

HYDROSTATIC PRESSURE ENHANCES CHONDROGENIC DIFFERENTIATION OF HUMAN MULTIPOTENTIAL MESENCHYMAL CELLS IN ALGINATE DISKS

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INTRODUCTION

The poor intrinsic healing capacity of articular cartilage and the limited number of cells available for transplantation have engendered much interest in the development of an alternative source of chondrocytes for tissue engineering applications. One such cell population is multipotential mesenchymal cells (MMCs), which are readily obtained from bone marrow and may be induced to differentiate along a number of different lineages [1,3]. These cells are expandable in culture, and can be grown in sufficient numbers to populate tissue-engineered constructs. MMCs in pellet or micromass culture can be differentiated towards a chondrogenic phenotype with the application of growth factors, such as those from the TGF superfamily, including TGF- β 1 [2] and - β 3 [3], and BMP-2 [4]. More recently it has been shown that these cells can differentiate *in situ* in scaffolds such as fibrin and alginate hydrogels [3,5,6] as well as in PGA felts [7] and PLA/alginate amalgams [8].

While the process of chemically induced differentiation is well understood, the role of mechanical forces in the differentiation process has yet to be fully characterized. Studies of embryonic development in chick eggs have long documented the role of physical forces in initiating and maintaining joint development [9]. More recently, studies using limb bud cells have shown that dynamic loading may encourage the chondrogenic differentiation of a greater number of these cells than would otherwise occur [10]. Moreover, recent theoretical studies have suggested a role for physical forces (such as hydrostatic pressure) in the growth of developing limbs [11]. In this study, we examined the role of one mechanical signal, dynamic hydrostatic pressurization, in the chondrogenic differentiation of human MMCs in an alginate disk culture system.

MATERIALS AND METHODS

Cell Culture: Human multipotential mesenchymal cells (hMMCs) were isolated from bone marrow aspirates of adults as described previously [12]. hMMCs were expanded in monolayer culture in α -MEM supplemented with 10% FBS and 1% antibiotic/antimycotic. Fourth passage cells were trypsinized, counted, and resuspended in 2%

medium viscosity alginate (Sigma Chemical, St. Louis, MO) in PBS at a concentration of 40×10^6 cells/ml. Cell-alginate suspensions were cast into wells in a custom flexible mold (2.2 deep x 4.76 mm diameter), and sandwiched between two rigid platens overlaid with sterile filter paper wetted with 100 mM CaCl_2 . Gelling was allowed to proceed for 30 minutes at room temperature, after which individual disks were removed and cultured in chemically defined media (CDM). CDM consisted of high glucose DMEM supplemented with 100 mM dexamethasone, 50 $\mu\text{g/ml}$ ascorbic acid, 100 $\mu\text{g/ml}$ sodium pyruvate, 50 $\mu\text{g/ml}$ proline, 1% ITS-premix and antibiotics and antimycotics. In some cases, CDM was further supplemented with either 10 ng/ml

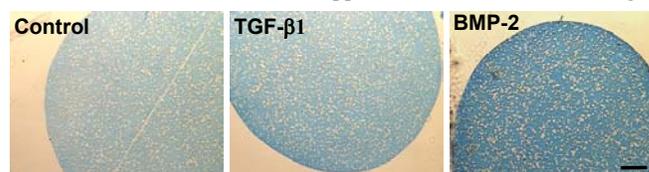


Figure 1 – Alcian Blue staining of alginate disks on day 28 with and without growth factor addition. Scale bar: 250 μm .

TGF- β 1 or BMP-2. In initial studies, disks were cultured as above with thrice weekly media changes, with disks removed for histology after 28 days of free swelling (FS) culture. **Dynamic Hydrostatic Pressurization:** To investigate the role of hydrostatic pressure in chondrogenic differentiation, MMCs were embedded in alginate disks as above and cultured for 14 days with or without TGF- β 1 supplementation. Dynamic hydrostatic pressurization (DHP) was carried out in a custom pressurization bioreactor at 37°C for five days per week with a magnitude of ~ 3 MPa and a frequency of 0.33 Hz for one hour per day. During pressurization, disks were placed in sterile plastic bags with 5 ml of CDM with supplementation. Free swelling controls were maintained similarly, with bags placed adjacent to the bioreactor. **mRNA extraction and RT-PCR:** Total cellular RNA from three disks for each experimental group was isolated by dissolving alginate disks in sodium citrate, washing the cells in PBS, and then solubilizing with Trizol reagent (Life Technologies,

