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Maturational growth of self-assembled, functional menisci as a result of TGF-β1 and enzymatic chondroitinase-ABC stimulation

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ABSTRACT

Replacement of the knee meniscus requires a material possessing adequate geometrical and biomechanical properties. Meniscal tissue engineering attempts have been unable to produce tissue with collagen content and biomechanical properties, particularly tensile properties, mimicking native menisci. In an effort to obtain the geometric properties and the maturational growth necessary for the recapitulation of biochemical and, thus, biomechanical properties, a scaffoldless cell-based system, the self-assembly process, was used in conjunction with the catabolic enzyme chondroitinase-ABC and TGF-β1. We show that combinations of these agents resulted in maturational growth as evidenced by synergistic enhancement of the radial tensile modulus by 5-fold and the compressive relaxation modulus by 68%, and additive increases of the compressive instantaneous modulus by 136% and Col/WW by 196%. This study shows that tissue engineering can produce a biomaterial that is on par with the biochemical and biomechanical properties of native menisci.

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1. Introduction

The knee menisci are fibrocartilaginous tissues specialized to protect the underlying articular cartilage of the knee joint via load distributive and shock absorptive capabilities [1,2]. Intrinsic repair capacity is limited to the peripheral region of the tissue, and injuries to other portions result in a loss of tissue functionality leading to osteoarthritic changes in the underlying articular cartilage [3–6]. The prevalent clinical treatment, partial meniscectomy, does not prevent the degenerative changes to articular surfaces resulting from non-physiological loading [1–3]. These deleterious changes may be able to be mitigated by engineering a mature tissue with tailored geometric and functional properties capable of replacing the meniscus.

The self-assembly process (SAP) has recently emerged as a useful technique for tissue engineering [7–11]. In this method of 3D construct formation, cells seeded into a non-adherent agarose well are guided by the Differential Adhesion Hypothesis to limit their free energy by binding to one another via N-cadherins [11,12]. This novel process is the only scaffoldless method being employed to engineer the meniscus and avoids issues associated with scaffold usage such as toxic degradation products, loss of mechanical properties through degradation, and stress shielding. Bovine cells were judiciously chosen for use in this attempt to engineer rabbit menisci because they have been successful and well-characterized in the SAP [7,8,11]; reports suggest cartilaginous cells and tissue may be capable of xenotransplantation due to a level of immunoprivilege [13,14], and, if immunogenicity exists, decellularization of tissue constructs can be performed [15]. While constructs created with the SAP have compressive properties and GAG content that mimic native menisci, the tensile properties and collagen content of native tissue have been more difficult to obtain [7,16]. Though chondroitinase-ABC (C-ABC) and TGF-β1 have emerged as stimuli capable of enhancing cartilage tensile properties, these agents have not been studied in combination for the engineering of cartilaginous tissue [8,10].

The use of C-ABC as a means to enhance construct properties is counterintuitive as it is an enzyme that degrades chondroitin and dermatan sulfate GAGs [17,18]. Previous work with chondrocytes in the SAP found that a one-time treatment of C-ABC after 2 or 4 wks of culture resulted in a 32% increase in the tensile modulus [10]. While this study proved the benefits of C-ABC application to self-assembled constructs, earlier application of C-ABC must be studied as constructs formed by the SAP have shown to be the most sensitive to stimulation between 1 wk and 2 wks [19].

TGF-β1 is a growth factor that has been extensively studied in many culture systems due to its ability to increase mechanical properties and production of cartilaginous ECM. Application of this growth factor has exhibited the greatest capability to enhance both collagen and GAG production and tensile and compressive biomechanical properties in both the self-assembly modality [8] and

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other culture systems [20–23]. A recent study has investigated the temporal application of TGF-β1 and noted that non-continuous stimulation can lead to improved results as compared to continuous stimulation [24].

The purpose of this present study is to examine the full factorial combinations of C-ABC (after 2 wk of culture, after 1 wk of culture, none) and TGF-β1 application (continuous, intermittent, none). We hypothesize that 1) additive or synergistic increases to functional properties will result from combined C-ABC and TGF-β1 stimulation, 2) early C-ABC treatment will be more beneficial than later treatment due to the level of construct "naivety," and 3) the enhancements due to intermittent TGF-β1 application will exceed those of continuous application.

