Analysis of Chondrocyte Metabolism *In Vitro* Utilizing Different Culture Methodology

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Introduction

Osteoarthritis (OA) is a painful and debilitating disease that is the number one cause of disability in the United States. Many *in vitro* models of OA have been used to better understand this disease, including cartilage tissue explant culture, chondrocyte monolayer culture, and chondrocyte 3-D culture. The metabolism of chondrocytes in monolayer cell culture has been shown to be different from chondrocytes in cartilage *in vivo*. Chondrocytes in monolayer cell culture lose the classic cartilage phenotype (aggrecan and collagen II expression) and de-differentiate into a more fibroblastic phenotype (collagen I expression). The process of de-differentiation occurs within a few passages in monolayer cell culture. It is not known how the process of de-differentiation affects basal chondrocyte metabolism or the chondrocytes' responses to various stimuli often used to mimic osteoarthritis *in vitro*. Therefore, it is possible that the data collected using cells propagated in monolayer cell culture is unsuitable for the study of osteoarthritis and normal cartilage metabolism. Chondrocyte 3-D culture has been shown to rescue the chondrocyte phenotype, with respect to collagen expression, but it is not clear how 3-D culture affects basal and cytokine stimulated chondrocyte metabolism. Therefore, more data needs to be collected focusing on the metabolic responses of chondrocytes in different types of *in vitro* culture to stimuli (like Interleukin (IL)-1β) commonly used for the analysis of cartilage metabolism and to model osteoarthritis. Once the metabolic responses of chondrocytes cultured under different *in vitro* conditions are better understood, the utility of various *in vitro* models to study specific aspects of cartilage metabolism in health and disease can be determined.

Objectives

- **Characterize the temporal basal and IL-1β stimulated production of nitric oxide (NO), prostaglandin E2 (PGE_2), matrix metalloproteinase (MMP)-2, MMP-3, MMP-9 and MMP-13 of chondrocytes in cartilage explant culture, monolayer culture, and 3-D agarose culture.**
- **Compare the production of MMPs, NO, and PGE_2 in response to IL-1β between cultured chondrocytes and cartilage explant tissues. Identify the time point during culture at which significant differences in the metabolism of chondrocytes in cell cultures and cartilage explant cultures occur *in vitro*.**

Hypotheses

- **The basal and IL-1β stimulated production of MMP-2, MMP-13, NO, and PGE_2 will all be significantly different between chondrocyte monolayer culture, chondrocyte 3-D culture, and tissue explant culture.**
- **Chondrocytes in 3-D culture will behave more like those in cartilage tissue explant culture than those in monolayer culture with respect to the metabolic factors studied.**

Methods

Tissue harvest and culture: Full thickness articular cartilage was harvested from the humeral heads of 6 skeletally mature canine cadavers euthanatized for reasons unrelated to this study. Tissue explants (CE) were prepared sterilely using a 4 mm dermal biopsy punch. Two explants were cultured in a 24 well plate in 1 ml of supplemented DMEM media, with or without (NEG) recombinant human (rh)IL-1β (100 ng/ml). For monolayer (M) and 3-D (3D) cell culture, chondrocytes were isolated from cartilage tissue by collagenase digestion. Isolated chondrocytes were counted and tested for cell viability using the trypan blue exclusion assay, and 1 ml (70,000 cells/ml) was used to seed 24 well plates. Once confluent, cells were either used for monolayer cell culture (3 wells per dog) and stimulated with rhIL-1β (0 (NEG), 10, or 100 ng/ml); used to create 3D cell culture constructs (3 wells per dog) and cultured with rhIL-1β (0, 10, or 100 ng/ml); or passaged (3 wells per dog) at a 1:4 split ratio for up to 4 passages. For 3-D construct preparation, a 4% agarose solution was created in DMEM media and kept liquid at 37°C. One well of cells was mixed with agarose and allowed to gel at 4 C to make a final 2% agarose construct for culture. The gel constructs were placed in a 24 well plate and cultured in 1ml of media as described above. All 3 culture types were incubated at 37°C with 5% CO_2 and 95% humidity for 9 days of culture. Media was changed and collected every 3 days and stored at -20°C for later testing. On day 9, cells, tissues, and 3-D constructs were digested with papain solution and stored at -20°C for DNA content testing.

Media analysis: Media was tested for NO by Griess Reagent (Promega), PGE_2 by EIA assay (Cayman Chemical), and MMP-2, 3, 9, and 13 using the Flourokine MAP Multiplex Human MMP panel (R&D Systems). Tissues and cell cultures harvested on day 9 were analyzed for total DNA content using the Quant-iT PicoGreen dsDNA Kit (Invitrogen) according to the manufacturer's protocol.

Data was analyzed for significance by One-Way ANOVA with significance set at $p<0.05$.

