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BIOTECHNOLOGY and BIOENGINEERING

Effects of Co-Cultures of Meniscus Cells and Articular Chondrocytes on PLLA Scaffolds

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ABSTRACT: The knee meniscus, a fibrocartilaginous tissue located in the knee joint, is characterized by heterogeneity in extracellular matrix and biomechanical properties. To recreate these properties using a tissue engineering approach, co-cultures of meniscus cells (MCs) and articular chondrocytes (ACs) were seeded in varying ratios (100:0, 75:25, 50:50, 25:75, and 0:100) on poly-L-lactic acid (PLLA) scaffolds and cultured in serum-free medium for 4 weeks. Histological, biochemical, and biomechanical tests were used to assess constructs at the end time point. Strong staining for collagen and glycosaminoglycan (GAG) was observed in all groups. Constructs with 100% MCs were positive for collagen I and constructs cultured with 100% ACs were positive for collagen II, while a mixture of collagen I and II was observed in other co-culture groups. Total collagen and GAG per construct increased as the percentage of ACs increased (27 ± 8 μg, 0% AC to 45 ± 8 μg, 100% ACs for collagen and 12 ± 4 μg, 0% ACs to 40 ± 5 μg, 100% ACs for GAG). Compressive modulus (instantaneous and relaxation modulus) of the constructs was significantly higher in the 100% ACs group (63 ± 12 and 22 ± 9 kPa, respectively) when compared to groups with higher percentage of MCs. No differences in tensile properties were noted among groups. Specific co-culture ratios were identified mimicking the GAG/DW of the inner (0:100, 25:75, and 50:50) and outer regions (100:0) of the meniscus. Overall, it was demonstrated that co-culturing MCs and ACs on PLLA scaffolds results in functional tissue engineered meniscus constructs with a spectrum of biochemical and biomechanical properties.

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KEYWORDS: meniscus cells; articular chondrocytes; PLLA; co-cultures; tissue engineering

Introduction

Knee menisci are semi-lunar-shaped fibrocartilaginous discs located in between the tibia and the femur in the knee joint. They contain a heterogeneous cell population with the outer third containing predominantly elongated fibroblast-like cells and the inner two thirds containing predominantly rounded chondrocyte-like cells (McDevitt et al., 2002). The outer meniscus is mainly fibrous with collagen type I, while the inner meniscus contains both collagen I and II in a 2:3 ratio (Sweigart and Athanasiou, 2001). In addition, the meniscus also contains several glycoproteins including aggrecan, biglycan, decorin, and COMP that aid in the constructs compressive and tensile properties (Nakano et al., 1997). The mechanical properties of menisci vary significantly between regions with the inner meniscus possessing higher compressive strength when compared to the outer meniscus (Sweigart et al., 2004). In addition, tensile properties of the meniscus are greater in the circumferential direction when compared to the radial direction (Sweigart and Athanasiou, 2005). Menisci play an important role in knee mechanics and stability, but unfortunately are prone to damage, and do not heal due to low tissue vascularity. Several attempts are underway to tissue engineer the meniscus; however, much work remains before a functional construct mimicking the heterogeneities of the native meniscus is achieved (Schoenfeld et al., 2007).

The limited supply of allogenic or autologous meniscus cells (MCs) for tissue engineering experiments necessitates the need for monolayer cell expansion to attain high cell numbers. This approach can lead to significant changes in the cells' phenotype and matrix production capability. Cartilaginous cells, such as articular chondrocytes (ACs), MCs, and temporomandibular joint (TMJ) disc fibrochondrocytes, are especially susceptible to monolayer dedifferentiation and have shown rapid changes in gene expression over passage for important cartilaginous markers (Allen and Athanasiou, 2007; Benya and Shaffer, 1982; Gunja and Athanasiou, 2007; Lin et al., 2008). For instance, in bovine meniscal fibrochondrocytes, collagen II expression has been shown to drop 70-fold over four passages with a dramatic 5,800-fold increase in collagen I expression (Gunja and Athanasiou, 2007). Several different redifferentiation vehicles have been instituted to reverse losses in gene expression for cartilaginous cells with mixed results; these include exogenous growth factor addition into the media, cell

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plating on surface protein coating, and cell encapsulation in agarose or alginate gels (Adesida et al., 2006; Allen and Athanasiou, 2006a; Allen et al., 2008; Darling and Athanasiou, 2005; Gunja and Athanasiou, 2007). Recovery of collagen II expression back to native values has posed a significant challenge, with only two studies reporting a retention of matrix phenotype in passaged MCs using a combination of hypoxia and a growth factor, bFGF (Adesida et al., 2006, 2007). Both studies, however, utilized gene expression and cell aggregates to determine the matrix forming capabilities of passaged cells. Recent work has investigated the potential of using primary ACs to treat defects in the inner meniscus with the goal of increasing collagen II and glycosaminoglycans (GAGs) content (Weinand et al., 2006a,b). Primary chondrocytes express high levels of collagen II and GAGs and if co-cultured with MCs on a scaffold may result in constructs resembling various regions of the knee meniscus (Darling and Athanasiou, 2005; Hoben et al., 2007). Additionally, primary chondrocytes have been used as a redifferentiation tool for passaged chondrocytes in monolayer (Gan and Kandel, 2007). Such an approach, however, has not been attempted with MCs.

Thus, this experiment investigated the effects of co-culturing various ratios of passaged MCs and primary ACs on poly-L-lactic acid (PLLA) scaffolds. It was hypothesized that constructs with a spectrum of histological, biochemical, and biomechanical properties would be achieved depending on the MC:AC co-culture ratio.