2. Methods

2.1. Cell isolation

Knee joints from 1 wk old calves (Research 87) were obtained and both medial and lateral menisci and the femoral articular cartilage were sterilely isolated. While keeping meniscal and articular cartilages separate, tissue was diced into 1 mm pieces and digested in 0.2% collagenase type II (Worthington) in cell culture medium. The medium formulation follows: Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), 10% fetal bovine serum (FBS) (Benchmark), 1% non-essential amino acids (NEAA) (Invitrogen), 25 μg of L-ascorbic acid (Sigma) and 1% penicillin/streptomycin/fungizone (PSF) (Biowhittaker/Cambrex). Following an 18 h digestion, cells were isolated by multiple PBS dilution and centrifugation iterations and finally filtration through a 70 μm mesh. Articular chondrocytes and meniscus cells were then frozen in the aforementioned culture media with an additional 10% FBS and 10% DMSO (Fisher Scientific). Following controlled freezing to −80 °C, cells were stored in liquid nitrogen until needed.

2.2. Construct seeding

Constructs were generated by seeding ACs and MCs into elliptical agarose wells replicating the complex geometry of the meniscus. Following analysis of native rabbit menisci, the idealized construct was modeled in AutoCAD (AutoDesk) such that if it was dived through the minor diameter it would result in the generation of two inner-meniscus construct. Positive dies were then rapid prototyped (ZPrinter) based the model of the idealized construct and coated with latex. After the latex coating on the positive dies had dried, they were plunged into molten 2% agarose (Fisher Scientific) and the agarose was allowed to set. Following removal of the positive dies, the agarose wells were placed in chondrogenic medium (CHG) and allowed to equilibrate for 1 wk. CHG media formulation follows: DMEM with GlutaMAX (Invitrogen), 100 nM dexamethasone (Sigma), 1% NEAA, 1% PSF, 1% ITS + premix (BD Biosciences), 50 mg/mL ascorbate-2-phosphate (Sigma), 40 mg/mL L-proline (Sigma), and 100 mg/mL sodium pyruvate (Fisher Scientific). ACs and MCs were rapidly thawed, combined together in CHG so that 50% ACs and 50% MCs were present and 200 μL aliquots of 20 million cells total were seeded into the meniscus-shaped agarose wells. After 7 days of culture, constructs were removed from the agarose wells and placed into wells coated with a thin layer of agarose to prevent cell migration or adhesion to the plastic surface. This was done to ensure constructs did not deform due to outgrowing their well and to facilitate C-ABC treatment.

2.3. Construct chemical stimulation

Two chemical stimulants, C-ABC (Sigma) and TGF-β1 (Peprotech), were applied at three levels each to meniscus constructs. TGF-β1 at 10 ng/mL was applied continuously throughout the entire duration of the 4 wk study (TC), intermittently (TI) (only during the first and third wks of culture), or never (T0). C-ABC was applied after 1 wk of culture (C1), after 2 wks of culture (C2), or never (C0). At 1 and 2 wks, groups of constructs were treated for 4 h with 2 U/mL C-ABC in CHG with 60 mM sodium acetate for C-ABC activation. After treatment, constructs were washed five times with CHG.

2.4. Construct processing

After 4 wks of culture, constructs were weighed to obtain wet weights, photographed to obtain geometric properties, and divided to obtain samples for further testing. For compression, ELISA, and biochemical assessments 2 mm punches were taken. Samples for circumferential tensile testing were taken from one of the long edges of the construct. Samples for radial tensile testing were taken from the other long edge in an attempt to minimize any effects of construct inhomogeneity. Histology was performed on the remaining pieces of the construct with careful attention paid to orientation so that radial and circumferential sections could both be obtained. For all tests, except histology, 5 samples from each experimental group were used.

