GROWTH FACTOR SUPPLEMENTATION AND DYNAMIC HYDROSTATIC PRESSURIZATION FOR ARTICULAR CARTILAGE TISSUE ENGINEERING

Robert L. Mauck*, Mandy M.Y. Ho*, Clark T. Hung*, Gerard A. Ateshian*+

*Department of Biomedical Engineering, Columbia University, New York, NY

+Department of Mechanical Engineering, Columbia University, New York, NY

INTRODUCTION

Articular cartilage transmits the stresses (5-12 MPa) that occur in joints with loading [1]. *In vivo*, a number of mechano-electrochemical signals arise with deformation of cartilage [2]. One of these signals, hydrostatic pressurization, occurs as a result of the high water content of the tissue and the small pore size. With deformation, fluid is constrained from rapidly leaving the tissue, and so pressurizes. This fluid pressurization has been shown both theoretically and experimentally to support upwards of 90% of the applied stress [3]. *In vitro*, chondrocytes respond to pressurization by altering their biosynthetic rates; dynamic pressurization generally leads to increases, while static pressure leads to decreases [4,5]. In monolayer culture of chondrocytes, dynamic pressurization (10 MPa, 1 Hz, 4 hours/day for 4 days) increased matrix gene expression [6]. Long-term growth of chondrocytes seeded in PGA felts demonstrated that intermittent hydrostatic pressure increased matrix deposition [7]. Based on these findings, we developed a custom bioreactor for applying dynamic hydrostatic pressurization (DHP) to chondrocyte-seeded agarose hydrogels [8]. With this device, we have recently reported a ~2 fold increase in the aggregate modulus compared to free swelling control, with similar increases in proteoglycan content after one month of DHP culture (3 MPa, 0.33 Hz) [8]. In other studies, using deformational loading, we had also previously shown that growth factors (IGF-1 and TGF-β1) interact synergistically with mechanical signals to increase tissue growth [9]. In the present study, we examined the growth of chondrocyte-seeded agarose hydrogels with growth factor supplementation for long term culture in free swelling conditions or with dynamic hydrostatic pressurization.

MATERIALS AND METHODS

Cell Culture: Chondrocyte-seeded agarose hydrogels were prepared as previously described [10]. Briefly, immature bovine chondrocytes were suspended in 2% agarose (Type VII, Sigma) at 60 million cells/ml. Disks (Ø 4.76 x 2.25 mm) were cored, and cultured in petri dishes (15 to 20 disks) with 30 ml of high glucose DMEM (supplemented with 10% fetal bovine serum, buffers, antibiotics, amino acids, and 50 μg/ml fresh ascorbic acid) at 37°C and 5% CO_2. Media, supplemented with the growth factors TGF-β1 (10 ng/ml), IGF-1 (300 ng/ml), or their combination, were changed daily. **Dynamic Hydrostatic Pressurizaton**: Dynamic hydrostatic pressurization (DHP) was applied using a custom-feedback controlled pressure bioreactor [8]. For pressurization, constructs were placed in sealed sterile plastic bags with 7 ml of fully supplemented DMEM (with growth factors where appropriate). Control samples were similarly sealed, and placed inside the incubator adjacent to the pressure bioreactor. DHP was applied with a triangular waveform with a peak pressure of 3 MPa and a frequency of 0.33 Hz for four hours per day, five days per week for four weeks. After loading, constructs were returned to free swelling culture in 30 ml of DMEM supplemented as above. Every two weeks, 3-4 samples were removed from culture for analysis. **Mechanical Testing**: Mechanical testing was carried out on constructs and native tissue (n=5) using stress relaxation tests in unconfined compression with a ramp compressive strain to 10% of the measured thickness. After equilibrium was reached, a sinusoidal displacement of 40 um was applied at

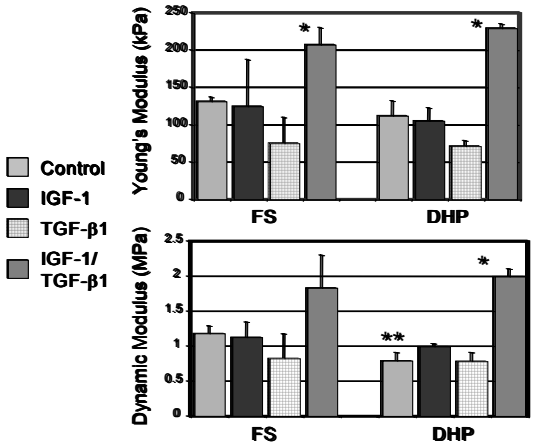

Figure 1 – Young's and dynamic modulus (at 1 Hz) on day 42. *indicates significant difference from CT in same group, ** indicates difference from CT in FS group ($p<0.05$, n=3-4).

