A Structured Co-culture Model for Stable Chondrogenic Differentiation of Human Mesenchymal Stem Cells in the Absence of Exogenous Growth Factors

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Introduction:
The biological and mechanical qualities of articular cartilage are lost in arthritis, leading to pain and joint dysfunction. A promising therapeutic approach is to regenerate cartilage tissue using adult autologous cells. A major challenge for this approach is the acquisition of a sufficient number of cells that synthesize proteoglycan and collagen II in an extracellular matrix. Autologous chondrocytes are not optimal because the harvesting procedure damages healthy cartilage, donor chondrocytes can only be obtained in small numbers, and these chondrocytes often dedifferentiate during in vitro expansion. By contrast autologous human mesenchymal stem cells (hMSC) can be harvested and expanded readily. However, hMSC require exogenous growth factors to promote their chondrogenic differentiation.

Chondrogenic induction of cultured hMSC is most commonly performed using transforming growth factor-β (TGFβ). Although TGFβ is a potent chondroinductive growth factor, it results in a hypertrophic phenotype in which the cells terminally differentiate and undergo apoptosis. This process normally occurs during the development of long bones in endochondral ossification, but is problematic in cartilage tissue engineering.

To overcome the hMSC requirement for exogenous growth factors, we developed a model that provides multiple physical and biochemical cues to induce chondrogenesis. We cultured hMSC in a pellet surrounded by a layer of instructive chondrocytes, which provided a totality of biochemical signals to direct hMSC differentiation. In addition, the bilaminar structure established physical interactions among and between mesenchymal precursors and the surrounding instructive chondrocytes that may enhance chondrogenesis. We hypothesized that structured co-culture of hMSC with instructive cells (IC) in a bilaminar cell pellet (BCP) will self-promote optimal chondrogenesis of the BCP in the absence of exogenous TGFβ.

Methods:
Cell culture: Commercially available bone marrow-derived human MSC (Lonza) were expanded to the seventh passage in growth media (DMEM low glucose, 1% Antibiotic/Antimycotic and 10% FBS). Human chondrocytes derived from juvenile organ donors (ISTO Technologies, Inc.) were used as the instructive cell population (IC).

Pellet formation: Three types of pellets were formed: single cell type pellets (hMSC or IC) and pellets of hMSC and IC organized into a bilaminar cell pellet (BCP). To form single cell type pellets, 500,000 cells were centrifuged in a 15mL polypropylene tube at 300g for 5 min. To create BCPs, 375,000 hMSC were centrifuged in a 15mL polypropylene tube at 300g for 5 min. Then, 125,000 IC were added to the same tube and centrifuged at 300g for 5 min. Pellets were cultured for 3 days in 2 mL media during which time the pellet became spherical. Pellets were transferred to ultra-low attachment 24 well plates (Corning) and cultured in normoxia or in hypoxia incubator (2% O2) for 21 days before harvesting. Media conditions consisted of: chondrogenic media (DMEM high glucose, 1% pen/strep, 1% NEAA, 1% hepes, 160μM l-proline, 200μM ascorbic acid, 0.1μM Dexamethasone, 3x10^{-3} M sodium selenite, 10μg/mL transferrin, 10μg/mL insulin) or growth media, with or without 10ng/mL TNFα and 10ng/mL IL1β or 5ng/mL TGFβ3.

Sulfated glycosaminoglycan (GAG) and DNA Content: The pellets were removed from culture media, digested in papain (200μg/mL in PBS), and assayed with dimethylmethylen blue to quantify GAG content. Digested pellets were also assayed with a Quant-IT PicoGreen kit (Invitrogen) to quantify DNA content.

Gene expression: Total RNA was isolated, purified and reverse transcribed. Quantitative RT-PCR assessed gene expression using established primer sets for each cDNA.

