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Cell–matrix interactions regulate mesenchymal stem cell response to hydrostatic pressure

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ABSTRACT

Both hydrostatic pressure (HP) and cell–matrix interactions have independently been shown to regulate the chondrogenic differentiation of mesenchymal stem cells (MSCs). The objective of this study was to test the hypothesis that the response of MSCs to hydrostatic pressure will depend on the biomaterial within which the cells are encapsulated. Bone-marrow-derived MSCs were seeded into either agarose or fibrin hydrogels and exposed to 10 MPa of cyclic HP (1 Hz, 4 h per day, 5 days per week for 3 weeks) in the presence of either 1 or 10 ng ml^{-1} of TGF-β3. Agarose hydrogels were found to support a spherical cellular morphology, while MSCs seeded into fibrin hydrogels attached and spread, with clear stress fiber formation. Hydrogel contraction was also observed in MSC–fibrin constructs. While agarose hydrogels better supported chondrogenesis of MSCs, HP only enhanced sulfated glycosaminoglycan (sGAG) accumulation in fibrin hydrogels, which correlated with a reduction in fibrin contraction. HP also reduced alkaline phosphatase activity in the media for both agarose and fibrin constructs, suggesting that this stimulus plays a role in the maintenance of the chondrogenic phenotype. This study demonstrates that a complex relationship exists between cell–matrix interactions and hydrostatic pressure, which plays a key role in regulating the chondrogenic differentiation of MSCs.

1. Introduction

Environmental cues, both biochemical and biophysical, regulate chondrogenesis of mesenchymal stem cells (MSCs). Various forms of mechanical loading, such as dynamic compression, tension, hydrostatic pressure (HP) and fluid flow have been shown to play a key role in determining the differentiation pathway of MSCs [1]. This has led to increased interest in the use of HP in the field of cartilage tissue engineering [2]. HP has been shown to increase both chondrogenic gene expression (e.g. Sox9, aggrecan, collagen type II) and matrix production (proteoglycan, collagen) in MSCs maintained in pellets, collagen I sponges, and synthetic scaffolds [3–8]. Furthermore, HP has been shown to play a role in the maintenance of the chondrogenic phenotype for chondrocytes and joint-tissue-derived stem cells by suppressing the expression of hypertrophic markers [9,10]. In contrast, other studies have demonstrated that HP has little to no effect on chondrogenic gene expression and matrix accumulation [11,12]. Therefore, uncertainty exists in the literature as to the role HP plays in regulating chondrogenic differentiation of MSCs.

Cell–matrix interactions also play a key role in the chondrogenic differentiation of MSCs [13]. Arginine–glycine–aspartic acid (RGD) is an amino acid sequence that integrins are known to bind to, and it is commonly used to allow cells to adhere to scaffolds that do not have any binding sites. Previous studies have demonstrated that when MSCs are seeded in RGD-modified alginate hydrogels chondrogenic gene expression and matrix accumulation are inhibited relative to arginine–glycine–glutamic acid (RGE)-modified controls, to which MSCs cannot adhere [14]. The inhibitory effect of RGD can be blocked with the addition of soluble RGD or cytochalasin D (an F-actin cytoskeleton inhibitor), demonstrating a role for cell attachment and actin cytoskeleton formation in suppressing chondrogenic differentiation [15]. While the interplay between MSCs and the extracellular environment may inhibit chondrogenesis, such interactions and the development of a functional actin cytoskeleton may also be necessary for mechanotransduction to occur [16–18]. For example, an intact dynamic actin cytoskeleton under tension has been shown to be necessary for fluid flow-induced changes in Sox9 gene expression in MSCs [19]. Furthermore, chondrocytes in pellet culture respond more favorably to HP relative to cells embedded in alginate hydrogels [20], suggesting that cellular interactions with the local environment also regulate stem cell response to HP. These studies would suggest that the response of MSCs to HP might depend on cell–matrix interactions unique to the scaffold or hydrogel within which they are embedded.

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The objective of this study was to examine the interplay of cell–matrix interactions and hydrostatic pressure on chondrogenesis of MSCs. We specifically sought to compare the response of MSCs to HP following encapsulation in fibrin, where cells are known to directly adhere and spread within the hydrogel [21,22], to that in agarose, where cells do not adhere and hence maintain a spherical morphology when encapsulated within the hydrogel [23–25]. Our hypothesis was that cells seeded in fibrin hydrogels would be more mechanosensitive and hence demonstrate a more robust response to HP, while cells seeded in agarose hydrogels would show little to no response to HP.

