Biomaterials 32 (2011) 2052–2058

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

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

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ARTICLE INFO

Article history:
Received 5 October 2010
Accepted 18 November 2010
Available online 10 December 2010

Keywords:
Cartilage tissue engineering
Knee meniscus
TGF (transforming growth factor)
C-ABC (chondroitinase-ABC)
ECM
Mechanical properties

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.

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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0142-9612/$ see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2010.11.041

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 contin uous 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

graph. As above, the significantly highest group is denoted with the letter a, the next highest with b, this pattern continues up to the letter e, if needed. Once again, groups not sharing a common letter are significantly different from each other. For comparison of circumferential and radial tensile moduli a paired *t*-test was used to determine if the direction of testing significantly ($p < 0.05$) altered the tensile modulus.

3. Results

3.1. Gross morphology and histology

Representative pictures of the constructs after 4 wks culture are shown in Fig. 1. While neotissue size varied among groups, all of the constructs maintained the elliptical ring shape and possessed a curved wedge profile. Due to the constructs being removed from the confinement of the agarose well at 1 wk, differences in the expansive and contractile growth due to chemical stimulation are apparent. TC treatment led to significantly smaller construct dimensions (Table 1), wet weight (WW), and hydration than both TI and T0 (Fig. 2). C1 and C2 treatments significantly decreased construct dimensions and WW as compared to C0. C1 treatment led to a decrease in hydration compared to both C0 and C2. The combined treatment C1TC resulted in an additive decrease in construct hydration (Fig. 2) and constructs best recapitulating the desired geometric properties (Table 1). The qualitative changes in opacity of the constructs are interesting to note as this may be predictive of matrix density. Constructs receiving a combination of TGF β1 and C ABC appear non translucent particularly compared with C0T0. To account for the construct contraction, immunohis tochemical assessment of α smooth muscle actin (α SMA) was performed. α SMA is expected to be present within or immediately surrounding the cells. From this staining, it was confirmed that TGF β1 treatment resulted in a graded increase in the frequency of cells positively stained for α SMA (Fig. 3). Confirmed by biochem ical analysis, following C ABC treatment GAGs were completely eliminated from neotissue (data not shown). Elimination of GAGs resulted in a decrease in construct size and WW, not only due to the physical removal of matrix, but also because of the decrease in swelling pressure due to the water attraction properties of nega tively charged GAGs. While the swelling pressure would be expected to increase when GAGs returned to the construct, there appears to have been a permanent change in the matrix that resulted in a long term decrease in WW and hydration. Taken together, these gross morphological characteristics and observa tions show that the application of TGF β1 and C ABC allow for constructs of the correct size to be generated and result in the formation of an apparently ECM dense construct potentially due to enhanced matrix production or remodeling.

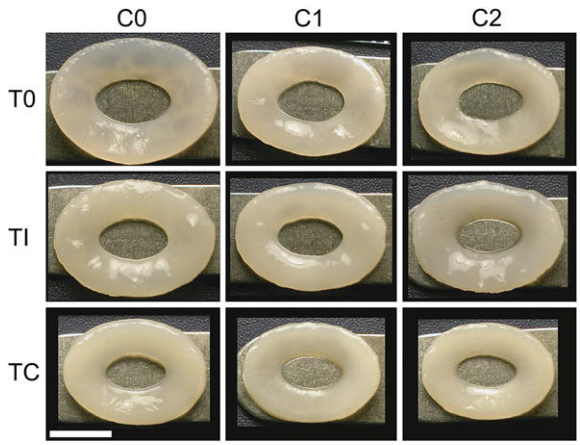

Fig. 1. Gross morphological images of meniscal constructs. Clear differences exist among groups in terms of the geometric properties and construct opacity. The length of the white scale bar in the lower left corner is equal to 5 mm.

3.2. Biochemistry

Construct biochemical analysis confirmed the observations regarding matrix density as both collagen per wet weight (Col/WW) and GAG per wet weight (GAG/WW) were significantly increased by the chemical stimulants alone and additively or synergistically increased through combinations (Fig. 2). Analysis of a two factor ANOVA showed that Col/WW was significantly enhanced following C1, C2, or TC treatments compared to their respective no treatment controls. Furthermore, the combined treatment C1TC yielded an additive increase in Col/WW to a value of 22%. Significant increases were also observed for GAG/WW following TI, TC, or C1 treatments compared to their respective no treatments controls. Synergistic increases were obtained for GAG/WW with the combined C1TI, C1TC, and C2TI treatments. Since the high levels of ECM density that were measured in this study sug gested that the functional properties of the neotissue should be on par with those of native tissue, a full spectrum of biomechanical testing ensued.