Materials and Methods

Cell Harvesting, Culture, and Passage

ACs and MCs were aseptically harvested from the knee joint of 10 skeletally mature New Zealand white rabbits less than 12 h after slaughter. For the meniscus, care was taken to isolate the avascular middle and inner regions of the tissue while the vascular outer region was discarded. The tissues were minced and digested overnight using 0.2% collagenase II (Worthington, Lakewood, NJ) dissolved in Dulbecco's modified culture medium (DMEM; Gibco, Grand Island, NY). The medium was supplemented with 10% fetal bovine serum (FBS) (Biowhittaker, Walkersville, MD), 4.5 g/L-glucose and L-glutamine (Gibco), 1% penicillin/streptomycin/fungizone (Sigma, St Louis, MO), 1% non-essential amino acids (Invitrogen, Grand Island, NY), and 50 μg/mL L-ascorbic acid (Sigma). Post-digestion, ACs, and MCs were pooled separately and counted using a hemocytometer. Cell viability was assessed using a trypan blue exclusion test and found to be over 95% for both cell types.

ACs were then placed in culture media supplemented with 20% FBS and 10% dimethyl sulfoxide and stored in liquid nitrogen. The MCs were plated on T-225 flasks at approximately 25% confluence in chemically defined serum-free DMEM media containing 4.5 g/L-glucose and L-glutamine, 40 μg/mL L-proline, 100 nM dexamethasone, 1% penicillin/streptomycin/fungizone, 50 μg/mL, ascorbate-2-phosphate, 100 μg/mL sodium pyruvate, and 1% insulin–transferrin–selenium (ITS+) (BD Biosciences, San Jose, CA). MCs were expanded to the second passage and then counted using a hemocytometer. ACs were thawed post-MC expansion and counted using a hemocytometer. AC viability was calculated to be over 95%.

Scaffold Preparation and Cell Seeding

Non-woven PLLA scaffolds (molecular weight = 100 kDa) (Biomedical Structures, Warwick, RI), 2 mm thick and 3 mm wide, were obtained from a PLLA sheet using a 3 mm dermal punch. Scaffolds were sterilized using ethylene oxide, pre-wetted using ethyl alcohol (70%), and then washed twice with phosphate-buffered saline (PBS). Prior to cell seeding, scaffolds were housed in serum-free media in 12-well plates that were previously coated with 0.5 mL of 2% sterile agarose. ACs and MCs were pooled into five different co-culture ratio groups ($n = 17$ for each group): (1) 100% MCs; (2) 75% MCs + 25% ACs; (3) 50% MCs + 50% ACs; (4) 25% MCs + 75% ACs; and (5) 100% ACs. Cells were injected into scaffolds at a density of 1 million cells/scaffold. The 12-well plates, containing cell-seeded scaffolds were placed on an orbital shaker (80 rpm) in the incubator for 3 days followed by static culture in the incubator for an additional 2 days.

Tissue Culture

Post-seeding, five constructs from each of the five co-culture groups were removed and processed for several biochemical assays to determine cell seeding numbers and total collagen and GAG levels at $t = 0$. The remaining constructs were transferred to six-well plates, each well housing one scaffold. Six milliliters of serum-free media was added to each well and changed once in every 2 days. Constructs were kept in culture for a period of 4 weeks.

Histology and Immunohistochemistry (IHC)

At $t = 4$ weeks, two samples from each group were frozen using HistoPrep (Fisher Scientific, Pittsburg, PA) and sectioned at 14 μm. GAG distribution was determined using a Safranin-O and fast grain stain (Rosenberg, 1971). Collagen distribution was determined using a Picro-sirius red stain (Battlehner et al., 1996). Collagen I and II distributions were determined using a Biogenex i6000 autostainer. Briefly, samples were fixed in chilled acetone and rinsed with IHC buffer. They were then treated with hydrogen peroxide/methanol and blocked with horse serum (Vectastain ABC kit). Slides were incubated with mouse anti-COL 1 (Accurate Chemicals, Westbury, NY) or mouse

anti-COL 2 (Chondrex, Redmond, WA) antibodies. Post-incubation, a secondary mouse IgG antibody (Vectastain ABC kit) was added and color was developed using the Vectastain ABC reagent and DAB (Vector Labs, Burlingame, CA).

Quantitative Biochemistry

Five samples collected at $t=0$ (5 days post-seeding) and $t=4$ weeks (33 days post-seeding) were processed for biochemical analysis post-compressive testing. The samples from each group at $t=4$ weeks were digested overnight at 65°C with 125 μg/mL papain in 50 mM phosphate buffer (pH 6.5) containing 2 mM *N*-acetyl cysteine and 2 mM EDTA. Total DNA content/sample was determined using a picogreen cell proliferation assay kit (Molecular Probes, Eugene, OR). Total GAG/sample was quantified using the Blyscan Glycosaminoglycan Assay kit (Pietila et al., 1999). Total collagen/sample was determined using a modified chloramine-T hydroxyproline assay (Woessner, 1961).

Compressive Tests

Prior to biochemical testing of 4-week samples, five samples from each group were subjected to biomechanical analysis. Viscoelastic compressive properties of samples from each group were examined by performing incremental stress relaxation tests at 10%, 20%, and 30% strain levels using an Instron 5565 (Allen and Athanasiou, 2006b). Each sample was held at the chosen strain level for 20 min with a 10% strain step. The strain rate was kept constant throughout the test at 0.5 mm/s. Data obtained from each test were fitted using MATLAB to an incremental stepwise viscoelastic stress relaxation solution for a standard linear solid as described elsewhere (Allen and Athanasiou, 2005). The parameters obtained from the model were converted to instantaneous modulus (E_i), relaxation modulus (E_r), and coefficient of viscosity (μ) for each strain level. Compressive properties of unseeded PLLA constructs at $t=4$ weeks after culture were also determined and served as a control.

