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Characterization of a Cartilage-Like Engineered Biomass Using a Self-Aggregating Suspension Culture Model: Molecular Composition Using FT-IRIS

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Abstract

Maintenance of chondrocyte phenotype and robust expression and organization of macromolecular components with suitable cartilaginous properties is an ultimate goal in cartilage tissue engineering. We used a self-aggregating suspension culture (SASC) method to produce an engineered cartilage, “cartilage tissue analog” (CTA). With an objective of understanding the stability of phenotype of the CTA over long periods, we cultured chondrocytes up to 4 years and analyzed the matrix. Both early (eCTAs) (6 months) and aged (aCTAs) (4 years) showed type II collagen throughout with higher concentrations near the edge. Using Fourier transform-infrared imaging spectroscopy (FT-IRIS), proteoglycan/collagen ratio of eCTA was 2.8 times greater than native cartilage at 1 week, but the ratio was balanced to native level ($p = 0.017$) by 36 weeks. Surprisingly, aCTAs maintained the hyaline characteristics, but there was evidence of calcification within the tissue with a distinct range of intensities. Mineral/matrix ratio of those aCTA with “intensive” calcification was significantly higher ($p = 0.017$) than the “partial,” but when compared to native bone the ratio of “intensive” aCTAs was 2.4 times lower. In this study we utilized the imaging approach of FT-IRIS and have shown that a biomaterial formed is compositionally closely related to natural cartilage for long periods in culture. We show that this culture platform can maintain a CTA for extended periods of time (4 years) and under those conditions signs of mineralization can be found. This method of cartilage tissue engineering is a promising method to generate cartilaginous biomaterial and may have potential to be utilized in both cartilage and boney repairs.

Keywords

cartilage tissue engineering; biomaterials; self-aggregating suspension culture; cartilage tissue analog; Fourier transform-infrared imaging spectroscopy

Articular cartilage is the articulating surface covering the end of bone and provides a flexible support to normal load bearing and shear stress.^{1,2} Cartilage has unique tensile strength and resistance against compressive force due to the interaction of its the two most abundant extracellular matrix molecules, fibrillar collagen type II, and proteoglycan (PG). Repair of this tissue is challenging and many tissue engineering approaches have been taken to aid in the restoration of damaged cartilage with the necessary biochemical and mechanical properties.³ However, tissue engineered cartilage generated by isolated chondrocytes often loses hyaline phenotype in vitro culture condition, and eventually turns to fibrocartilage. Thus, maintenance of chondrocyte phenotype and robust production and organization of macromolecular components in the correct balance of Extracellular Matrix (ECM) with suitable cartilage-like properties is ultimate goal in cartilage tissue engineering.^{4,5} Many culture methods and scaffolds have been tested with as many various outcomes.⁶⁻⁹ Interestingly, most studies show that PG synthesis in tissue engineered cartilage is more robust than collagen synthesis although PG content is four to five times less than collagen content in normal cartilage.¹⁰⁻¹² A proper ratio of PG and collagen in the ECM is crucial for effective absorption and distribution of mechanical loads.¹³ Many culture platforms including those scaffold-based and those established with pellet culture have supported growth of chondrocytes or mesenchymal stem cells and produced cartilaginous materials with mechanical and biochemical properties approaching those of cartilage.¹⁴⁻¹⁷ However few extend the culture time beyond a few weeks to several months. In one longer study, Freed et al.¹⁸ reported a production of tissue engineered cartilage during a period of up to 7 months. However most studies demonstrate outcomes based on a short-term culture. In fact, there is no definitive evidence as to whether tissue engineered cartilage can continue to grow and maintain hyaline phenotype over time in vitro, or be ossified as a result of chondrocyte hypertrophy.¹⁹ Our objective was to determine if maturing chondrocytes in engineered cartilage could become hypertrophic and the ECM mineralized in vitro. While calcification would be a concern in engineered biomaterial used in surface cartilage defects such biphasic material might have potential to be utilized in osteochondral defects.²⁰ Our study was designed to assess the maintenance of phenotype over extended periods in culture and was designed on the hypothesis that longer pre-culture periods may alter cell function and result in changes in the cartilage-like properties. Our study was a fore-runner to in vivo studies where the true maintenance of phenotype will be challenged.

