Characterization of *Ex Vivo*–Generated Bovine and Human Cartilage by Immunohistochemical, Biochemical, and Magnetic Resonance Imaging Analyses

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Osteoarthritis (OA) is a prevalent age-associated disease involving altered chondrocyte homeostasis and cartilage degeneration. The avascular nature of cartilage and the altered chondrocyte phenotype characteristic of OA severely limit the capacity for *in vivo* tissue regeneration. Cell- and tissue-based repair has the potential to revolutionize treatment of OA, but those approaches have exhibited limited clinical success to date. In this study, we test the hypothesis that bovine and human chondrocytes in a collagen type I scaffold will form hyaline cartilage *ex vivo* with immunohistochemical, biochemical, and magnetic resonance (MR) endpoints similar to the original native cartilage. Chondrocytes were isolated from 1- to 3-week-old calf knee cartilage or from cartilage obtained from human total knee arthroplasties, suspended in 2.7 mg/mL collagen I, and plated as 300 μ L spot cultures with 5×10^6 each. Medium formulations were varied, including the amount of serum, the presence or absence of ascorbate, and treatments with cytokines. Bovine chondrocytes generated metachromatic territorial and interstitial matrix and accumulated type II collagen over time. Type VI collagen was confined primarily to the pericellular region. The *ex vivo*–formed bovine cartilage contained more chondroitin sulfate per dry weight than native cartilage. Human chondrocytes remained viable and generated metachromatic territorial matrix, but were unable to support interstitial matrix accumulation. MR analysis of *ex vivo*–formed bovine cartilage revealed evidence of progressively maturing matrix, but MR-derived indices of tissue quality did not reach those of native cartilage. We conclude that the collagen-spot culture model supports formation and maturation of three-dimensional hyaline cartilage from active bovine chondrocytes. Future studies will focus on determining the capacity of human chondrocytes to show comparable tissue formation.

Introduction

TISSUE ENGINEERING OF cartilage constructs is an area of active investigation, motivated by the potential for routine generation of functional human articular cartilage *ex vivo* to provide a novel and highly effective mode of treatment for degenerative cartilage defects. The methodologies to support the *ex vivo* formation of cartilage include bioreactors,^{1,2} alginate beads and other scaffold-based systems,^{3–5} and suspension culture.⁶ However, regeneration of three-dimensional cartilage from human chondrocytes remains a challenge, most likely owing to the limited regenerative capacity of the articular chondrocytes obtained from arthroplasty. Stimulating these chondrocytes to re-synthesize extracellular matrix (ECM) molecules and assemble them into a correctly organized and biomechanically functional

cartilage will most likely require a complex strategy, including growth factor provision and possibly application of mechanical stimulus. However, some success with human cells has been reported. Chondrocytes from a 14-year-old girl suffering from knee osteoarthritis (OA) after meniscectomy were suspended in a collagen I matrix and incubated in the culture medium for 3 weeks, after which time the construct was implanted into the girl's femoral defect. Arthroscopy performed 1 year after surgery revealed full integration of the construct into the surrounding tissue.⁷ A similar *ex vivo* model system is employed in the present study to assess potential tissue formation as compared to native tissue.

With the inherent difficulties involved in stimulating aged and/or diseased human chondrocytes to re-synthesize ECM, the study of readily available and easily replicable animal models will be essential toward the development of tissue

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regeneration protocols using human cells. Of course, animal models such as cows, rabbits, and mice do not exhibit the same amount of physiological loading that is experienced in human knee articular cartilage, and loading has been shown to stimulate ECM synthesis and secretion.⁸ Mechanical stimulation of *ex vivo*-generated human cartilage may become an essential component for successfully engineering cartilage constructs. However, the generation of animal models for basic research remains important. Studies with both embryonic chick sternal chondrocytes^{1,9,10} and bovine articular chondrocytes, as a mammalian source,^{8,11,12} have been reported.

Noninvasive analysis of *ex vivo*-developing constructs permits ongoing evaluation of tissue regeneration protocols. In particular, magnetic resonance imaging (MRI) outcome measures reflect a variety of tissue characteristics. Those most commonly used include (1) T_2 , which reflects overall macromolecular content; (2) the magnetization transfer ratio (MTR) and rate, which is particularly sensitive to tissue collagen content; and (3) the derived fixed charge density (FCD), which reflects proteoglycan (PG) content. Characterization of scaffold-free engineered cartilage constructs using these MR parameters has already been reported.^{1,9} The aims of the present work were to directly compare MR endpoints along with additional biochemical and protein markers between native cartilage explants and scaffold-based tissue-engineered constructs. Our hypothesis was that bovine and human chondrocytes in a collagen type I scaffold would form hyaline cartilage *ex vivo* with immunohistochemical, biochemical, and MR endpoints similar to the original native cartilage.

