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Homologous structure–function relationships between native fibrocartilage and tissue engineered from MSC-seeded nanofibrous scaffolds

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ABSTRACT

Understanding the interplay of composition, organization and mechanical function in load-bearing tissues is a prerequisite in the successful engineering of tissues to replace diseased ones. Mesenchymal stem cells (MSCs) seeded on electrospun scaffolds have been successfully used to generate organized tissues that mimic fibrocartilages such as the knee meniscus and the annulus fibrosus of the intervertebral disc. While matrix deposition has been observed in parallel with improved mechanical properties, how composition, organization, and mechanical function are related is not known. Moreover, how this relationship compares to that of native fibrocartilage is unclear. Therefore, in the present work, functional fibrocartilage constructs were formed from MSC-seeded nanofibrous scaffolds, and the roles of collagen and glycosaminoglycan (GAG) in compressive and tensile properties were determined. MSCs deposited abundant collagen and GAG over 120 days of culture, and these extracellular molecules were organized in such a way that they performed similar mechanical functions to their native roles: collagen dominated the tensile response while GAG was important for compressive properties. GAG removal resulted in significant stiffening in tension. A similar stiffening response was observed when GAG was removed from native inner annulus fibrosus, suggesting an interaction between collagen fibers and their surrounding extrafibrillar matrix that is shared by both engineered and native fibrocartilages. These findings strongly support the use of electrospun scaffolds and MSCs for fibrocartilage tissue engineering, and provide insight on the structure–function relations of both engineered and native biomaterials.
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1. Introduction

The overarching goal of tissue engineering is to develop strategies by which cells (generally in combination with biocompatible materials) can be appropriately instructed to form new tissues to replace damaged or diseased ones. This task is quite challenging for load-bearing soft tissues of the musculoskeletal system, where the combination of composition and microstructural organization gives rise to anisotropic and nonlinear mechanical behaviors that are necessary for tissue function. Despite this challenge, recent advances in the engineering of orthopaedic soft tissues such as meniscus [1,2], tendon and ligament [3–6], cartilage [7,8], and the annulus fibrosus of the intervertebral disc [9–12] have shown promise in the generation of functional replacement tissues. Outcome measures in such studies are often focused on compositional analyses to measure the accumulation of collagen and glycosaminoglycan (GAG), organizational analyses to determine how these constituents are assembled, and ultimately, mechanical testing to determine how these tissues perform functionally with respect to their native counterparts. While many testing modalities are available and germane to native tissue function, two of the most commonly employed methods include uniaxial tension [13–15] and unconfined compression [16–18].

A number of load-bearing tissues exemplify the interrelation between composition, structure, and function. For instance, tissues subjected to large tensile stresses, such as ligaments and tendons, contain a large amount of collagen, while tissues subjected primarily to compressive loading, such as articular cartilage and the nucleus pulposus of the intervertebral disc, contain large amounts of proteoglycan containing charged GAG chains [19]. Fibrocartilage tissues are subjected to both tension and compression and therefore contain relatively large amounts of both collagen and GAG, and include such tissues as the annulus fibrosus of the intervertebral disc and the knee meniscus.

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To arrive at function from composition, it is necessary to consider structural organization. Fibrocartilages like the annulus fibrosus and knee meniscus are highly ordered structures, densely packed with collagen fibers that are locally aligned. Therefore, engineering successful replacements for these tissues necessitate replication of structural organization in order to ultimately achieve similar mechanical function. We have recently shown that electrospinning permits the fabrication of ordered nanofibrous assemblies that direct cell alignment and extracellular matrix deposition [20]. These scaffolds have been employed to engineer the annulus fibrosus across multiple length scales, from a single lamella [14,21] to angle-ply laminates [9] and most recently, whole disc composites containing an angle-ply annulus fibrosus surrounding a central nucleus pulposus [17]. In each case, mesenchymal stem cells seeded on nanofibrous scaffolds deposited GAG and collagen. Concomitant with extracellular matrix deposition, mechanical properties improved, and in the case of engineered angle-ply laminates, matched the uniaxial tensile modulus of the native annulus fibrosus after 10 weeks of *in vitro* culture [9]. While, clearly, matrix deposition and mechanical properties are related in these engineered tissues, the nature of this relationship, and how it compares to native tissue, is unknown. Therefore, the objective of the present study was to determine the functional role of GAG and collagen in engineered fibrocartilage formed from aligned electrospun nanofibrous poly(ε-caprolactone) (PCL) scaffolds seeded with mesenchymal stem cells (MSCs), and to determine whether these constituents are integrated in a manner consistent with their function in native fibrocartilage. While digestion studies have demonstrated the importance of GAG for the compressive properties of annulus fibrosus [22], their precise functional role in tension is not known. The inner annulus fibrosus, which contains large amounts of both GAG and collagen, was investigated as a model for native fibrocartilage, and therefore, a second objective of the present work was to understand the role of GAG in the tensile behavior of inner annulus fibrosus.

