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Engineered cartilage using primary chondrocytes cultured in a porous cartilage-derived matrix

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Abstract

Aim—To investigate the cell growth, matrix accumulation and mechanical properties of neocartilage formed by human or porcine articular chondrocytes on a porous, porcine cartilage-derived matrix (CDM) for use in cartilage tissue engineering.

Materials & methods—We examined the physical properties, cell infiltration and matrix accumulation in different formulations of CDM and selected a CDM made of homogenized cartilage slurry as an appropriate scaffold for long-term culture of human and porcine articular chondrocytes.

Results—The CDM scaffold supported growth and proliferation of both human and porcine chondrocytes. Histology and immunohistochemistry showed abundant cartilage-specific macromolecule deposition at day 28. Human chondrocytes migrated throughout the CDM, showing a relatively homogeneous distribution of new tissue accumulation, whereas porcine chondrocytes tended to form a proteoglycan-rich layer primarily on the surfaces of the scaffold. Human chondrocyte-seeded scaffolds had a significantly lower aggregate modulus and hydraulic permeability at day 28.

Conclusions—These data show that a scaffold derived from native porcine articular cartilage can support neocartilage formation in the absence of exogenous growth factors. The overall characteristics and properties of the constructs depend on factors such as the concentration of CDM used, the porosity of the scaffold, and the species of chondrocytes.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Keywords

articular cartilage; collagen; decellularized; extracellular matrix; osteoarthritis; proteoglycan; stem cell; tissue engineering

The limited self-repair capacity of cartilage has posed significant clinical challenges in the fields of orthopedic and plastic surgery. For example, it is estimated that more than 1 million procedures to treat defects of knee cartilage are performed per year [1]. Approximately half of these defects are sufficiently severe to require the replacement or regeneration of damaged articular cartilage [2]. The lack of consistent therapeutic success in the conventional treatment has stimulated cell-based approaches such as autologous chondrocyte implantation [3,4]. A recent randomized controlled study, however, suggested little difference in the efficacy of autologous chondrocyte implantation over microfracture of the subchondral bone [5].

Nonetheless, the tissue engineering approach remains a promising avenue to repair cartilage defects in a functional manner [6–8]. For such applications, primary chondrocytes have been combined with various scaffolds or hydrogels capable of supporting the synthesis of functional extracellular matrices with encouraging results [9–18]. Examples of naturally occurring sea-derived scaffolds include agarose, alginate and chitosan [9–18]. Some other commonly used biomaterials are fibrin [19], collagen matrix/alginate composites [20], hyaluronic acid [8,21], polyester elastomers [22], polyethylene glycol [23,24], nonwoven porous α -hydroxy esters of polylactic acid, polyglycolic acid and copolymers of the two [16,22,25,26]. While the use of such biomaterials have yielded tissue engineered constructs that demonstrate the formation of cartilage-specific extracellular matrix (ECM) molecules, they have required the presence of exogenous growth factors or prolonged periods of *ex vivo* culture to achieve the appropriate structure and functional properties before implantation. Thus, it would be advantageous for a biomaterial scaffold to support chondrocyte biosynthetic activity both *in vitro* and *in vivo* in a manner that accelerates tissue growth and associated functional biomechanical properties of the engineered construct.

In this regard, it has been shown that ‘bioactive’ scaffolds that provide environmental cues to the cells seeded within them can better control, and in some cases accelerate, the synthesis and assembly of a functional neo-tissue. In particular, there has been growing interest in the use of processed native tissues in this context, with the hypothesis that native tissues contain growth factors and ECM molecules that can strongly influence cell activity. For example, Urist first proposed the use of demineralized bone matrix to induce ectopic bone formation *in vivo* [27]. Recent studies have also indicated that ECM derived from various tissues can provide a bioactive scaffold [28–32]. Such tissue-derived scaffolds may provide a natural microenvironment for cell migration and differentiation to contribute to tissue regeneration. However, tissue-derived scaffolds must be processed to decrease immunogenicity, and, while some of the focus has been on human allograft tissues, many of these tissue-processing methods can usually be applied to xenogenic transplants owing to the highly conserved nature of ECM components maintained between species [33].