Materials and Methods

Isolation of native cartilage samples

All studies involving human tissue were approved by the NEUOCOM IRB. Human cartilage samples were isolated from tibial plateaus and femoral condyles of patients undergoing total knee arthroplasty within 3 h of surgery (patient $n = 22$, average age 67.2 years). Cells from these samples were used to generate spot cultures (SCs), with tissue from two to five patients pooled before cell isolation to maximize yield. Healthy, non-OA knee cartilage isolated from patients found to have chondro- and osteosarcomas, along with hip and ankle cartilage resected secondary to nondegenerative diseases such as alcoholism and amputation caused by fracture, were taken as normal cartilage (patient $n = 11$, average age 42.4 years) for comparison with the SCs. Owing to the limited number of patients and the number of cells acquired, normal patients were not used as a source of chondrocytes for SCs. All human SCs were generated from cartilage isolated from total knee arthroplasties due to degenerative cartilage defects. Bovine knees of 1–3-week-old Holstein calves were transported from the slaughterhouse (Mahan Packing Co., Bristolville, OH) in Betadine and on ice to maintain sterility and tissue viability. Articular cartilage was dissected from the femoral condyles and tibial plateaus, and placed in phosphate-buffered saline with 100 $\mu\text{g}/\text{mL}$ Primocin (Invivogen, San Diego, CA) on ice.

Generation of bovine and human SCs

The human or bovine cartilage was minced and digested in 4% collagenase (Worthington Biochemical, Lakewood, NJ)

in Hank's balanced salt solution (Gibco, Carlsbad, CA) with 100 $\mu\text{g}/\text{mL}$ Primocin overnight at 37°C for chondrocyte isolation. Collagen I solution (rat tail tendon; BD Biosciences 354236) was prepared according to the manufacturer's instructions at 2.7 mg/mL. Chondrocytes were suspended without expansion in monolayer in the collagen I solution at 5×10^6 cells per 300 μL collagen (1.67×10^7 cells/mL) and plated into 100 mm² tissue culture dishes to form the SCs. Preliminary data not shown here indicated that SCs with high concentrations of chondrocytes resulted in greater tissue formation than SCs seeded with lower concentrations of cells. The collagen in the hydrogel gelatinized after approximately 10 min at 37°C, after which time the medium was applied. SCs were incubated at 37°C in Opti-MEM I Reduced-Serum Medium or Ham's F-12 (Gibco). As indicated in the figure legends, SCs were incubated at 37°C in Opti-MEM I Reduced-Serum medium alone or with one of the following: 50 $\mu\text{g}/\text{mL}$ ascorbate, 10 ng/mL tumor necrosis factor- α (TNF- α), and 10 ng/mL interleukin-1 β (IL-1 β). In separate studies the SCs were incubated with Hams F-12 medium with either 2% fetal bovine serum (FBS) or 10% FBS. Distinct sets of SCs were evaluated at 24 h intervals over the first 7 days of culture, and then after 2, 4, and 6 weeks of growth, to assess the characteristics of the developing tissue.

Cytokine experimental design

The effects of cytokine treatment on *ex vivo*-generated cartilage were evaluated through the following groups of SCs. First, a set of SCs was allowed to develop under control conditions for 3 weeks and then treated for 1 week with the medium containing 10 ng/mL IL-1 β and 10 ng/mL TNF- α . This group was sacrificed immediately after cytokine exposure to determine the direct effect of cytokines on ECM composition, and is designated "No Recovery." Second, to determine the chondrocyte capacity for ECM recovery after cytokine-induced matrix degradation, an additional set of SCs were subjected to the same conditions as above (3 weeks of growth followed by 1 week of cytokine treatment), with the cytokine-containing medium then being replaced by the control medium for an additional 2 weeks of growth (designated "2wk Recovery"). Finally, for comparison, SCs were maintained in the control medium for 4 and 6 weeks as controls.

Both *ex vivo* and native cartilage were analyzed in a similar manner as described in the following sections.

Immunohistochemistry

Tissue samples were fixed in 10% neutral-buffered formalin (Fisher Scientific, Pittsburgh, PA), processed, and paraffin embedded according to standard laboratory procedures. Paraffin blocks were sectioned at 6 μm (native cartilage) or 10 μm (SCs) and stained with 0.1% thionin for basic histological analysis. Primary antibodies for immunofluorescence were as follows: collagen II mouse monoclonal (1:50; NeoMarkers MS-235, Fremont, CA), collagen VI rabbit polyclonal (1:100; Fitzgerald, Acton, MA, RDI-600401108), and collagen I mouse monoclonal (1:100; Santa Cruz, Santa Cruz, CA, sc-59772). Secondary antibodies were purchased from (Molecular Probes, Carlsbad, CA) and used at 1:200 concentration: goat anti-rabbit Alexa Fluor 568 (A11011), and goat anti-mouse Alexa Fluor 488 (A11001). Dimethylamino-

benzaldehyde reaction was performed using the ABC staining kit (Santa Cruz).