Previously, we showed a self-aggregating suspension culture (SASC) method that is scaffold-free and high cell density and forms a biomass with hyaline cartilage chondrocyte phenotype and remarkable matrix productivity.²¹⁻²³ This simple method results in scaffold-free and high cell density cartilage-like material we refer to as a “cartilage tissue analog” (CTA). The CTAs grown for short and long-term were defined as early (eCTA; 6 months) and aged (aCTA; 4 years). While our method bears some similarities to other techniques including pellet cultures using high cell densities,²⁴⁻²⁶ our method is fundamentally different than most in that cells never attach to any culture surface or artificial substrate due to a culture dish coating of the hydrogel poly-2-hydroxyethyl methacrylate (poly-HEMA). In this model, chondrocytes are cultured at high densities, self-aggregate, float above the hydrogel coating, and quickly produce robust ECM providing a natural scaffold in which to grow. We have used this model to produce a CTA with phenotypically appropriate gene

expression as evident with extended culture periods.²² This SASC method is free from potential problems related for scaffold degradation and toxicity and we demonstrate that this SASC model is also able to be customized to size and dimensions by adjusting an initial cell density, volume of culture media, and using different size of culture well.

This study seeks to characterize biochemical content of CTA after in vitro culture for up to 4 years using Fourier transform-infrared imaging spectroscopy (FT-IRIS) and other assessment techniques. The novel technique has extensively been utilized to identify composition, structure, and changes of biological tissues such as bone and cartilage at the molecular level.²⁷

Methods

Cartilage Tissue Analog Culture

Cartilage was obtained from the femoral condyle and head of young Yorkshire cross pigs (1–3 months old). Minced cartilage pieces were placed in a plastic Petri dish with collagenase (type II, Worthington, Lakewood, NJ) solution, and chondrocytes were isolated by digestion overnight at 37°C in a 5% CO₂. Extensively washed and suspended chondrocytes were plated at 2×10^7 cells/ml/well in a 24-well plate coated with poly-HEMA (Low cluster plates, Corning, Corning, NY) to prevent cell attachments. In some cases, wells were custom coated with 1% poly-HEMA (Polysciences, Warrington, PA) in 90% ethanol. The cells formed a biomass within a couple of hours post-inoculation, and were cultivated from day 0 up to 6 months (eCTA) and 4 years (aCTA). All CTA were cultured in DMEM (Mediatech, Inc., Manassas, VA) added with 10% fetal bovine serum and supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (2.5 µg/ml), L-glutamine (2 mM) (Lonza, Walkersville, MD), 1% MEM vitamin (Mediatech, Inc.), ascorbic acid (50 µg/ml) (Gibco-Invitrogen, Carlsbad, CA), and HEPES buffer (25 mM) (Gibco-Invitrogen). CTAs were fed twice a week with the media described above by a complete change of media (2 mL per each well). Constructs were harvested at 2 weeks (n = 3), 12 weeks (n = 2), 24 weeks (n = 4), 36 w (n = 3), and 4 years (n = 10).

Fourier Transform-Infrared Imaging Spectroscopy

CTA were fixed in 80% EtOH/1× cetylpyridinium chloride (CPC) for 24 h and embedded in paraffin and sectioned at 7 µm. Section mounted onto barium fluoride window was dehydrated completely to avoid signal interference from water molecules. FT-IR data acquisition was carried out using a Spectrum Spotlight 300 spectrometer (Perkin-Elmer, Waltham, MA), and the data were analyzed using ISys 5.0 software (Malvern Instruments Ltd., Worcestershire, UK). FT-IRIS methods were followed as previously described²⁷ and macromolecular characteristics of ECM components, type II collagen and aggrecan, were identified based on pure components mixtures.²⁸ Characteristic absorbance peaks of bone and cartilage were observed at specific frequencies (wavenumber, cm⁻¹); collagen-amide I (1,650 cm⁻¹), PG-sugar (1,050 cm⁻¹), mineral-phosphate (1,034 cm⁻¹), carbonate (870 cm⁻¹) respectively. The area ratios of PG-to-collagen-amide I (PG/collagen), mineral-to-matrix (mineral/matrix, same as phosphate/amide I) and carbonate-to-phosphate (carbonate/phosphate) demonstrated the relative amounts of PG, mineral, carbonate,²⁹ respectively. The

peak height ratios (PHR) of 1,660/1,690 cm^{-1} and has been shown to be related to collagen maturity and crystallinity, respectively.²⁷ To examine the relative amount of PG (PG/collagen) in CTAs compared to native cartilage tissues, we utilized previously processed FT-IRIS data from juvenile bovine and human OA cartilage with a low Mankin grade of 3.5 as normal/natural cartilage controls. In addition, the ratios of mineral-to-matrix, carbonate-to-phosphate, collagen maturity and, crystallinity were determined to identify calcification of early and aged CTAs, and these parameters were compared to the FT-IR values from normal cortical bone. These components determined by FT-IRIS were considered as a representative of mineralized tissue, respectively.

