CO-CULTURE OF PRIMARY AND PASSEDGE ARTICULAR CHONDROCYTES ALLOWS USE OF MONOLAYER EXPANDED CHONDROCYTES TO FORM CARTILAGE TISSUE IN VITRO

Gan L, Anderca M, Kandel RA
CHR-BioEngineering of Skeletal Tissues Team, Mount Sinai Hospital, Toronto, Canada, M5G 1X5
rkandel@mtsniail.on.ca

INTRODUCTION
Insufficient chondrocyte numbers is one of the limitations preventing clinical application of tissue engineering approaches to cartilage repair. Although use of stem cells would circumvent this problem, they have other limitations suggesting that chondrocytes may be the preferred cell source. However, in vitro monolayer expansion to obtain large numbers of chondrocytes results in dedifferentiation of these cells [1]. To redifferentiate the passaged chondrocytes, a variety of approaches have been explored including culture in a three-dimensional (3D) environment such as a gel, a pellet, or a 3D scaffold [2]. However, studies have shown that these redifferentiated chondrocytes still produce collagen type I and small proteoglycans to some extent, indicating that the cartilage phenotype has not been fully restored suggesting the need to identify alternative methods. Co-culture of stem cells with osteoblasts has been shown to induce differentiation of stem cells raising the possibility that co-culture may be one way to affect the differentiation state of passaged chondrocytes [3]. In this study, we examined whether chondrocytes which had been grown in monolayer culture to expand cell number could be induced to form cartilage tissue in vitro when co-cultured with small numbers of primary differentiated articular chondrocytes.

MATERIALS AND METHODS
Monolayer Culture: Bovine articular cartilage (6-9 months old) was harvested from metacarpal-phalangeal joints and chondrocytes isolated by enzymatic digestion [4]. The cells were placed in monolayer culture in Ham's F12 supplemented with 5% FBS at a cell density of 2000 cells/cm². Cells were passaged twice and seeded each time at the same cell density resulting in approximately 200-fold expansion (P2).

Co-culture Conditions: The P2 cells (1.6x10⁶ cells) were mixed with primary chondrocytes (autologous, cryopreserved, 0.4x10⁶ cells) and seeded onto filter inserts (Millicell) at a final cell number of 2x10⁶ cells/filter (P2/P0=4:1) in Ham's F12 supplemented with 5% FBS. The serum concentration was increased to 20% on day 5, and on day 7 ascorbic acid (100 µg/ml) was added. As controls, primary (either 0.4x10⁶ or 1x10⁶ cells) or passaged cells (2x10⁶ cells) alone were placed on the filters.

Histological Examination: In vitro-formed tissue at 4 weeks was fixed in 10% formalin, and paraffin embedded. Sections were cut and stained with toluidine blue (TB) or hematoxylin and eosin (H&E).

Determination of Proteoglycan, Collagen, and DNA Content: The tissue was papain digested at 4 weeks. The proteoglycan content was estimated by the dimethylmethylen blue dye-binding assay and spectrophotometry. The collagen content was determined by hydrolyzing the papain digest in 6 N HCl (110°C, 18h) and OH-proline quantified using chloramine-T/Ehrlich’s reagent and spectrophotometry. The DNA content was determined using the Hoechst dye 33258 dye binding assay and fluorometry.

Gene expression: Total RNA was isolated from in vitro-formed tissues by Trizol extraction, reverse-transcribed (Superscript II), and relative gene expression semi-quantified following PCR using sequence specific primers.

Proteoglycan Synthesis: The cells were incubated with [35S] SO₄ (4uCi/con) for the final 24 h of the 1 week culture period to label newly synthesized proteoglycans. The tissues were harvested, papain digested and proteoglycan synthesis estimated by quantifying radioisotope incorporation using a β-liquid scintillation counter.