In general, unmasking was achieved using chondroitinase ABC (Sigma, St. Louis, MO) for 20 min at 37°C. Primary antibodies were incubated overnight at 37°C, and secondary antibodies were applied for 1 h at room temperature. Slides were counterstained with DAPI (Vectashield Hard Set Mounting Medium with DAPI; Vector Laboratories, Burlingame, CA). Control slides were prepared by performing every step of the procedure except for the substitution of the primary antibody with blocking serum. Images were obtained using Bioquant Osteo II (v.8.00.20; Bioquant Image Analysis Corporation, Nashville, TN), and background exposure was normalized to the associated minus-primary control slide, with any additional staining considered positive.

Quantitative biochemistry

SCs were desiccated in a vacuumed centrifuge and digested overnight in proteinase K solution. Chondroitin sulfate (CS) content was quantified by metachromasia via optical density: sulfated glycosaminoglycans (sGAGs) were reacted with dimethylmethylene blue and absorbance was read against a standard curve. Values are listed as $\mu\text{g sGAG}/\mu\text{g dry weight}$. Unseeded collagen gels were tested for sGAG content and found to have no significant values. Hydroxyproline (HP) content was quantified by using dimethylaminobenzaldehyde to observe chloramine T-oxidized HP, and absorbance was read against a standard curve. HP values of SC samples represent total content without normalizing to an unseeded collagen gel. With the exception of the treatment of 2% FBS+ascorbate, SC values represent the mean of three separate animals or patients $\pm$ the standard deviation from the mean. Results for native cartilage represent the results from seven samples.

MRI methods

MRI data were acquired at 9.4 T using a Bruker DMX system operating at 400 MHz (Bruker Biospin, Billerica, MA) and equipped with 1000 mT/m three-axis microimaging gradients. Standard imaging sequences were implemented as described below separately for the native tissue and the SCs, including measurement of matrix FCD via the gadolinium (Gd) exclusion assay.

Native human and bovine tissue. Osteochondral plugs (7 mm diameter) were immersed in a solution consisting of Sigma general-purpose protease inhibitor concentrate in 100 mL Dulbecco's phosphate buffered saline (DPBS, Sigma, St. Louis, MO) with 12 mM of the MMP inhibitor GM6001 (Chemicon, Billerica, MA) titrated to pH 7.5 ± 0.1 . Each osteochondral plug sample was immobilized within agarose gel in a 9 mm O.D. glass tube. Four such tubes were placed in a susceptibility-matched sample holder and imaged simultaneously with sagittal-coronal oblique slices passing through the center of each plug. Images were acquired with a 2×2 cm field of view (FOV), 0.5 mm slice thickness, 256×256 matrix size, and two averages. T_2 was measured using 32 echoes with repetition time (TR)/TE = 5 s/12.8 ms. Pre-Gd T_1 values were measured using TR ranging from 15 s to 400 ms in seven steps. Post-Gd TR values ranged between 1.6 s and 50 ms in 11 steps. MT was measured

using the same spin-echo sequence preceded by a 6 kHz off-resonance saturation pulse of amplitude $B_1 = 12 \mu\text{T}$ and pulse length t_p incremented from 0.1 to 4.6 s in eight steps. Results for the MRI evaluation of cartilage explants are reported in terms of a shallow, rather than superficial, zone, because of the limited spatial resolution of the MRI measurement.

Spot cultures. At each designated time point, the SCs and 15 mL of control media were placed into a 20 mm O.D. NMR tube. Imaging was performed using a Micro 2.5 probe body (Bruker Biospin) with a 20 mm transmit/receive proton birdcage resonator, with sagittal-coronal oblique slices passing through the center of each sample. Parameters included 3×2 cm FOV, 1 mm slice thickness, 256×256 matrix size, and two averages. T_2 was measured using 64 echoes with TR/TE = 5 s/12 ms. Pre-Gd T_1 values were measured using TR values ranging from 15 s to 100 ms in 12 steps. Post-Gd TR values ranged between 1.6 s and 25 ms in 12 steps. MT measurements were made using the same spin echo sequence preceded by a 6 kHz off-resonance saturation pulse of amplitude $B_1 = 12 \mu\text{T}$ and pulse length t_p incremented from 0.1 to 4.6 s in eight steps.

Statistics

Data are reported as mean $\pm$ standard deviation. Significance was assessed using one-way analysis of variance except at Figure 8A, where a two-way analysis of variance was used. Student-Newman-Keuls or Tukey *post hoc* tests were performed, depending upon whether the distribution of the pertinent data was consistent with normality.

