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Structured bilaminar co-culture outperforms stem cells and disc cells in a simulated degenerate disc environment

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Abstract

Study Design—This study explores the use of bilaminar coculture pellets of mesenchymal stem cells (MSC) and Nucleus Pulposus cells (NPC) as a cell-based therapy for intervertebral disc regeneration. The pellets were tested under conditions that mimic the degenerative disc.

Objective—Our goal is to optimize our cell-based therapy in vitro under conditions representative of the eventual diseased tissue.

Summary of Background Data—Harnessing the potential of stem cells is an important strategy for regenerative medicine. Our approach seeks to direct the behavior of stem cells by mimicking embryonic processes underlying cartilage and intervertebral disc development. Prior experiments have shown that bilaminar co-culture can help differentiate MSC and substantially improve new matrix deposition.

Methods—We have designed a novel spherical bilaminar cell pellet (BCP) where MSC are enclosed in a shell of NPC. There were three groups: MSC, NPC, and BCP. The pellets were tested under three different culture conditions: in a bioreactor that provides pressure & hypoxia (mimicking normal disc conditions), with inflammatory cytokines (IL-1b and TNF-a), and a bioreactor with inflammation (mimicking painful disc conditions).

Results—When cultured in the bioreactor, the NPC pellets produced significantly more glycosaminoglycan (GAG)/cell than the other groups: 70-80% more than the BCP and MSC alone. When cultured in an inflammatory environment, the MSC and BCP groups produced 30-34% more GAG/cell than NPC ($p < 0.05$). When the pellets were cultured in a bioreactor with inflammation, the BCP made 25% more GAG/cell than MSC and 57% more than NPC ($p < 0.05$).

Conclusion—This study shows that BCP outperform controls in a simulated degenerated disc environment. Adapting inductive mechanisms from development to trigger differentiation and restore diseased tissue has many advantages. As opposed to strategies that require growth factor supplements or genetic manipulations, our method is self-sustaining, targeted, and minimally invasive injection.

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Keywords

Mesenchymal stem cell; bilaminar pellet; coculture; induction; differentiation; bioreactor; inflammation

Introduction

Tissue engineering is a growing and dynamic field with the potential to provide patients with minimally-invasive treatments that repair or replace dysfunctional musculoskeletal tissues. For the intervertebral disc, the goal is to re-establish pain-free motion by restoring the disc's physical and biochemical properties [1-3]. This may be accomplished by stimulating host cells to resume matrix synthesis (particularly aggrecan) and/or by introducing new, more synthetically-active cells [4-8]. This work focuses on the latter option.

Pellet culture systems may have benefits as part of a tissue engineering strategy as demonstrated in the literature [9-13]. Furthermore, co-culture of stem cells and disc cells is a promising strategy for cell based tissue regeneration as it makes use of signaling interaction between the two cell types to promote MSC differentiation [14-20]. We explored the benefits of co-culturing nucleus pulposus cells (NPC) and adult MSC using a 3D system that exploit the processes of tissue induction and condensation. We report here on a novel “bi-laminar cell pellet” approach that structures cell-cell signaling between MSCs and non-degenerative NPCs [21, 22]. A spherical bi-laminar cell pellet (BCP) consists of an inner sphere of one cell type enclosed within a shell formed by another cell type. It has been previously shown that BCP composed of 75% MSC as the inner sphere and 25% NPC forming the outer shell produce 30-50% more matrix than the MSC and NPC controls in vitro. This indicates an advantageous synergy between the two cell types in this spatially organized configuration [22].

A fundamental challenge to functional disc tissue engineering is the lack of an established, validated pre-clinical model that simulates the pathologic/painful disc condition. Still, it is valuable to challenge and optimize investigational treatments with conditions anticipated in vivo since environmental cues can dramatically affect the therapeutic performance [23-28]. In the degenerate disc these three key components are pressure, hypoxia, and inflammation [28].

The healthy disc supports substantial compressive load while remaining pliant to facilitate motion. It is the body's largest avascular organ, making nutrient and oxygen transport limiting factors to cell survival [29]. Mechanical and hypoxic stress can be adverse to viability and matrix synthesis of mesenchymal stem cells (MSCs) and nucleus pulposus cells (NPCs) [30-31]. Additionally, painful degeneration also includes elevated levels of inflammatory mediators such as IL-1 and TNF-alpha that are byproducts of chronic wound healing [34-36].

In order to anticipate the therapeutic effects of BCPs in painful discs, we mimicked these key features of the degenerate disc environment in vitro. BCP cell viability and matrix synthesis were measured in the presence of inflammatory cytokines (IL-1beta and TNF-alpha each at 10ng/ml; mimicking levels seen in degenerative discs), under pressure (0.1 MPa) and hypoxia (4.5% oxygen; mimicking human disc conditions at rest), and finally under pressure and hypoxia with inflammatory cytokines.