3.3. Biomechanics

The results obtained from the compressive and tensile biome chanical assessments of the meniscal neotissue are shown in Fig. 4. C1TC stimulation resulted in synergistic increases to the radial tensile Young's modulus (E_{Yr}) (2.1 MPa) and compressive relaxation modulus (E_R) (0.32 MPa). Also, C1TC treatment led to a compressive instantaneous modulus (E_∞) of 2 MPa and not only had a signifi cantly higher E_∞ than all other groups but also resulted in an additive increase in this metric. Both C1TC and C2TC treatments led to additive increases in the circumferential tensile Young's modulus (E_{Yc}) to obtain values of 2.5 MPa and 3.2 MPa, respectively. From two factor ANOVAs, the TC treatment was shown to significantly increase all functional properties, C1 increased all properties except E_∞, and C2 increased both E_{Yc} and E_{Yr}. Via a paired *t* test, E_{Yc} was shown to be significantly greater than E_{Yr} suggesting collagen orientation in the circumferential direction. This was confirmed by polarized light visualization of picrosirius red stained sections (Fig. 5).

4. Discussion

Replication the salient mechanical properties, particularly the tensile modulus, of the meniscus is one of the greatest challenges in engineering this complex tissue. Thus, this experiment was con ducted in an effort to increase these critical mechanical properties by studying the temporal application of TGF β1 in conjunction with C ABC. The central hypothesis of this work was that combinations of these two chemical stimulants would result in synergistic increases in the functional properties of meniscal constructs. This hypothesis was proven as C1TC treatment synergistically enhanced the E_{Yr} by 5 fold and E_R by 68%, and additively increased E_∞ by 136% and Col/WW by 196%. Furthermore, this treatment improved the biochemical properties through a synergistic increase in GAG/WW and an additive increase in Col/WW.

The secondary hypothesis regarding the efficacy of early C ABC treatment was proven in some metrics via a two factor ANOVA. Early C ABC treatment was more beneficial then later in terms of GAG/WW and relaxation modulus, equivalent to later treatment in E_{Yr} and Col/WW, and worse than later treatment in E_{Yc}. However, the benefits of early C ABC treatment become more pronounced

Table 1
Geometric properties of meniscal neotissue and the SAP well in which they were formed.

1	COTO	C1T0	C2T0	COTI	C1TI	C2TI	C0TC	C1TC	C2TC	Well
Maj. outer (mm)	13.3 ± 0.2^{a}	11.6 ± 0.6^{d}	11.9 ± 0.3^{od}	13.3 ± 0.2^{a}	12.1 ± 0.3^{bc}	12.3 ± 0.3^{b}	11.7 ± 0.2^{cd}	10.9 ± 0.2^{e}	11.0 ± 0.1^{e}	9.5
Min. outer (mm)	10.0 ± 0.2^{a}	8.6 ± 0.5^{bc}	8.8 ± 0.3^{b}	$10.0 \pm 0.5^{\circ}$	8.8 ± 0.2^{b}	9.9 ± 1.1^{a}	8.6 ± 0.3^{bc}	8.0 ± 0.2^{c}	8.0 ± 0.2^{c}	6.9
Maj. inner (mm)	5.9 ± 0.2^{a}	5.0 ± 0.3^{cd}	5.1 ± 0.1^{c}	5.4 ± 0.2^{b}	5.2 ± 0.1^{c}	5.2 ± 0.1^{bc}	4.7 ± 0.1^{e}	4.7 ± 0.2^{e}	4.8 ± 0.2^{de}	4.1
Min. inner (mm)	3.3 ± 0.2^{ab}	3.2 ± 0.3^{abc}	3.2 ± 0.3^{abc}	3.4 ± 0.5^{a}	3.1 ± 0.2^{bcd}	3.2 ± 0.2^{abc}	2.9 ± 0.2^{cde}	2.7 ± 0.2^{e}	2.8 ± 0.2^{de}	2.5
Height (mm)	1.4 ± 0.2^{ab}	1.0 ± 0.1^{e}	1.0 ± 0.2^{de}	1.5 ± 0.1^{a}	1.1 ± 0.2^{cde}	1.2 ± 0.1^{bcd}	1.2 ± 0.2^{bc}	0.9 ± 0.2^{e}	1.0 ± 0.1^{e}	1

