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Dynamic compression can inhibit chondrogenesis of mesenchymal stem cells

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ABSTRACT

The objective of this study was to investigate the influence of dynamic compressive loading on chondrogenesis of mesenchymal stem cells (MSCs) in the presence of TGF-β3. Isolated porcine MSCs were suspended in 2% agarose and subjected to intermittent dynamic compression (10% strain) for a period of 42 days in a dynamic compression bioreactor. After 42 days in culture, the free-swelling specimens exhibited more intense alcian blue staining for proteoglycans, while immunohistochemical analysis revealed increased collagen type II immunoreactivity. Glycosaminoglycan (GAG) content increased with time for both free-swelling and dynamically loaded constructs, and by day 42 it was significantly higher in both the core (2.5 ± 0.21%w/w vs. 0.94 ± 0.03%w/w) and annulus (1.09 ± 0.09%w/w vs. 0.59 ± 0.08%w/w) of free-swelling constructs compared to dynamically loaded constructs. This result suggests that further optimization is required in controlling the biomechanical and/or the biochemical environment if such stimuli are to have beneficial effects in generating functional cartilaginous tissue.

Articular cartilage has a limited capacity for repair. This has motivated the development of cell based therapies such as autologous chondrocyte implantation for the repair of cartilage defects [1,2]. A major limiting factor in extending the use of such therapies is obtaining sufficient numbers of differentiated autologous chondrocytes, particularly in elderly and more osteoarthritic (OA) patients. An age-related loss in chondrogenic capacity has been observed in culture-expanded chondrocytes [3], while collagen synthesis is lower in chondrocytes obtained from OA patients [4]. This may be partially explained by changes in the responsiveness of aged or OA chondrocytes to cytokines and growth factors [5–7]. In addition, OA chondrocytes are likely to have significant DNA damage among other cellular degenerative alterations [8].

Mesenchymal stem cells (MSCs) are a promising alternative cell source for cartilage repair due to both their ease of isolation and expansion, and their chondrogenic differentiation potential [9]. Chondrogenic differentiation of MSCs from different tissue sources has been demonstrated in the presence of growth factors from the transforming growth factor-β (TGF-β) superfamily [10–15]. A tissue engineering approach to repair damaged cartilage tissues would involve seeding MSCs into a scaffold and either implanting following minimal in vitro pre-culture, or to culture these constructs for longer periods of time to engineer a more functional cartilaginous tissue. While chondrogenesis does occur in these different systems, it has been demonstrated that the amount of cartilage matrix production and the subsequent mechanical properties of the tissue is lower with MSCs compared to chondrocytes [16], leading to the suggestion that further optimization may be required if MSCs are to be used to engineer cartilaginous tissues with similar functional properties to that obtainable with healthy chondrocytes.

It is well established that the application of appropriate levels of dynamic compressive loading can enhance the biosynthetic activity of chondrocytes [17–23]. It is also believed that the differentiation pathway of MSCs is at least partially regulated by the local mechanical environment. In the absence of chondrogenic growth factors, it has been demonstrated that dynamic compressive loading can enhance chondrogenesis of bone marrow derived MSCs [24–26]. For example, increased aggrecan gene expression has been observed with as few as three 4 hour loading cycles [25], however, other studies have observed little dynamic compression induced stimulation of gene expression or matrix synthesis in the absence of chondrogenic growth factors [27]. The combined effects of dynamic compression and chondrogenic growth factors (TGF-β) on chondrogenesis of MSCs are generally more complex [24,27]. Dynamic compression has been shown to up-regulate aggrecan gene expression in the absence of TGF-β, but to down-regulate it in the presence of TGF-β [24]. In both the presence and absence of chondrogenic growth factors, it has also been demonstrated that short-term exposure ($\leqslant$1 week) to intermittent dynamic

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compression leads to enhanced GAG synthesis in the proceeding weeks of culture compared to free swelling controls [26,28]. What remains unclear is what influence long-term application of dynamic compression has on the differentiation of MSCs in a chondrogenic environment. This is an important question not only for engineering functional cartilaginous tissues from MSCs, but also to understand how MSCs will respond once implanted into load bearing defects. The goal of this study was to determine how the daily application of dynamic compressive loading influences cartilage specific matrix production by MSCs undergoing chondrogenic differentiation in long-term agarose culture. Our initial hypothesis was that dynamic compression would enhance matrix synthesis, leading to increases in the mechanical properties of MSC seeded agarose hydrogels.

Methods

Cell isolation, expansion and agarose hydrogel encapsulation: Femora from a 4-month-old porcine donor (~50 kg) were sawn and the gelatinous bone marrow removed under sterile conditions. Porcine MSCs were isolated and expanded according to a modified method developed for human MSCs [29]. Cultures were expanded in high-glucose Dulbecco's modified Eagle's medium (DMEM GlutaMAX) supplemented with 10% foetal bovine serum (FBS), and penicillin (100 U/ml)-streptomycin (100 μg/ml) (all GIBCO, Biosciences, Ireland). Culture-expanded MSCs (3rd passage) were encapsulated in agarose (Type VII) at ~40 °C, to yield a final gel concentration of 2% and a cell density of 15×10^6 cells/ml. The agarose–cell suspension was cast in a stainless steel mould to produce cylindrical discs (Ø 6 × 4 mm thickness). Constructs were maintained in a chemically defined chondrogenic medium (CM) consisting of DMEM GlutaMAX supplemented with penicillin (100 U/ml)-streptomycin (100 μg/ml) (both GIBCO, Biosciences, Ireland), 100 μg/ml sodium pyruvate, 40 μg/ml L-proline, 50 μg/ml L-ascorbic acid-2-phosphate, 1 mg/ml BSA, 1 × insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma–Aldrich, Ireland) and 10 ng/ml recombinant human transforming growth factor-β3 (TGF-β3; R&D Systems, UK). Constructs were allowed to equilibrate for 4 days before the addition of dexamethasone and TGF-β3, and the initiation of mechanical compressive loading.