Immunohistochemistry and Histology

Procedures to obtain the histological sections were described in FT-IRIS method section above. To assess type II collagen, tissue sections were deparaffinized, and to ensure complete blocking of nonspecific staining, sections were blocked using 10% normal goat serum in 2.5% bovine serum albumin (BSA; Roche Diagnostics Corp., Indianapolis, IN), and 0.2% nonidet (BDH Chemicals Ltd., Poole, England) in 1× TBS solution. Endogenous peroxidase activity was neglected pretreating sections with hydrogen peroxide and ultra V-block solution (Ultravision, Labvision, Fremont, CA). Type II collagen immunohistochemistry was performed using a commercially available antibody that works with several species (including porcine) by utilizing a conserved epitope (Chondrex, Redmond, WA) with dilution of 1:750 for 2 h in a humid chamber at room temperature. A HRP Polymer detection system with AEC chromogen (Labvision) was used following the manufacturer's specifications. Serial sections were stained with Alcian blue and von Kossa to examine PG and for mineralization, respectively. Sections were examined at low (2×) and high (40×) magnification, and representative images were presented.

Statistical Analysis

One-way analysis of variance (ANOVA) with Holm–Sidak test was carried out to examine statistical significance ($p < 0.05$) in FT-IRIS parameters using SigmaStat 3.5 software (SYSTAT Software, Inc., Chicago, IL).

Results

Histology

Establishment of CTA using mature porcine chondrocytes was carried out. We show that by modifying the starting cell number and dimensions of the culture vessel this suspension culture method can result in biomasses of different sizes, both in diameter and thickness. This is shown in Figure 1 where distinctly different size CTAs are formed after 6 months using 100×10^6 cells in a 6-well plate (top and bottom left) and 20×10^6 cells in a 24-well (top and bottom right). For the remainder of the study 20×10^6 were used in a 24-well culture plate. The CTAs were formed by self-assembly within several hours and were cultivated for 6 months (eCTA) to 4 years (aCTA) (Fig. 2). Appearance by gross examination shows a single opaque disk formed with dimensions of 4–5 mm in diameter by 2–3 mm in thickness. Histological sections of CTAs (Fig. 3) demonstrated distribution of cells within an abundant ECM. Both eCTA and aCTA showed abundant type II collagen by

immunohistochemistry, and the collagen appeared higher in concentration near the edges (Figs. 3A,B and 4). Alcian blue staining showed that eCTA (Fig. 3C) had greater PG content than aCTAs (Fig. 3D), and the PG content was distributed near the center, which was opposite to the collagen distribution. No evidence of calcification occurred at an early time point (eCTA) as shown using von Kossa stain (Fig. 3E) while aCTAs showed calcification as a result of long-term culture. The calcification was more intensive near the edge (Fig. 3F). In general, there were chondrocytes of various sizes throughout ECM of all aCTAs (Fig. 4B).

Fourier Transform-Infrared Imaging Spectroscopy

To determine how similar the compositional properties of CTAs were compared to normal cartilage, we examined ratio of PG to collagen-amide I (PG/collagen) using FT-IRIS. The FT-IRIS-determined PG content normalized by collagen-amide I (PG/collagen) is shown in Figure 5. It is observed that CTAs produced relatively significant levels of collagen and that compositional changes occurred with time in culture. While the PG/collagen ratio of eCTAs (eCTA: 0.46) was 2.8 times higher than that of normal cartilage (human OA Mankin 3.5 grade: 0.15 and bovine: 0.18) at 2 weeks, the ratio of eCTAs was nearly similar to that to the native levels by 24 weeks (eCTAs: 0.22). With growth, the PG/collagen ratio in CTAs decreased over time, and became equivalent to the normal cartilage level at 36 weeks ($p = 0.017$). At the later time point (4 year), however, the ratio showed a slight increase. Based on the FT-IRIS analysis and associated histological analysis, no mineralization occurred in eCTAs at all time tested (Fig. 6A). Conversely, three ($n = 3/10$) of aCTAs showed both characteristics of cartilage and bone, with partial calcification in a distinct region (Fig. 6B,C), while seven ($n = 7/10$) of aCTAs were more intensively calcified on the entire section without any remaining un-mineralized region (Fig. 6D). FT-IRIS determined intensity and distribution of mineral content (mineral/matrix area ratio) in both partially and intensively calcified aCTAs was supported by von Kossa stain (Fig. 6E–G). In addition, the mineral content (mineral/matrix area ratio) of the intensively mineralized aCTAs was significantly higher ($p = 0.017$) than partially mineralized aCTAs (Fig. 7A). Further, the carbonate content (carbonate/phosphate area ratio) ($p = 0.025$) (Fig. 7B) and crystallinity ($p = 0.017$) (Fig. 7D) of intensively mineralized aCTAs were also significantly different from eCTA values. However, all the parameters of aCTAs were less when compared to the parameters of native cortical bone (dotted line).

