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The use of bioinspired alterations in the glycosaminoglycan content of collagen–GAG scaffolds to regulate cell activity

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ABSTRACT

The design of biomaterials for regenerative medicine can require biomolecular cues such as growth factors to induce a desired cell activity. Signal molecules are often incorporated into the biomaterial in either freely-diffusible or covalently-bound forms. However, biomolecular environments *in vivo* are often complex and dynamic. Notably, glycosaminoglycans (GAGs), linear polysaccharides found in the extracellular matrix, are involved in transient sequestration of growth factors via charge interactions. Biomaterials mimicking this phenomenon may offer the potential to amplify local biomolecular signals, both endogenously produced and exogenously added. GAGs of increasing sulfation (hyaluronic acid, chondroitin sulfate, heparin) were incorporated into a collagen–GAG (CG) scaffold under development for tendon tissue engineering. Manipulating the degree of GAG sulfation significantly impacts sequestration of growth factors from the media. Increasing GAG sulfation improved equine tenocyte metabolic activity in normal serum (10% FBS), low serum (1% FBS), and IGF-1 supplemented media conditions. Notably, previously reported dose-dependent changes in tenocyte bioactivity to soluble IGF-1 within the CG scaffold were replicated by using a single dose of soluble IGF-1 in scaffolds containing increasingly sulfated GAGs. Collectively, these results suggest that CG scaffold GAG content can be systematically manipulated to regulate the sequestration and resultant enhanced bioactivity of growth factor signals on cell behavior within the matrix.

1. Introduction

A major focus in the field of tissue engineering is the development of biomaterials able to mimic critical features of the extracellular matrix (ECM), the three-dimensional microenvironment surrounding cells in the tissues and organs of the body. Beyond the use of scaffold mechanical, structural, and compositional signals to impact cell fate, the addition of growth factors into the biomaterial is often a primary way of providing instructive signals within the matrix [1]. Methods for biochemical supplementation include providing factors free in solutions [2–6], covalently tethering factors in random and specific orientations to the materials [7–10], and growth factor release vectors [11–13]. However, growth factor activity within the native ECM is often dictated by non-covalent interactions with ECM biomolecules such as proteins and proteoglycans that mediate transient immobilization and release.

Glycosaminoglycans (GAGs) are linear polysaccharides found in the native ECM and are known to play a critical role in sequestering growth factors within the matrix [14–22]. Along with structural variations in their carbohydrate backbone, GAGs can present varying levels of negative charges depending on their degree of sulfation [14,15], making them attractive for developing growth factor sequestering biomaterials. In addition to the nonspecific, electrostatic growth factor–GAG interactions facilitated by the sulfate groups, it has also been shown that the sulfation code, the positions of the sulfate groups on the carbohydrate backbone, has an impact on growth factor binding [15]. Recently *Hudalla* et al. immobilized heparin-binding peptides on a self-assembled monolayer to demonstrate sequential binding of first heparin and then heparin-binding growth factors to the substrate in order to enhance human mesenchymal stem cell (hMSC) bioactivity [18]. Similar work has also shown that TGF-β1 can be adsorbed onto biomaterials composed of type I collagen and a sulfated hyaluronan [19]. Considering that charged moieties have been shown to

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sequester biomolecules [15,18–22], systematic incorporation of differentially-charged GAGs within a biomaterial to selectively impact growth factor sequestration represents a promising avenue for tuning biomolecular signals. The efforts described here are therefore targeted at exploring whether the degree of GAG sulfation of a collagen–GAG scaffold could be modified to impact the scaffold's capacity to transiently sequester activity-impacting molecules within the scaffold network.