Results

Histological and immunohistochemical characterization of native cartilage

Analysis of the cartilage explants was performed as a basis for comparison with the *ex vivo* cartilage constructs described above. As expected, both bovine and human native cartilage displayed metachromasia indicative of the presence of sGAGs and PGs, and this metachromasia increased with the depth of cartilage (Fig. 1). Bovine cartilage expressed collagen type II in both the territorial (arrowheads) and interstitial matrix (arrows), along with limited intracellular staining (Fig. 1). Type II collagen within the human cartilage was preferentially localized to the interstitial matrix (Fig. 1, arrows). Both bovine and human cartilage exhibited collagen type VI localized to the territorial matrix (arrowheads), as has been previously described^{13,14} (Fig. 1), though some interstitial matrix staining was observed in the young bovine cartilage (arrows). Collagen type I was localized within the superficial zone of the articular surface in both the bovine and human cartilage, as is also consistent with previous literature¹⁵ (Fig. 1, arrows).

Histological and immunohistochemical analysis of ex vivo-generated cartilage

As described above, sets of SCs were evaluated at 24-h intervals over the first 7 days of culture, and then after 2, 4, and 6 weeks of growth (not all time points shown). Intracellular metachromasia was observed after 1 day in culture,

FIG. 1. Histological characterization of native bovine and human cartilage. Sections were stained with thionin to estimate proteoglycan distribution via metachromasia, and reacted with antibodies against collagen II, collagen VI, and collagen I. In both bovine and human cartilage, metachromasia increased with depth from the articular surface. Collagen II was found in both the territorial (arrowheads) and interstitial matrix (arrows). As expected, collagen VI was localized in the pericellular regions within human articular cartilage (arrowheads). Collagen VI was found in both the pericellular (arrowheads) and interstitial (arrows) zones in native bovine cartilage. Collagen I was localized to a small band in the superficial zone in both samples (arrows). Asterisk indicates articular surface. Color images available online at www.liebertonline.com/ten.

with progressive development of metachromatic ECM staining thereafter (Fig. 2). Lacunae formation around chondrocytes, associated with the development of territorial and interstitial ECM, was observed after 3–4 days, with further development of these features at later time points. Collagen type II progressively accumulated in the interstitial matrix with increased time in culture (Fig. 2). Collagen type VI reached maximal expression after 2–3 days in culture and exhibited pericellular localization throughout all time points (Fig. 2). After 6 weeks in culture, SCs did not display collagen I at levels above the minus-primary control (not shown), suggesting that the original collagen I gel had been fully remodeled.

Human SCs were grown in a variety of medium conditions to promote synthesis and accumulation of ECM. Histological results representing the results of four separate experimental conditions and time points are shown in Figure 3. Under each set of conditions, human chondrocytes remained viable

throughout the 4-week culture period and displayed a limited degree of intracellular and territorial metachromasia (Fig. 3A–D). However, human SCs did not accumulate significant interstitial ECM, and did not appear to be undergoing active synthesis of type II collagen (Fig. 3E–H).

Biochemical analysis of native and ex vivo-generated cartilage

Biochemical assays were performed to quantify sGAG content and HP content of native and *ex vivo*-generated cartilage. Bovine SCs grown in the absence of ascorbate developed approximately the same amount of sGAGs per unit dry weight as the native bovine cartilage after ~4 weeks in culture, with some decline in sGAG content observed at 6 weeks. Bovine SCs grown in the presence of ascorbate exceeded the sGAG amount of native bovine cartilage after ~2 weeks and continued to accumulate sGAGs up to 6 weeks in culture (Fig. 4A).

FIG. 2. Histological characterization of bovine SCs grown in Opti-MEM medium with ascorbate. Histological sections were analyzed with thionin, along with antibodies against collagen II, collagen VI, and collagen I. There was increased extension of proteoglycan and collagen II into the interstitial matrix with time in culture. Collagen VI reached maximal staining at 2–3 days in culture, and retained its pericellular localization. Collagen I staining diminished after ~5 days in culture, and was nearly undetectable at 6 weeks. d, day; wk, week; SCs, spot cultures. Color images available online at www.liebertonline.com/ten.

The total HP content of SCs grown in the medium with or without ascorbate did not approach the value seen in native bovine cartilage (Fig. 4B). However, SCs grown in the presence of ascorbate displayed increased HP content compared with SCs grown without ascorbate. Neither the sGAG nor HP content of human SCs approached the total content of native human cartilage (Table 1).

MRI analysis of native and engineered cartilage

Figure 5 shows the average T_2 , k_m , and MTR values for native bovine and human cartilage by zone, and for ascorbate-treated bovine SCs over time. Native bovine and human cartilage both showed a shorter T_2 in the middle and deep zones compared with the value in the shallow zone (Fig. 5A),

FIG. 3. Efficacy of various medium formulations to induce matrix synthesis from human SCs. (A, E) Ham's F-12+2% FBS+50 μ g/mL Asc; (B, F) Ham's F-12+10% FBS; (C, G) Ham's F-12+10% FBS+50 μ g/mL Asc; (D, H) Opti-MEM+50 μ g/mL Asc; time points as indicated. Thionin staining (A–D) illustrates little metachromasia outside small regions of territorial matrix. Immunostaining for collagen II (E–H) also displays little matrix staining, though minimal territorial matrix staining appears in the (F) and (G) groups. Asc, ascorbate; FBS, fetal bovine serum; wk, week. Color images available online at www.liebertonline.com/ten.

consistent with the increase in PG content with depth seen in Figure 1. As seen in Figure 5B, T_2 values for the SCs decreased with time, indicating development of matrix, but remained higher than those in the explant tissue. This is consistent with the fact that the matrix content in the SCs, while increasing, did not reach the levels of native cartilage. Values for k_m and MTR, generally reflective of collagen content, were greater in the middle and deep zones of native tissue than in the shallow region. SCs showed an increase in k_m and MTR with time, consistent with the denser collagen II immunostaining shown in Figure 2. Human SCs did not develop sufficient tissue integrity to permit MR evaluation.

