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Targeted proteomics effectively quantifies differences between native lung and detergent-decellularized lung extracellular matrices

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ABSTRACT

Extracellular matrix is a key component of many products in regenerative medicine. Multiple regenerative medicine products currently in the clinic are comprised of human or xenogeneic extracellular matrix. In addition, whole-organ regeneration exploits decellularized native organs as scaffolds for organotypic cell culture. However, precise understanding of the constituents of such extracellular matrix-based implants and scaffolds has sorely lagged behind their use. We present here an advanced protein extraction method using known quantities of proteotypic ^{13}C-labeled peptides to quantify matrix proteins in native and decellularized lung tissues. Using quantitative proteomics that produce picomole-level measurements of a large number of matrix proteins, we show that a mild decellularization technique ("Triton/SDC") results in near-native retention of laminins, proteoglycans, and other basement membrane and ECM-associated proteins. Retention of these biologically important glycoproteins and proteoglycans is quantified to be up to 27-fold higher in gently-decellularized lung scaffolds compared to scaffolds generated using a previously published decellularization regimen. Cells seeded onto this new decellularized matrix also proliferate robustly, showing positive staining for proliferating cell nuclear antigen (PCNA). The high fidelity of the gently decellularized scaffold as compared to the original lung extracellular matrix represents an important step forward in the ultimate recapitulation of whole organs using tissue-engineering techniques. This method of ECM and scaffold protein analysis allows for better understanding, and ultimately quality control, of matrices that are used for tissue engineering and human implantation. These results should advance regenerative medicine in general, and whole organ regeneration in particular.

Statement of Significance

The extracellular matrix (ECM) in large part defines the biochemical and mechanical properties of tissues and organs; these inherent cues make acellular ECM scaffolds potent substrates for tissue regeneration. As such, they are increasingly prevalent in the clinic and the laboratory. However, the exact composition of these scaffolds has been difficult to ascertain. This paper uses targeted proteomics to definitively quantify 71 proteins present in acellular lung ECM scaffolds. We use this technique to compare two decellularization methods and demonstrate superior retention of ECM proteins important for cell adhesion, migration, proliferation, and differentiation in scaffolds treated with low-concentration detergent solutions. In the long term, the ability to acquire quantitative biochemical data about biological substrates will facilitate the rational design of engineered tissues and organs based on precise cell-matrix interactions.

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1. Introduction

Decellularized scaffolds for regenerative medicine are created by removing intact cells from tissues or organs using detergents, nucleases, and other reagents [1]. The resulting scaffold is comprised of extracellular matrix (ECM) proteins that have important physical and biochemical roles in organ function. Use of such scaffolds has rapidly expanded in both the laboratory and in the clinic [2], yet precise knowledge of the components of these matrices remains elusive. Standard proteomics techniques do not render reproducible or quantitative information about crosslinked and highly insoluble matrix elements. Immunostaining, ELISA, and immunoblotting are confounded by antibody cross-reactivity and difficulties in sample preparation, as well as limits on precise quantification. Identification and quantification of the individual components of these bioactive matrix materials is important to understand outcomes following *in vitro* or *in vivo* use, and for designing strategies to improve scaffolds for tissue engineering. Using recently developed proteomic techniques [3], we present a detailed, quantitative comparison of the ECM that results from two different decellularization regimens that are used in whole lung engineering. These quantitative proteomics analyses exceed, both in precision and in completeness, all other similar scaffold analyses published to date [4–7] and serve to illustrate the power of this novel method for ECM characterization.

In total, 71 ECM proteins were quantified in native and decellularized lungs. We present the results in 7 categories: proteoglycans; matricellular proteins; other basement membrane and ECM-associated proteins; laminins and fibronectin; elastin-related proteins; cellular proteins; and collagens. In some cases, immunofluorescence and Western blotting were used to corroborate the proteomic results of individual proteins, to visualize the differences between scaffolds, and to add spatial information for key basement membrane proteins. Lungs that are decellularized using the mild method described herein retain basement membrane and basement membrane glycoproteins and proteoglycans to a greater degree than our previously published method (6). We observed near-native retention of a large variety of matrix components that bind cells and support biological activity. However, a trade-off was noted, in that mild decellularization resulted in an increase in remnant cellular proteins that persisted within the scaffold, particularly cytoskeletal proteins. Overall, the results of this study show the feasibility of deriving absolute quantification of a large number of matrix molecules within whole tissues. Armed with this information, it will be possible to tune decellularization and organ culture strategies to better mimic the structural complexities of native tissues.