Collagen–GAG (CG) scaffolds have been used for a wide variety of applications for skin, peripheral nerve, and cartilage tissue engineering as well as 3D environments for *in vitro* studies of cell behavior [2,23–28]. Early development of the CG scaffold platform for skin regeneration included comparison of the effects of the type and weight percent of GAG contained in the scaffold [29], though these studies did not consider biomolecule sequestration. Based on results from *in vivo* kinetics of wound contraction and quality of regeneration studies, CG scaffolds have traditionally included a 11:1 (wt:wt) collagen:GAG ratio employing chondroitin sulfate [30]. Recent efforts in our lab have described modification of the CG scaffold platform for tendon repair applications. As tendon is composed primarily of type I collagen arranged into aligned fibrils [31–33], we described a directional solidification method to fabricate CG scaffolds with highly anisotropic (aligned) morphology composed of longitudinally-aligned ellipsoidal pores [34]. Notably, scaffold anisotropy was found to improve equine tenocyte alignment as well as long-term maintenance of a pro-tenogenic phenotype [34,35]. Further, incorporation of growth factor signals within the anisotropic scaffold in either freely-soluble or covalently-immobilized forms has been shown to impact tenocyte bioactivity in a dose-dependent manner [3]. In particular, soluble or covalently-bound insulin-like growth factor 1 (IGF-1) was found to enhance tenocyte proliferation but at the expense of tenocyte phenotype [34]. Similarly, soluble growth/differentiation factor 5 (GDF-5) was used to increase expression of tenogenic-specific genes within the CG scaffolds [3].

This manuscript described the manipulation of the degree of GAG sulfation within the CG scaffold to promote transient, non-covalent sequestration of growth factors for applications in tendon tissue engineering. As prior work has shown dose-dependent tenocyte responses to growth factor within the CG scaffold [3,34], this work investigated whether alterations of GAG content within the scaffold could replicate dose-dependent effects using a single growth factor dose. Notably, it was hypothesized that scaffolds containing a highly sulfated GAG (heparin) would show an increase in transient growth factor sequestration and enhanced bioactivity of cells seeded within these scaffolds relative to less sulfated GAGs such as chondroitin sulfate, the GAG traditionally used in CG scaffolds, or non-sulfated hyaluronic acid. The response of equine tenocytes and human mesenchymal stem cells (hMSCs) to pro-proliferation (IGF-1) and pro-tenocyte phenotype (GDF-5) factors in the culture media as well as metabolically limited culture environments (low serum) was examined to explore the impact of GAG-mediated non-covalent sequestration on cellular bioactivity [3].

2. Materials and methods

2.1. Fabrication of anisotropic CG scaffolds

2.1.1. Preparation of CG suspension

A suspension of collagen and a defined glycosaminoglycan was made by homogenizing type I collagen from bovine Achilles tendon (Sigma–Aldrich, St. Louis, MO) and one of three glycosaminoglycans (GAGs): hyaluronic acid from *Streptococcus equi* (Sigma–Aldrich #53747, St. Louis, MO), chondroitin sulfate from shark cartilage (Sigma–Aldrich #C4384, St. Louis, MO) or heparin from porcine intestinal mucosa (Sigma–Aldrich #H4784, St. Louis, MO) in 0.05 M acetic acid [23]. A constant collagen concentration (1.5% w/v) and collagen:GAG ratio (11.28:1) was used for all experiments. The suspension was stored at 4 °C and degassed prior to use [36].

2.1.2. Fabrication of CG scaffolds via freeze drying

CG scaffolds were fabricated as previously described [34]. Briefly, the scaffolds were produced via directional solidification using a polytetrafluorethylene (PTFE)-copper mold. The mismatch in thermal conductivity between the mold materials promotes unidirectional heat transfer through the copper bottom when the mold is placed on a precooled freeze-dryer shelf (VirTis, Gardiner, NY). The CG suspension was added to the cylindrical wells of the mold and frozen at −10 °C for 2 h prior to the sublimation of the resulting ice crystals at 0 °C and 200 mTorr. This resulted in a dry, porous scaffold 6 mm in diameter and 20 mm in length with constant pore size along its length [34].