2. Materials and methods

2.1. Scaffold fabrication

Nanofibrous PCL scaffolds were electrospun as described previously [1,9,14]. Eight grams of poly(ε-caprolactone) (PCL, Sigma Aldrich, batch # 00702CE) was dissolved at 37 °C overnight in 56 mL of equal parts of tetrahydrofuran and *N*,*N*-dimethylformamide. The PCL solution was ejected via syringe pump at 2.5 mL/h through a spinneret charged to +13 kV, generating a nanofibrous jet that was collected on a grounded mandrel rotating at 10 m/s at a distance of 20 cm from the spinneret tip. The spinneret was fanned axially along the mandrel to ensure uniform fiber deposition. Aluminum shields on either side of the spinneret were charged to +9 kV in order to focus the jet toward the mandrel [23]. Fibers were collected onto the mandrel for 6–8 h, resulting in an aligned nanofibrous mesh of approximately 1 mm thickness. Rectangular scaffolds (5 mm × 30 mm) were excised from the mesh with the long axis parallel to the prevailing fiber direction.

2.2. Isolation of bovine mesenchymal stem cells (MSCs) and seeding on nanofibrous scaffolds

MSCs were isolated from bone marrow obtained from juvenile (3–6 month old) bovine knee joints as described previously [9,20] and expanded to passage 2 in basal medium containing 10% fetal bovine serum and 1% penicillin streptomycin and fungizone in DMEM. Scaffolds were hydrated by sequential washes in 100%, 70%, 50% and 30% ethanol and finally phosphate buffered saline (PBS). Before seeding, scaffolds were incubated overnight in 20 μg/mL fibronectin at 25 °C. MSCs were trypsinized, washed, and concentrated to 1×10^7 cells/mL in basal medium. 50 μL of cell solution were applied to each side of the scaffold, allowing 1 h at 37 °C between applications. After an additional 2 h incubation, samples were transferred to chemically defined media (DMEM, 0.1 μM dexamethasone, 40 μg/mL L-Proline, 100 μg/mL Sodium Pyruvate, 1% Insulin, Transferrin, Selenium/Premix, and 1% penicillin, streptomycin and fungizone supplemented with 10 ng/mL Transforming Growth Factor β3) [24]. Media was replaced twice weekly for 120 days of *in vitro* culture. This culture duration was chosen to ensure formation of a mature tissue with abundant extracellular matrix, so that the relative functional roles of collagen and GAG could be assessed.

2.3. Enzymatic digestion of mature engineered fibrocartilage constructs

Enzymatic digestions were performed in order to assess the roles of GAG and collagen in engineered fibrocartilage mechanics. All digestions were performed in PBS at 37 °C for 36 h. Collagen depletion was achieved by digestion with collagenase (COL-ASE, 10 U/mL) and GAG depletion was achieved by digestion with chondroitinase ABC (ChABC, 0.125 U/mL). A control group was subject to the digestion protocol, but without the addition of enzyme (PBS). A fourth group was tested directly from culture (Fresh) in order to determine whether the digestion protocol alone (PBS) had any effects on the mechanics or composition of the native and engineered fibrocartilages. Additional acellular scaffolds maintained in culture under identical conditions throughout the 120 day culture period were also tested in order to establish baseline mechanical properties in the absence extracellular matrix deposition (SCAF). All digestion protocols were developed in preliminary studies to confirm effective removal of GAG and collagen. Because in preliminary experiments protease inhibitors were not necessary to maintain engineered construct biochemistry in PBS, none were included here; in native tissue experiments (below), however, protease inhibitors were used.