2.5. Histology

Radial or circumferential orientation was accordingly noted when samples were frozen at −20 °C in HistoPrep™ (Fisher Scientific). Samples were cut to 14 μm sections, placed onto glass slides, and warmed overnight at 37 °C. Slides were formalin-fixed, then stained with picrosirius red for collagen, and viewed under polarized light to visualize collagen fibril orientation. Immunohistochemistry for α-SMA was performed by fixing sections in chilled acetone, quenching peroxidase activity, and then using the protocols associated with the Vectastain ABC and DAB Substrate kit (Vector Laboratories) in conjunction with a mouse anti-α-SMA antibody (Dako).

2.6. Biochemistry

Portions of constructs designated for biochemical evaluation were weighed, lyophilized, and weighed again. At this point, samples were digested in phosphate buffer with 5 mM EDTA, 5 mM N-acetyl-cysteine, and 125 μg/mL papain (Sigma) for 18 h at 65 °C. Collagen was quantified by a modified colorometric hydroxyproline assay [25]. GAG was quantified by the Blyscan GAG assay kit (Biocolor). DNA was quantified using the PicoGreen® dsDNA reagent (Invitrogen) and a conversion factor of 7.7 pg DNA/cell.

2.7. Compression testing

Punches designated for compression testing were photographed to determine the diameter and underwent unconfined, stepwise stress relaxation testing on an Instron 5565. Samples were compressed to 20% and 30% strain in a PBS bath and the force data was recorded. This data was analyzed with the MatLab curve fitting toolbox (Math Works) and a custom program to determine the viscoelastic properties relaxation modulus (E_0), instantaneous modulus (E_∞), and coefficient of viscosity.

2.8. Tension testing

Circumferential and radial tensile samples were cut into a dog bone shape. Photographs of tensile specimens were taken from both the top and side views to enable the determination of geometrical properties. Small pieces of paper with a gap of consistent length were created to ensure consistence of gauge length. Samples were adhered to the strips of paper with cyanoacrylate glue, secured into the grips of an Instron 5565, and the paper was cut so that only the constructs would be subjected to tension. At this point, the constructs were strained at 1% of the gauge length per second until failure. The sample specific load, elongation, and geometric data was loaded into Matlab and analyzed with a custom program to isolate the linear region of the curve to determine the Young's modulus in both the radial (E_{Yr}) and circumferential directions (E_{Yc}).

2.9. Statistical analysis

Each group consisted of $n = 5$ for biochemical, compression, and tensile testing. Results of these tests were analyzed with a two-factor ANOVA. The two factors, TGF-β1 stimulation and C-ABC stimulation, each had three levels. When the main effects test showed significance ($p < 0.05$), Tukey's *post hoc* test was performed to determine significant differences among the levels of a particular factor or among all groups. Also, the interaction term obtained from the two-factor ANOVA involving the four groups of interest was used to determine synergy between treatments with $p < 0.05$ defined as significant [26]. In subsequent Figures illustrating biochemical and biomechanical data, statistical significance between levels of a given factor or individual groups is present when a letter is not shared. For C-ABC treatment, if the two-factor ANOVA indicates significant differences between treatment levels, the results of Tukey's HSD are presented with the characters α, β, and γ in parenthesis following the label of the treatment level. The character α is given to the level of C-ABC treatment that results in the significantly highest value, β is given to the treatment level with the next highest resultant value, and, if needed, γ is used to denote the treatment level that resulted in the significantly lowest values. For example, if one treatment level is denoted with α and another is denoted with β, the former treatment level resulted in a statistically significant increase over the latter treatment level. However, if once treatment level is denoted with αβ and another with β there is not a statistically significant difference between the treatments. For TGF-β1 treatment, if the two-factor ANOVA indicates significant differences between treatment levels, the results of Tukey's HSD are presented with the letter A, B, and C in parenthesis following the label of the treatment level. The letter A is given to the level of TGF-β1 treatment that results in the significantly highest value, B is given to the treatment level with the next highest resultant value, and, if needed, C is used to denote the treatment level that resulted in the significantly lowest values. The examples provided above for the denotation of C-ABC application also apply to TGF-β1 stimulation except with the letters A, B, and C instead of α, β, and γ. For comparisons among the 9 treatment groups, if the interaction term of the two-factor ANOVA indicates significant differences between groups, the results of Tukey's HSD are presented with the letters a, b, c, d, and e over the corresponding column of the

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