Results

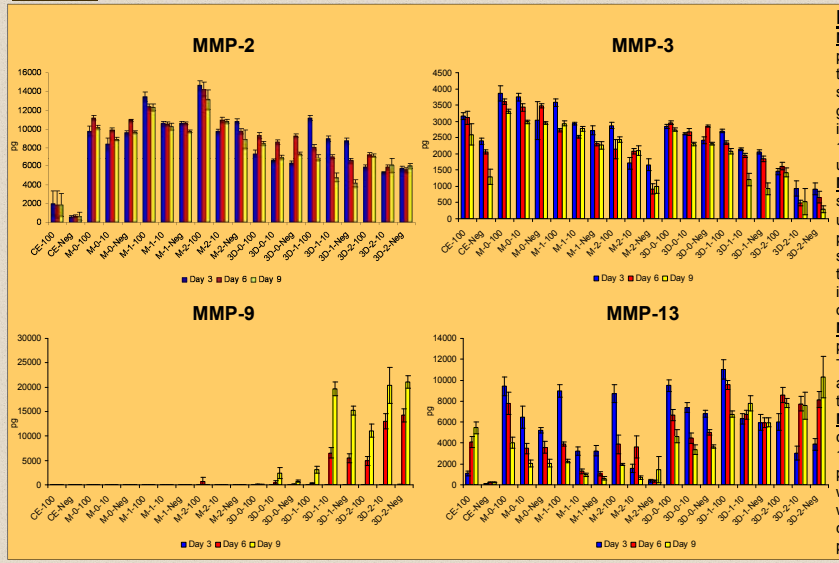

Figure 2: MMPs

MMP-2: There was significantly more MMP-2 production in the M and 3D groups compared to the CE group, and the M group produced significantly more MMP-2 compared to the 3D group at all time points. IL-1β treatment increased production of MMP-2 only in the M-1-100 and 3D-1-100 groups compared to untreated controls.

MMP-3: The CE-100 group produced significantly more MMP-3 compared to untreated controls at all time points. The production of MMP-3 in the M group was significantly higher than the 3D group at all time points. There was a significant decrease in MMP-3 production in the M and 3D groups over time by passage.

MMP-9: The 3D group was the only consistent producer of MMP-9 starting on day 6 of culture. This production increased with cell passage and time in culture, but not with IL-1β treatment.

MMP-13: The CE-100 and M-100 groups consistently produced significantly more MMP-13 compared to the negative controls at all time points, and the fold increase in the CE group was higher than the M group. The 3D groups were significantly higher than the corresponding CE groups for MMP-13 production at most time points.

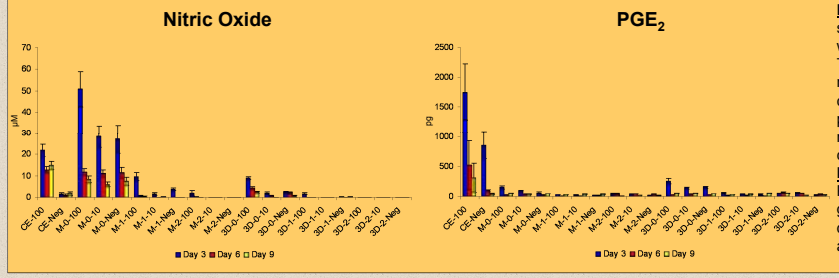

Figure 3: Inflammatory Markers

Nitric Oxide: There was a dramatic and significant loss of NO production associated with the M and 3D groups after passage 0. The IL-1β treated CE and 3D-0-100 groups released significantly more NO than untreated controls at all time points. However, the production of NO by the M-0-100 group was not significantly different than the untreated control.

PGE_2: The CE group had significantly higher PGE_2 production compared to the M and 3D groups. However, there were no consistent differences between the IL-1β treated groups and the untreated controls.

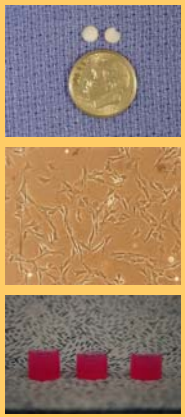

Figure 1

Tissue Explants

Monolayer Cell Culture

3-D Cell Culture Constructs

Discussion

This study indicates that there is a significant change in the metabolism and phenotype of chondrocytes in monolayer cell culture compared to tissue explant culture. The basal production of MMPs and inflammatory markers and the responses to cytokine stimulation are notably different between the two culture types. The loss of nitric oxide production, the increase in MMP-2 and MMP-13 production, and the lower fold-increase of MMP-13 in response to IL-1β indicate a loss of chondrocytic phenotype, which is progressive with cell culture passage. Although it was hypothesized that the 3-D culture would restore the phenotype by allowing the chondrocytes to retain their shape, the phenotype was not restored in the time allowed for this study. This indicates that the phenotypic change is not simply a result of cell shape change. It is possible that over time, the 3-D culture could develop an extracellular matrix, and restore the chondrocytic phenotype observed in tissue explant culture.

Conclusions

- **The basal and IL-1β stimulated metabolic responses of chondrocytes are significantly different between tissue explant culture and monolayer cell culture.**
- **3-D culture did not allow for recovery of chondrocyte phenotype after monolayer culture as assessed during the time frame of this study.**

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