Figure 6 shows the FCD results for the native cartilage by zone, and for the SCs over time. As expected, the magnitude of FCD increased with depth in both explant systems (Fig. 6A). In the SCs (Fig. 6B), the magnitude of the FCD increased in the SCs through 4 weeks, but decreased at the 6-week time point.

Effects of cytokines on neocartilage growth and recovery

Histology and immunohistochemistry of the control SCs displayed intense territorial and interstitial metachromasia,

along with extensive staining for collagen type II (Fig. 7A, B, E, F). Cytokine treatment for 1 week resulted in nearly a complete loss of metachromasia and collagen II immunostaining (Fig. 7C, D), consistent with the significantly reduced total sGAG content in cytokine-treated SCs compared to control SCs (Fig. 8A, No Recovery). Bovine chondrocytes in SCs were not able to regain comparable levels of sGAGs and FCD (Fig. 8A, B, 2wk Recovery); indeed, they were unable to initiate ECM neosynthesis even after 2 weeks of recovery following 1 week of cytokine exposure (Figs. 7G, H and 8A). No intracellular metachromasia or neosynthesis of collagen II was observed. The level of sGAGs in the 2wk Recovery samples was not significantly different from SCs sacrificed immediately after cytokine treatment (No Recovery, Fig. 8A).

Discussion

Healthy, native cartilage represents a rational standard for comparison of tissue-engineered cartilage. Therefore, it is important to develop experimental paradigms that examine specific characteristics of native tissue compared with *ex vivo*-generated cartilage, including biochemical, biomechanical properties, and MR properties.

FIG. 4. (A) Biochemically determined sulfated glycosaminoglycan (sGAG) content of bovine SCs grown in Opti-MEM medium with or without 50 µg/mL ascorbate. sGAG content of native bovine cartilage is also presented for comparison. SCs grown without ascorbate reached similar levels of sGAG content to native bovine cartilage after 4 weeks in culture. SCs grown with ascorbate reached similar levels of sGAG content to native bovine cartilage after 2 weeks in culture, and exceeded native cartilage after 4 weeks (* $p < 0.05$). d, day; wk, week. (B) Biochemically determined hydroxyproline (HP) content of bovine SCs grown in Opti-MEM medium with or without 50 µg/mL ascorbate. Results for native bovine cartilage are also presented for comparison. Numerical results reflect both the underlying collagen I gel scaffold and the collagen elaborated by neocartilage, but can, nevertheless, be compared between the two groups presented. Ascorbate-treated SCs accumulated significantly higher HP content at the indicated time points (* $p < 0.05$); however, HP values did not reach those of the native tissue. d, day; wk, week.

The findings in this study support the use of collagen I as a scaffold for the *ex vivo* generation of neocartilage from isolated chondrocytes. In comparison to native cartilage, bovine *ex vivo*-generated cartilage was hyaline in appearance and exhibited typical major ECM proteins localized to their proper regions either in the territorial (collagen VI) or in the interstitial matrix (collagen II and sulfated PGs). This indicates that the young bovine chondrocytes remained differentiated,

even in the presence of type I collagen. Additionally, the presence of ascorbate had a profound impact on the ability of bovine constructs to accumulate sGAGs over time. Ascorbate increased the total amount of HP at each time point, although HP did not increase over time. We hypothesize that this is caused by the turnover of collagen I and its replacement with newly synthesized type II collagen, as the HP assay does not discriminate between the two collagens. Ascorbate has been

TABLE 1. BIOCHEMICAL CONTENT OF HUMAN SPOT CULTURES AND NATIVE HUMAN CARTILAGE

Tissue source	Treatment	Time in culture	µg sGAG/µg dry weight	µg HP/µg dry weight
Human cartilage	n/a	n/a	0.104 ± 0.045	0.024 ± 0.001
Human SCs	2% FBS+Asc	2 weeks	0.000 ($n = 1$)	0.002 ($n = 1$)
Human SCs	10% FBS	4 weeks	0.011 ± 0.012	0.009 ± 0.001
Human SCs	10% FBS+Asc	4 weeks	0.003 ± 0.009	0.008 ± 0.0001
Human SCs	Opti+Asc	4 weeks	0.052 ± 0.086	0.006 ± 0.003