Tensile Tests

Five samples were used for tensile tests at $t=4$ weeks. Tests were performed using an Instron 5565 with a 50 N load cell. To ensure testability, samples were carefully carved into a dog-bone shape with a 2-mm gauge length. The ends of the samples were glued to paper tabs using cyanoacrylate. After a 0.05 N tare load was applied, the construct was pulled to failure at a constant strain rate of 0.02 s^{-1}. Stress–strain curves generated from the test yielded the tensile stiffness and the ultimate tensile strength (UTS) of each construct. Tensile properties of unseeded PLLA constructs at $t=4$ weeks were also determined and served as a control.

Statistical Analysis

The quantitative biochemical and biomechanical data were compared using an analysis of variance (ANOVA) test. If significant differences were observed, a Tukey's post hoc test was performed to determine specific differences among groups. A significance level of 95% with a *P*-value of 0.05 was used in all statistical tests performed. All values are reported as mean ± standard deviation.

Results

Gross Morphology, Histology, and IHC

At $t=4$ weeks, all constructs showed presence of ECM inside the scaffold. As the percentage of ACs increased, pockets of ECM were observed on the periphery of the scaffold (Fig. 1A). Construct diameter increased significantly as the percentage of ACs increased from 3.0 ± 0.4 mm for the 100% MC group to 3.2 ± 0.3 mm for the 100% AC group (Table I) ($P=0.02$). Similar significant increases were observed for the thickness of constructs from 2.0 ± 0.1 mm for the 100% MC group to 2.3 ± 0.1 mm for the 100% AC group (Table I) ($P=0.003$). Collagen and GAG staining was positive in all groups, with stronger staining for both ECM components observed in 100% AC group (Fig. 1A). Positive staining for collagen I was observed in constructs cultured

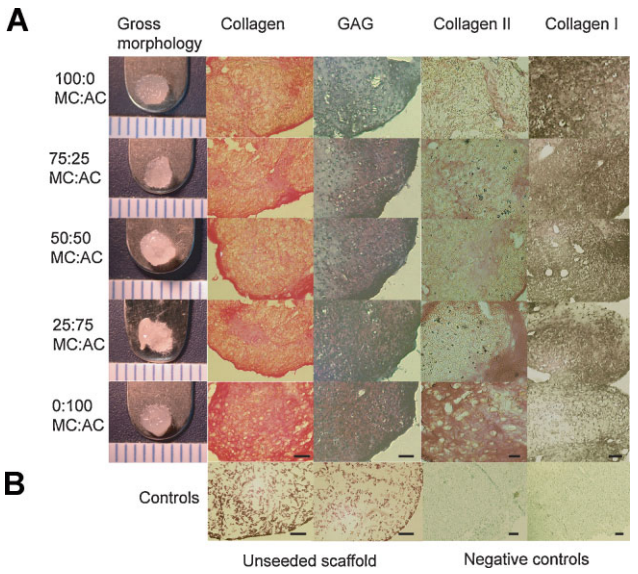

Figure 1. **A**: Gross morphological, histological, and immunohistochemical sections of constructs at $t=4$ weeks. Scale bar is 200 μm. Strong staining for collagen and GAG was observed in all groups. Constructs with 100% MCs were positive for collagen I and constructs cultured with 100% ACs were positive for collagen II, while a mixture of collagen I and II was observed in other co-culture groups. **B**: Representative controls. Unseeded scaffolds did not stain for collagen or GAG content. Negative controls for collagen I and II (no primary antibodies). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Table I. Wet weight, dry weight, thickness, diameter, and cell number of constructs at t = 4 weeks.

MC:AC	Wet weight (mg)	Dry weight (mg)	Thickness (mm)	Diameter (mm)	Cell number (millions)
100:0	12.3 ± 2.4	1.3 ± 0.3^{b}	2.0 ± 0.1^{b}	3.0 ± 0.1^{b}	0.8 ± 0.2
75:25	12.4 ± 2.4	1.3 ± 0.4^{b}	2.0 ± 0.2^{b}	3.0 ± 0.2^{b}	0.9 ± 0.1
50:50	12.5 ± 2.2	1.3 ± 0.5^{b}	2.1 ± 0.2^{b}	3.0 ± 0.1^{b}	0.8 ± 0.2
25:75	12.9 ± 1.7	1.4 ± 0.4^{b}	2.1 ± 0.1^{b}	3.1 ± 0.1^{b}	0.8 ± 0.2
0:100	13.1 ± 1.5	1.5 ± 0.1^{a}	2.3 ± 0.1^{a}	3.2 ± 0.3^{a}	0.7 ± 0.2
P-value	0.31	0.002	0.003	0.02	0.35

A P-value <0.05 was considered significant. Letters denote significant differences among groups using a 1-way ANOVA.

with 100% MCs, while positive staining for collagen II was observed in constructs cultured with 100% ACs. A mixture of collagen I and II was observed in other co-culture groups (Fig. 1A). Unseeded scaffolds did not stain positively for collagen or GAG (Fig. 1B). Collagen I and II staining was absent in the negative controls where the collagen I and II primary antibody was not added (Fig. 1B).

Biochemistry

The dry weight of samples cultured with 100% ACs (1.6 ± 0.1 mg) was significantly higher than all other groups ($P = 0.002$) (Table I); however, no significant differences were observed in the wet weight of constructs ($P = 0.31$) (Table I). At $t = 0$, a cell seeding density of 0.67 ± 0.23 million cells/scaffold was obtained. Over the 4-week culture period, no significant increases were observed among groups for cell number/construct with cell numbers ranging from 0.85 ± 0.14 to 0.68 ± 0.19 million ($P = 0.35$) (Table I). Total collagen (Fig. 2) and GAG (Fig. 3) per construct increased significantly as the percentage of ACs increased (27 ± 8 μg, 0% AC to 45 ± 8 μg, 100% ACs for collagen ($P = 0.03$) and 12 ± 4 μg, 0% ACs to 40 ± 5 μg, 100% ACs for GAG ($P = 0.006$)). When collagen content was normalized to dry weight, groups with 25%, 50%, 75% ACs were not significantly different from each other, but were significantly higher than the 100% MC and 100% AC groups ($P = 0.03$) (Fig. 2). Similar trends were observed for collagen per wet weight with 100% MC constructs (~2 μg collagen/mg DW) containing significantly less collagen than the 100% AC group (~3.5 μg collagen/mg DW) ($P = 0.03$). For GAG/dry weight, constructs exposed to 50%, 75%, or 100% ACs were significantly different from the other groups ($P = 0.002$) (Fig. 3). GAG per WW mirrored total GAG values with significant increases observed in constructs with higher percentage of ACs ($P = 0.02$).