Values shown as mean ± SD. Data not sharing a common superscript letter indicate significant difference.

when applied with TGF β1 as seen with the synergistic increase in the E_{∞} with C1TC or C1TI treatment and the additive increase in Col/WW seen with the C1TC treatment. Application of C ABC did not enhance total collagen or GAG normalized to dry weight (DW) (Fig. 6), thus increases in Col/WW and GAG/WW must be attributed to the decrease in hydration. While rationale for the immediate reduction in hydration due to removal of negatively charged GAGs is straightforward, GAGs eventually returned to levels of GAG/DW of untreated constructs. This suggests during the time in which constructs were devoid of GAGs changes occurred to permanently alter the construct's ability to resist hydration. Following removal of GAGs, the remaining neotissue components were placed in much closer proximity to one another allowing potential interaction to occur that would not occur in untreated constructs due to the steric hindrances of GAGs and increased distance to due swelling pres sure. C ABC treatment may be able to increase collagen–collagen interactions via crosslinking, but an alternate reason for treated constructs' ability to resist hydration may be due to enhanced access of ACs and MCs to collagen fibrils. This enhanced access would lead to a greater number of cell to collagen bonds and would allow for a cell based stabilization of the condensed collagen network.

The results of C ABC treatments observed in this study agree with the other reports of the application of this enzyme [10,27–29]. Immediately following C ABC treatment, Schmidt observed a 37% increase in the tensile modulus of articular cartilage explants [29]. While this result does indicate C ABC is effective at enhancing tensile properties it did not determine if this was due to the temporary elimination of GAGs from the tissue or due to a lasting change in the matrix. A later study by Asanbaeva cultured cartilage explants following C ABC application for 13 days to assess tensile properties after GAG recovery had occurred [27]. They determined that the increase in tensile modulus observed immediately following C ABC treatment remained even after GAG/WW returned to values similar to those of non treated controls. These observa tions along with the concomitant decrease in WW and hydration observed 13 days following C ABC treamtent are in agreement with the results obtained in the current study. Natoli applied C ABC to self assembled articular chondrocyte constructs and observed a similar response in terms of enhanced tensile properties and Col/WW and decreased WW and GAG/WW 2 wks after C ABC application [10]. In a study by Bian, C ABC was applied as an additive to the culture media instead of the one time treatment that was used in the current study [28]. Although the application of the enzyme was quite different, increases in Col/WW and tensile properties were observed. These studies, in addition to the results presented in the current study, are beginning to form a foundation for the support of C ABC application for the enhancement of tissue engineered cartilage.

The secondary hypothesis regarding the benefits of intermittent application of TGF β1 was disproven as GAG/WW, Col/WW, E_{Yc}, E_{Yr}, E_{R}, and E_{∞} were shown to be significantly higher with continuous TGF β1 treatment. The reason for the enhancement in matrix quality seen with TC treatment is not due to increases in total