Cell Labeling and Flow Cytometry: To examine the distribution and the percent of the primary and passaged chondrocytes within the tissue over time, primary cells (P0) were labeled with the carboxyfluorescein diacetate succinimidyl ester (CFDA, green fluorescence) and the passaged cells (P2) were labeled with PKH26 (red fluorescence). The cells were co-cultured for various times up to 28 days. Confocal microscopy was used to visualize the cells within the tissue. The tissue was then digested with 1% collagenase, cells isolated and sorted using flow cytometry. The ratio of P0 to P2 cells was determined at different times in culture.

Statistics: Results were expressed as mean ± SD and analyzed by one-way ANOVA. Significance was assigned at p<0.05.

RESULTS
Histological examination showed that passaged cells alone did not form cartilage in vitro (Fig. 1 A, B). Co-culture of P2 cells with P0 cells resulted in cartilage tissue formation (Fig. 1 C, D) which was greater in amount than that formed by P0 cells alone at the same cell number used in the co-culture (0.4x10⁶ cells) (Fig. 1 E, F). This was confirmed biochemically as the tissue formed by co-cultured cells had significantly greater collagen and proteoglycan content than the tissue formed by P2 cells alone (p<0.05).

RT-PCR showed that passaged cells when grown alone expressed collagen type I and very low levels of collagen type II, aggrecan, and sox 9 in keeping with their dedifferentiated state. Growing P2 cells with primary cells (0.4x10⁶) resulted in up-regulation of collagen type II, aggrecan and sox 9 gene expression and down-regulation of collagen type I expression. Confocal microscopy showed that the P0 cells were distributed throughout the in vitro-formed tissue. Cell sorting studies confirmed that there was no overgrowth of one population of cells as the ratio of P2 to P0 cells (4:1) was maintained throughout the 4 weeks of culture. To determine which cells were synthesizing more matrix molecules and if cell-cell contact was required for this stimulatory effect, the primary cells (0.4x10⁶/cells/filter) and passaged cells (1.6x10⁶/cells/filter) were grown on separate filter inserts but were placed in the same culture well. This prevented cell-cell contact but would allow diffusion of a soluble factor. There was a significant increase in proteoglycan synthesis (p<0.05) by the passaged cells when compared to P2 cells grown in the absence of P0 cells at 1 week of culture. No difference in DNA content was detected in the side by side co-culture of either primary cells or passaged cells as compared to their corresponding control cultures, respectively, confirming that co-culture did not induce either P0 or P2 cell proliferation.

DISCUSSION
Passaged cells when grown in 3D culture on filter inserts did not form cartilage. However, co-culture of passaged cells with small numbers of primary chondrocytes under the same conditions resulted in cartilage tissue formation in vitro as determined histologically and biochemically. The amount of tissue that formed was greater than that generated by the same number of P0 cells as used in the co-cultures and grown on their own. P0 cells appeared to be inducing P2 cells to contribute to cartilage tissue formation as P2 cells synthesized more proteoglycans when grown with P0 cells. This stimulatory effect was due to a soluble factor as cell-cell contact was not required. The effect of co-culture on gene expression suggested that co-culture induced re-differentiation of the passaged cells as there was upregulation of chondrogenic genes (sox 9, aggrecan, and collagen type II) with down-regulation of the collagen type I a marker of dedifferentiation. The stimulatory effect was not a result of overgrowth of primary cells as these cells distributed throughout the tissue did not show preferential proliferation, and the ratio of P0 to P2 cells was maintained throughout the culture period. This method appears to be a promising approach to overcome the issue of limited cell numbers while still allowing use of chondrocytes. Studies are ongoing to identify the factor responsible for inducing redifferentiation of passaged chondrocytes as it may be possible to eliminate the requirement for primary cells and further decrease the number of cells needed to tissue engineer cartilage in vitro.

References:

ACKNOWLEDGEMENTS: Supported by CIHR, the Arthritis Society and Canadian Arthritis Network.

Poster No: 0428