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Remodeling by fibroblasts alters the rate-dependent mechanical properties of collagen

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ABSTRACT

The ways that fibroblasts remodel their environment are central to wound healing, development of musculoskeletal tissues, and progression of pathologies such as fibrosis. However, the changes that fibroblasts make to the material around them and the mechanical consequences of these changes have proven difficult to quantify, especially in realistic, viscoelastic three-dimensional culture environments, leaving a critical need for quantitative data. Here, we observed the mechanisms and quantified the mechanical effects of fibroblast remodeling in engineered tissue constructs (ETCs) comprised of reconstituted rat tail (type I) collagen and human fibroblast cells. To study the effects of remodeling on tissue mechanics, stress-relaxation tests were performed on ETCs cultured for 24, 48, and 72 h. ETCs were treated with deoxycholate and tested again to assess the ECM response. Viscoelastic relaxation spectra were obtained using the generalized Maxwell model. Cells exhibited viscoelastic damping at two finite time constants over which the ECM showed little damping, approximately 0.2 s and 10–30 s. Different finite time constants in the range of 1–7000 s were attributed to ECM relaxation. Cells remodeled the ECM to produce a relaxation time constant on the order of 7000 s, and to merge relaxation finite time constants in the 0.5–2 s range into a single time content in the 1 s range. Results shed light on hierarchical deformation mechanisms in tissues, and on pathologies related to collagen relaxation such as diastolic dysfunction.

Statement of Significance

As fibroblasts proliferate within and remodel a tissue, they change the tissue mechanically. Quantifying these changes is critical for understanding wound healing and the development of pathologies such as cardiac fibrosis. Here, we characterize for the first time the spectrum of viscoelastic (rate-dependent) changes arising from the remodeling of reconstituted collagen by fibroblasts. The method also provides estimates of the viscoelastic spectra of fibroblasts within a three-dimensional culture environment. Results are of particular interest because of the ways that fibroblasts alter the mechanical response of collagen at loading frequencies associated with cardiac contraction in humans.

1. Introduction

Interactions between living cells and their mechanical environments are central to a host of physiologic and pathologic events ranging from development to metastasis to wound healing. Fibroblasts remodel their extracellular neighborhood considerably when activated, stiffening and compressing their extracellular matrix (ECM) environment [1–9]. During remodeling, fibroblasts secrete ECM proteins, including collagens, proteoglycans, glycoproteins, and proteases, and cross-linking proteins and enzymes [1–4,10]. Fibroblasts exert traction on the ECM and each other [9,11–13], and secrete soluble factors that affect neighboring cells and tissues in a paracrine manner [14]. This remodeling changes the tissue as a whole by establishing a network to link and organize individual cells [15–20].

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Although several studies have estimated the effects of this remodeling on ECM elasticity [5,21], much less is known about the viscoelastic effects of ECM remodeling. Fibroblasts are known to affect viscoelastic tissue relaxation in pathologies such as congestive heart failure. However, these effects have not been quantified, and there is a pressing need for data on viscoelastic remodeling of tissues.

Additionally, the cells themselves change during remodeling. Cytoskeletal disposition is known to change in response to perturbations in mechanical loading [22–24]. During wound healing, cytoskeletal structure is regulated to develop force against the ECM to close the wound [25]. Cell transformation and tumorigenicity are associated with a decrease in cell viscosity and elasticity [26]. Although well established techniques exist for estimating cell elasticity within engineered tissues [27,5], protocols are still needed to acquire information about cell viscoelasticity.

Engineered tissue constructs (ETCs) comprised of reconstituted rat tail (type I) collagen and human dermal fibroblast cells serve as *in vitro* models of this remodeling, and provide simplified systems in which to assess how remodeling affects the mechanics of cells and ECM [28,29]. These systems have been applied to study both linear and nonlinear elasticity of cells and ECM, but variations of time-dependent tissue mechanics by ECM remodeling has not been well characterized quantitatively.

The goal of this study was to establish the ways that fibroblast cells modulate their ECM viscoelastically and contribute to ETC-level viscoelasticity over the course of ETC remodeling. We studied these effects by performing viscoelastic relaxation tests on ETCs at three different timepoints. Following techniques that are standard for evaluation of ECM elasticity, ECM viscoelasticity was evaluated by treating ETCs with deoxycholate, a mild detergent which dissolves cell membranes and disperses cytoplasmic structures including the cytoskeleton. Tests were interpreted using a discrete spectral generalized Maxwell approach [30], which yields both elastic moduli and viscoelastic relaxation finite time constants. Results showed that cells actively adapted the ECM, and that cells relaxed at multiple timescales, including one that is fast compared to those of the ECM.