Discussion

A high-density SASC method produces a cartilage-like biomass by essentially producing its own scaffold and organized matrix due to the high density and rapid coalescing of chondrocytes within the initial 24 h. In this model cells become biosynthetically very active within the first 3 days and continue to increase in mass for long periods.²¹ This long-term study demonstrates the macromolecular components of CTA using FT-IRIS, and assessed the changes in ECM production, phenotype stability, and included assessments for potential mineralization as a result of long-term cultivation. For the purpose of studying tissue engineered cartilage few bioengineered materials have been studied for these extended periods of time in culture. Our CTA contain abundant type II collagen and PG, in similar

ratio in comparison to natural cartilage and this study supports using the SASC model is a pertinent tool for cartilage tissue engineering particularly given its ease of use and lack of foreign materials. A previous study also proves that biomechanical properties of CTA are comparable to many scaffold-based engineered constructs.²² Distribution of type II collagen and PG of eCTA was observed through the tissue section and at all times, and characteristically type II collagen is more abundant near the edge and PG more concentrated in the core of the CTA. The ECM compositional distribution pattern was very consistent in all CTAs. An increase in collagen synthesis and cellular inhomogeneity near the edge may be due to a direct contact of nutrients to the tissue surface, or changes in oxygen tension. However, what causes the pattern is not fully understood, and although there are spatial differences in the staining for collagen II and PG both are expressed throughout the CTA. Different spatial distribution of collagen and PG in CTAs is consistent with other studies and with variations in native cartilage.³⁰ In the future studies, exploring how this SASC/CTA platform reorganizes over time in vivo is the next obvious step; testing this material in a cartilage defect in vivo.

In this present study, we demonstrate for the first time the macromolecular composition of CTA of various ages using FT-IRIS, an emerging powerful tool to study molecular change in biological tissues including cartilage and bone.²⁷ Proximity of PG/collagen ratio of CTA to native cartilage by 24 weeks indicates that robust collagen synthesis is progressive, and may improve tensile strength of CTA over time. As compared with CTA, a tissue engineered cartilage grown in a hollow fiber bioreactor (HFBR) was very different, and while the ratio of PG/collagen is seen like this in other engineered material it may in part be due to ascorbic acid which was lacking in the HFBR-grown material.¹¹ This supplement was not utilized at that time because the study was to optimize physical growth conditions including perfusion flow rate and cell density using mammalian chondrocyte in the HFBR system. Although the FT-IRIS determined ratio (PG/collagen) cannot imply that mechanical properties of CTAs are close to that of native cartilage, this remains to be determined by other studies. We have, however, previously published the mechanical properties of CTA grown in this manner for 8 and 16 weeks with remarkable properties given the system is based on cell aggregation and not a scaffold.¹⁸ The CTA model we describe here provides a model with an ability to grow cartilage-like material and as demonstrated after certain periods of time elaborates an ECM with PG/collagen ratio more similar to native cartilage which is in contrast to what is observed in many other engineered cartilage analogs which typically produce more PG than collagen.¹¹ FT-IRIS derived PG/collagen ratios at 4 years were slightly increased, and it could be due to the mineralization of the hypertrophic cartilage (Fig. 4). Although the precise timing of when mineralization initially occurred in CTA was not clear, the process began with focal areas of evidence of calcification in CTA. Since minute numbers of cells that could have been carried through the initial isolation and digestion which were close to or part of the growth plate so it can not be ruled out that these cells were there from the beginning. Regardless of where or when the cells appeared both characteristics of cartilage and bone were coexistent in distinct regions in the same tissue at 3.5 years time point, and the mineralization process was more complete by 4 years. Abrahamsson et al.²⁰ also observed hypertrophic mineralization from newly produced ECM in hMSC-based tissue-engineered cartilage. This long-term observation of CTA

demonstrates a potential utility of CTA that can be applied for cartilage and subchondral bone repair. FT-IRIS determination of mineralized CTAs compared to normal cortical bone as a control shows that the mineralization is in progress. Particularly, the disparity in mineral content (mineral/matrix) and carbonate content (carbonate/phosphate) indicates an intensity of mineralization between partially and intensively calcified aCTAs, but both relative quantities are much less than normal cortical bone.