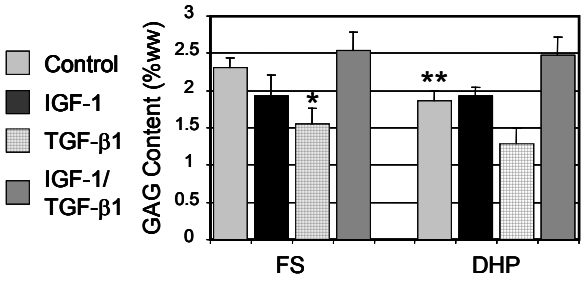

Figure 2 – GAG content (normalized to wet weight) on day 42. *indicates difference from CT in same group, ** indicates difference from CT in FS group (p<0.05, n=3-4).

frequencies ranging from 0.005-1.0 Hz. The Young's and dynamic moduli were calculated from the load/deformation profiles and specimen geometry. **Biochemistry and Histology**: After papain digestion, glycosaminoglycan (GAG) content was determined using the DMMB assay [11]. One half of each construct was fixed in acid/formalin/ethanol, dehydrated, embedded in paraffin, sectioned, deparaffinized, and stained with Safranin O. **Statistics**: Statistics were performed using one-way ANOVA with Fisher's LSD post-hoc tests, with α=0.05. Data is reported as the mean ± SD of 3-4 samples.

RESULTS

Supplementation with growth factors in free swelling culture had significant effects on the size, mechanical properties, and biochemical composition of chondrocyte-seeded agarose constructs. Over six weeks of culture, control constructs increased in both diameter (10.5%) and thickness (10.0%). Constructs supplemented with IGF-1 exhibited further increases in both diameter (24.0%) and thickness (22.4%) compared to control constructs on day 42 (**Table 1**). Supplementation with TGF-β1 or with IGF-1 and TGF-β1 together resulted in only slight changes in these values (<~6.0%). Constructs grew well under free swelling conditions for 42 days, with control (CT) constructs attaining a Young's modulus of ~131 kPa and a dynamic modulus of ~1.2 MPa (**Figure 1**) compared to starting values of ~10 kPa and ~0.1 MPa, respectively. Supplementation with IGF-1 had little effect on the mechanical properties, while TGF-β1 produced lower Young's and dynamic moduli. Interestingly, when IGF-1 and TGF-β1 were added together, a large increase in mechanical properties was observed, with constructs reaching a maximum Young's modulus of ~208 kPa (p<0.025, vs CT), and a dynamic modulus of ~1.8 MPa (p<0.10, vs. CT). GAG content of control constructs reached ~2.3% ww by day 42 (from a starting point of 0.2% ww). TGF-β1 supplementation led to significantly less GAG (1.6 %ww, p<0.025) while addition of IGF-1 and TGF-β1 together led to maximal increases to ~2.5% ww by day 42 (**Figure 2**). Safranin O staining for proteoglycans showed increases in intensity with IGF-1 and IGF-1 and TGF-β1 together, and showed clearly less staining intensity with TGF-β1 supplementation (**Figure 3**).

In this study construct growth was largely independent of applied dynamic hydrostatic pressurization. All measured parameters showed similar patterns in either mechanical environment (**Figure 1,2, Table 1**). DHP led to a smaller Young's modulus (~112 kPa), and a significantly smaller dynamic modulus (~0.79 MPa, p<0.025) versus free swelling controls. Furthermore, GAG content was significantly less, ~1.9 % ww (p<0.025), with DHP in control conditions. With DHP supplemented with IGF-1 and TGF-β1, constructs reached a Young's modulus of ~230 kPa and ~2.0 MPa, significantly higher than control DHP samples (p<0.01 and p< 0.001, respectively). These mechanical and biochemical properties compare favorably with the native tissue, which has a Young's modulus of ~277 kPa a dynamic modulus (at 1 Hz) of ~7.0 MPa, and a GAG content of ~3.0 % ww.