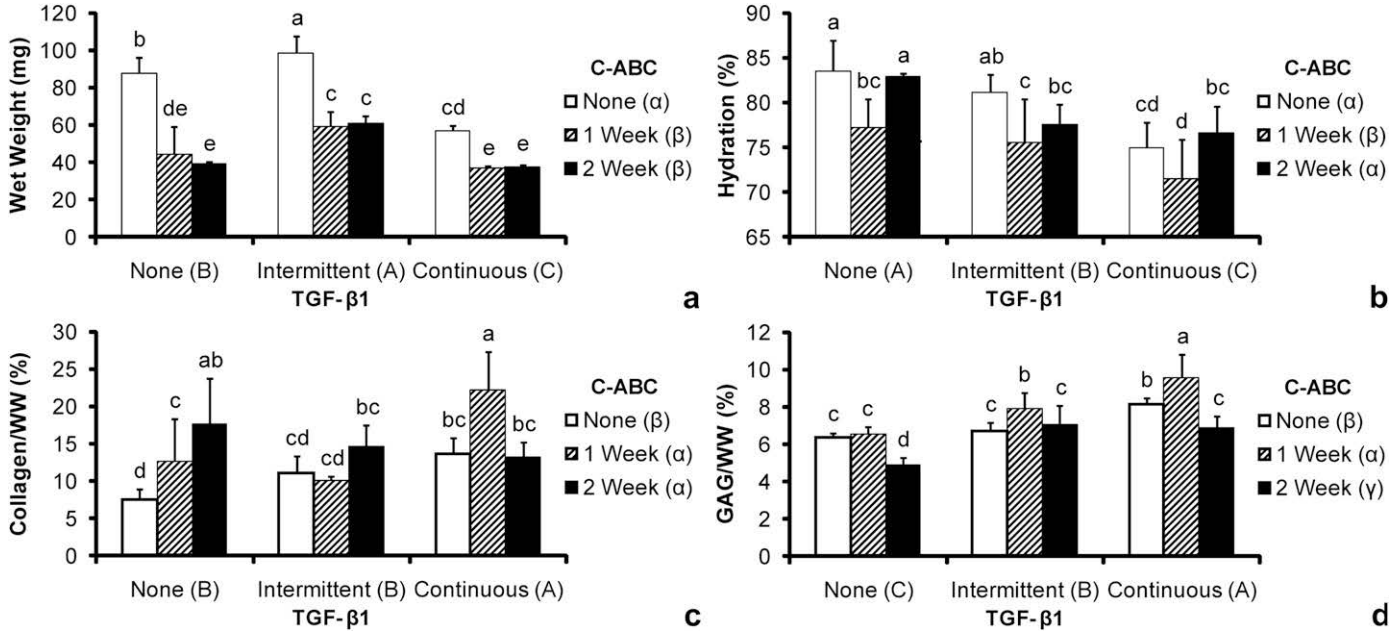

Fig. 2. Construct biochemical properties. (a) Construct wet weight (b) Hydration percentage (c) Collagen per wet weight (d) GAG per wet weight. All data is presented mean ± s.d. Significant difference among levels of the two factors were detected by a two-factor ANOVA and Tukey's HSD post hoc test ($p < 0.05$). Individual factor levels or groups not possessing a letter in common are significantly different. Additional information regarding the depiction of statistical significance can be found in the Statistical analysis section of the Methods.

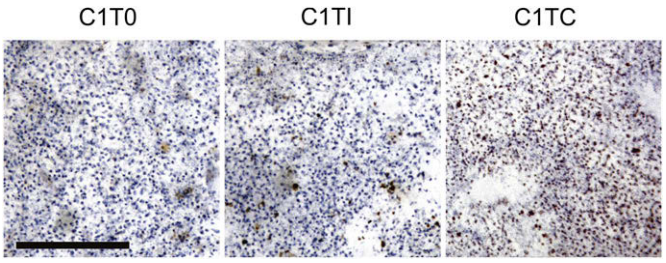

Fig. 3. Immunohistochemistry for α-smooth muscle actin. As α-SMA is expected to be localized within or immediately surrounding the cells, an increase in the frequency of brown-stained α-SMA positive cells is noted as the total time of TGF-β1 application increased. Constructs in both C0 and C2 groups exhibited a similar trend as C1 images that are shown. The scale bar in the lower left is equal to 500 μm.

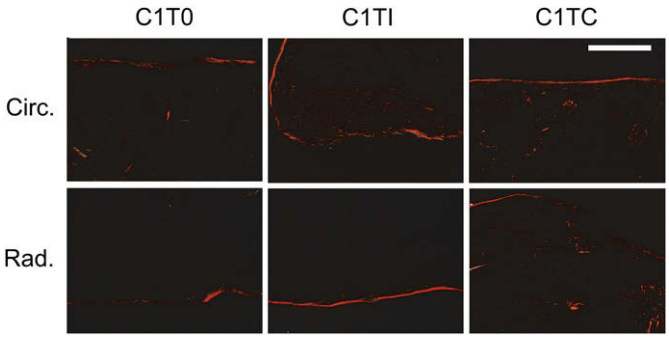

Fig. 5. Collagen orientation. Picrosirius red histological staining in both the circumferential and radial directions viewed under polarized light. Images are taken such that fiber orientation in the relevant direction will be horizontal in all images. Birefringence event frequency and intensity are decreased when viewing radial sections as compared to circumferential sections. This difference was observed in all groups. The scale bar in the upper right is equal to 500 μm.

