Can Dynamic Compression in the Absence of Growth Factors Induce Chondrogenic Differentiation of Bone Marrow Derived MSCs Encapsulated in Agarose Hydrogels?

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Can dynamic compression in the absence of growth factors induce chondrogenic differentiation of bone marrow derived MSCs encapsulated in agarose hydrogels?

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Abstract—**The objectives of this study were twofold; to determine if cartilage specific matrix synthesis by mesenchymal stem cells (MSCs) is regulated by the magnitude and/or duration of dynamic compression in the absence of growth factors, and to investigate if expanding MSCs in the presence of both fibroblast growth factor-2 (FGF-2) and transforming growth factor β-3 (TGF-β3) would influence their subsequent response to dynamic compression following encapsulation in agarose hydrogels. Porcine bone marrow derived MSCs were suspended in agarose and cast to produce cylinders (Ø5×3mm). Constructs were maintained in a chemically defined medium. Dynamic compression was applied at 1 Hz at strain amplitudes of 5%, 10% and 5% superimposed upon a 5% pre-strain for durations of 1, 3 and 12 hours. MSCs were also expanded in the presence of FGF-2 and TGF-β3. The biochemical constituents of constructs were analyzed. Under strain magnitudes of 5% and 10% and durations of 1 and 3 hours small increases in sGAG accumulation relative to unloaded controls were observed. However this was orders of magnitude lower than that induced by TGF-β3 stimulation. Expansion in FGF-2 and TGF-β3 did not positively modulate chondrogenesis of MSCs in either unloaded or loaded culture.**

Keywords—**Tissue engineering, Cartilage, Mesenchymal stem cells, Mechanobiology, TGF-β3.**

I. Introduction

Chondrogenic differentiation of MSCs can be induced by members of the transforming growth factor-β (TGF-β) superfamily [1-3]. The differentiation pathway and biosynthetic activity of MSCs is also somewhat regulated by their biophysical environment [4,5]. MSCs have been cultured three-dimensionally *in vitro* in bioreactors designed to imitate joint loading, most commonly hydrostatic pressure and dynamic compression [6-8]. It has been demonstrated that dynamic compressive loading in the absence of TGF-β family members can increase chondrogenic gene expression [7-13] and accretion of cartilage specific ECM constituents [8,11,14]. Nevertheless some uncertainty remains as despite increases in gene expression, a number of studies have also demonstrated no positive effect of loading on glycosaminoglycan accumulation in the absence of chondrogenic cytokines [13,15].

Expansion conditions can influence the subsequent chondrogenic differentiation of MSCs. Monolayer expansion in the presence of fibroblast growth factor-2 (FGF-2) has been shown to enhance proliferation during expansion and the subsequent chondrogenic potential of MSCs [16-19]. The addition of growth factors including FGF-2 and TGF-β1 to chondrocyte expansion media has been shown to modulate the cells potential to re-differentiate and respond to regulatory molecules on transfer to a 3D environment [20,21]. Similarly, media including these growth factors added to fat pad and synovial cells during expansion resulted in enhanced sulfated glycosaminoglycan (sGAG) deposition and collagen type II gene expression [22]. It remains unclear what role MSC expansion conditions will have on the cells subsequent response to biophysical stimulation.

As outlined, there is significant variability in the reported responses of MSCs to mechanical loading. This can potentially be attributed to numerous factors, one of which could be that MSCs are sensitive to the magnitude and duration of loading they experience, as suggested by a number of computational studies [23,24]. It has previously been demonstrated that the frequency of dynamic compression modulates MSC viability and chondrogenic differentiation in the absence of growth factors [25]. The first objective of this study was to determine if cartilage specific matrix synthesis by MSCs is also regulated by the magnitude and/or duration of dynamic compression in the absence of growth factors. Related to this question, it is unclear what effect MSC expansion conditions will have on their subsequent response to dynamic compression. The second objective of this study is therefore to investigate if expanding MSCs in the presence of both FGF-2 and TGF-β3 will influence their response to dynamic compression following encapsulation in agarose hydrogels.

II. Materials and Methods

A. Experimental Design

This study comprised of two parts. The first involved MSC expansion in normal conditions (Norm) with constructs undergoing various magnitudes and daily durations of dynamic compression (DC) for 6 days in the absence of TGF-β3 as outlined in table 1. Unloaded free-swelling controls (FS) were kept both in the absence and presence of TGF-β3. Constructs were taken for analysis at day 7 and

day 28. The second study involved MSC expansion either in normal conditions (Norm) or with the addition of FGF-2 and TGF-β3 (FGF/TGF). Constructs from both conditions were kept in the absence or presence of 10 ng/mL TGF-β3 (CM- or CM+), and loaded to 10% strain for 2 hours/day, 5 days/week for 3 weeks. Each part of the study was preformed independently with separate porcine donors; n=4-6 constructs per condition.