Along these lines, it has been demonstrated that chondrocytes produce suitable morphogenetic factors that induce chondrogenic differentiation of mesenchymal stem cells *in vitro* and *in vivo* [34]. We previously hypothesized that these cartilage morphogenetic proteins would be retained in the ECM of cartilage and could serve as a potential source of endogenous biologically active molecules that can promote cartilage formation. In this regard, we recently reported on the development of a porous, interconnected scaffold derived solely from articular cartilage for stem cell-based cartilage tissue engineering [35].

This cartilage-derived matrix (CDM) could provide a novel scaffold material for exogenous cell seeding and tissue growth for cartilage repair, either as an adjunct to autologous chondrocyte transplantation or microfracture. While these CDM scaffolds have been shown to support the chondrogenic differentiation of both adiposederived stem cells as well as bone marrow-derived mesenchymal stem cells [35,36], the influence of these scaffolds on the behavior of primary chondrocytes is not known.

The goal of this study was to determine the ability of porcine CDM to support chondrogenesis by human and porcine chondrocytes. Primary chondrocytes were seeded onto porous CDM scaffolds and maintained in culture for up to 4 weeks. The composition, histologic appearance and mechanical properties of the constructs were determined at different time points throughout the culture period.

Materials & methods

Preparation & characterization of scaffolds

Porous scaffolds were created from porcine articular cartilage as described previously [35]. Briefly, full-thickness porcine cartilage from femoral condyles of freshly sacrificed skeletally mature (2–3 years old) female pigs was harvested and finely minced using scalpels. The minced particles were suspended in distilled water and homogenized using a tissue homogenizer to form cartilage slurry, as described previously [35]. The homogenized tissue suspension was centrifuged, the supernatant removed and the precipitate tissue particles were resuspended in distilled water at concentrations of 0.05, 0.1 and 0.2 g/ml. Aliquots (0.75 ml) of the slurry were placed in wells of a 48-well plate, snap frozen in liquid nitrogen and then lyophilized for 24 h. The resulting ‘spongy’ constructs were cut using a biopsy punch and scalpel to form constructs 6 mm in diameter by approximately 1.5 mm thick. These specimens were sterilized using ethylene oxide before cell culture.

The lyophilized CDM scaffolds were pulverized into fine powder by a freezer mill (SPEX SamplePrep 6770, Metuchen, NJ, USA). The CDM powder was press-fit into a mold, and reflection Fourier transform infrared (FT-IR) data were acquired at 8 cm^{-1} spectral resolution using an attenuated total reflection-FT-IR imaging system (Varian, Palo Alto, CA, USA). Intact porcine cartilage harvested from the femoral condyle was also analyzed as a control.

For scanning electron microscopy, CDM samples with different concentration were fixed and sputter coated with gold. The prepared scaffolds were then viewed with a FEI XL30 Environmental Scanning Electron Microscope (ESEM). The pore size of the CDM constructs was determined on the ESEM pictures using Scion Image (Scion Corp., Frederick, MD, USA).

The porosity of the unseeded scaffolds was determined quantitatively by liquid displacement as we have reported previously [35]. To determine the swelling ratio of the CDM, three dry scaffolds from each group were weighed (W_d) and immersed into phosphate-buffered saline at room temperature for 24 h. After removing the unabsorbed solution using filter paper, the wet weight (W_w) of the CDM constructs was recorded. The swelling ratio of the scaffold was defined as the ratio of the weight increase ($W_w - W_d$) to the initial weight (W_d) [37].

Chondrocyte isolation & culture

Human chondrocytes were harvested from hip and knee cartilage of patients undergoing joint replacement surgery ($n = 5$, age range: 54–73 years). Porcine chondrocytes were harvested from femoral condyles of knee cartilage from adult female pigs ($n = 5$). The cells were isolated using sequential pronase (Calbiochem, San Diego, CA, USA) and collagenase

