institutional Review Board has considered these approaches exempt from oversight as all subjects are deceased upon lung recovery. Human lungs were sent to Yale University within 24hr post-explant. The right middle lobe was dissected from the whole lung, and processed immediately.

All donors were prescreened for lung disease prior to tissue procurement, and only non-smokers were used in this study. After tissue procurement, human lungs were subsequently examined for abnormalities (interstitial matrix deposition, inflammation, retention of lung architecture) prior to inclusion in this study. All cell experiments were preformed on lung tissue slices that were deemed healthy as ascertained by these metrics.

2.2. Decellularization

The decellularization process is described previously, with the addition of a benzonase rinse at the end of the decellularization process [24]. Briefly, tissues were perfused using a gravity feed at approximately 30–40 cm H_2O pressure. Infusion of decellularization solutions occurred via the vasculature, or via gentle manual flushing of the airway. Immediately following procurement, the airways were inflated with PBS containing antibiotics (10% penicillin/streptomycin, 4% amphotericin B, 2% gentamicin). Tissues were mounted within a bioreactor for subsequent decellularization steps. A sequence of low concentration detergents and endonuclease (benzonase, Sigma) were applied within a physiological pH range (pH 7–8) at either 4 °C or room temperature. The decellularization protocol concluded with a final PBS buffer and benzonase rinse of the lung vasculature and resulted in the generation of decellularized extracellular matrix scaffolds.

2.3. Histology and immunostaining

Randomly sampled areas (n = 3–5) from each native and decellularized lung were isolated and fixed with 10% neutral buffered formalin (Sigma) for 4 h at room temperature. Samples were then stored overnight in 70% ethanol, embedded in paraffin, and sectioned at 5 μm. Tissue slides were stained for hematoxylin and eosin (H&E), Masson's Trichrome staining (Trichrome), or Verhoff's Van Gieson (EVG) as described previously [24]. For immunofluorescence, section were blocked with PBS containing 3% BSA and 0.2% Triton X-100 for 45 min, and subsequently incubated in primary antibody against PCNA (Abcam, ab29, 1:1000) overnight at 4 °C. After washing slides with PBS, secondary antibody (Alexafluor 555) was used at 1:500 dilution for 45 min. The slides were visualized using a Leica DMI6000 B fluorescence microscope.

2.4. Collagen assay

Hydroxyproline assays were used to quantify collagen content, as previously described [25]. Samples were oxidized with chloramine-T, combined with *p*-dimethylaminobenzaldehyde (Mallinckrodt Baker, Phillipsburg, NJ), and absorbance was measured at a wavelength of 550 nm against a verified hydroxyproline standard curve. The total hydroxyproline was then quantified for each sample, and a 1:12 w/w ratio of hydroxyproline was used to calculate the collagen amount. Total collagen content was normalized to dry tissue weight.

2.5. Elastin assay

Elastin was measured by determining the desmosine crosslinks via an ELISA assay as described previously [26]. Briefly, desmosine crosslinks were measured by first lyophilizing the sample, hydrolyzing with 6 N HCL for 48 h, and reconstituting and filtering the tissue through a 0.45 μm filter. Total elastin was normalized to dry tissue weight.