Day	Loading	Condition	Thickness (mm)	Diameter (mm)
0	--	--	2.39 ± 0.02	4.76 ± 0.00
42	FS	CT	2.63 ± 0.11	5.26 ± 0.01
		IGF-1	3.22 ± 0.26	6.52 ± 0.31
		TGF-β1	2.57 ± 0.16	5.13 ± 0.04
		IGF-1/ TGF-β1	2.55 ± 0.04	5.58 ± 0.10
	DHP	CT	2.75 ± 0.16	5.28 ± 0.08
		IGF-1	3.58 ± 0.21	6.71 ± 0.28
		TGF-β1	2.45 ± 0.06	5.15 ± 0.21
		IGF-1/ TGF-β1	2.58 ± 0.03	5.61 ± 0.08

Table 1 – Construct dimensions on day 0 and day 42.

DISCUSSION

This study demonstrates that growth factor supplementation affects the size and the biochemical and mechanical properties of chondrocyte-seeded agarose constructs. Interestingly, the addition of either IGF-1 or TGF-β1 alone did not enhance tissue properties. This finding is in contrast to our previous study, which showed that each led to increases in mechanical and biochemical properties [9]. These differences may be accounted for by the differences in seeding density (10 vs 60 x 10^6 cells/ml) or by the difference in age of the tissue from which the cells were harvested (2-12 days vs 4-6 months). Different serum lots, which may contain variable levels of growth factors (and inhibitors), were employed in these studies, and may have also contributed to the observed differences. The second finding of this study, that dynamic hydrostatic pressure showed no beneficial effects on construct growth, is counter to our findings previously reported using the same DHP regime [8]. It is possible that the observed differences between these studies arise for reasons as stated above, such as differences in serum lots and/or cell populations from different digestions. These subtle alterations may result in different responses of similar cells to a given mechanical stimulus. The variable results with dynamic hydrostatic pressure, however, are unlike the more consistent elevations in tissue properties that we have observed with applied deformational loading [10]. Despite the uncertainty regarding the efficacy of DHP in our construct system, this study does demonstrate that growth factor addition to chondrocyte-seed agarose hydrogels can increase construct material properties, particularly when applied in combination. Current studies are underway to assess the effects of growth factor addition and dynamic hydrostatic pressurization in a well-defined low serum containing growth media, to minimize possible variations seen between studies.

ACKNOWLEDGEMENTS

This study was supported by the NIH [R01 AR46568 and R01 AR46532] and a graduate fellowship from the Whitaker Foundation.

REFERENCES

[1] Ateshian and Hung, 2003, *Functional Tissue Engineering*, in press. [2] Mow, et al., 1992, *Biomaterials*, 13:67-97. [3] Soltz and Ateshian, 1998, *J Biomech*, 31:927-934. [4] Hall, 1999, *J Cell Phys*, 178:197-204. [5] Parkkinen, et al., 1993, *Arch Biochem Biophys*, 300:458-465. [6] Smith, et al., 2000, *J Rehabil Res Dev*, 37:153-161. [7] Carver and Heath, 1999, *Biotech Bioeng*, 62:166-174. [8] Mauck, et al., 2003, *Trans ORS*, accepted. [9] Mauck, et al., 2003, *Tiss Eng*, in press. [10] Mauck, et al., 2000, *J Biomech Eng*, 122:252-260. [11] Farndale, et al., 1982, *Connect Tiss Res*, 9:247-248.

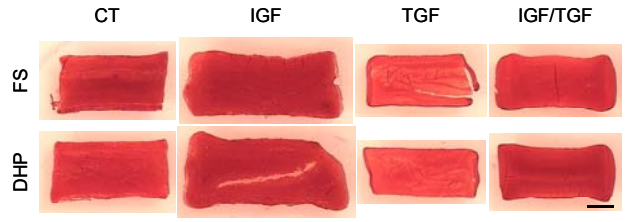

Figure 3 – Safranin O staining on day 42. Scale bar: 1 mm.