Asc, ascorbate; FBS, fetal bovine serum; HP, hydroxyproline; SCs, spot cultures; sGAG, sulfated glycosaminoglycan; n/a, non-applicable.

shown to stimulate ECM production and organization,¹⁶ as well as cell proliferation, differentiation, and metabolic activity,¹⁷ and our results substantiate the findings of others. Ibold *et al.* showed that despite the form or concentration of ascorbate applied, supplementation resulted in upregulation of collagen type II, aggrecan, and cartilage oligomeric matrix protein mRNA in high-density pellet cultures.¹⁸ Ronzière *et al.* showed that with high seeding density in three-dimensional collagen sponges, ascorbate induced chondrocyte proliferation and upregulated the expression of mRNAs coding for cartilage matrix proteins.¹⁹ In monolayer, ascorbate treatment results in the prevention of de-differentiation in a subpopulation

of chondrocytes, but not all chondrocytes. Round, undifferentiated cells immunostained positively for collagen type II, but not for collagen type I or fibronectin; nonround cells de-differentiated.²⁰ This indicates that ascorbate influences chondrocytes to remain in a differentiated state in monolayer, but also suggests that monolayer culture will not be optimal for generating *ex vivo* cartilage.

Young, native bovine cartilage used as a source material for chondrocytes exhibited typical cartilage matrix molecules such as sulfated PGs, collagen II, and collagen VI. The distribution of collagen II and collagen VI was different from the pattern observed in mature cartilage. Specifically, colla-

FIG. 5. (A) Magnetic resonance analysis of native bovine ($n = 12$) and human ($n = 7$) cartilage. T_2 is inversely correlated with overall macromolecular content; both bovine and human samples show a decrease in T_2 with distance from the articular surface. Magnetization transfer rate (MTR) and k_m correlate primarily with the collagen content of the matrix; both bovine and human samples show increasing values with depth. (B) Magnetic resonance analysis of ascorbate-treated bovine SCs ($n = 5$). SCs show a decrease in T_2 with increased time in culture, reflecting progressive deposition of matrix, but values remained significantly lower than those in native cartilage. SCs show an increase in k_m and MTR with time in culture, but values did not reach those seen in native bovine cartilage. Asterisks indicate statistical significance ($p < 0.05$).

FIG. 5. (Continued).

gen II was found within intra- and pericellular regions, whereas collagen VI expression was localized to the superficial zone. It is well documented in the literature that bovine articular cartilage undergoes biochemical changes throughout development and postnatal maturation, more so with respect to collagen composition and networking than PGs.^{21–24} Lee *et al.* showed that chondrons could not be isolated from fetal or juvenile bovine cartilage.²⁵ Sherwin *et al.* described the expression and localization of collagen VI in the developing bovine femoral head as strongest in the superficial zone and the pericellular region around the cells in this zone.²⁶ We have observed that bovine cartilage isolated from 2- to 4-year-old cows displayed collagen II localized to the interstitial matrix and collagen VI expressed throughout the depth of the cartilage and localized in the pericellular region (data not shown). In this regard, our bovine SC constructs more closely resembled mature bovine articular cartilage than the very young tissue from which the

chondrocytes were derived. We hypothesize that the matrix molecules in our SC constructs are undergoing a more rapid maturation process than would occur *in vivo*, which may be caused by a number of things such as culture conditions or the heterogeneity of chondrocytes in young bovine cartilage, some of which will undergo further hypertrophy and ossification at the subchondral bone interface.

OA and degenerative joint disease (DJD) affect at least 20 million people in the United States and cost billions in medical care and lost wages.²⁷ The successful generation of implantable constructs from mature cartilage biopsies or mesenchymal stem cells that achieve integration into the surrounding tissue has the potential to revolutionize DJD treatment and surgical options. Model systems under study for the tissue engineering of cartilage include the use of biodegradable polymeric scaffolds such as polyglycolic acid^{28,29} and poly-L-lactic acid,^{30,31} high-density pellet or suspension culture,^{6,18} bioreactors,^{1,32,33} and agarose,³⁴ collagen,^{35,36} and fibrin³⁷ scaffolds alone or in combination. Collagen, as a natural material, has some advantages over synthetic polymers in terms of tissue compatibility. Collagen is widely used as a scaffold for the tissue engineering of skin, lung tissue, and skeletal tissues such as bone and cartilage.^{38–41} However, the control of porosity available in polymeric and hybrid scaffolds^{42–44} is an important advantage that may prove to be a crucial component in the successful generation of human cartilage to be used surgically. With difficulties in obtaining sufficient numbers of human chondrocytes without monolayer expansion, the focus should be on maximizing the ECM formation and retention from smaller pools of chondrocytes, perhaps through the use of polymeric scaffolds. Currently, the most viable technique for treatment of advanced OA/DJD is the total knee arthroplasty. However, therapies such as autologous chondrocytes implantation (also known as the Carticel® procedure), where autologous chondrocytes that have been expanded in monolayer are injected beneath a periosteal flap that has been sewn over the cartilaginous defect, along with autologous allografts, subchondral drilling, and matrix-associated autologous chondrocytes transplantation are being used with mixed postsurgical success.⁴⁵