Compressive Properties

Compressive moduli (instantaneous and relaxation modulus) and coefficient of viscosity were measured at 10%, 20%, and 30% strain levels. At 10% strain, the instantaneous and relaxation modulus of constructs with 100% ACs

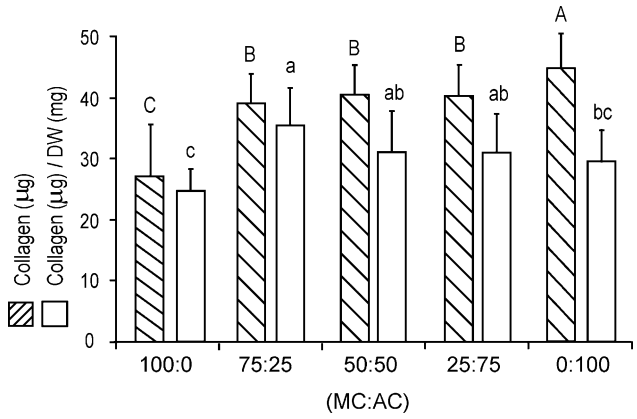

Figure 2. Total collagen per construct and collagen/DW. Total collagen per construct increased as the percentage of ACs increased. Capital letters denote significant differences among groups for total collagen per construct using a one-way ANOVA. Small letters denote significant differences among groups for collagen per DW using a one-way ANOVA. All values are reported as mean ± SD. An $n = 5$ was used to perform the assay.

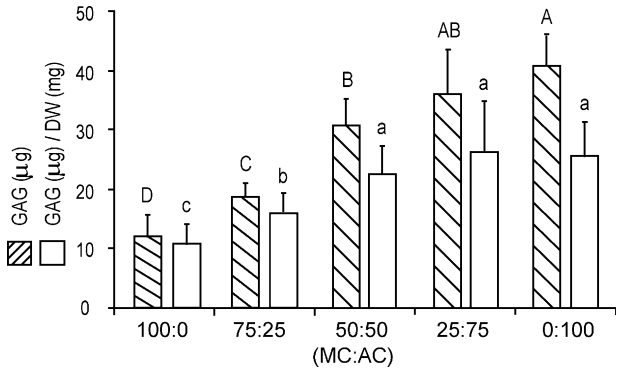

Figure 3. Total GAG per construct and GAG/DW. Total GAG per construct increased as the percentage of ACs increased. Capital letters denote significant differences among groups for total GAG per construct using a one-way ANOVA. Small letters denote significant differences among groups for GAG per DW using a one-way ANOVA. All values are reported as mean ± SD. An $n = 5$ was used to perform the assay.

(63 ± 12 and 22 ± 9 kPa, respectively) was significantly higher than the other groups ($P = 0.03$) (Fig. 4). No significant differences were observed among groups for the coefficient of viscosity. Similar results were observed at 20% and 30% strain levels. Compressive properties of unseeded PLLA constructs in medium at $t = 4$ weeks were found to be 12 ± 4 kPa for the instantaneous modulus, 6 ± 3 kPa for the relaxation modulus, and 3 ± 1 MPa s for the coefficient of viscosity.

Tensile Properties

No significant differences were observed among groups for tensile properties ($P = 0.31$) (Fig. 5). Tensile stiffness ranged from 690 ± 220 kPa for the 100% MC group to 595 ± 200 kPa for the 100% AC group. UTS ranged from 500 ± 250 kPa for the 100% MC group to 300 ± 250 kPa. Tensile properties of unseeded PLLA constructs at $t = 4$ weeks in media were found to be 300 ± 60 kPa for the tensile stiffness and 180 ± 80 kPa for the UTS.

Correlation Between Biochemical and Biomechanical Data

The compressive and tensile properties obtained were correlated with the GAG and collagen content, respectively. For compressive properties at 10% strain, univariate regression analysis showed a significant correlation between instantaneous modulus and GAG/construct ($P = 0.0001$, $R^2 = 0.71$), and collagen/construct ($P = 0.0002$, $R^2 = 0.39$). Similar results were obtained when correlating the relaxation modulus and GAG/construct ($P < 0.0001$, $R^2 = 0.74$), and collagen/construct ($P = 0.007$, $R^2 = 0.33$). Further, the coefficient of viscosity correlated significantly with