collagen and GAG production, as can be seen in Col/DW and GAG/DW values in Fig. 6. Instead cell induced contraction via α SMA was able to lower the WW and hydration while the production of these matrix components remained unchanged by TGF β1 application. This led to the higher GAG/WW and Col/WW which then led to the enhanced biomechanical properties. In tissue engineering attempts in which the contraction is so great that the resulting construct is no longer of clinically applicable dimensions, the presence of α SMA is seen as detrimental. However, in this study the presence of α SMA is beneficial because the contraction creates a more ECM dense construct with suitable geometric properties and, equally important, enhanced tensile properties.

The enhancement of Col/WW, GAG/WW, and compressive and tensile biomechanical properties obtained due to TGF β1 application are in agreement with previous studies involving the application of this growth factor [20–23,30–32]. Mauck found that the application of TGF β1 to chondrocyte seeded agarose gels increased compressive properties, GAG/WW, and Col/WW but did not result in decreased hydration or geometric properties as was seen in the current study [32]. This discrepancy is likely due to the method of construct formation. Due to the spatial isolation of chondrocytes within the agarose, constructs formed by this method are not continuous, ECM rich structures. Thus, the possibility of macroscopic cell induced matrix contraction is limited. Supporting the idea that presence of a scaffold alters the effect of TGF β1, Giovannini applied TGF β1 to chondrocyte pellets and reported a significant reduction in pellet diameter compared to non treated pellets with a concomitant decrease in GAG per pellet [31]. However, neither GAG/WW nor Col/WW was reported so it is difficult to determine the quality of the matrix created. Also, Elder reported increased Col/WW and GAG/WW and decreased construct thickness following TGF β1 application of scaffold free chondrocyte constructs [30]. With regards to native meniscal tissue, Imler found that TGF β1 was a potent stimulator of both total protein and proteoglycan synthesis [20] and Lietman demonstrated that an increase in proteoglycan synthesis accompanied TGF β1 stimulation [21]. Gunja and Pangborn found that TGF β1 application to scaffold based meniscal constructs resulted in the enhancement of both