Table 1 Loading conditions investigated in Study A

Strain amplitude	Duration	TGF-β3
0%	0 hrs	+/-
5%	1, 3 hrs	-
10%	1, 3, 12 hrs	-
5%(pre-strain) + 5%	1, 3, 12 hrs	-

B. Cell isolation and expansion

MSCs were isolated from the femora of two 4 month old porcine donors (~50 kg) within 3 hours of sacrifice as described [26]. Mono-nuclear cells were plated at a seeding density of 2.5×10^6 cells/10cm dish for colony forming unit fibroblast assay, or 10×10^6 cells/75cm^2 flask for expansion in high-glucose Dulbecco's modified eagles medium (4.5 mg/mL D-Glucose, 200mM L-Glutamine; hgDMEM) supplemented with 10% foetal bovine serum (FBS) and penicillin (100 U/mL)-streptomycin (100 µg/mL) (GIBCO, Biosciences, Dublin, Ireland) (Norm). FGF-2 (5 ng/mL) and TGF-β3 (0.5 ng/mL) (ProSpec-Tany TechnoGene Ltd., Israel) were added to a number of flasks from each animal (FGF/TGF) as described above. At the first passage colony's were stained with crystal violet (Sigma-Aldrich, Arklow, Ireland) and counted to obtain the colony-forming cell fraction. Cells were subsequently plated at 5×10^3 cells/cm^2 and expanded to passage two (18.5-20 population doublings) in a humidified atmosphere at 37°C and 5% CO_2.

C. Agarose encapsulation and dynamic compression

MSCs from 2 donors were pooled, suspended in hgDMEM and mixed with 4% agarose (Type VII, Sigma-Aldrich) in phosphate buffered saline (PBS) at a ratio of 1:1 at ~40°C, to yield a final gel concentration of 2% and a cell density of either 30×10^6 cells/mL (Study A) or 15×10^6 cells/mL (Study B). The agarose-cell suspension was cast between two plates and cored to produce cylindrical constructs (Ø 5mm×3mm thickness). Intermittent dynamic compression was carried out as described previously [27]. The dynamic compression protocol consisted of strain amplitude and duration as described above at 1 Hz.

D. Biochemical analysis

The biochemical content of constructs (n=3-4) was assessed at each time point as described previously [27]. Constructs were cored using a 3mm biopsy punch and digested with papain (125µg/ml; Sigma–Aldrich) at 60°C for 18 hours. This enabled spatial variations in biochemical content of the core and annulus of the construct to be determined. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay [28]. Sulphated glycosaminoglycan (sGAG) was quantified using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Carrickfergus, UK). Total collagen content was determined through measurement of the hydroxyproline content [29] with a hydroxyproline-to-collagen ratio of 1:7.69 [30]. Samples of cell culture medium taken for analysis at each media exchange (n=3) were analyzed for sGAG and hydroxyproline secreted to the media.

E. Statistical analysis

Statistics were preformed using MINITAB 15.1 software package (Minitab Ltd., Coventry, UK). Groups were analysed for significant differences using a general linear model for analysis of variance. Tukey's test for multiple comparisons was used to compare conditions. Significance was accepted at a level of $p\leq0.05$. Numerical and graphical results are presented as mean ± standard error.

III. Results

A. Study A: The influence of compression magnitude and duration

In the absence of TGF-β3 construct DNA content dropped with time in FS culture and was significantly less at day 28 than at either day 0 or day 7 (p<0.05). Conversely, with TGF-β3 addition, DNA content increased with time (p<0.05). FS controls in the presence of TGF-β3 demonstrated robust chondrogenesis, with sGAG accumulation of 1.03±0.05 %ww with TGF-β3 supplementation as opposed to 0.02±0.0003%ww in its absence. While increases in sGAG were slight, statistical increases were seen above FS controls in construct annuli for both 5% and 10% strain for durations of 1 and 3 hours (p<0.05; Fig. 1). Dynamic compression periods of 12 hours were detrimental to accumulation of both sGAG and collagen in construct cores. In the absence of TGF-β3, ECM accumulation was greatest in the annulus region (p<0.0001), while in its presence ECM accumulation was greatest in the core (p=0.0003).

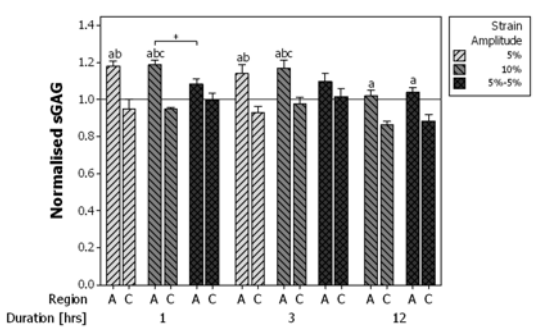

Fig. 1 Day 28 construct sGAG [%wet-weight] normalized to FS control. A: annulus, C: core. $p<0.05$, a vs. core; b vs. FS control; c vs. 12 hrs.