(Worthington, Lakewood, NJ, USA) digestion, as previously described [38]. Chondrocytes were plated on 225 cm² culture flasks (Corning, Corning, NY, USA) and cultured overnight at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 15 mM HEPES (Gibco, Grand Island, NY, USA) and 0.1 mM nonessential amino acids (Gibco). On the second day, porcine chondrocytes were pooled and seeded onto the scaffolds immediately after cell harvest. Human chondrocytes were frozen prior to use to allow time for collection of five specimens. Once five specimens were obtained, the human cells were passaged once more and pooled before seeding onto the CDM scaffolds. A previous study showed no significant dedifferentiation of the chondrocytes after one passage [39]. The cells were resuspended in culture medium at 500,000 cells per 30 µl and seeded by pipetting 30 µl directly on the scaffolds, which were placed in 24-well low-attachment plates (Corning). The constructs were incubated at 37°C for 1 h to allow the cells to diffuse into and attach to the scaffolds before adding 1 ml of culture medium in each well. The culture medium consisted of Dulbecco's modified Eagle's medium-high glucose (Gibco), 10% fetal bovine serum (Atlas Biologicals, Ft. Collins, CO, USA), 1% penicillin–streptomycin (Gibco), l-ascorbic acid 2-phosphate (37.5 µg/ml; Sigma, St. Louis, MO, USA), 0.1 mM nonessential amino acids and 10 mM HEPES. The medium was changed every 2–3 days. Cultures were terminated at defined time points (day 2, 7, 14 and 28) during the evaluation of cell proliferation and chondrogenesis.

At day 2 and 7, porcine and human chondrocyte-seeded 0.05, 0.1 and 0.2 g/ml CDM constructs (n = 3 per group) were harvested and viewed using confocal microscopy (LSM 510, Zeiss, Thornwood, NY, USA) after staining with calcein AM (Invitrogen, Carlsbad, CA, USA) to view live cells, and Texas Red[®] C2-dichlorotriazine (Invitrogen) to view the CDM scaffold. Confocal microscopic images of blank 0.1 g/ml CDM scaffolds were also obtained as a control.

Assays for chondrogenesis

At each time point (7, 14 and 28 days), chondrocyte–CDM constructs (n = 3) were digested by incubating in 1 ml of papain (papain [125 µg/ml; Sigma] and 100 mM phosphate buffer, 10 mM cysteine, 10 mM EDTA, pH 6.3) for 24 h at 65°C. Total DNA content was measured fluorometrically using the PicoGreen[®] fluorescent dsDNA assay (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol (excitation wavelength: 485 nm; emission wavelength: 535 nm). Glycosaminoglycan (GAG) content was determined using bovine chondroitin sulfate as a standard and measuring sample content with the dimethylmethylene blue assay [40]. Total collagen content was determined by measuring the hydroxyproline content of the scaffolds after acid hydrolysis and reaction with *p*-dimethylaminobenzaldehyde and chloramine-T, using 0.134 as the ratio of hydroxyproline to collagen [41].

Histology & immunohistochemistry

For histology and immunohistochemistry, three constructs from each group were fixed overnight at 4°C in a solution containing 4% paraformaldehyde in a 100 mM sodium cacodylate buffer (pH 7.4), dehydrated in graded ethanol solutions, embedded in paraffin, cut into 6-µm thick sections in the center of the constructs and mounted on SuperFrost[®] microscope slides (Microm International AG, Volketswil, Switzerland). To stain for sulfated GAGs, a minimum of three sections were treated with hematoxylin for 3 min, 0.02% fast green for 3 min and 0.1% aqueous Safranin-O solution for 5 min, rinsed with distilled water and dehydrated with xylene. Immunohistochemical analysis was also performed on 6-µm sections, using monoclonal antibodies to type I collagen (ab6308, Abcam, Cambridge, MA, USA), type II collagen (II-II6B3; Developmental Studies Hybridoma Bank, University of Iowa, IA, USA), type X collagen (C7974, Sigma) and chondroitin 4-sulfate (2B6; gift from

Dr Virginia Kraus, Duke University Medical Center, NC, USA). Digest-All™ (Zymed, South San Francisco, CA, USA) was used for pepsin digestion on sections for type I, II and X collagen. Sections to be labeled for chondroitin 4-sulfate were treated with trypsin and then with soybean trypsin inhibitor and chondroitinase (all from Sigma). The Histostain-Plus ES Kit (Zymed) was used on all sections for serum blocking before secondary antibody labeling (antimouse IgG antibody, Sigma Catalog No. B7151), and subsequent linking to horseradish peroxidase. Aminoethyl carbazole (Zymed) was used as the enzyme substrate/chromogen. The appropriate positive controls for each antibody were prepared and examined to ensure antibody specificity (human cartilage for type II collagen and chondroitin 4-sulfate, calcified zone of cartilage for type X collagen and ligament for type I collagen). Negative controls (without primary antibody) were also prepared to detect any nonspecific labeling.