2. Materials and methods

2.1. Organ procurement

All animal work was performed in accordance with AAALAC guidelines and was approved by the Yale Institutional Animal Care and Use Committee.

Lungs were harvested from adult (~3 months old) Fischer 344 male rats [8] and prepared for decellularization.

2.2. Decellularization – Triton/SDC

Lungs were perfused with a series of reagents including antibiotics/antimycotics, phosphate buffered saline (PBS) containing Ca^{2+} and Mg^{2+}, and 0.0035% Triton X-100. This was followed by a benzonase endonuclease step, wherein lungs were inflated with the enzyme and incubated at room temperature for 30 min a rinse with a 1 M NaCl solution was performed, followed by rinsing with PBS without divalent ions. A series of washes with increasing concentrations of sodium deoxycholate (0.01%, 0.05%, 0.1%) was applied, followed by a 1 h benzonase incubation after perfusion of the enzyme into the lungs via the pulmonary artery. Finally, the lung was perfused with 0.5% Triton X–100, followed by extensive rinsing with PBS and antibiotics/antimycotics for 48 h at room temperature (~25 ºC). Lungs were perfused with the antibacterial/antifungal solution for 48 h at 37 °C.

2.3. Decellularization – CHAPS

Lungs were perfused at 37 °C via the pulmonary artery with 500 ml of CHAPS-based decellularization solution [8], pH 12.4. Upon conclusion of detergent application, lungs were treated with benzonase endonuclease (90 U/ml) by inflation of the lungs with the benzonase solution via the trachea and incubated for 1 h at 37 °C. Following benzonase, lungs were rinsed extensively with PBS via the pulmonary artery. Fully rinsed lungs were mounted in a sterile bioreactor and perfused with antibiotics/antimycotics, as described for Triton/SDC.

2.4. Histology and immunofluorescence

Fixed, dehydrated tissues were processed by standard histological techniques at the histological core facilities. Unstained sections were deparaffinized and stained for laminin-γ1, fibronectin, and proliferating cell nuclear antigen (PCNA) by indirect immunofluorescence. Tissue designated for frozen sections was fixed with formalin and embedded in optimal cutting temperature (OCT) gel. The embedded tissue was frozen over dry ice for ~25 min, then transferred to the −80C freezer until sectioned. Sections were thawed to room temperature and rehydrated in PBS for 10 min to stain for collagen IV alpha (α) chains. See Detailed Online Methods.

2.5. Western blot

Whole lower right lobe lung samples were lysed from native and decellularized lungs (both Triton/SDC and CHAPS preparations) with 500–1000 μL of RIPA Buffer (Invitrogen) containing protease inhibitors (Roche), rocked for 1 h at 4 °C, and centrifuged at 14,000 rpm to collect the supernatant. Reducing Laemmli buffer was added to all lysed lungs samples. After normalization for differences in buffer volume for each sample, equal volumes of each lung sample were loaded on a 4–12% SDS-page gel (Bio-Rad) for separation and transferred to polyvinylidene (PVDF) membrane for Western blot analysis. a-actin (Abcam) 1:500, MHC-1 (BD transduction laboratories) 1:500, MHC-2 (Abcam) 1:1000 overnight at 4 °C to probe for proteins of interest.

2.6. Repopulation and culture of seeded scaffolds

Decellularized lung extracellular matrix scaffolds were prepared as described and mounted in biomimetic bioreactors as described previously [9]. After terminal sterilization with antibiotics and antimycotics, 100–150 million rat lung microvascular endothelial cells were introduced into the decellularized lung scaffolds via the pulmonary artery and pulmonary veins in series. Mixed lung cell populations isolated from neonatal rats were subsequently introduced into the scaffold via the trachea [8]. Pulsatile flow via the pulmonary artery was begun 1 h after seeding the mixed lung cell population at ~1 mL/min and constructs were maintained in 50% DMEM + 10% FBS/50% MCDB-131 with 1% penicillin/streptomycin for 4 days. Half the volume of media was