Gaithersburg, MD). Reverse transcription was performed using the First Strand cDNA Synthesis kit (Invitrogen) according to the manufacturer's instructions with oligo(dT) primers. PCR amplification was performed with thermal cycling parameters of 94°C for 1 minute, annealing at 55°C (aggrecan) or 60 °C (β-actin) for 1 minute, and 72°C for 1 minute for 32 cycles, followed by a final extension at 72°C for 7 minutes. Primers for human aggrecan were as published [8] while those for β-actin were designed using a computer-aided software package based on the mRNA sequence deposited in Genbank. PCR products were resolved on a 2% agarose gel and visualized with ethidium bromide. **Histology:** Specimens were permanently cross-linked in 100 mM BaCl₂ for 10 minutes, fixed in acid-formalin/ethanol, dehydrated in a graded series of alcohol, and embedded in paraffin for sectioning. Sections were stained with either Alcian Blue (pH 1.0) for proteoglycans or with monoclonal antibodies to type II collagen as described previously [13]. **Statistics:** Two-tailed unpaired t-tests assuming unequal variance were carried out using the Microsoft Excel analysis package.

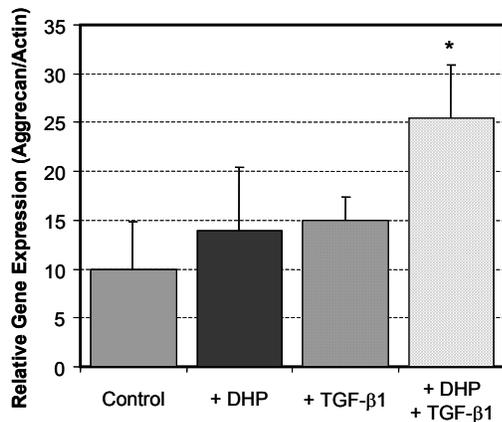


Figure 2 - Aggrecan mRNA levels (normalized to β-Actin) on day 14 (*indicates p<0.025, n=3).

RESULTS

This study confirms the finding that human multipotential mesenchymal cells in alginate disks are capable of differentiating along a chondrogenic pathway, given the proper chemical stimulus. Exposure of MMCs to TGF-β1 or BMP-2 increased chondrogenic differentiation as evidenced by increases in Alcian Blue staining of extracellular matrix (Figure 1). Culture in the presence of dynamic hydrostatic pressure (DHP) or TGF-β1 for 14 days elevated aggrecan mRNA levels by ~40% and ~50%, respectively (Figure 2). The combination of DHP with TGF-β1 led to a greater ~150% increase in aggrecan gene expression (p<0.025 vs. FS controls, Figure 2). Staining these constructs for type II collagen demonstrated a low level of cartilage specific matrix gene expression in control conditions (FS culture in CDM, Figure 3), with increases in type II collagen in the pericellular region with the addition of TGF-β1. Culture in CDM with DHP increased type II collagen deposition, resulting in more diffuse and fibrous deposition. Finally, the combination of DHP and TGF-β1 resulted in both intense staining pericellularly as well as diffuse staining in regions further removed from cells.

DISCUSSION

The differentiated state of cells is determined by a number of different factors, including their chemical and mechanical environment. Recent studies have shown that even fully differentiated tissues may alter their local phenotype with changes in the mechanical

environment [14]. MMCs have been successfully employed to aid in the in vivo repair of fibrous tissue [15]. Furthermore, applied tensile strain and torsion have been shown to induce MMCs to differentiate along a fibrous tissue lineage when cultured on collagen gels [16]. Previous studies have also shown that human dermal fibroblasts increase both gene expression and production of fibrocartilaginous matrix components in response to an applied dynamic hydrostatic pressure [17]. Additionally, studies employing primary cells for functional tissue engineering using dynamic hydrostatic pressurization [18] and dynamic deformational loading [19] have generally resulted in increases in construct mechanical properties. These findings, coupled with the results of this study, suggest that mechanical preconditioning may create an environment favorable for both chondrogenic differentiation of MMCs and the long-term development of tissue-engineered constructs. This finding may provide one solution to the problem of cell source for cartilage repair and provide a new context for functional tissue differentiation.

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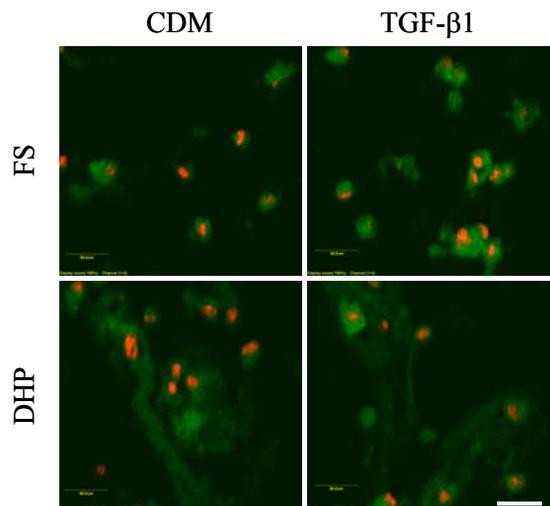


Figure 3 - Type II Collagen staining (green) with propidium iodide stained nuclei (red) on day 14. Scale bar: 50 μm.