Chondrocyte-CDM constructs were analyzed at two time points (7 and 28 days) for mechanical testing ($n = 3-5$ per group). Cylindrical plugs were punched from the central regions of the engineered cartilage tissue using a biopsy punch to ensure circular geometries for mechanical testing. Creep experiments were conducted in confined compression using an electromechanical materials testing system (ELF 3200, Bose, Minnetonka, MN, USA) [42]. Briefly, specimens were placed in a confining chamber in a phosphate-buffered saline bath at room temperature, and compressive loads were applied using a rigid porous platen. Following equilibration of a small tare load (1–5 gf), a step compressive load (5–25 gf, dependent on time point such that final equilibrium strain was between 15 and 20%) was applied to the sample and allowed to equilibrate for 2000 s. The biphasic compressive aggregate modulus (H_A) and hydraulic permeability (k) were determined using a three-parameter, nonlinear least-squares regression procedure [42,43].

Statistical analysis

One-way analysis of variance with Scheffé's *post-hoc* test was applied to compare pore size and swelling ratio of different CDM scaffolds, and biochemical data of chondrocyte-seeded CDM at different time points. Student's *t*-test was applied to compare mechanical properties of chondrocyte-seeded CDM at two time points. The analyses were performed using STATA software (Stata Inc., College Station, TX, USA). Statistically significant values were defined as $p < 0.05$.

Results

Characterization of the scaffolds

A representative FT-IR spectrum obtained from pig knee articular cartilage is shown with labeled absorbance bands of interest demonstrated by previous studies [44,45]. Tissues were dehydrated prior to analysis to avoid masking the absorbance bands of the CDM or cartilage. The CDM absorbance bands matched the bands found in the infrared spectrum of intact porcine cartilage (Figure 1), suggesting the processing steps for creating CDM did not alter the chemical composition of cartilage ECM.

By liquid displacement analysis, the porosity of the 0.05 g/ml CDM ($97.5 \pm 1.0\%$), 0.1 g/ml CDM ($95.3 \pm 2.8\%$) and 0.2 g/ml CDM ($95.9 \pm 0.8\%$) were determined (mean $\pm$ standard deviation, $n = 3$). No significant difference in the porosity among the three CDM scaffolds was detected. We also evaluated the pore size of the scaffolds using ESEM (Figure 2). The 0.2 g/ml CDM scaffold had an average pore size of $98.2 \pm 15 \mu\text{m}$, significantly smaller than those of 0.05 g/ml CDM ($191 \pm 63 \mu\text{m}$) or 0.1 g/ml CDM ($175 \pm 70 \mu\text{m}$) (mean $\pm$ standard deviation, $n = 30$; Figure 3A).

In swelling experiments, all specimens could absorb 20- to 40-fold of physiological fluid and still maintain their form stability. However, the swelling ratio significantly differed with concentration, with 0.05 g/ml CDM exhibiting the highest swelling ratio of 40.4 (Figure 3B).

Cell penetration in scaffolds with different density

Staining of blank 0.1 g/ml CDM scaffolds demonstrated no cell survival after the homogenization and lyophilization process to make CDM from native cartilage. Following chondrocyte seeding, cross-sectional microscopic pictures of the constructs showed poor cell penetration in 0.2 g/ml CDM but good penetration in 0.1 g/ml and 0.05 g/ml CDM scaffolds (Figure 4A & B, images of 0.05 g/ml CDM not shown). To balance between enhancing native cartilage content and promoting cell penetration, 0.1 g/ml CDM was chosen for use in the further experiments. Good cell penetration was also observed in human chondrocyte-seeded 0.1 g/ml CDM (Figure 4C).