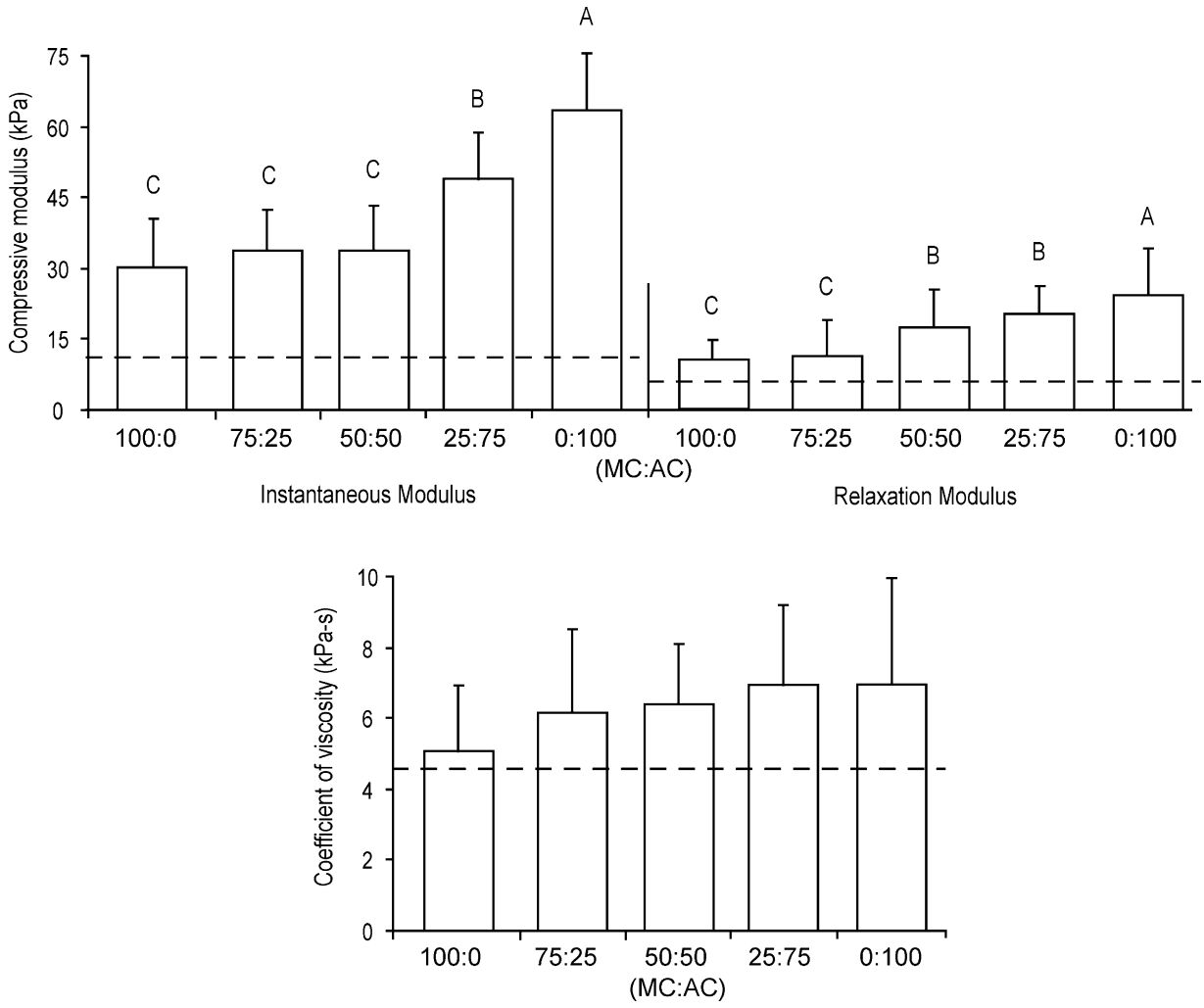

Figure 4. Compressive properties of the constructs at 10% strain. Compressive moduli of the constructs were significantly higher in the 100% ACs group when compared to groups with higher percentage of MCs. No significant differences were observed among groups for the coefficient of viscosity. Dotted line represents compressive properties of unseeded PLLA scaffolds. Groups with different letters are significantly different from each other. All values are reported as mean ± SD. An $n = 5$ was used to perform the assay.

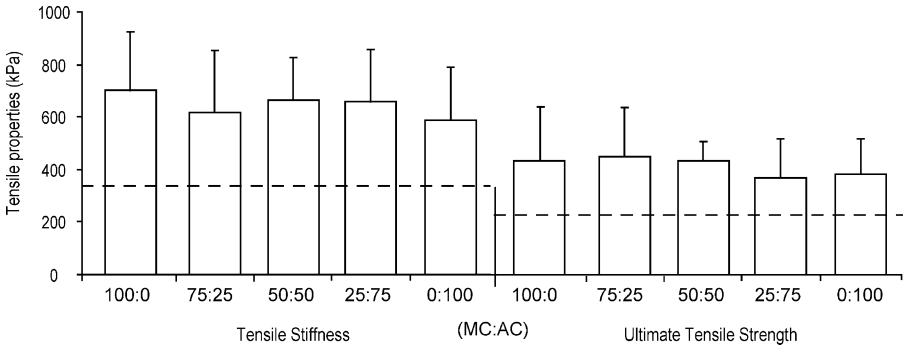

Figure 5. Tensile properties of the constructs. No differences in tensile properties were noted among groups. Dotted line represents tensile properties of unseeded PLLA scaffolds. All values are reported as mean ± SD. An $n = 5$ was used to perform the assay.

GAG/construct ($P = 0.0001$, $R^2 = 0.50$), and collagen/construct ($P = 0.0001$, $R^2 = 0.42$) as well. Similar significant correlations were obtained at 20% and 30% strain levels. For tensile properties, a significant correlation was observed between the tensile stiffness and collagen/construct ($P = 0.008$, $R^2 = 0.53$), and GAG/construct ($P = 0.002$, $R^2 = 0.21$). In addition, the UTS was also found to correlate significantly with collagen/construct ($P < 0.0001$, $R^2 = 0.45$), and GAG/construct ($P < 0.001$, $R^2 = 0.19$).

Discussion

This study employed five different co-culture ratios of passaged MCs and primary ACs on PLLA scaffolds to identify co-culture ratios most closely resembling the biochemical and biomechanical properties of the inner and outer regions of the knee meniscus. Qualitative and quantitative tests showed clear differences between the ECM and biomechanical properties of the constructs in each group. Specifically, constructs cultured with a higher percentage of ACs had enhanced compressive properties and contained significantly higher levels of collagen I, collagen II, and GAG. Constructs cultured with higher percentage of MCs contained higher levels of collagen I. Overall, we were able to demonstrate that co-culturing MCs and ACs on PLLA scaffolds resulted in functional tissue engineered fibrocartilage constructs with a spectrum of biochemical and biomechanical properties.