B. Study B: Expansion conditions

Greater consistency in colony forming units between donors was observed for cells expanded in the presence of FGF-2 and TGF-β3 (Fig 2). Colonies tended to be smaller and stained more intensely under FGF/TGF expansion than Norm conditions (Fig 2). On analysis of expansion media, FGF/TGF expanded MSCs were found to have secreted substantial quantities of sGAG while non-measurable levels were found in the Norm expansion media group.

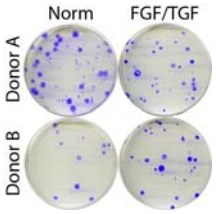

Fig. 2 Representative CFU-F assay plates.

Dynamic compression in CM+ conditions inhibited matrix accumulation, although increases were seen over day 0 sGAG and collagen levels. In the absence of TGF-β3, loading had no effect on sGAG or collagen accumulation (Fig. 3). Overall, sGAG accumulation was greatest in the Norm expansion FS constructs; however this was not significantly different than under FGF/TGF expansion (Fig 3A). These same trends were also apparent for collagen accumulation (Fig. 3B). Despite low collagen accumulation levels under CM- conditions, a considerable quantity was secreted to the media (2.45±0.29 µg/construct accumulated vs. 56.79±2.62 µg/construct secreted; not shown). Significantly more collagen was secreted to the media than remained in the construct for all CM+ groups bar FS Norm expansion. A greater proportion of total collagen production was secreted to media for loaded constructs in comparison to FS (p=0.003), although levels remained lower than FS groups.

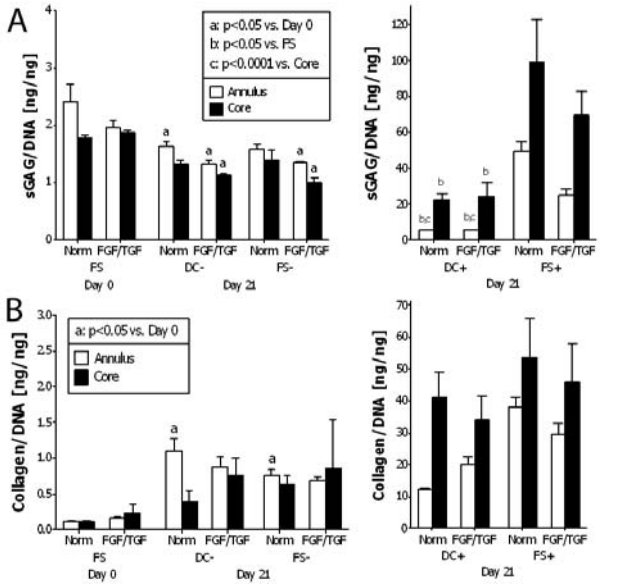

Fig. 3 A: sGAG/DNA. B: Collagen/DNA. Left: CM-, Right: CM+.

IV. Discussion

Previously, Huang et al. showed that dynamic compression alone applied to rabbit MSCs in agarose induced similar levels of aggrecan and collagen type II gene expression as TGF-β1 stimulation [7]. Under certain magnitudes and durations we saw an increase in sGAG accumulation relative to FS controls. However this was orders of magnitude lower than that affected through TGF-β3 stimulation as seen here and elsewhere [8,14]. In the absence of growth factors, dynamic compression has been shown to increase chondrogenic gene expression without a consequent increase in accumulated extra cellular matrix (ECM) [13]. This may suggest a disparity between gene expression results and accumulated ECM.

MSC expansion conditions did not affect the subsequent response to loading. Expansion with FGF-2 and TGF-β3 reduced sGAG and collagen accumulation in unloaded culture in the presence of TGF-β3, although this was not significant. This result is in contrast with previous work where fat pad and synovium cells were treated with FGF-2 and TGF-β1 [22]. This may be due to different concentrations of TGF-β used, the fact that they also added platelet-derived growth factor-bb, or simply variation between cell types. FGF-2 and TGF-β3 supplementation during expansion failed to illicit a more positive response to dynamic compression than found in cells expanded under Norm conditions. Future work will examine other scaffold systems where cells adhere to the substrate. This may alter the response of MSCs to mechanical stimuli.

V. CONCLUSIONS

Dynamic compression in the absence of growth factors can augment sGAG accumulation by bone marrow MSCs in agarose hydrogel in comparison to unloaded controls; however this remains orders of magnitude lower than that induced due to TGF-β3 stimulation. Expansion in FGF-2 and TGF-β3 did not positively modulate chondrogenesis of MSCs in either loaded or unloaded culture.

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