Materials and Methods

Bioreactor Design and Construction

The bioreactor is a simple design composed of easily available components. This permits easy fabrication of many replicate units that can be used simultaneously. Each bioreactor is composed of a dialysis cassette (10 kDa pore size, Slide-A-Lyzer, Pierce) modified to withstand internal pressurization (Figure 1A). The cassette cavity (300 mL) is filled with a 15% hyaluronan gel (MW 80,000, Genzyme, Cambridge, MA) that is entrapped within the device by a gasket and semi-permeable membranes.

A 15% hyaluronan (HA) gel was made by dissolving sodium hyaluronate powder in growth media. Three hundred microliters of this gel were injected through the self-sealing silicone port of a dialysis cassette and excess air was removed. The cassette was then incubated in growth media (Dulbecco's modified eagle medium (DMEM) low glucose, 1% antibiotic/antimycotic, and 10% Fetal Bovine Serum (FBS)) for four hours to allow for gel volume expansion by osmotic swelling. An autoclavable porous metal filter surrounds the device to permit fluid exchange and prevent membrane rupture due to internal pressurization (Figure 1B).

Bioreactor Oxygen and Pressure Measurements

Bioreactor oxygen and pressure were measured after cassettes were incubated in growth media at 37°C with 5% CO₂ and 21% O₂ as described previously [28]. Under these conditions the oxygen tension and pressure inside the bioreactor reach a steady-state of 4.8% and 0.12 MPa respectively, which are comparable with physiologic levels of the human disc at rest.

Cell Culture

Bovine NPC were isolated from caudal discs of healthy adult cows within 48 hours of sacrifice. The NP tissue was carefully separated by gross dissection and digested in 0.5% collagenase/dispase and 2% antibiotic/antimycotic in low glucose DMEM at 37°C for 4-6hrs with constant stirring. The cells were then plated in tissue culture flasks and expanded to the fourth passage in NPC Media (DMEM low glucose, 1% antibiotic/antimycotic, 1.5% 400m Osmolarity, and 5% FBS) at 37°C with 5% CO₂. Culture media was changed twice a week.

Commercially available human MSC were purchased (Lonza, Switzerland) and expanded to the sixth passage in monolayer culture using growth media at 37°C with 5% CO₂. Culture media was changed twice a week.

Pellet formation and culture

Human MSC and bovine NPC were used to make coculture pellets. It has previously been reported that when using controls of human MSC with human NPC pellets as well as bovine MSC with bovine NPC pellets that there are no significant cross-species interactions [22].

Two different pellet types were formed, each consisting of 500,000 cells total: pellets of 100% one cell type and BCPs of 75% MSC and 25% NPC organized into a bilaminar. This ratio was selected based on previous studies [16, 17, 21]. To produce the single cell type pellets, 500,000 cells were pipetted into a 15mL polypropylene tube and centrifuged at low speeds (300g) for 5min. In order to form the bilaminar organized pellets, 375000 MSCs were added to a 15mL polypropylene tube and centrifuged at low speed for five minutes. Subsequently, 125000 NPCs were gently added to the same tube. The cells were then centrifuged again at low speed for 5 min. All pellets were cultured in 2 mL of growth media

for three days with caps loosened to allow for gas exchange. After three days, the pellets became spherical and were transferred to ultra-low attachment 24 well plates (Corning).

All pellets were cultured in growth media for a total of one week. At the end of the first week, pellets in the bioreactor group (BG) were injected into the bioreactor using a Radiology Angiography needle (Beckton Dickinson, NJ; Figure 1C). The pellets in the inflammatory cytokine group (ICG) were cultured in growth media supplemented with IL-1 β and TNF- α at 10ng/mL each (Peprotech, NJ; Figure 1D). The cytokine concentration was selected to reflect physiologic levels based on prior reports [37-40]. The pellets in the bioreactor with inflammatory cytokines group (BICG) were injected into a bioreactor that contained the same cytokines as the ICG (Figure 1E). Five replicates were used per pellet type per culture group.

DNA and DMMB assays for proteoglycan quantification

At the end of two weeks, the pellets were harvested and digested overnight at 60 degrees in a papain solution (20 U/mL in PBS). Digested pellets were assayed with a Quanti-iTPicoGreen kit (Invitrogen, CA) to measure DNA content. Measurements were made using a spectrophotometer, with the excitation at 488nm and absorption at 525 nm.