Biochemical results

The mean dsDNA content of human chondrocyte-seeded CDM scaffolds was approximately 2.5-fold higher than the porcine chondrocyte-seeded constructs on day 7 and 14. However, as the dsDNA content decreased to 1.90 μg in the human chondrocyte group and increased to 1.76 μg in the porcine chondrocyte group, the difference was not obvious by day 28. In the human chondrocyte group, the GAG content decreased from 109.7 μg (day 7) to 83.4 μg (day 14), followed by an increase to 108.2 μg (day 28); the collagen content also increased to 100.8 μg (day 28) after a decrease from 105.7 μg (day 7) to 66.0 μg (day 14). However, the collagen and GAG content in the porcine chondrocyte group demonstrated opposite trends over the same period. The GAG content increased from 129.7 μg (day 7) to 142.5 μg (day 14), followed by a decrease to 116.0 μg (day 28); the collagen content decreased to 114.4 μg (day 28) after an increase from 109.1 μg (day 7) to 129.2 μg (day 14). For either group, the difference of dsDNA, GAG and collagen content among different time points was not statistically significant (Figure 5).

Histology & immunohistochemistry

From histological analysis, significant deposition and accumulation of ECM components was evident within the human and porcine chondrocyte-seeded constructs during *in vitro* culture from day 14 to 28 (Figure 6). On day 28, gross morphologic differences were noted among the CDM scaffolds seeded with human or porcine chondrocytes. In general, the human chondrocyte-seeded scaffolds appeared more opaque than the porcine chondrocyte-seeded scaffolds. Safranin-O staining at day 28 showed abundant GAG formation of the porcine chondrocyte-seeded CDM scaffolds, especially at the periphery of the constructs. Human chondrocyte-seeded CDM scaffolds also exhibited intense GAG staining, but in contrast to that observed with the porcine chondrocyte-seeded CDM, the GAG-rich regions were present throughout the construct (Figure 6). Immunohistochemical analysis further showed that the pores of the CDM scaffold were filled with cartilaginous tissue by day 28 (Figure 7). By day 28 in culture, both human and porcine chondrocyte-seeded CDM scaffolds were rich in collagen type II and chondroitin 4-sulfate. The immunohistochemical labeling pattern of collagen corresponded to that of Safranin-O staining for both cell types at all time points. In addition, all constructs stained slightly positive for type I collagen, and were absent for type $\times$ collagen (Figure 7).

Mechanical properties

The compressive aggregate modulus (H_A) of the human chondrocyte-seeded scaffolds decreased significantly from a value of 0.040 MPa (range: 0.031–0.056 MPa) on day 7 to

0.021 MPa (range: 0.015–0.028 MPa) on day 28, while the modulus of porcine chondrocyte-seeded scaffolds slightly increased from 0.042 MPa (range: 0.031–0.046 MPa) to 0.045 MPa (range: 0.037–0.057 MPa). From day 7, the hydraulic permeability (k) of the human chondrocyte-seeded scaffolds decreased approximately fivefold by day 28 in culture to a value of $0.0055 \text{ mm}^4/(\text{N}\cdot\text{s})$, while the permeability of the porcine chondrocyte-seeded scaffolds decreased approximately 1.5-fold to a value of $0.0182 \text{ mm}^4/(\text{N}\cdot\text{s})$ (Figure 8). However, the hydraulic permeability did not exhibit statistical difference between day 7 and 28 data in either the human or porcine chondrocyte group.

Discussion

The findings of this study indicate that a scaffold composed purely of homogenized and reconstituted native cartilage can support the growth and matrix accumulation of neocartilage by primary chondrocytes *in vitro*. As no exogenous growth factors were required to support tissue formation, these findings suggest that a CDM scaffold may have potential in augmenting chondrocyte-based cartilage tissue repair approaches, as has been shown with techniques such as matrix-induced autologous chondrocyte implantation [46]. Ultimately, the success of such procedures will require a more thorough understanding of the interaction of cells, the biomaterial scaffold and growth factor signaling [47], as the cellular response will depend largely on the chosen cell type and the nature and structure of material used [36,48].

The ECM not only mediates cell attachment and provides key cues to cells, but it also binds growth factors and subsequently limits their diffusion [49]. Therefore, several previous studies have examined the potential for ECM-based biomaterials, including cartilage-derived ECM scaffolds, to support tissue growth [35,50,51]. An important question that remains involves the potential mechanism by which such scaffolds support chondrogenesis. For example, chondrocyte growth and biosynthetic activity may be promoted by direct interactions with cartilage ECM molecules that are present in the CDM scaffold [52]. Alternatively, the cartilage ECM may serve as a source of bound growth factors such as TGF- β , bone morphogenetic protein-2 or FGF [53,54], which then may be released over time during remodeling of the CDM. Further studies will be necessary to determine the factors by which CDM affects cartilage formation by primary chondrocytes or stem cells.