2. Materials and methods

2.1. Engineered tissue construct (ETC) preparation

ETCs were synthesized using procedures described in detail elsewhere [25]. Briefly, human dermal fibroblasts (Lonza, Allendale, NJ, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) at 37 °C and 5% CO_2. The media were changed every 3–4 days and the cells were split when cell confluency reached >80% of the dish surface. The cells were used for culturing ETCs at the 7th-10th passage. 0.5 million cells were mixed with 1 ml of a solution consisting of DMEM and 0.5 mg/ml type I rat tail collagen (harvested in our laboratory). The pH of this mixture was brought to neutrality using 0.2 M NaOH. 0.5 ml of this mixture was poured into hollow, cylindrical Teflon molds; the molds contained a central rod to create an annular well with outer and inner diameters of 14.9 mm and 9.5 mm, respectively. The final mixture was incubated at 37 °C with 5% CO_2 for 30 min to allow the collagen to polymerize. Then, the molds were filled with DMEM supplemented with 5% fetal bovine serum (FBS) and were kept in an incubator for 24, 48 or 72 h to allow the cells to remodel the collagen. Three specimens were tested at each of the three remodeling times, a total of nine specimens.

2.2. Stress-relaxation testing apparatus and protocol

Stress-relaxation tests were performed on the ring-shaped ETCs. ETCs were mounted within glass organ baths filled with HEPES-buffered DMEM (pH 7.4) and 5% FBS, and kept at 37 °C, conditions standard for culturing engineered tissues [31]. One end was attached to an actuator connected to a stepper motor, and the other to a force transducer, as described elsewhere [25]. Tissues were allowed one hour to accommodate to the new media before the stress-relaxation test. The protocol started with 10 min of force monitoring to establish a baseline. This was followed by tissue preconditioning consisting of 5 sequential cycles of a 20% axial stretch, followed by a 30 min recovery interval. A preconditioning protocol is standard in testing of collagenous tissues [32]. Using a strain rate analogous to that of the subsequent characterization experiments and a strain magnitude twice that used in the characterization experiments yields repeatable results in the testing of ETCs [27,33].

In the characterization experiments, ETC rings were stretched 10% at 20%/s then held isometrically for 3600 s while force was recorded at 50 Hz. Nominal stress data were inferred from force data by dividing the force by the cross-sectional area measured for each specimen at the conclusion of the test, as described below. Note that, despite the preconditioning, the specimens likely experienced some permanent deformation over the course of the loading; by considering a transversely isotropic specimen with a Poisson ratio of 0.5 locked into its deformed configuration, the difference between the actual first Piola Kirchoff stress and that we report can be estimated to be less than 10%. The strain amplitude of 10% strain was chosen because it represents the upper end of the linear range for a tendon [34]. Achieving this over 0.5 s is representative of strains in response to a brisk walking cadence and to stretching by the cardiovascular system. The stretch rate and prolonged monitoring were furthermore suitable for characterizing the temporal range of physiological responses [30,35].

2.3. Deoxycholate treatment

The specimens were returned to their baseline configurations and allowed to recover for 30 min to prepare for testing the contribution of the remodeled ECM to viscoelastic behavior of the ring constructs. For this purpose, DMEM + HEPES was replaced with 0.05% w/v deoxycholate in PBS (pH 7.4), and specimens were allowed to incubate for 60 min. The stretch-and-hold protocol was then repeated. Deoxycholate was chosen over inhibitors such as cytochalasin D and latrunculin because it enables lysis of cells without altering the mechanics of the protein structure in the remaining porous ECM [36,27].

2.4. Measurement of ETC dimensions

After the end of each experiment, specimens were mounted on spacers and stretched to their reference length, then fixed in 4% formaldehyde for 20 min at room temperature. Afterwards, specimens were cut into two equal pieces and placed within four-well plates filled with PBS. The width and thickness of each tissue were measured using Confocal microscopy (LSM 510, Zeiss). The thickness was measured near the upper and lower borders as well as in the middle of the tissue. The cross-sectional area of the tissue was calculated as the mean of the three measurements. All measurements were conducted by the same person.

2.5. DNA quantification

We synthesized an additional 9 specimens (three each at 24, 48, and 72 h of incubation) to estimate the final cell concentration in ETCs using a total DNA quantification assay. Right after the measurement of tissue dimensions, the constructs were centrifuged with 2 ml PBS in capped tubes. PBS was removed and, after 1 ml of lysis buffer was added, the tubes were sonicated. 30 μl of this sample was mixed with 3 ml of Hoechst solution (30 nM of