Digested pellets were also analyzed using a dimethylmethylene blue (DMMB) assay to quantify GAG content. A standard curve was made with chondroitin sulfate isolated from bovine trachea (Sigma, MO). Absorption was measured at 525nm using a spectrophotometer.

The results obtained from GAG quantification were normalized to the cell number as calculated from the DNA content assuming 6 μ g DNA/cell.

In situ hybridization

In situ hybridization was performed to localize cell synthetic activity [41]. Sections were hybridized with ³⁵S-labeled human riboprobes to *aggrecan* and *col2a1* (fibrillar collagen). Sections were counterstained with Hoechst dye (Sigma, MO). Hybridization signals were detected using illuminated darkfield and the nuclear stain with epifluorescence.

Statistical Analyses

All statistical analyses were performed using JMP statistical software (JMP V 8.0, SAS Institute Inc.). Analysis of variance (ANOVA) procedures were used to compare group means and to estimate the effect of the sample variables (pellet type and culture conditions entered as categorical predictors) on the measured parameters of interest (cell density and GAG content). Standard p-values were calculated for assessing statistical significance. When indicated, Tukey post-hoc tests were performed to identify group differences. Probabilities between 0.05 < p < 0.10 were defined as 'trends' with near statistical significance [42].

Results

DNA and DMMB assays for proteoglycan quantification

In the BG, where pellets are cultured under pressure and hypoxic conditions, the NPC pellets produced significantly more GAG per cell than the other groups: 70-80% more than the BCP and MSC alone (p<0.05; Figure 2). The BCP displayed a trend towards producing 53% more GAG per cell than MSC (p<0.1) and having more cells than other groups (p<0.1; Figure 3). There was no significant difference in overall GAG produced per pellet, though

the MSC groups trended towards producing the least amount of GAG per pellet ($p < 0.1$; Figure 4).

In the ICG, the MSC and BCP groups produced 30-34% more GAG per cell than NPC ($p < 0.05$; Figure 2). The NPC also had approximately 160% fewer cells per pellet than the MSC and BCP groups ($p < 0.05$; Figure 3). This led to 113% and 157% more GAG per pellet for the BCP and MSC groups respectively than the NPC group ($p < 0.05$; Figure 4). There was no statistically significant difference between the MSC and BCP for any of the measures.

In the BICG, the BCP secrete 25% more GAG per cell than MSC and 57% more than NPC ($p < 0.05$). The MSC produced 42% more GAG per cell than NPC ($p < 0.05$; Figure 2). There was no significant difference in DNA content between the groups (Figure 3). Ultimately, the BCP produced 113% more GAG per pellet than MSC and 157% more GAG per pellet than NPC ($p < 0.05$; Figure 4).

In situ hybridization

The MSC pellets did not exhibit any significant levels of collagen II expression regardless of their environment (Figure 5 A,G,M). However, the levels of aggrecan expression did vary with the environmental conditions. There are low levels of expression in both the BG and ICG, where expression does appear to be homogeneously spread (Figure 5H).

For the NPC pellets, collagen and aggrecan expression varied with environmental condition. In the BG, the aggrecan and collagen II expression was very strong and homogeneous (Figure 5C,D). In the ICG, aggrecan and collagen II expression both dropped significantly compared to the levels in BG (Figure 5 I,J). In addition, the expression was localized to the outer layer of the pellet in the ICG. In the BICG, expression of aggrecan and collagen II dropped further below levels in the ICG, particularly for collagen II expression that was extremely faint (Figure 5 O). Aggrecan expression was again localized to the outer periphery of the pellet (Figure 5 P).

For the BCP group, gene expression also varied with environmental conditions. In the BG, aggrecan and collagen II expression were high and homogeneous (Figure 5E,F). These levels decreased somewhat in the ICG, but remained homogeneous as opposed to the NPC group (Figure 5 K,L). Finally, in the BICG, aggrecan and collagen II expression both had a strong signal and remained homogeneously expressed (Figure 5 Q,R).

Discussion

The goal of this study was to determine the environmental effects on cell pellet matrix synthesis and gene expression in order to anticipate the in vivo behavior. Our data demonstrate that GAG production, cell proliferation, and gene expression vary significantly with culture conditions and have different effects on MSCs and NPCs. We observed that NPC produced the most GAG/cell in the BG that simulates the disc's resting pressure and oxygen tension. The in situ hybridization data also indicate that NPC expressed higher levels of aggrecan and collagen II than MSC and BCP. These results are expected since NPC are adapted to survive and produce matrix under these conditions. Interestingly, the BCP did show high levels of aggrecan and collagen II expression, which may be an indication that the MSCs (that compose 75% of the BCP) had differentiated. This was not the case in the MSC-only group, where expression levels were very low. The BCP also tended to have the most cell proliferation in the BG (though this was not significant) and considerable levels of aggrecan and collagen II expression. Under conditions of pressure and hypoxia the NPC

produce the most amount of GAG/cell, and BCP are able to proliferate and produce GAG as well.