We employed FT-IR to analyze the porous biomaterial scaffold used in this study as FT-IR has emerged as a valuable technique for the characterization of cartilage composition over the past few years [45,55]. One of the advantages of this technique in cartilage research is its ability to determine the content and spatial distribution of specific molecular components in cartilage. The characteristic spectral features for the primary cartilage components, such as collagen and proteoglycans, have been well described using this technique [55,56]. The absorbance spectrum of CDM corresponded with the spectrum of intact pig articular cartilage, from which CDM is made. Since the material used for making CDM consisted of pig cartilage only, other naturally derived materials, such as polysaccharides, which have similar IR absorbance values of approximately 1640 and 1550 cm^{-1} , would not account for the IR absorbance measured in the CDM. The corresponding FT-IR spectra between CDM and native cartilage thus served to strongly suggest that the major ECM components of cartilage have been minimally manipulated and preserved in the manufacture of CDM.

By varying the cartilage ECM concentration before lyophilization, we obtained highly porous CDM scaffolds that had different physical properties, including pore size and swelling ratio. Scaffolds with smaller pores have a greater surface area, which provides increased sites for cell attachment after seeding [57]. However, scaffold permeability decreases with decreasing pore size, which may hinder the initial cell penetration and

subsequent nutritional diffusion into the scaffold [58]. Hence, the pore size can play an important role in the biological function of CDM. Although CDM with a higher concentration may provide more chondrogenic signals as well as cell attachment sites, cells were only able to penetrate approximately 800 μm in 0.2 g/ml CDM, suggesting that the smaller pore size impeded cell penetration throughout the scaffold. Pore size alone is not the only factor in determining cell penetration and distribution within the matrix. Indeed, it is quite possible that the morphology of the scaffold and the formed pores as a function of CDM density may have also contributed to the final penetration and distribution of the cells within the scaffold.

Large animal models such as sheep and swine play an important role in the development and evaluation of tissue engineered cartilage repair strategies [59]. It is therefore interesting to investigate the difference in chondrocyte biology among species and test the transferability of animal data to human clinical studies. However, previous interspecies comparison studies regarding primary chondrocytes have been performed in monolayer or pellet culture [59–61] and thus may have limitations in their application and predictive capabilities when applying these studies to functional tissue engineering in a human clinical setting. Since we previously showed encouraging results with our CDM scaffold using adult stem cells [35,62,63], we also investigated potential species variation and phenotype differences when culturing chondrocytes on this bioactive CDM scaffold. In monolayer culture, it has previously been reported that proliferation was higher in animal-derived chondrocytes compared with human chondrocytes [59,61], although our data revealed higher dsDNA content in human chondrocyte-seeded CDM on day 7 and 14. The discrepancy may be explained by the application of different culture models and the absence of exogenous growth factors in our culture system. With respect to ECM synthesis after monolayer expansion, previous studies suggested that human chondrocytes shared more similarity with porcine than with ovine or equine chondrocytes [59]. Nevertheless, our results demonstrated some differences in the spatial distribution of cartilage-specific ECM in CDM scaffolds, despite comparable overall quantities of matrix formation. It is also noteworthy that overall collagen and GAG content remained relatively constant over the culture period in both groups. However, abundant cartilaginous ECM deposition was demonstrated by histology and immunohistochemistry on day 28, and it potentially alludes to the degradation rates of the native CDM scaffolds and synthesis rates by the seeded cells being equal, thereby resulting in a net-zero change in collagen and GAG content as measured by the hydroxyproline and dimethylmethylene blue assays, respectively.

The histology and immunohistochemistry also revealed some differences in the pattern of tissue formation between the CDM constructs of each cell type. Despite confocal microscopy showing good penetration of pig and human chondrocytes into CDM at an early stage of culture, day 28 histology showed the preference of pig chondrocytes to grow on the surface of CDM scaffolds. A layer of cartilage-like tissue, rich in GAG and collagen type II, was noted at the periphery of the CDM scaffold, while the center of the construct remained relatively porous without significant cartilaginous ECM deposition. It is possible that the newly formed GAG-rich tissue in the scaffold periphery prevented nutrient diffusion, resulting in poor cell proliferation and differentiation in the central part. Confocal microscopy also showed complete penetration of human chondrocytes into CDM at day 7. However, the resultant histology and immunohistochemistry showed some differences in comparison to those of pig chondrocyte-seeded CDM. GAG and collagen type II were distributed throughout the entire construct in human chondrocyte-seeded CDM, but the microstructure of the new matrix was more fibrous, not resembling the dense structure of native cartilage. The fibrous structure of human chondrocyte-deposited ECM appeared more porous, suggesting that the different ECM deposition patterns derived from human and porcine chondrocytes subsequently determined the tissue distribution in CDM scaffolds.