The approach used in this tissue engineering study was unconventional in that it utilized a co-culture of second passaged MCs and primary ACs. Second passage MCs were used in this experiment to address the limitation of the number of primary rabbit MCs available and with the knowledge that MCs retain their ability to produce extracellular matrix up to the second passage (Uthamanthil and Athanasiou, 2005). Primary ACs were judiciously chosen based on their ability to enhance collagen II and GAG content, both of which are found in abundance in the inner regions of the meniscus (Sweigart and Athanasiou, 2001) and are down-regulated in passaged MCs (Gunja and Athanasiou, 2007). The IHC data showed that, as the percentage of ACs increased in the constructs, a concomitant increase in collagen II and GAG production was observed. In addition, for the first time, we were able to mimic certain compositions of the meniscus using co-cultures of MCS and ACs seeded on PLLA. The GAG/DW for the 100%, 75%, and 50% AC groups was found to be in the range of the inner region of the rabbit meniscus (~20–25 μg GAG/mg dry tissue) while the GAG/DW for the 100% MC group was in the range of the outer region of the rabbit meniscus (~5–10 μg GAG/mg dry tissue) (Sweigart, 2004). The collagen content/DW in all groups (~25–35 μg collagen/mg dry tissue) was found to be lower than native values (600–700 μg collagen/mg dry tissue) (Sweigart, 2004). It should be noted, however, that the dry weight data presented are likely underestimated, as approximately 65–75% of the constructs' dry weights are that of the PLLA scaffold. If the weight of the scaffold is excluded, and the data re-normalized, the new collagen per dry weights approach those of native tissue with values ranging from 70 to 100 μg collagen/mg dry tissue. A limitation of this study was that specific collagen levels were not determined. In future studies, techniques such as enzyme-linked immunosorbent assay (ELISA) may be used to quantify collagen I and II within the constructs and compare the results to native tissue.

Primary ACs were also used in the experiment to aid in enhancing the capabilities of passaged MCs to produce relevant ECM. It has been shown that a low percentage of primary ACs (up to 20%), cultured with passaged ACs can aid in the reversal of gene expression of the passaged ACs (Gan and Kandel, 2007). Specifically, the authors observed increases in expression of chondrogenic markers, collagen II, SOX-9, and aggrecan, and decreases in expression of the fibroblastic marker collagen I in the passaged cells. Akin to that experiment, we examined whether low percentages of ACs would significantly increase the ability of MCs to

produce relevant meniscus markers, especially those pertaining to the inner meniscus, such as collagen II. IHC images of collagen II distribution, however, did not allude to possible redifferentiation of MCs towards a chondrogenic phenotype. Gene expression analyses may provide a better understanding of the cells' phenotype post-co-culture. In future experiments, ACs and MCs can be separated by flow-associated cell sorting and examined for gene expression levels of relevant ECM markers (Ahmed et al., 2008).

The compressive biomechanical properties were evaluated at 4 weeks with increases observed in the instantaneous and relaxation modulus for constructs cultured with higher percentage of ACs. The instantaneous modulus, relaxation modulus, and the coefficient of viscosity correlated strongly with the amount of GAG in the construct. GAGs are negatively charged particles that attract water molecules into the constructs and increase the overall compressive stiffness. Weaker but statistically significant correlations were observed for collagen/construct and the three tested variables for the compressive tests. The compressive properties obtained were in the range of 30–75 kPa, approaching those of the rabbit inner meniscus (~120 kPa) (Sweigart and Athanasiou, 2005).

The tensile properties, UTS, and tensile stiffness, at 4 weeks were found to correlate strongly with the collagen content in the scaffolds. Collagen provides tensile resistance to the tissue (Skaggs et al., 1994) and, thus, this result was not unexpected. Weaker but statistically significant correlations were observed for the tensile properties with GAG per construct. Much work remains, however, to enhance the tensile properties of the cell-seeded constructs towards native tissue (approximately 150 MPa for the tensile stiffness and 20 MPa for the UTS) (Sweigart and Athanasiou, 2005). Chemical agents such as chondroitinase-ABC can be utilized during culture to temporarily deplete GAGs from the constructs while enhancing collagen alignment (Natoli et al., 2009). In addition, strategies to enhance collagen content need to be employed, perhaps by the addition of growth factors such as TGF-β1 into the culture medium or by the application of mechanical stimuli, such as hydrostatic pressure (Elder and Athanasiou, 2008; Gunja et al., 2009).

In this experiment, the absence of serum did not appear to hinder ECM production or decrease cell attachment to the PLLA constructs. Previous experiments in the laboratory have shown that 1% FBS in the culture medium is necessary for MC attachment and proliferation in monolayer culture (Gunja et al., 2009). However, to enhance the clinical translatability of our approach, we employed a serum-free medium containing ITS+ and dexamethasone in this experiment. The serum-free formulation obviates concerns of possible immune rejection and batch-to-batch variations in animal serum (Mandl et al., 2002; Yaeger et al., 1997). This cocktail has been used previously for both meniscus and articular cartilage tissue engineering to maintain cell phenotype and increase proteoglycan synthesis (Chua et al., 2005; Giannoni et al., 2005; Hoben and Athanasiou, 2008; Yates et al., 2005).

