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Local micromechanical properties of decellularized lung scaffolds measured with atomic force microscopy

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ABSTRACT

Bioartificial lungs re-engineered from decellularized organ scaffolds are a promising alternative to lung transplantation. Critical features for improving scaffold repopulation depend on the mechanical properties of the cell microenvironment. However, the mechanics of the lung extracellular matrix (ECM) is poorly defined. The local mechanical properties of the ECM were measured in different regions of decellularized rat lung scaffolds with atomic force microscopy. Lungs excised from rats ($n = 11$) were decellularized with sodium dodecyl sulfate (SDS) and cut into ~7 μm thick slices. The complex elastic modulus (G^*) of lung ECM was measured over a frequency band ranging from 0.1 to 11.45 Hz. Measurements were taken in alveolar wall segments, alveolar wall junctions and pleural regions. The storage modulus (G', real part of G^*) of alveolar ECM was ~6 kPa, showing small changes between wall segments and junctions. Pleural regions were threefold stiffer than alveolar walls. G' of alveolar walls and pleura increased with frequency as a weak power law with exponent 0.05. The loss modulus (G'', imaginary part of G^*) was 10-fold lower and showed a frequency dependence similar to that of G′ at low frequencies (0.1–1 Hz), but increased more markedly at higher frequencies. Local differences in mechanical properties and topology of the parenchymal site could be relevant mechanical cues for regulating the spatial distribution, differentiation and function of lung cells.

1. Introduction

Respiratory diseases are a leading cause of death worldwide. Lung transplantation is the only treatment currently available in end-stage severe respiratory diseases, including chronic obstructive pulmonary disease, alpha-1-antitrypsin deficiency, pulmonary fibrosis and pulmonary arterial hypertension [50]. However, lung transplantation achieves only ~50% survival at 5 years and is hampered by a severe shortage of donor organs [30,50]. Bioartificial lungs re-engineered from decellularized lung matrix scaffolds are a promising alternative to lung transplantation. Recently, a bioreactor has been used to culture pulmonary epithelium and vascular endothelium cells seeded on acellular lung matrices [31,34]. These biological scaffolds displayed a remarkable repopulation of epithelial and endothelial compartments. Moreover, the re-engineered lungs participated in gas exchange when implanted into rats [34,43]. Cortiella and co-workers [7] reported a first attempt to use whole acellular lung as a biologic scaffold to support the development of engineered lung tissue from murine embryonic stem cells. In constructs produced in whole acellular lungs, these authors found organization of differentiating stem cells into three-dimensional (3-D) structures reminiscent of complex tissues. These works provide proof of the concept of generation of transplantable recellularized lungs as a viable strategy for lung regeneration, either as a part or as the entire organ. However, this approach is currently limited by low repopulation efficiency. A more efficient repopulation of lung scaffolds requires better understanding of the intricate combination of biophysical and biochemical factors that modulate cell engraftment, proliferation and differentiation.

There is compelling evidence that cells sense and respond to the mechanical properties of their microenvironment [9,28,32,48]. By seeding cells on a gradient of substrate elasticity, Lo and co-workers [26] showed that cells accumulate on stiffer substrates, in a process called durotaxis. Engler and co-workers [12] observed that embryo-derived cardiomyocytes maintained their spontaneous beating on substrates with elasticity less than or equal to that of

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normal heart tissue, but the cells stopped beating on rigid matrices that mechanically mimic a fibrotic scar. Stiffness of the microenvironment also mediates stem cell differentiation. Engler and co-workers [11] cultured naive mesenchymal stem cells on two-dimensional synthetic matrices of varying elasticity. They reported that soft matrices mimicking brain are neurogenic, stiffer matrices that mimic muscle are myogenic, and more rigid matrices that mimic collagenous bone are osteogenic. Saha and co-workers [40] found that neuron differentiation is favored on soft matrices mimicking normal brain, whereas differentiation into glia is promoted on harder matrices that typify glial scars. These findings suggest that cell engraftment, proliferation and differentiation in the lung are modulated by the mechanical properties of the cell microenvironment. A further understanding of this cell–microenvironment interplay requires a precise knowledge of the local mechanical properties of the acellular lung extracellular matrix (ECM).

Mechanical properties of acellular lung tissue have been probed in air-filled decellularized murine lungs by recording quasi-static pressure–volume curves during inflation and deflation [34] and by measuring respiratory impedance with the forced oscillation technique [46]. This approach does not provide accurate estimation of lung parenchymal ECM, since mechanical measurements performed on air-filled whole lung scaffolds depend on several other factors, including the 3-D architecture of lung parenchyma, the mechanical properties of airways and blood vessels embedded in the alveolar structure, and the surface tension of the alveolar air–liquid interface. The contribution of airways, vessels and surface tension can be avoided by using liquid-filled lung parenchymal strips obtained from peripheral regions of decellularized lungs. By subjecting strips to uniaxial stretching, the elastic modulus and tensile strength of 3-D lung tissue can be measured [34], but the actual mechanical parameters of the alveolar wall cannot be determined. Atomic force microscopy (AFM) provides a direct approach to measuring the local mechanics of alveolar ECM. AFM uses a sharp tip to indent the surface of the sample with nanometer resolution, and simultaneously measure the applied force. The complex elastic modulus (G^*) of the parenchymal lung ECM can be measured at different frequencies by vertically oscillating the AFM tip. Moreover, ECM mechanics can be locally determined at the microscale at which alveolar cells sense their mechanical microenvironment.