Despite the difference in ECM architecture, the chondrogenic state of both types of chondrocyte was largely maintained, evidenced by the deposition of collagen type II and cartilage-specific proteoglycans, and rounded cell morphology. However, light collagen type I staining was noted on day 28 immunohistochemistry of both cell types, suggesting mild dedifferentiation of chondrocytes occurring over the culture period. It is noteworthy that we did not add any chondrogenic factors, such as TGF- β , in the medium, so a certain degree of dedifferentiation of chondrocytes may be inevitable, particularly under conditions that allow chondrocyte proliferation *in vitro*.

Human chondrocytes filled the highly interconnected pores of CDM and deposited a heterogeneous ECM throughout the construct by week 4 as measured by histology. Despite abundant ECM deposition, the aggregate modulus of human chondrocyte-seeded CDM decreased to 0.021 MPa, representing a twofold decrease relative to day 7 samples. On the contrary, the aggregate modulus of pig chondrocyte-seeded CDM increased slightly (0.040–0.045 MPa) from day 7 to 28. Our histology and immunohistochemistry revealed that newly deposited cartilaginous ECM was distributed throughout the entire construct in human chondrocyte-seeded CDM. However, the microstructure of the new matrix was more fibrous and porous, not resembling the dense structure of pig chondrocyte-seeded CDM. The porous structure of human chondrocyte-deposited ECM may have exhibited less mechanical strength. Thus, as the original CDM scaffold degraded in culture medium over time, the overall aggregate modulus of the human chondrocyte-seeded CDM decreased from day 7 to 28. These aggregate moduli values are an order of magnitude lower than those of native articular cartilage (0.5–0.9 MPa) [64], but are on the same range as the aggregate moduli reported in previous studies of 0.100–0.015 MPa after 4 weeks in mechanically stimulated and static culture, respectively [14], and are similar to the data we previously published for the aggregate modulus of human adipose stem cell-seeded CDM (0.079 MPa at day 28) [35]. We obtained our human chondrocyte samples from osteoarthritis patients, so it is not known whether these cells exhibit the same characteristics as those from healthy subjects. However, previous studies have shown that chondrocytes from control and patients with osteoarthritic cartilage express the same ECM molecules at comparable levels when grown on a 3D hyaluronan-based scaffold [65]. Our results are also consistent with a previous study that showed human chondrocytes and mesenchymal stem cells exhibited differential chondrogenic responses due to interactive effects with the scaffold material (chitosan or silk) [66].

Extracellular matrix accumulation in the scaffold also significantly affected the permeability of the scaffolds as the permeability decreased over the culture period in both groups. At 28 days, the porcine chondrocyte-seeded CDM had a significantly higher permeability value than that of the human chondrocyte-seeded scaffolds (0.0182 vs 0.0055 mm⁴/(N·s)), with the latter comparable to that of native articular cartilage. As histology demonstrated that porcine chondrocytes mostly resided in the periphery of the scaffold at the later time points, the porous central portion could help maintain a relatively higher overall permeability value. Human chondrocytes, on the other hand, deposited ECM throughout the scaffold, suggesting more homogenous subsequent neo-tissue generation, and resulting in lower permeability at 28 days compared with porcine chondrocytes. Low permeability may prevent adequate nutrient supply, which is necessary for cell proliferation and ECM production. It may further contribute to the decreased aggregate moduli of human chondrocyte-seeded scaffolds from day 7 to 28.