2.1.3. Crosslinking of CG scaffold

Following lyophilization, scaffolds were sterilized and dehydrothermally crosslinked in a vacuum oven (Welch, Niles, IL) at 105 °C under vacuum for 24 h [23]. 5 mm long sections were cut from the scaffold and used for all experiments [34]. Prior to use, these scaffolds were hydrated in 100% ethanol overnight and washed in phosphate-buffered saline (PBS) for 24 h. Scaffolds were subsequently crosslinked using carbodiimide chemistry to make them resistant to tenocyte contraction [30,37]. Scaffolds were immersed in 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (NHS) at a molar ratio of 5:2:1 EDC:NHS:COOH for 2 h under shaking at room temperature. Following crosslinking, scaffolds were washed with PBS and stored in fresh PBS at 4 °C.

2.2. SEM analysis

Scanning electron microscopy (SEM) was used to visualize the scaffold microstructure. Dry, uncrosslinked sections from the center of the scaffold were used for analysis. Samples were sputter-coated with gold–palladium and imaged with a JEOL JSM-6060LV scanning electron microscope using secondary electron and backscattered electron detectors under high vacuum.

2.3. Evaluation of CG scaffold microstructure

Microstructural features (pore size, aspect ratio) of the aligned CG scaffold variants were calculated using previously described stereology approaches [34]. Briefly, serial longitudinal and transverse sections were generated from glycolmethacrylate (Polysciences, Warrington, PA) embedded scaffolds using a microtome (Leica Microsystems, Germany) and mounted on slides. Sections were then stained with aniline blue to facilitate the visualization of the scaffold struts on an optical microscope (Leica Microsystems, Germany). Multiple images were captured per section and then analyzed using MATLAB equipped with a linear intercept method which outputs parameters used to calculate pore diameter and aspect ratio [38]. For each GAG variant a minimum of 6 scaffold sections were analyzed (3 longitudinal, 3 transverse) with a minimum of 5 fields of view captured per section.

2.4. Pull down sequestration assay

The degree of growth factor sequestration by CG scaffold variants was determined via a pull down assay. Ten hydrated crosslinked scaffolds were incubated overnight at 37 °C in a single well of an ultra-low attachment 6-well plate (Fisher, Waltham, MA) in 4 mL of a pH 7.4 PBS solution with 500 ng/mL IGF-1 (ProSpec, Israel) and 1% bovine serum albumin (BSA). Scaffolds fabricated from each GAG were tested separately, with wells containing the IGF-1 solution but no scaffolds used as controls. Following incubation, the amount of IGF-1 remaining in solution was measured via an ELISA kit (R&D Systems, Minneapolis, MN). Relative pull down, the amount of IGF-1 trapped within the CG scaffolds, was calculated from the difference in IGF-1 remaining in the media of the experimental versus control wells. Pull down for each CG variant was reported as a percentage of the total IGF-1 concentration in the loading solution.

2.5. Cell culture

2.5.1. Tenocyte isolation and culture

Tenocytes (tendon cells) were isolated from 2 to 3 year old horses that were euthanized for reasons not related to tendinopathy using previously described methods [39]. Tenocytes were expanded in standard culture flasks in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 1% Antibiotic–Antimycotic (Invitrogen, Carlsbad, CA), 1% L-glutamine (Invitrogen, Carlsbad, CA), and 50 μg/mL ascorbic acid (Wako, Richmond, VA). The tenocytes were cultured to confluence at 37 °C and 5% CO_2 and the media was changed every 3 days. Passage 4 cells were used for all culture experiments.

2.5.2. hMSC culture

Human mesenchymal stem cells (hMSC) from human bone marrow (Lonza, Switzerland) were cultured in standard culture flasks in low glucose Dulbecco's modified Eagle's medium supplemented with 10% MSC FBS (Invitrogen, Carlsbad, CA), 1% Antibiotic–Antimycotic (Invitrogen, Carlsbad, CA), 1% L-glutamine (Invitrogen,