In this work, the local mechanical properties of the ECM of different regions of decellularized scaffolds of rat lungs were measured by AFM. Lungs excised from rats were decellularized and cut into thin slices. The complex elastic modulus of the ECM was measured over a wide frequency range by applying low-amplitude indentation oscillations with the AFM tip. Measurements were taken in alveolar wall segments, alveolar wall junctions and pleural regions of the scaffold. The frequency dependence of G^* was interpreted in terms of a rheological model defined by a linear superposition of two power laws.

2. Materials and methods

2.1. Lung decellularization

The study was carried out on lungs ($n = 11$) excised from young (8–9 weeks old) adult Sprague–Dawley rats (250–300 g) (Charles River, Wilmington, MA). The animal protocol was approved by the Ethical Committee for Animal Experimentation of the University of Barcelona. The lungs were decellularized following a procedure described previously [7]. The whole trachea, esophagus and lungs were excised from exsanguinated rats and washed with phosphate buffered saline (PBS). The lungs were frozen at −80 °C, thawed in a water bath at 40 °C and frozen again. The freezing–thawing cycle was carried out with the lungs uninflated (zero transpulmonary pressure) and repeated four times to enhance the formation of intracellular ice crystals that disrupt cellular membranes and cause cell lysis. The lungs were flushed with 1% sodium dodecyl sulfate (SDS) through the trachea using a syringe coupled to a catheter. Flushing was repeated three times per day to remove the disrupted cellular material with the lungs submerged in 1% SDS in continuous agitation for 5 days. Then the lungs were washed with Dulbecco's PBS (DPBS) with antibiotics (streptomycin (90 μg ml^{-1}) and penicillin (50 U ml^{-1})) and antimycotic (amphotericin B (25 μg ml^{-1})) to eliminate the detergent. DPBS was flushed through the trachea three times per day with the lungs submerged in DPBS in continuous agitation for 3 days. The upper lobe of the left decellularized lung was snap frozen with liquid nitrogen in tissue freezing medium (OCT compound, Sakura, Torrance, CA) until use. For AFM measurements, 7-μm-thick lung sections were cut using a cryostat and placed on top of positively charged glass slides (Thermo Fisher Scientific, Waltham, MA). The matrix sections were rinsed, immersed in DPBS and placed on the AFM sample holder.

2.2. AFM measurements

Mechanical measurements were performed on three different lung parenchyma regions: segments of alveolar walls; junctions of alveolar walls; and pleural membrane. Measurements were carried out with a custom-built AFM attached to an inverted optical microscope (TE2000, Nikon, Tokyo, Japan), using a previously described method [2,39]. Lung matrix samples were probed with a Si_3N_4 V-shape Au-coated cantilever with a four-sided pyramidal tip on its apex with a semi-included effective angle (θ) of ∼20° and a nominal spring constant (k) of 0.1 N m^{-1} (MLCT, Bruker, Mannheim, Germany). The cantilever was displaced in 3-D with nanometric resolution by means of piezoactuators coupled to strain gauge sensors (Physik Instrumente, Karlsruhe, Germany) to measure the displacement of the cantilever (z). The defection of the cantilever (d) was measured with a quadrant photodiode (S4349, Hamamatsu, Japan) using the optical lever method. The slope of a d–z curve obtained from a bare region of the coverslip was used to calibrate the relationship between the photodiode signal and cantilever deflection. The force (F) on the cantilever was computed as $F = kd$. To correct force measurements for the hydrodynamic drag force (F_d) on the cantilever, the cantilever was sinusoidally oscillated (16 Hz, 75 nm amplitude) at different tip–substrate distances (h) [1]. The drag factor $b(h)$ was computed at different tip–substrate distances as $b(h) = F_d/s$, where s is the relative cantilever-liquid velocity ($s = \dot{d} - \dot{z}$, where dots indicate time derivative). Drag factor data were fitted with a scaled spherical model.

At each measurement point, five force–displacement (F–z) curves were first recorded by vertically oscillating the cantilever with triangular displacement at 1 Hz and peak-to-peak amplitude of 5 μm to reach a maximum indentation of ∼2 μm (approaching velocity = 10 μm s^{-1}). The indentation of the sample (δ) was computed as $\delta = (z - z_c) - (d - d_{off})$, where z_c is the position of the contact point, and d_{off} is the offset of the photodiode. The position of the contact point was determined from the last recorded F–z curve. Subsequently, the tip was placed at an operating indentation (δ_0) of ∼500 nm, and a small amplitude (75 nm) multifrequency oscillation [39] composed of five sine waves (0.1, 0.35, 1.15, 3.55, 11.45 Hz) was applied for 140 s. This multifrequency signal is cyclical, with a period of 20 s. The chosen frequencies were logarithmically distributed over two decades, and each component was non-sum and non-difference of the others to avoid harmonic cross-talk. All sinusoidal components were taken of equal amplitude, except the lowest frequency component, which was taken