Application of dynamic compression: Intermittent dynamic compression (DC) was carried out in a custom pneumatic based compressive loading bioreactor with the constructs immersed in CM. The dynamic compression protocol consisted of ~10% strain amplitude superimposed on a 0.01 N preload at a frequency of 0.5 Hz. This loading regime was employed for a period of 1 h, 5 days/week. Free swelling (FS) controls were maintained in the same amount of medium adjacent to the loading device during loading periods. A 50% medium exchange was performed every 2–3 days.

Mechanical and biochemical analysis: Constructs were mechanically tested in unconfined compression between impermeable platens using a standard materials testing machine with a 5N load cell (Zwick Z005, Roell, Germany). Stress relaxation tests were performed, consisting of a ramp and hold cycle with a ramp displacement of 1 μm/s until 10% strain was obtained and maintained until equilibrium was reached (~30 min). Dynamic tests were performed immediately after the stress relaxation cycle. A cyclic strain amplitude of 1% superimposed upon the 10% strain was applied for 10 cycles at 1 Hz. The compressive equilibrium modulus and dynamic modulus were determined from these tests.

The biochemical content of constructs was assessed at each time point (0, 14 and 42 days); constructs were cored using a 3 mm biopsy punch, the wet mass of both annulus and core was recorded and then frozen for subsequent analyses. Annuli and core samples were digested with papain (125 μg/ml) in 0.1 M sodium acetate, 5 mM L-cysteine–HCl, 0.05 M EDTA, pH 6.0 (all from Sigma–Aldrich, Ireland) at 60 °C under constant rotation for 18 h. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay as described previously [30], with a calf thymus DNA standard. Proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycan (GAG) in constructs using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content [31], using a hydroxyproline-to-collagen ratio of 1:7.69 [32].

Histology and immunohistochemistry: At each time point, at least one sample per group was fixed in 4% paraformaldehyde overnight, rinsed in PBS, and embedded in paraffin. The constructs were embedded such that sectioning at 8 μm produced a cross section perpendicular to the disc face. Sections were stained with 1% alcian blue 8GX (Sigma–Aldrich, Ireland) in 0.1 M HCl which stains sGAG, and picro–sirius red to stain collagen. The deposition of collagen type II was indentified by immunohistochemical analysis. A rabbit anti-human polyclonal antibody (concentration 3 g/l; MD Biosciences, Switzerland) that binds to collagen type II was used. The presence of the collagen type II antibody was subsequently detected by the secondary antibody, biotin-labelled goat anti-rabbit polyclonal (concentration 1 g/l; Sigma–Aldrich, Ireland). Negative and positive controls, trachea and cartilage respectively, were included.

Statistical analysis: Statistical analyses were preformed using GraphPad Prism (Version 4.03) software with 3–4 samples analysed for each experimental group. Two-way ANOVA was used for analysis of variance with Bonferroni post-tests to compare between groups. Numerical and graphical results are displayed as means ± standard deviation. Significance was accepted at a level of $p < 0.05$.

Results

There were no statistical differences in the DNA content between the FS and DC groups at any time point; or between core and annular regions within any group of constructs (Fig. 1A).

The GAG content was significantly higher after both 14 and 42 days in culture compared to the corresponding samples on day 0 (Fig. 1B). No difference was found between the FS and DC groups for annulus and core respectively at day 14. However, at day 42, both annulus (1.09 ± 0.09%w/w vs. 0.59 ± 0.08%w/w) and core (2.5 ± 0.21%w/w vs. 0.94 ± 0.03%w/w) regions for FS constructs were significantly greater ($p < 0.001$) than DC constructs. GAG content in the core region of all construct groups was significantly greater than that in the annulus (Fig. 1B). Collagen content was also higher in FS constructs compared to DC constructs at day 42 (Fig. 1C).

Staining of construct sections with alcian blue for sulphated proteoglycan revealed a steady accretion of positive staining for both FS and DC conditions (Fig. 2). At both days 14 and 42 the FS constructs exhibited more intense positive staining for GAG than the DC constructs. Similar trends were seen in the Picro–sirius staining for collagen (data not shown). This staining corresponded to biochemical results, with a less intense staining around the periphery of the construct. The collagen type II immunohistochemistry (Fig. 2) provides evidence of chondrogenic differentiation, again showing stronger staining away from the construct edge. The DC constructs exhibit a more homogeneous spatial staining in each case than the FS constructs.

The equilibrium and dynamic mechanical properties were dependent on time in culture ($p < 0.001$) and mechanical stimulation ($p < 0.001$) (Fig. 3). However, significant differences between FS and DC were only seen at day 42 ($p < 0.001$); the equilibrium modulus of day 42 FS constructs reached 49.9 ± 0.6 kPa compared to 24.4 ± 1.5 kPa for the DC constructs. The 1 Hz dynamic modulus