Generation of cartilage-like constructs using continuous expansion culture primary chondrocytes seeded in dense collagen gels.

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INTRODUCTION

Following injury, adult articular cartilage has a poor regenerative capacity often resulting in development of degenerative joint disease (1, 2). Cell-based therapies such as autologous chondrocyte implantation (ACI) are increasingly used clinically to replace damaged cartilage and suppress the progression of joint disease. This procedure requires biopsies of healthy tissue from which cells can be harvested and expanded in monolayer culture prior to application (3, 4). Large populations of cells can be achieved; however, standard culture techniques require multiple passaging of cells with degradative enzymes which leads to rapid loss of chondrogenic phenotype termed dedifferentiation (5, 6). Dedifferentiation often leads to a more fibrotic phenotype in cultured chondrocytes thus rendering them undesirable for tissue engineering applications. To circumvent this caveat, major efforts are being made to devise new culture strategies for enhanced chondrocyte phenotype in monolayer culture.

In an attempt to circumvent chondrocyte dediffentiation, we recently developed a continuous expansion surface culture procedure which effectively retains chondrogenic phenotype in monolayer culture (7). The device uses high extension silicone rubber dishes which are chemically modified for cell adhesion. The dishes are coupled to an iris-like mechanical device which slowly expands to 600% of the initial surface area thereby avoiding passaging of cells. We have found that this culture system retains chondrocyte phenotype by limiting exposure to degradative enzymes and promotes enhanced cartilage-like tissue regeneration. Moreover, the silicone culture dishes can be modified with other biomolecules to further enhance chondrocyte phenotype (8).

In addition to our continuous expansion culture, other culture techniques can aid in chondrocyte phenotype retention. In vivo, chondrocytes grow in a three-dimensional environment and maintain a rounded morphology. In vitro, cells flatten and adhere to rigid culture surfaces. Culture methods using three dimensions, like alginate beads, have shown positive results for enhanced chondrogenic properties (9). Moreover, growth of chondrocytes in three dimensional collagen scaffolds which mimic native matrix seems even more promising for tissue regeneration. We have been developing the use of dense collagen gels in bone and cartilage tissue engineering (10, 11), and such gels may prove useful in applications such as matrix assisted ACI. Here we tested if continuous expansion chondrocytes exhibit enhanced phenotype when seeded in dense collagen gels. Furthermore, we explored whether continuous expansion chondrocytes could produce superior cartilage-like matrix when grown in the dense gels.

METHODS

<u>Chondrocyte isolation and continuous expansion culture.</u>

5g of articular cartilage was dissected from the femoropatellar groove of skeletally mature cows and digested overnight in collagenase type II (1.5mg/mL) as described (7). Continuous expansion (CE) and static silicone (SS) culture dishes were prepared as previously described (7). 10,000 cells/cm^2 were seeded to CE and SS culture dishes and populations were expanded for 10.5 days where SS cultures underwent two passages (30mm to

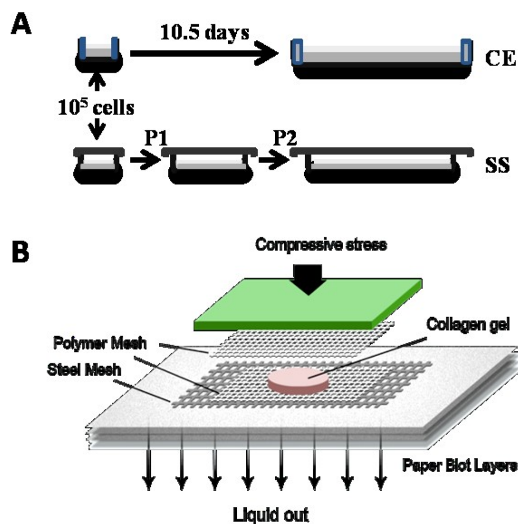

Figure 1. A) Schematic representation of continuous expansion (CE) and static silicone (SS) culture. B) Representation of dense collagen gel production.

60mm to 100mm dishes)(Figure 1A). Media was changed every three days.

Dense collagen gel preparation.

Sterile, rat-tail type I collagen (2.10 mg/ml in 0.6 % acetic acid) was used for collagen self-assembly - achieved by mixing the acidic collagen solution with 10 X MEM (Sigma Aldrich, Ottawa, ON, CA) at a ratio of 4:1 and neutralized with 5 M NaOH (Fisher Scientific, Ottawa, ON, CA). For cellular gels, bovine chondrocytes were seeded within the hydrogels at a density of $3x10^5$ cells/ml of collagen solution, immediately prior to gel polymerization.