The MSC and BCP groups produced significantly more GAG and had more cell proliferation in the ICG than the NPC. The literature indicates that MSC may be immune-privileged cells and tolerant of inflammatory conditions [43]. Also, considering that the BCP are 75% MSC, it is not surprising that the BCP performed well in this environment. Importantly, NPC did not perform well under ICG, with GAG/cell dropping 86% from the BG levels ($p < 0.05$). Aggrecan and collagen II gene expression in the NPC group was lower in the ICG than in the BG and located on the periphery. These data indicate that NPC are very sensitive to inflammatory cytokines that have a strong debilitating effect on gene expression and aggrecan production, while MSC and BCP are more resilient.

The BCP produced the most matrix (GAG/cell as well as GAG/pellet) in the BICG. Consistent with this, the BCP group had the highest aggrecan expression levels. These data suggest that BCP would outperform NPC or MSC alone when used as part of a regenerative therapy. Interestingly, we also observed that MSC produced significantly more GAG than NPC in the BICG, indicating that the inflammatory cytokines have a more dominant effect on NPC than pressure and hypoxia. The combined exposure to pressure, hypoxia, and cytokines did not alter cell proliferation but did have an effect on all the pellets' ability to produce GAG at the protein and gene expression levels.

This study is limited due to the use of an in vitro system that only partially mimics the in vivo disc environment. The disc degenerative process is complex and many biological and biomechanical stressors impact the ability to rejuvenate nucleus pulposus tissue [30]. Even more so, pain mechanism for degenerate discs are multifactorial and poorly defined. In addition to pressure, hypoxia, and pro-inflammatory cytokines studied here, other factors such as nerve and blood vessel ingrowth are often-reported findings unique to painful discs [44]. Consequently, whether the BCP behaviors reported constitute disease-modifying activity for low back patients remains to be studied. While pre-clinical studies of BCPs for disc repair in small animals are promising [45], large animal models are an important next step to assess therapeutic activity relative to other approaches being investigated such as growth factors [46-47], bioactive scaffolds [48], and gene therapy [49]. We also haven't addressed the mechanisms by which structured coculture leads to the beneficial behaviors. Cell contact and/or paracrine signaling across the cell boundary may be important, and identification of the signaling pathways using quantitative gene expression analyses may allow further optimization of this tissue engineering approach.

Ultimately, our goal is to develop cell-based therapies for painful disc degeneration. In this study we isolated the effects of the major features in degenerative discs (inflammation, pressure, and hypoxia) on different cell types. Our results indicate that the BCP configuration may exploit inductive signaling between and within organized cell layers to impart therapeutic advantages in the harsh, painful disc environment over NPC and MSC alone.

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Figure 1.

A) The bioreactor is filled with HA gel which swells due to osmotic pressure. The pellet is then injected into the chamber. B) Once the bioreactor is swollen, an autoclaved porous metal clip is placed around the membrane so that it will not continue to swell and burst. C) Some of the pellets were cultured in the bioreactor group (BG), inducing hypoxia and pressure. D) Some of the pellets were in the inflammatory cytokines group (ICG). E) Some of the pellets were cultured in the bioreactor with inflammatory cytokines (BICG).

Figure 2. Graph of μgGAG secreted per 10^6 cells by each of the groups in the three different environmental conditions. Nucleus cells performed best under pressure and hypoxic conditions (BG), while BCPs performed best when inflammatory disc conditions were mimicked with pressure, hypoxia, and cytokines (BICG).

Figure 3.
Graph of the number of cell per pellet in the three different environmental conditions.
Inflammatory conditions stimulated the greatest cell proliferation, principally in MSCs.

Figure 4.

Graph of μgGAG produced per pellet by each of the groups in the three different environmental conditions. Both MSC and BCP pellets had significantly increased GAG secretion under inflammatory conditions. Under simulated inflammatory disc conditions the BCP synthesized significantly more GAG than either cell type alone.

Figure 5. Localization of aggrecan and collagen II gene expression (white), counterstained with Hoechst dye (blue). A-F are of pellets cultured in a bioreactor. G-L were cultured with inflammatory cytokins. M-R were culture in a bioreactor with inflammation. MSC pellets did not demonstrate significant Col II gene expression while the aggrecan gene expression matched GAG trends. Under inflammatory conditions (ICG and BICG) the aggrecan and Col II expression was limited to the pellet periphery. Aggrecan and Col II expression was homogeneous under all conditions.