Conclusion

A proper scaffold material with adequate stiffness, geometry and bioactivity is necessary for generating functional tissue engineered constructs [67,68]. Processed ECM products and

synthetic ECM mimics are promising candidates of such biomaterials for tissue engineering [28,29]. In the present study, we found that a native cartilage-derived ECM scaffold supports chondrogenesis and tissue formation *in vitro* by articular chondrocytes, without the use of exogenous growth factors. Our data further revealed some interspecies difference in human and porcine chondrocytes when cultured in CDM scaffolds, including proliferation rate, mechanical properties, histology and immunohistochemistry. Additional strategies, such as the use of bioreactors or controlled mechanical stimuli, may also accelerate tissue growth and improve the biomechanical properties of these scaffolds [63,69–71].

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Figure 1. Infrared spectra of (A) cartilage-derived matrix and (B) porcine cartilage
Infrared spectrum of porcine articular cartilage showed typical absorbance bands for vibrations of collagen amide bonds (Am I, Am II and Am III) and PG sugar ring absorbance, and these bands are preserved in the infrared spectrum of cartilage-derived matrix. PG: Proteoglycan.

Figure 2. Scanning electron micrographs showed different 3D structures of cartilage-derived matrix scaffolds made from homogenized cartilage slurry with different concentrations (A) 0.2 g/ml; (B) 0.1 g/ml; and (C) 0.05 g/ml.

Figure 3. Physical properties of cartilage-derived matrix scaffolds

(A) Pore size measurement and (B) swelling ratio of CDM scaffolds with different concentrations. Data presented as mean $\pm$ standard deviation (data not sharing the same letter are statistically different from each other, $p < 0.05$).

CDM: Cartilage-derived matrix.

Figure 4. Fluorescent labeling of the scaffold and viable cells

Confocal microscopic pictures of porcine chondrocytes seeding in (A) 0.1 g/ml and (B) 0.2 g/ml cartilage-derived matrix (CDM) scaffolds. Left panel: cross-section view of the day 2 CDM scaffolds; middle panel: cross-section view of the day 7 CDM scaffolds; right panel: surface view of the day 7 CDM scaffolds. Poor penetration of porcine chondrocytes in 0.2 g/ml CDM scaffolds was observed on the cross-section views, and it was further evidenced by high cell density at the CDM surface on day 7. (C) Confocal microscopic pictures of blank CDM and human chondrocytes seeding in 0.1 g/ml CDM scaffolds. Good penetration of human chondrocytes in 0.1 g/ml CDM scaffolds was observed.

Figure 5. Biochemical composition of human and porcine chondrocytes cultured in cartilage-derived matrix scaffolds

(A) dsDNA content, (B) collagen content and (C) GAG content (data presented as mean $\pm$ standard deviation; n = 3). GAG: Glycosaminoglycan.

Figure 6. Histologic images of safranin-O/fast green stain of human and porcine chondrocyte-seeded cartilage-derived matrix scaffolds after 14 and 28 days of culture

(A) Gross view of chondrocyte-seeded constructs on day 28. (B) Day 14 and (C) day 28 histologic low power view (scale = 200 μ m). The red glycosaminoglycan-rich regions are dispersed throughout the cartilage-derived matrix construct in the human chondrocyte group, while they are concentrated at the periphery of the cartilage-derived matrix scaffold in the porcine chondrocyte group. (D) In the day 28 higher power view (scale = 100 μ m), the neo-matrix made by human chondrocytes appeared more fibrous and porous, as compared with the more cartilaginous and dense matrix secreted by porcine chondrocytes.

Figure 7. Immunohistochemistry of human and porcine chondrocytes cultured in cartilage-derived matrix after 28 days of culture

(A) Blank cartilage-derived matrix (CDM) scaffolds; (B) human chondrocyte-seeded CDM; (C) porcine chondrocyte-seeded CDM; and (D) positive controls (human ligament for collagen 1 antibody; human osteochondral sections for other antibodies). Intense staining of chondroitin 4-sulfate and collagen type II were observed in human or porcine chondrocyte-seeded CDM scaffolds, although the distribution of the heavily stained regions was quite different. Scale bar: 200 μ m.

Figure 8. Mechanical properties of chondrocyte-seeded cartilage-derived matrix scaffolds (A) Aggregate modulus (H_A) and (B) hydraulic permeability (k) of human and porcine chondrocyte-seeded cartilage-derived matrix scaffolds on days 7 and 28. Data presented as mean $\pm$ standard deviation; * $p < 0.05$ compared with day 7 data of human chondrocyte-seeded cartilage-derived matrix; $n = 3-5$.