2. Materials and methods

2.1. Cell isolation, expansion, and encapsulation

Bone marrow was harvested from the femoral diaphysis of three 4-month-old pigs (~50 kg) under sterile conditions. MSCs were isolated and expanded according to a modified method developed for human MSCs [26]. Cultures were expanded in high-glucose Dulbecco's modified Eagle's medium (hgDMEM GlutaMAX) supplemented with 10% fetal bovine serum (FBS), and penicillin (100 U ml^{-1})–streptomycin (100 μg ml^{-1}) (all GIBCO, Biosciences). After expansion (third passage) MSCs were encapsulated in either agarose (Type VII, Sigma-Aldrich) or fibrin hydrogels at a density of 1.5×10^7 cells ml^{-1}. MSCs were mixed with agarose at ~40 °C to yield a final gel concentration of 2%. The agarose–cell suspension was cast in a stainless steel mold, and cored using a biopsy punch to produce cylindrical scaffolds (∅5 × 3 mm thickness). Fibrinogen (Sigma-Aldrich, Ireland) was dissolved in aprotinin solution (Nordic Pharma, UK). Cells were mixed with this solution and thrombin added to crosslink the gel. The solution was immediately injected into an agarose mold to yield fibrin cylindrical hydrogels (50 mg ml^{-1} fibrinogen, 5000 KIU ml^{-1} aprotinin, and 2.5 U ml^{-1} thrombin final concentrations) with the same dimensions as the agarose hydrogels. Constructs were maintained in 5 ml per construct of a chemically defined chondrogenic media consisting of hgDMEM GlutaMAX supplemented with penicillin–streptomycin (GIBCO), 100 μg ml^{-1} sodium pyruvate, 40 μg ml^{-1} L-proline, 50 μg ml^{-1} L-ascorbic acid-2-phosphate, 1.5 mg ml^{-1} BSA, 1 × insulin–transferrin–selenium, 100 nM dexamethasone (all Sigma-Aldrich, Ireland) and either 1 or 10 ng ml^{-1} recombinant human transforming growth factor-β3 (TGF-β3;ProSpec-Tany TechnoGene Ltd., Israel). Constructs were allowed to equilibrate overnight before the initiation of hydrostatic pressure.

2.2. Application of hydrostatic pressure

Constructs were sealed into sterile bags with 2 ml of medium per construct during the loading period, and then returned to culture dishes containing 5 ml of medium per construct. Cyclic HP was applied in a custom bioreactor filled with water within a 37 °C incubator as described previously [27]. The sealed bags exposed to HP were placed into the pressure vessel while the free swelling (FS) controls were placed into an open water bath next to the pressure vessel. HP was applied at an amplitude of 10 MPa at a frequency of 1 Hz, 4 h per day, 5 days per week for 3 weeks. Half-medium exchanges were performed every 3–4 days and media samples were collected for biochemical analysis.

2.3. Biochemical analysis

Constructs' ($n = 4$) wet weight and diameters were measured, and digested with papain (125 μg ml^{-1}) in 0.1 M sodium acetate, 5 mM L-cysteine-HCl, and 0.05 M EDTA (pH 6.0, all Sigma-Aldrich) at 60 °C under constant rotation for 18 h. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay as described previously [28], with a calf thymus DNA standard. Sulfated glycosaminoglycan (sGAG) content was quantified using the dimethylmethylene blue dye-binding assay (DMMB; Blyscan, Biocolor Ltd., Northern Ireland) with a chondroitin sulfate standard. Collagen content was determined by measuring the hydroxyproline content. Samples were hydrolyzed at 110 °C for 18 h in 38% HCl and assayed using a chloromine-T assay with a hydroxyproline:collagen ratio of 1:7.69 [29,30]. Media samples were also analyzed using the DMMB and hydroxyproline assays, and subsequently added to that accumulated within constructs to yield the total sGAG and collagen produced. Total sGAG and hydroxyproline values were normalized to DNA values; subsequently the HP groups were normalized to the FS groups. Media samples were analyzed for alkaline phosphatase (ALP) activity using a *p*NPP ALP colorimetric assay (Cambridge Bioscience Ltd., UK).

2.4. Confocal microscopy, histology, and immunohistochemistry

At day 21, constructs ($n = 2$) were cut in half and fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight at 4 °C and rinsed with PBS. In order to examine cellular morphology and the F-actin cytoskeleton, samples were permeabilized in a 0.5% Triton-X solution, washed in PBS, incubated in a solution containing 1.5% BSA and 5 U ml^{-1} rhodamine 110-phalloidin (VWR International, Ireland) for 1 h, and then imaged using a Zeiss 510 Meta confocal microscope at 20× magnification.

The remaining halves were dehydrated and embedded in paraffin wax. Constructs were sectioned perpendicular to the disc face yielding 5 μm thick sections. Sections were stained with either 1% Alcian blue 8GX (Sigma-Aldrich, Ireland) in 0.1 M HCl for sGAG, picro-sirius red for collagen, or Fast Red TR Salt 1,5-naphthalenedisulfonate (Sigma-Aldrich, Ireland) for ALP enzyme activity. Collagen types I, II, and X were further identified through immunohistochemistry. Sections were treated with peroxidase, followed by chondroitinase ABC (Sigma-Aldrich) in a humidified environment at 37 °C for 1 h to permeabilize the extracellular matrix. Samples were then blocked with goat serum, and afterwards the primary antibodies for collagen types I, II, and X (mouse monoclonal, Abcam) were applied for 1 h. Next, the secondary antibody (Anti-Mouse IgG biotin conjugate, Sigma-Aldrich) was added for 1 h followed by incubation with ABC reagent (Vectastain PK-4000, Vector Labs) for 45 min. Finally the slides were developed with DAB peroxidase (Vector Labs) for 4 min. Samples were washed with PBS between each step, and negative and positive controls of porcine ligament (positive for type I collagen, negative for type II collagen) and cartilage (positive for type II collagen, negative for type I collagen) were also assessed.

2.5. Experimental design

Porcine bone marrow from one donor was cultured, exposed to HP, and analyzed. In order to examine the effects of possible donor-to-donor variability, the experiment was independently repeated two more times with bone-marrow-derived MSCs from two additional donors. Data presented in Figs. 1–5 are from the first donor. Fig. 6 is a compilation comparing key data for all three donors. Fig. 7 compares ALP activity in the media from donors 1 and 3 (insufficient media prevented such an analysis for donor 2).

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.00, GraphPad Software) with 3–4 samples analyzed for each experimental group for every donor. Biochemical results, both