2.4. Mechanical, histologic, and biochemical analyses

Samples were either subjected to uniaxial tensile testing followed by biochemical analyses ($n = 5$), unconfined compression testing ($n = 3$), or histologic examination ($n = 2$). Cross-sectional area was measured using a custom laser device that provided noncontact measurements of thickness and width [25]. For tensile testing, samples were lightly airbrushed with black enamel paint to generate texture for strain analysis (below). Testing in uniaxial tension was performed using an Instron 5542. All samples were placed into customized serrated grips and maintained in a PBS bath during the testing protocol: 5 min preload of 0.1 N, followed by 15 cycles of preconditioning to 0.1% at 0.5%/s, and finally a quasi-static constant elongation until failure at a rate of 0.1% strain/s [26]. During the test, strain control was performed via cross-head displacement and images of the sample midsubstance were collected per 0.5% strain increments. Images were analyzed by texture correlation to determine the local surface deformations, and two-dimensional components of Lagrangian strain were calculated (Vic-2D, Correlated Solutions Inc.). Modulus was calculated by a linear regression to either the toe or linear region of the stress–strain response, and similarly Poisson's ratio was computed from the negative slope of longitudinal (applied) vs. transverse strain within the linear range of the stress–strain curve. After tensile testing, water content was determined by measuring the change in weight with overnight lyophilization. Dried samples were digested with papain, and biochemical analyses were conducted to determine total GAG and collagen content via 1,9-dimethylmethylene blue (DMMB) dye binding assay and measurement of ortho-hydroxyproline (OHP) following acid hydrolysis, respectively [24].

Compression testing was performed on 4 mm diameter samples cored from three separate constructs. Unconfined compression was performed by first applying a 2 g creep preload for 5 min, followed by a ramp to 10% axial strain applied at 0.05% per second and then relaxation for 1000 s [15].

For histologic analyses, samples from each group ($n = 2$) were embedded in OCT freezing medium and flash frozen in liquid nitrogen. Embedded samples were cryosectioned in the plane perpendicular to the fiber direction (i.e. in cross-section) and stained to visualize GAG (Alcian Blue) or collagen (Picrosirius Red) as described previously [24].

2.5. Native inner annulus fibrosus preparation and mechanical testing

Intervertebral discs were excised from adult bovine caudal spines. Inner annulus fibrosus samples were harvested from each disc. The bovine caudal disc has previously been established as a valid model for disc research due to its functional and geometric similarities with the human disc [27,28]. Samples were taken circumferentially from the discs and sectioned to 1.5 mm thickness by freezing-stage microtome. Samples were further cut to uniform dimensions of $14 \times 3 \times 1.5$ mm^3 (length × width × thickness) as described previously [26], and wrapped with PBS-soaked gauze for storage at −20 °C until testing.

To parallel the above experiments on engineered fibrocartilage, native inner annulus fibrosus specimens were subjected to GAG depletion by chondroitinase digestion (ChABC, $n = 6$ each) or were used in one of two control conditions: those tested after being freshly thawed (Fresh, $n = 7$) and those subjected to the digestion protocol but lacking chondroitinase (PBS, $n = 6$). The digestion protocol consisted of 6 h incubation with gentle agitation at 37 °C in PBS with protease inhibitors (10 mM *N*-ethylmaleimide, 5 mM benzoic acid, 1 mM phenylmethane-sulfonylfluoride) either with (ChABC) or without (PBS) the addition of chondroitinase ABC (1 U/ml). To control swelling, samples were hydrated in PBS for 30 min prior to tensile testing. Uniaxial tensile testing was performed as described above for engineered fibrocartilage. Effects of enzymatic digestion on the compressive properties of native annulus fibrosus have been investigated elsewhere [22] and were therefore omitted

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