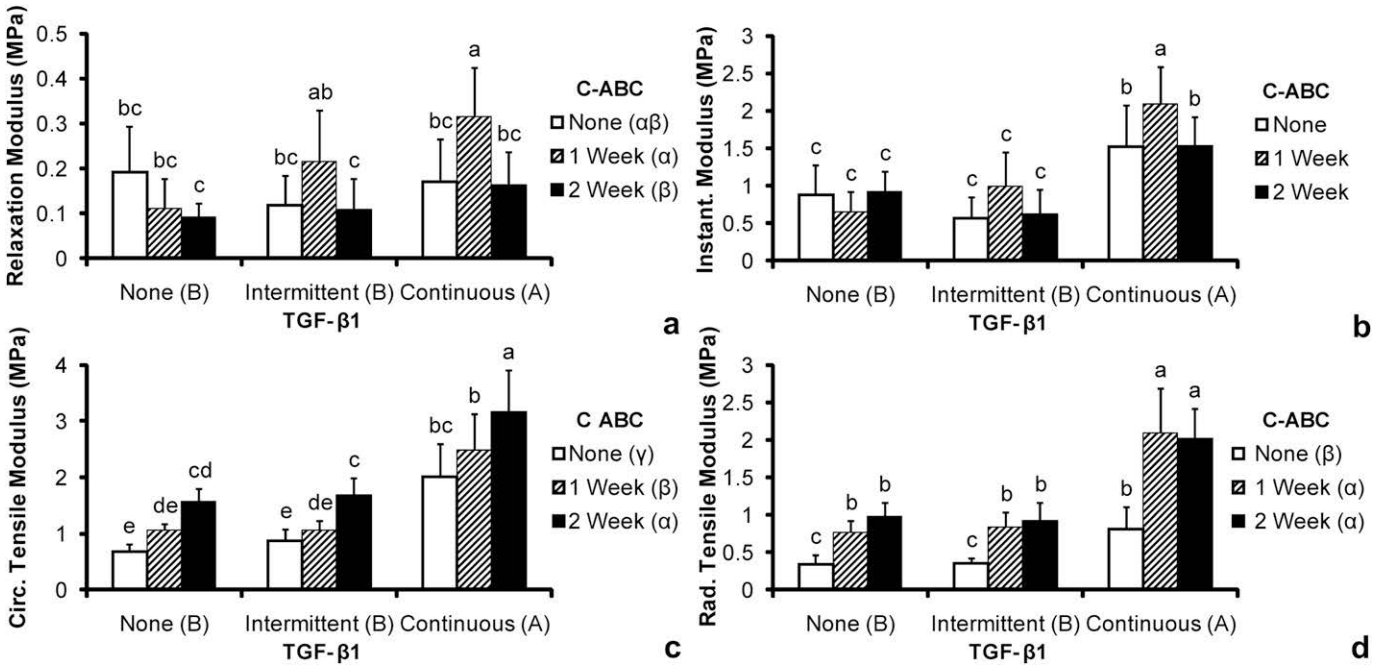

Fig. 4. Construct biomechanical properties. (a) Compressive relaxation modulus (b) Compressive instantaneous modulus (c) Circumferential Young's tensile modulus (d) Radial Young's tensile modulus. All data is presented mean ± s.d. Significant difference among levels of the two factors were detected by a two-factor ANOVA and Tukey's HSD post hoc test ($p < 0.05$). Individual factor levels or groups not possessing a letter in common are significantly different. Additional information regarding the depiction of statistical significance can be found in the Statistical analysis section of the Methods.

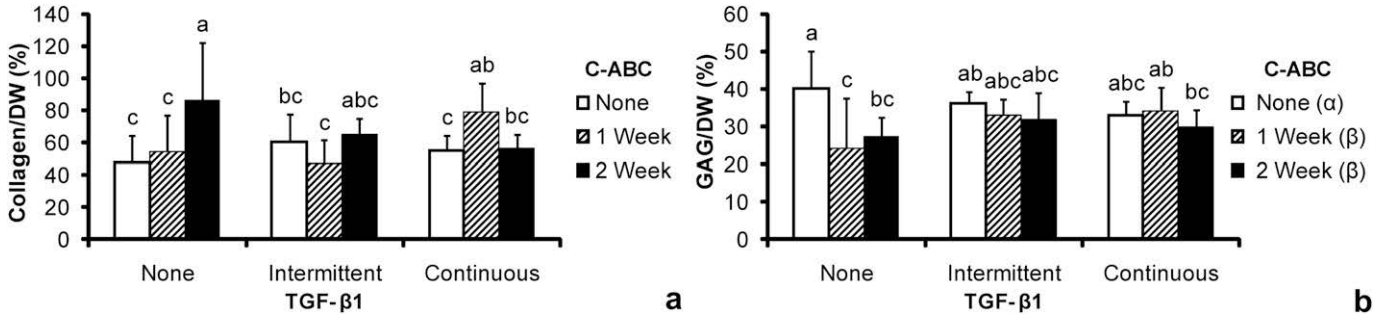

Fig. 6. Additional construct biochemical properties. (a) Collagen per dry weight (b) GAG per dry weight. All data is presented mean ± s.d. Significant difference among levels of the two factors were detected by a two-factor ANOVA and a post hoc Student's *t*-test ($p < 0.05$). These differences are denoted with capital letters with the significantly highest level marked with an A and lower groups marked with letters in descending alphabetical order. Significant differences in a one-factor ANOVA were defined as $p < 0.05$ and are denoted with lowercase letters also in descending alphabetical order.

collagen and GAG synthesis and increased compressive properties but did not result in decreased construct geometric properties [22,23]. Together the body of work on TGF β1 stimulation indicates that this is an effective growth factor to achieve enhancement of biochemical and functional properties. Interestingly, this enhancement appears to be not only due to increased matrix synthesis but also contraction of the protein rich matrix obtained in scaffold free methods of construct formation.