An interesting observation in this study was the presence of small nodules of tissue growing atop the PLLA constructs with higher percentage of ACs. Several studies have shown that chondrocytes prefer to bind to other chondrocytes when placed in a 3D environment, possibly to minimize their surface free energy (Hu and Athanasiou, 2003a). This process is likely mediated by cadherin–cadherin interactions that are described by the differential adhesion hypothesis (Foty and Steinberg, 2005). In addition, the surface of PLLA is hydrophobic and does not possess physiologic activity, which may further hinder chondrocyte cell adhesion (van Wachem et al., 1985). Although, nodules were observed in constructs with a high percentage of ACs, they were conspicuously absent in groups with a higher percentage of MCs. In previous experiments, primary and passaged MCs have been shown to attach, proliferate, and migrate inwards on PLLA scaffolds as evidenced by histological examination (Gunja et al., 2009). The phenotype of the cell may dictate its attachment preferences to a substrate. The elongated or spindle-shaped MCs may reduce their free surface energy by attaching to the PLLA scaffold and spreading, while the spherical chondrocytes stay rounded and form nodules (Hu and Athanasiou, 2003b; McDevitt et al., 1992).

Constructs in all groups stained positively for GAG and collagen. Interestingly, the intensity of staining increased as the percentage of ACs increased with stronger staining observed at the periphery of the construct and weaker staining towards the center. The disparity in staining intensity may be an indication of cell location within the tissue. Primary ACs are known to produce GAGs and collagen II in large amounts while passaged MCs produce mainly collagen I (Gunja and Athanasiou, 2007). Based on this and our histological findings, we can speculate that over the 4-week culture period, ACs migrate towards the periphery of the construct while MCs populate the center. The differential expression of cadherins on the cell surface has been shown to drive cell sorting (Niessen and Gumbiner, 2002) and it is likely that passaged MCs and primary ACs express different levels of cadherins prior to and during co-culture. Further, MCs are known to exert contractile forces in scaffoldless meniscus constructs and may migrate towards the center of the scaffold (Aufderheide and Athanasiou, 2007). In future experiments, cell-tracking agents such as Cell Tracker Orange can be utilized to examine cell sorting. In addition, cells can be stained for cadherin levels and alpha-smooth muscle actin to document cell migration. A gradual cell separation from a fibroblastic phenotype to a chondrocytic phenotype would be ideal for a meniscus tissue engineering strategy, as it would mimic the native MC distribution.

Conclusions

In conclusion, this study used co-cultures of passaged MCs and primary ACs to form robust constructs with a spectrum of biochemical and biomechanical properties. Collagen,

GAG, and compressive properties increased significantly as the percentage of ACs increased. Excitingly, we were able to achieve GAG levels close to native values for the inner and outer meniscus using a variety of co-culture ratios. Collagen levels approached native meniscus as well if the dry weight normalizations excluded the weight of PLLA. It is envisioned that this study will provide a baseline for future meniscus tissue engineering studies attempting to regenerate different regions of the meniscus.

References

Adesida AB, Grady LM, Khan WS, Hardingham TE. 2006. The matrix-forming phenotype of cultured human meniscus cells is enhanced after culture with fibroblast growth factor 2 and is further stimulated by hypoxia. Arthritis Res Ther 8(3):R61.

Adesida AB, Grady LM, Khan WS, Millward-Sadler SJ, Salter DM, Hardingham TE. 2007. Human meniscus cells express hypoxia inducible factor-1alpha and increased SOX9 in response to low oxygen tension in cell aggregate culture. Arthritis Res Ther 9(4):R69.

Ahmed N, Gan L, Nagy A, Zheng J, Wang C, Kandel RA. 2008. Cartilage tissue formation using redifferentiated passaged chondrocytes in vitro. Tissue Eng Part A (E-pub ahead of print).

Allen KD, Athanasiou KA. 2005. A surface-regional and freeze-thaw characterization of the porcine temporomandibular joint disc. Ann Biomed Eng 33(7):951–962.

Allen KD, Athanasiou KA. 2006a. Growth factor effects on passaged TMJ disk cells in monolayer and pellet cultures. Orthod Craniofac Res 9(3):143–152.

Allen KD, Athanasiou KA. 2006b. Viscoelastic characterization of the porcine temporomandibular joint disc under unconfined compression. J Biomech 39(2):312–322.

Allen KD, Athanasiou KA. 2007. Effect of passage and topography on gene expression of temporomandibular joint disc cells. Tissue Eng 13(1): 101–110.

Allen KD, Erickson K, Athanasiou KA. 2008. The effects of protein-coated surfaces on passaged porcine TMJ disc cells. Arch Oral Biol 53(1):53–59.

Aufderheide AC, Athanasiou KA. 2007. Assessment of a bovine co-culture, scaffold-free method for growing meniscus-shaped constructs. Tissue Eng 13(9):2195–2205.

Battlehner CN, Carneiro Filho M, Ferreira Junior JM, Saldiva PH, Montes GS. 1996. Histochemical and ultrastructural study of the extracellular matrix fibers in patellar tendon donor site scars and normal controls. J Submicrosc Cytol Pathol 28(2):175–186.

Benya PD, Shaffer JD. 1982. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30(1):215–224.

Chua KH, Aminuddin BS, Fuzina NH, Ruszymah BH. 2005. Insulin-transferrin-selenium prevent human chondrocyte dedifferentiation and promote the formation of high quality tissue engineered human hyaline cartilage. Eur Cell Mater 9:58–67, discussion 67.

Darling EM, Athanasiou KA. 2005. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. J Orthop Res 23(2):425–432.

Elder BD, Athanasiou KA. 2008. Synergistic and additive effects of hydrostatic pressure and growth factors on tissue formation. PLoS ONE 3(6):e2341.

Foty RA, Steinberg MS. 2005. The differential adhesion hypothesis: A direct evaluation. Dev Biol 278(1):255–263.

Gan L, Kandel RA. 2007. In vitro cartilage tissue formation by co-culture of primary and passaged chondrocytes. Tissue Eng 13(4):831–842.

Giannoni P, Crovace A, Malpeli M, Maggi E, Arbico R, Cancedda R, Dozin B. 2005. Species variability in the differentiation potential of in vitro-expanded articular chondrocytes restricts predictive studies on cartilage repair using animal models. Tissue Eng 11(1–2):237–248.