Histology: Pellets were fixed in 4% paraformaldehyde at 4°C, dehydrated, embedded in paraffin and sectioned at 7 micron thickness. Immunohistochemistry was performed (Misch 4 Universal HRP-Polymer Kit with DAB, Biocare Medical) with primary mouse anti-aggrecan antibody (SC-73693, Santa Cruz Biotechnology, CA).

Statistics: Calculated using ANOVA and Tukey’s tests.

Results:
TGFβ induced chondrogenic differentiation of the hMSC pellets. The hMSC pellets cultured with TGFβ had greater expression of the chondrogenic gene, Sox9 (p<0.0001) compared to pellets without TGFβ. Aggrecan immunohistochemistry and proteoglycan findings were consistent with these results. However, hMSC pellets cultured with TGFβ also had greater expression of genes indicative of hypertrophy: MMP13, Runx2, Col I and Col X, (p<0.007), by comparison to pellets without TGFβ. Thus, TGFβ induces chondrogenesis but also hypertrophy in hMSC pellets.

Compared to hMSC pellets cultured with TGFβ, the BCP co-culture had greater expression of the chondrogenic genes: Sox9 (3.81-fold increase, p=0.0004), Coll I (949-fold increase, p=0.0021) and Aggrecan (353-fold increase, p=0.0001). Immunohistochemistry and proteoglycan findings are consistent with these results. Moreover, the BCP co-culture had less expression of the hypertrophic genes: Runx2 (59.5% decrease, p=0.0001), Coll X (99.6% decrease, p<0.0001) and MMP13 (89.5% decrease, p=0.001). These results suggest that the BCP co-culture can induce chondrogenic differentiation without upregulating the expression of hypertrophic genes, as occurred in pellets induced by TGFβ.

To determine whether the BCP co-culture can retain its chondrogenic potential in osteoarthritic conditions, we assessed the proteoglycan production of the pellets cultured in hypoxia and with inflammatory cytokines, or in hypoxia alone or with inflammatory cytokines alone. The BCPs produced more proteoglycan than the hMSC and IC pellets in all three conditions (p<0.05). As expected, the production of proteoglycan by the IC pellets was impaired by inflammatory cytokines. These results suggest that the BCP co-culture may overcome significant limitations to cartilage regeneration in osteoarthritis.

Discussion:
Our study supports the feasibility of a novel approach to cartilage tissue engineering. The results of this study demonstrate that the BCP co-culture promotes chondrocyte differentiation and cartilage matrix synthesis. We have found that the production of proteoglycans and the expression of chondrogenic genes induced by BCPs are nearly equivalent to IC pellets even though the BCPs contain only 25% IC. These results suggest that the interaction between IC and hMSC in the BCP co-culture is promoting chondrogenesis in the absence of exogenous TGFβ.

Specifically, compared to hMSC pellets cultured with TGFβ, BCPs have greater expression of the chondrogenic genes Sox9, Coll II and Aggrecan, and less expression of hypertrophic genes MMP13, Runx2 and Coll X. These results suggest that the BCP co-culture uses the instructive chondrocytes to induce differentiation of a stable adult cartilage phenotype, in contrast to the undesirable hypertrophic phenotype that results from chondrogenic induction of hMSC by TGFβ. Furthermore, BCPs maintain their chondrogenic potential under hypoxic and inflammatory conditions, which are recognized challenges to the success of cartilage tissue engineering for the treatment of osteoarthritis.

To conclude, this study shows that the preparation of instructive chondrocytes with hMSC in a bilaminar cell pellet supports chondrogenic differentiation without the addition of TGFβ. hMSCs are plentiful; accordingly, they may be used to expand the limited supply of chondrocytes for cartilage bioengineering. Although IC pellets express genes indicative of a chondrogenic phenotype, instructive chondrocytes are limited in supply and also have impaired production of proteoglycan under inflammatory conditions that are seen in osteoarthritis. In contrast, we have shown that the production of proteoglycan by BCPs is not negatively affected when exposed to inflammatory and hypoxic conditions. Thus, BCPs may provide significant advantages as a therapeutic approach for cartilage regeneration.

References:

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