Cartilaginous tissue has been shown to have two distinct growth modalities when cultured in vitro, appositional, characterized by tissue expansion, and maturational, characterized by growth within the tissue [27]. Appositional growth can occur by both accumulation of water and ECM whereas maturational growth involves accumulation of ECM and tissue remodeling and reorganization. Maturational growth has been very difficult to obtain in the engineering of cartilaginous tissues due to an overproduction of GAG and a collagen network that is unable to restrain tissue swelling. It is these two challenges associated with appositional growth that result in decreased functional properties and ECM quality. This study shows that the application of TGF β1 and C ABC can alter the growth pattern of meniscal neotissue from appositional to maturational. Both of these methods, through different means, were able to enhance the strength of the collagen network, as indicated by tensile biomechanics, which enabled the constructs to resist the swelling pressure imparted by GAGs. Because C ABC and TGF β1 are able to enhance construct maturational properties via two distinct methods, it is easy to see why combined treatments with C ABC and TGF β1 resulted in additive or synergistic increases in tissue maturational growth. With C ABC treatment, the cells likely had the ability to develop more integrin adherence points to the collagen network. Thus, when combined with TGF β1, cell induced contraction of the matrix, through α SMA, would be more pronounced because the cells would have been bound to a larger number of collagen fibrils. Maturational growth due to this combined treatment was manifested by the additive decreases in hydration and WW and synergistic and additive increases in biomechanical properties and Col/WW seen with C1TC and C2TC treatments.

In order to assess the ability of TGF β1 and C ABC stimulation to create high quality meniscal constructs suitable for eventual *in vivo* implantation into the rabbit knee, comparison of construct biochemical and biomechanical properties to native rabbit menisci is critical. Constructs receiving C1TC stimulation possessed GAG/WW of 9% which slightly exceeds native tissue (5%) and Col/WW of 22% which matches native tissue [6]. In terms of compressive properties, E_R for native tissue is 0.25 MPa and E_∞ is 3 MPa [6]. These values compare very well to the values obtained for constructs following C1TC stimulation in terms of both E_R (0.32 MPa) and E_∞ (2.1 MPa). Following C1TC stimulation, E_{Yc} increased to 2.5 MPa which is below native values of 160 MPa [6]. However, this reported value for native tissue E_{Yc} is obtained from samples taken from the outer more fibrous region of the meniscus. Thus, it is likely that the E_{Yc} of the inner meniscus, which is the goal of this study, is considerably lower.

Although the tissue generated in this study is of high quality, the E_{Yc} is still significantly lower than native tissue. This indicates that even though collagen content was appropriate, collagen maturation and orientation need to be enhanced. Unfortunately, tissue engineers do not yet know how to control the process in which collagen fibrils are organized into collagen fibers. Intensive studies into this area would not only greatly enhance meniscus tissue engineering but all fields that require the tensile strength afforded by collagen fibers. However, the issue of collagen orientation could be addressed in two ways. Compression of the meniscus constructs with an appropriately shaped platen would result in physiological meniscus loading in which both compression and circumferential tension would be applied. This circumferential tensile stimulation would likely result in increased collagen orientation and thus, tensile properties. Also, the meniscus specific SAP well has been shown to impart collagen orientation and a study with ACs in a cylindrical SAP model has shown that optimization of the duration of well confinement can enhance collagen orientation [19]. This suggests that confinement time should be optimized for meniscus constructs as well. Another means by which tensile properties could be enhanced is through multiple C ABC treatments and increased TGF β1 application. Studies employing one or the other of these two techniques have been performed in the cylindrical SAP model and been found to be beneficial to tensile properties [8,10]. Despite the one major hurdle, below native value E_{Yc}, this study has shown that with the appropriate temporal combination of C ABC and TGF β1 maturation growth of meniscal neotissue can be significantly enhanced to near native meniscus properties.

5. Conclusions

This study showed that maturational growth of meniscal neotissue occurs with either TGF β1 or C ABC stimulation and synergistic and additive increases to maturational properties are achieved with combined treatments. By enabling construct maturational growth both biochemical and biomechanical properties approaching or meeting native meniscus values were obtained. The generation of a high quality meniscal construct without the use of a scaffold is not only exciting but highly desirable because concerns with scaffold usage, particularly loss of mechanical properties and lack of cellular infiltration following implantation, are potentially mitigated. The level of maturational growth obtained in this study, as seen by Col/WW and tensile properties, has never been obtained in meniscal

tissue engineering and will hopefully open the door to eventual *in vivo* applications of this scaffoldless, cell generated biomaterial.

Acknowledgement

The authors would like to acknowledge NIH NIAMS, R01 AR047839 and NFL Charities for funding this work.

Appendix

Figures with essential colour discrimination. Figs. 1, 3 and 5 of this article may be difficult to interpret in black and white. The full colour images can be found in the online version at doi:10.1016/j.biomaterials.2010.11.041.

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