The neutralized collagen solution was pipetted into a four-well plate (0.9 ml/well of 4.5 mm height x 16 mm diameter) and placed at 37 °C in a 5 % CO_2 incubator for 30 min. Hydrogels were removed from the moulds and dense gels were produced by plastic compression (PC) as previously reported (12). Briefly, highly hydrated gels were placed on a stack of blotting paper, nylon mesh and metal mesh, and subjected to PC using an unconfined compressive stress of 1 kPa for 5 min, to remove the excess casting fluid (Figure 1 B). After compression, circular gels were cultured under static conditions for 21 days with medium changes at 2-day intervals.

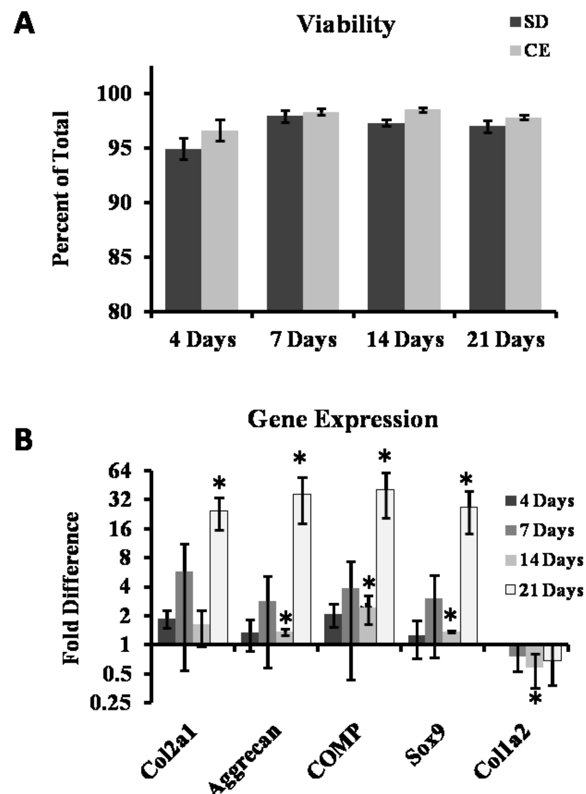

Figure 2. A) Viability was quantified in the seeded hydrogels by LIVE/DEAD assay. B) Gene expression analysis of CE versus SS seeded gels. * indicates $p<0.05$, Student's t-test.

Gene Expression analysis.

RNA was extracted from chondrocyte-seeded hydrogels using TRIzol Reagent (Invitrogen). 500 ng of total RNA was converted to cDNA using a qScript cDNA synthesis kit (Quanta Biosciences). Consequently, 1 mL of each cDNA sample was loaded per reaction (in duplicate) using PerfeCTa SYBR Green FastMix (Quanta Biosciences). Quantitative real-time PCR was carried out using the ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems) with primers exactly as described (7, 8).

Immunofluorescence.

10 μm thick sections were cut using a Leica CM3050 S cryomicrotome. Samples were blocked in permeabilization buffer for 30 min (PBS, 0.1% Triton X-100 and

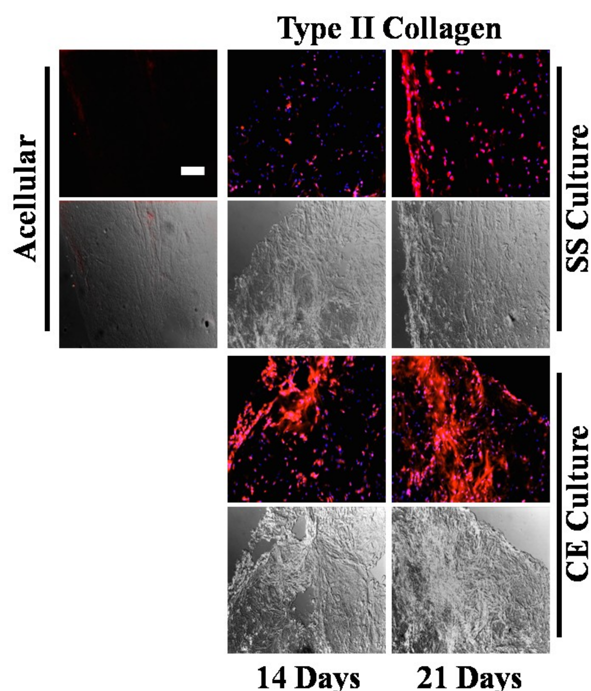

Figure 3. Immunofluorescence detection of collagen type II (red) overlaid on stained cell nuclei (blue, DAPI). DIC images directly below samples. Scale bar: 200μm.