In summary, the utility of the SASC model for cartilage tissue engineering is promising and the study is unique in that it examines CTA at multiple time points up to 6 months and for 4 years, an exceptionally long period of time for any culture study. Incorporation of FT-IRIS along with more routine outcome measures makes this study unique given the time frame of engineered material studied. FT-IRIS provides the identification of minute alterations and molecular characteristics of CTA. This study shows evidence of phenotype duplicity that emerge over time, which could result from a change in phenotype in culture or result from cells that begin with that phenotype. Further study of this model using known mixed and ultra purified cell populations will help us to understand the mechanism of the in vitro calcification. Knowing how to manipulate the phenotype of chondrocytes in this culture model may provide new ways to engineer cartilage constructs that contain bone portions that ultimately are useful in clinical interventions related to full thickness defects such as those resulting from traumatic injury and disease.

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Figure 1. Size customization of cartilage tissue analog (CTA). Top view (A) and side view (B) of CTAs. CTA formation using 100×106 cells in a 6-well plate (top and bottom left) and 20×106 cells in a 24 well (top and bottom right) after 6 months culture. CTA can be customized in size and thickness by adjusting cell density, tissue culture, or media volume. Scale bar: 1.0 mm.

Figure 2. Early CTA (eCTA) and aged CTA (aCTA). Gross appearance of CTA generated from porcine chondrocytes and matured in culture for 6 months (eCTA) (A) and 4 years (aCTA) (B). Scale bar: 1.0 mm.

Figure 3. Histological evaluation of CTA. CTA were stained for type II collagen by immunohistochemistry (A,B), Alcian blue (C,D), and von Kossa stain (E,F). Scale bar: 1.0 mm.

Figure 4.

Cellular heterogeneity of CTA. Regions from eCTA (A) and aCTA (B) show the differences in cellularity of the two age groups. Images are higher magnifications of identical tissue shown in Figure 2A and B, respectively. Chondrocytes appear smaller and heterogeneous the early time point (A), while the chondrocytes near the edge were larger in diameter than near the center in later culture (B). Scale bar: 60 μ m.

Figure 5.

Maturation of ECM in CTA: PG/collagen ratio by FT-IRIS. FT-IRIS-determined data of PG/collagen ratio of CTA are shown from 2 weeks to 4 years. Normal juvenile bovine (§) and human OA cartilage (with a low Mankin grade 3.5) (§) were compared to CTA, $n = 3$ (2 weeks), $n = 2$ (12 weeks), $n = 4$ (24 weeks), $n = 2$ (36 weeks), and $n = 3$ (4 year) ($p < 0.05$).

Figure 6.

Temporal changes of ECM composition in CTA. FT-IRIS was used to demonstrate that all eCTAs and some aCTAs maintained cartilage-like characteristics through long-term culture periods. In CTA of 36 weeks (A) cartilage-like characteristics are seen without evidence of calcification (E). Some aCTAs ($n = 3/10$) also maintained cartilage-like characteristics (B), with minimal calcification (C) identified peripherally. Other aCTAs ($n = 7/10$) representatively shown in (D) were intensively calcified throughout. FT-IRIS-determined calcification (partial or intensive) of aCTAs and parallel sections were assessed sections with von Kossa stain (F,G). All FT-IR spectrum are from CTAs (panel H). Solid spectrum was from eCTA (A) and partially cartilaginous portion of aCTA (B), and dotted spectrum was from intensively calcified aCTAs (C and D). [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/jor>].

Figure 7.

FT-IRIS-determined bone-like characteristics of aCTA. FT-IRIS-determined data are presented of the mineral content (mineral/matrix) of intensively calcified aCTAs was significantly greater than partially calcified aCTAs ($p < 0.05$) (A) while carbonate content (carbonate/phosphate) was significantly lower ($p < 0.05$) (B). No differences were determined for both partially and intensively calcified aCTAs on the collagen cross-linking parameter (C), and the intensively calcified aCTAs were also significantly greater in crystallinity ($p < 0.05$) (D). (Dotted line indicates FT-IRIS-determined values of normal cortical bone.)