Human chondrocytes in the SC model remained viable and exhibited some territorial ECM, but never formed extensive three-dimensional tissue. It has been shown that human chondrocytes in monolayer derived from OA patients can be stimulated to upregulate ECM molecules and down-regulate catabolic molecules such as matrix metalloproteinase-13 through medium treatments such as insulin-like growth factor-1 and transforming growth factor-beta1.⁴⁶ In our study, the relative youth and metabolic activity of the bovine chondrocytes most likely allowed for the effective turnover of the gelatinous collagen type I and the neosynthesis of collagen type II. However, the aged and diseased human chondrocytes used in this study likely did not have the catabolic or anabolic capacity to turnover and re-generate ECM proteins to the extent that would be required for surgical implantation and integration into the surrounding tissue. It is possible that with the proper stimulation, these cells could support the interstitial accumulation of ECM molecules based on their viability and synthesis of territorial matrix. Although the use of younger, healthier human chondrocytes would be optimal, autologous implantation of *ex vivo*-generated cartilage will

FIG. 6. (A) Magnetic-resonance-determined fixed charge density (FCD) of native bovine and human cartilage. FCD is a negative quantity, since the matrix-fixed charges are negative. An increase in the magnitude of FCD reflects increasing sGAG deposition. The magnitude of FCD increases with depth from the articular surface in both native bovine ($n = 12$) and human cartilage ($n = 7$). (B) FCD of ascorbate-treated bovine SCs ($n = 5$). The magnitude of FCD increases through 4 weeks in culture, with a decline seen 6 weeks. The FCD never reaches the level seen in the explant tissue. Asterisks indicate statistical significance ($p < 0.05$).

mostly likely occur in older patients whose chondrocytes may already be undergoing a disease process. The increased study of mesenchymal stem cells in both natural and synthetic constructs may prove to be key if these older, fully differentiated chondrocytes cannot be stimulated to form new cartilage on a replicable basis.

The noninvasive analysis of cartilage is an important adjunct in the diagnosis of joint disease, and biochemical data that can be derived from MR parameters will be useful in prescribing the appropriate treatment as matrix-specific therapies become available. While the T_2 relaxation time of cartilage is dependent upon a number of tissue parameters, it has been demonstrated to decrease with the development of more solid matrix in engineered constructs. Thus, T_2 can be regarded as an approximate marker for cartilage quality in this setting. In our studies, there was in fact a decrease in cartilage T_2 over development time of the SCs, consistent with our biochemical findings of progressive matrix development. However, the T_2 of SCs remained higher than the value for the native tissue, which is consistent with a lower

level of overall macromolecular content in the SCs compared to the native tissue. In native bovine and human cartilage, interpretation of the depth-wise variation of T_2 in terms of matrix composition is complicated by the layered structure of native cartilage. In particular, the dependence on depth of collagen fiber orientation⁴⁷ results in a depth dependence of T_2 , as well as a dependence of T_2 values on the orientation of the sample within the MRI magnet.⁴⁸ Additional complexities apply to both native tissue and the SCs. These include the fact that T_2 is dependent upon both CS and collagen content.⁴⁹ Further, the measurement of tissue T_2 values is based on a fit of MRI-derived relaxation data to a single decaying component, whereas in reality there is the potential for multiple compartments within tissue, each exhibiting its own T_2 relaxation time. Indeed, a multiexponential analysis has revealed such multiple compartments within cartilage, with provisional assignments to collagen, PG, and free water.⁵⁰

Investigation of observed MT ratios and apparent rate (k_m) of cartilage matrix has demonstrated a strong dependence on

FIG. 7. Thionin staining and collagen II immunohistochemistry of control (Opti-MEM medium with ascorbate) and cytokine-treated (Opti-MEM medium with ascorbate, interleukin-1 β , and tumor necrosis factor- α as indicated in Materials and Methods section) SCs. (A, B) Control SCs grown for 4 weeks; (E, F) control SCs grown for 6 weeks, without the addition of cytokines. SCs sacrificed immediately after 1 week of cytokine treatment (C, D), while SCs exposed to cytokines for 1 week and then grown in the absence of cytokines for a further 2 week recovery period (G, H); see text for experimental details. Extracellular matrix depletion resulting from the cytokine treatment is evident in all treated samples (C, D, G, H). Even after 2 weeks of recovery, bovine chondrocytes in SCs are unable to re-initiate extracellular matrix neosynthesis (G, H). Color images available online at www.liebertonline.com/ten.