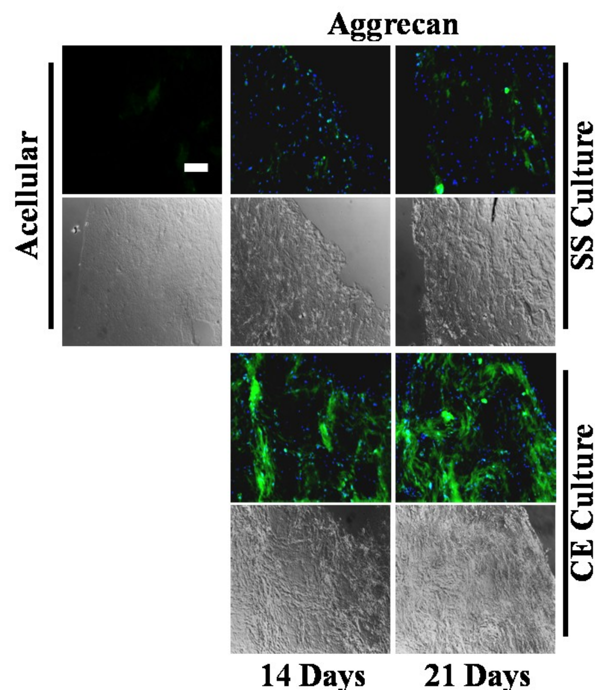

Figure 4. Immunofluorescence detection of aggrecan (green) overlaid on stained cell nuclei (blue, DAPI). DIC images directly below samples. Scale bar: 200μm.

1% BSA) and then incubated with antibodies against collagen type II (1:200; Abcam) and aggrecan (1:200, Developmental Studies Hybridoma Bank, Iowa) overnight at 4^0C. Samples were washed in PBS and incubated with either Alexa Fluor 488 Goat anti-Mouse IgG (1:250; Invitrogen) or rhodamine-conjugated Goat anti-Rabbit IgG (1:250; Sigma) for 1.5 h at room temperature. The samples were washed and mounted with Fluoroshield with DAPI (Sigma) and visualized on an Olympus IX81 inverted fluorescence microscope. Viability of cell in collagen gels was assessed using LIVE/DEAD assay according to the manufacturer's instructions.

RESULTS

<u>CE culture chondrocytes are phenotypically superior when seeded in dense collagen gels.</u>

Chondrocytes were cultured on CE and SS dishes for 10.5 days and then seeded in dense collagen gels as described in Methods, and cultured for 21 days. No differences in viability were detected between CE and SS seeded gels (Figure 1 A). Gene expression analysis revealed significantly higher expression of chondrogenic markers in CE seeded gels compared to SS seeded gels, particularly after 14 and 21 days (Figure 1 B). CE seeded gels also displayed significantly less fibrotic gene expression (Col1a2).

<u>CE culture chondrocytes produce superior chondrogenic matrix when seeded in dense collagen gels.</u>

Significant differences in gene expression were after 14 and 21 days in culture, so these two time points were used to assess production of cartilage-like matrix. Immunofluorescence probing of frozen sections revealed significantly more collagen type II (Figure 3) and aggrecan (Figure 4) matrix deposition in CE seeded gels as compared to SS seeded gels. We also probed for GAG content using histological stains, however, did not detect any differences between CE and SS seeded gels at either time point (data not shown).

DISCUSSION

Growth of chondrocytes in monolayer culture using standard methods often results in loss of chondrogenic phenotype. We have previously shown that chondrocytes grown in CE culture are superior to cells grown in standard culture and redifferentiate more efficiently to form cartilage-like tissue (7, 8). Here we tested if CE culture cells were also superior when seeded in three dimensional dense collagen hydrogels, a biodegradable medium suitable for tissue engineering.

Both CE and SS cultured cells maintained high levels of viability within the hydrogels. Significantly higher chondrogenic gene expression in CE seeded gels indicated a stronger potential for producing cartilage matrix, while suppressing collagen type I. This indicated CE seeded gels retain chondrogenic phenotype while in three dimensions, while SS culture cells remain dedifferentiated. Immunofluorescence analysis indicated that CE seeded gels qualitatively produced more collagen type II and aggrecan, supporting the gene expression data. This data indicated that CE seeded gels produce superior cartilage-like matrix, making them highly suitable for tissue engineering applications. Further testing of gel mechanical properties are required, though, to understand the relationship of our engineered tissue to native cartilage.

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