Gunja NJ, Athanasiou KA. 2007. Passage and reversal effects on gene expression of bovine meniscal fibrochondrocytes. Arthritis Res Ther 9(5):R93.

Gunja NJ, Uthamanthil RK, Athanasiou KA. 2009. Effects of TGF-beta1 and hydrostatic pressure on meniscus cell-seeded scaffolds. Biomaterials 30(4):565–573.

Hoben GM, Athanasiou KA. 2008. Creating a spectrum of fibrocartilages through different cell sources and biochemical stimuli. Biotechnol Bioeng 100(3):587–598.

Hoben GM, Hu JC, James RA, Athanasiou KA. 2007. Self-assembly of fibrochondrocytes and chondrocytes for tissue engineering of the knee meniscus. Tissue Eng 13(5):939–946.

Hu JC, Athanasiou KA. 2003a. Effects of culture conditions on agarose and PLLA cell-seeded scaffolds in a bioreactor. New Orleans, LA (Conference abstract).

Hu JCY, Athanasiou K. 2003b. Chapter 4: Structure and function of articular cartilage. In: An YH, Martin KL, editors. Handbook of histology methods for bone and cartilage. Totowa, NJ: Humana Press, p. xviii, 587.

Lin Z, Fitzgerald JB, Xu J, Willers C, Wood D, Grodzinsky AJ, Zheng MH. 2008. Gene expression profiles of human chondrocytes during passaged monolayer cultivation. J Orthop Res 26(9):1230–1237.

Mandl EW, van der Veen SW, Verhaar JA, van Osch GJ. 2002. Serum-free medium supplemented with high-concentration FGF2 for cell expansion culture of human ear chondrocytes promotes redifferentiation capacity. Tissue Eng 8(4):573–580.

McDevitt CA, Miller RR, Spindler KP. 1992. The cells and cell matrix interactions of the meniscus. In: Mow VC, Arnoczky SP, Jackson DW, editors. Knee meniscus: Basic and clinical foundations. New York: Raven Press, pp. 29–36.

McDevitt CA, Mukherjee S, Kambic HE, Parker R. 2002. Emerging concepts of the cell biology of the meniscus. Curr Opin Orthop 13(5):345–350.

Nakano T, Dodd CM, Scott PG. 1997. Glycosaminoglycans and proteoglycans from different zones of the porcine knee meniscus. J Orthop Res 15(2):213–220.

Natoli RN, Responte DJ, Lu BY, Athanasiou KA. 2009. Effects of multiple chondroitinase ABC applications on tissue engineered articular cartilage. J Orthop Res (E-pub ahead of print).

Niessen CM, Gumbiner BM. 2002. Cadherin-mediated cell sorting not determined by binding or adhesion specificity. J Cell Biol 156(2):389–399.

Pietila K, Kantomaa T, Pirttiniemi P, Poikela A. 1999. Comparison of amounts and properties of collagen and proteoglycans in condylar, costal and nasal cartilages. Cells Tissues Organs 164(1):30–36.

Rosenberg L. 1971. Chemical basis for the histological use of safranin O in the study of articular cartilage. J Bone Joint Surg Am 53(1):69–82.

Schoenfeld AJ, Landis WJ, Kay DB. 2007. Tissue-engineered meniscal constructs. Am J Orthop 36(11):614–620.

Skaggs DL, Warden WH, Mow VC. 1994. Radial tie fibers influence the tensile properties of the bovine medial meniscus. J Orthop Res 12(2): 176–185.

Sweigart MA. 2004. Characterization of the meniscus for future tissue engineering efforts [Ph.D.]. Houston: Rice University.

Sweigart MA, Athanasiou KA. 2001. Toward tissue engineering of the knee meniscus. Tissue Eng 7(2):111–129.

Sweigart MA, Athanasiou KA. 2005. Tensile and compressive properties of the medial rabbit meniscus. Proc Inst Mech Eng [H] 219(5):337–347.

Sweigart MA, Zhu CF, Burt DM, DeHoll PD, Agrawal CM, Clanton TO, Athanasiou KA. 2004. Intraspecies and interspecies comparison of the compressive properties of the medial meniscus. Ann Biomed Eng 32(11):1569–1579.

Uthamanthil RK, Athanasiou K. 2005. Effect of passage and expansion on the tissue engineering potential of meniscal fibrochondrocytes. Baltimore, MD (Conference abstract).

van Wachem PB, Beugeling T, Feijen J, Bantjes A, Detmers JP, van Aken WG. 1985. Interaction of cultured human endothelial cells with polymeric surfaces of different wettabilities. Biomaterials 6(6):403–408.

Weinand C, Peretti GM, Adams SB, Jr., Bonassar LJ, Randolph MA, Gill TJ. 2006a. An allogenic cell-based implant for meniscal lesions. Am J Sports Med 34(11):1779–1789.

Weinand C, Peretti GM, Adams SB, Jr., Randolph MA, Savvidis E, Gill TJ. 2006b. Healing potential of transplanted allogeneic chondrocytes of three different sources in lesions of the avascular zone of the meniscus: A pilot study. Arch Orthop Trauma Surg 126(9):599–605.

Woessner JF. 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 93:440–447.

Yaeger PC, Masi TL, de Ortiz JL, Binette F, Tubo R, McPherson JM. 1997. Synergistic action of transforming growth factor-beta and insulin-like growth factor-I induces expression of type II collagen and aggrecan genes in adult human articular chondrocytes. Exp Cell Res 237(2):318–325.

Yates KE, Allemann F, Glowacki J. 2005. Phenotypic analysis of bovine chondrocytes cultured in 3D collagen sponges: Effect of serum substitutes. Cell Tissue Bank 6(1):45–54.