collagen concentration. Seo *et al.* reported a significant increase in MTR with increasing collagen concentration in solution, as well as a relative lack of dependence of MT on CS content.⁵¹ Laurent *et al.* showed an increase in MT with increasing concentration of both collagen I and collagen II in solution, with the former exhibiting a somewhat stronger effect.⁵² The authors attribute this difference to the sample preparation, which may have resulted in a greater degree of crosslinking in the collagen I sample. Indeed, this effect of crosslinking on MT and k_m has been studied directly.⁵³ We found that native human cartilage exhibited a greater MTR and k_m than bovine cartilage in spite of its lower collagen content, consistent with a greater degree of crosslinking in human as compared to bovine cartilage.⁵⁴ MTR and k_m increased with time in the SC system, although never reaching the values observed in the explants. This is consistent with the substantially lower collagen content of the SCs. Of note is that while MTR and k_m increased with culture time in the SC system, the HP content remained relatively unchanged. This again is consistent with development of a more heavily crosslinked collagen matrix.

Fixed negative charges responsible for osmotic activity in cartilage are primarily associated with sGAGs.⁵⁵ Because negative FCD is the functional constituent of the matrix responsible for attracting water and thus imparting resistance to loading, nondestructive quantification of FCD through use of the MRI Gd exclusion method has been widely used to evaluate cartilage quality. In terms of tissue characteristics,

MRI evaluation of FCD is complimentary to biochemically derived sGAG content, particularly because different disaccharides differ with respect to their ability to contribute to FCD. For example, CS contributes -2 moles of charge, while keratan sulfate contributes -1 mole of charge per mole of disaccharide.⁵⁶

Of note is the fact that while native bovine cartilage was found to have more than twice the amount of CS by dry weight than did human cartilage, the MRI-derived FCD was only $\sim 10\%$ greater. This may be attributable to the greater water content in bovine cartilage,⁵⁷ as FCD represents the number of charged species per wet sample volume. For both types of tissue, our finding of increasing FCD with distance from the articular surface is consistent with previous MRI measurements using dGEMRIC,⁵⁸ which have recapitulated earlier biochemical measurements of this depth dependence.⁵⁹

In spite of the fact that the sGAG content normalized to dry weight of the SC system after 4 weeks exceeded that of native bovine cartilage, the FCD remained substantially lower. This could be accounted for by the much greater degree of hydration in the SCs. In addition, little is known regarding the nature of the disaccharides produced by chondrocytes in collagen I scaffolds.

Remodeling of the ECM demonstrates the chondrocyte's capacity to respond to physiological signals. We determined here that bovine chondrocytes in the SC model system have the capability to upregulate matrix degrading enzymes in

FIG. 8. (A) Biochemical determination of sGAG content of cytokine-treated SCs (control and cytokine treatments as specified in Fig. 7). Both cytokine-treated groups (No Recovery and 2wk Recovery, labeled +Cytok) showed significantly decreased sGAG content as compared with control SCs grown in the absence of cytokines (labeled -Cytok, $*p < 0.05$). Even after 2 weeks of control growth postcytokine treatment, there is no quantifiable increase in sGAG content compared with SCs sacrificed immediately after cytokine treatment ($^{\wedge}p = 0.58$). (B) Magnetic-resonance-determined FCD in SCs as assessed after 2 weeks of recovery following 1 week of cytokine exposure (2wk Recovery), and values for corresponding control SCs (6 weeks of control growth, $n = 5$). See text for full experimental details. As seen, there was a persistent deficit in FCD in the cytokine-exposed SCs.

response to treatment with the cytokines IL-1 β and TNF- α , as evidenced by the histological breakdown of PGs and collagen II, and diminished biochemical sGAG content. These cellular responses have long been described in chondrocytes,^{60,61} so it is not surprising that bovine chondrocytes in the SC system were able to receive and respond to these signals. In fact, chondrocytes in cartilage explants from pigs responded to similar concentrations of IL-1.⁶² However, even after 2 weeks of recovery time, the chondrocytes did not show neosynthesis of ECM molecules. Whether this is the result of irreversible damage, phenotype alteration, or insufficient time allowed for recovery cannot be determined without further study. These results suggest that inserting healthy constructs into diseased joints might not be effective, because of the wealth of degradative signals already present and active within the joint.

There are some limitations to the techniques used in this study; for example, the immunohistochemical data were

observed and described rather than quantified through computer software. Additionally, these biochemical assays cannot differentiate between different types of GAGs and collagens. Further quantitative analysis of these *ex vivo*-generated cartilage constructs is currently underway, including qRT-PCR determination of ECM gene expression. As yet, it is unclear whether the human SCs are limited in their secretion of ECM molecules due to decreased mRNA expression.

In summary, we have presented a model of *ex vivo*-generated bovine cartilage with properties similar to its native tissue. However, we have also demonstrated that despite similar biochemical characteristics, *ex vivo*-generated bovine cartilage does not exhibit a spatial distribution of MR parameters comparable to that of native bovine cartilage. Human chondrocytes in this model remained viable but did not produce ECM to a degree that would permit surgical implantation studies.

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Disclosure Statement

No competing financial interests exist.

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