Proceedings of the 28th IEEE
EMBS Annual International Conference
New York City, USA, Aug 30-Sept 3, 2006

The Effect of Applied Compressive Loading on Tissue-Engineered Cartilage Constructs Cultured with TGF-β3

Eric G. Lima[1], Liming Bian[1], Robert L. Mauck[2], Benjamin A. Byers[3], Rocky S. Tuan[3], Gerard A. Ateshian[1], and Clark T. Hung[1+]

I. INTRODUCTION

The application of dynamic loading (DL) within appropriate ranges of magnitude and frequency can be a beneficial tool for the functional tissue engineering of articular cartilage. Using a variety of culture systems, physiologic dynamic loading *in vitro* has been shown to increase synthesis of cartilage extracellular matrix components such as proteoglycans, collagens and other matrix elements compared to control constructs maintained in free-swelling (FS) culture [2, 3, 9, 12, 13, 22]. Many factors influence the degree of tissue development. These include scaffold properties, media components, cell age and species, cell-seeding density, as well as loading parameters such as magnitude, duration, and frequency [8, 10, 11, 24]. Previously, our lab has demonstrated that the application of 10% strain at 1 Hz, for 3 hrs/day for 56 days to bovine chondrocyte-seeded agarose hydrogels cultured in media containing 20% fetal bovine serum can lead to Young's Modulus (E_Y) values of greater than 250 kPa (30% of the parent articular cartilage), or a five fold increase over FS controls [17, 18].

Chondrocytes *in vivo* also respond strongly to chemical stimuli. One such biochemical regulator of matrix biosynthesis found in articular joints is transforming growth factor β3 (TGF-β3) [1, 14, 19-21]. Recent work using the temporal supplementation of TGF-β3 in free-swelling, serum-free cultures of chondrocyte-laden agarose hydrogel constructs has shown great promise. In those studies, a 2-week exposure to TGF- β3 followed by six further weeks of culture in its absence resulted in the development of constructs possessing cartilage-like compressive mechanical properties [4].

It is the aim of the present study to extend our earlier work on the mechanical preconditioning of engineered cartilage constructs to include transient supplementation with TGF- β3 in a clinically-relevant, chemically-defined, serum-free media formulation.

Manuscript submitted April 24th, 2006. This work was supported in part by the NIH grant AR46568.

[1.] Department of Biomedical Engineering, Columbia University, New York, NY; [2.] McKay Laboratories for Orthopaedic Research, University of Pennsylvania, Philadelphia, PA; [3.] Cartilage Biology and Orthopaedics Branch, NIAMS, NIH, Bethesda, MD 20892; [+.] Corresponding author. Phone: 212-854-6542; fax: 212-854-8725; e-mail: cth6@columbia.edu).

II. MATERIALS AND METHODS

A. Cell Isolation:

Articular cartilage was harvested from bovine carpo-metacarpal (CMC) [18] joints of freshly slaughtered 4-6 month old calves. Cartilage was rinsed in high-glucose Dulbecco's Modified Essential Medium (hgDMEM) supplemented with 10% fetal bovine serum (FBS), amino acids (0.5X minimal essential amino acids, 1X non-essential amino acids), buffering agents (10 mM Hepes, 10 mM sodium bicarbonate, 10 mM TES, 10 mM BES), and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). The cartilage chunks were then combined and digested in DMEM with 0.5 mg/ml collagenase type II (Sigma Chemicals, St. Louis, MO) for 11 hours at 37°C with stirring. The resulting cell suspension was then filtered through a 70 μm pore size mesh and sedimented in a bench top centrifuge for 10 minutes at 1000 × *g*. Viable cells were counted using a hemacytometer and trypan blue. One volume of chondrocyte suspension (at 60 x 10^6 cells/ml) was then mixed with an equal volume of 4% low-melt agarose (Type VII, Sigma) at 37°C to yield a final cell concentration of 30 x 10^6 in 2% agarose. The chondrocyte/agarose mixture was cast into slabs and cored using a sterile disposable punch (Miltex, York, PA) to final dimensions of 0.5 cm diameter and 0.23 cm thickness (0.045 cm^3). Constructs were maintained in culture in a chemically defined serum-free growth medium for up to 56 days. Growth medium consisted of hgDMEM supplemented with 1X PSF, 0.1 uM dexamethasone, 50 ug/mL ascorbate 2-phosphate, 40 ug/mL L-proline, 100 ug/mL sodium pyruvate, and 1X ITS+ (Becton Dickinson). Growth medium was changed every three days and maintained at a cell/media volume ratio of less than 1 million cells/ml media. As detailed below, in the latter two experiments, growth medium was further supplemented with 10 ng/mL TGF- β3 (R&D Systems, Minneapolis, MN) for either the first 14 or 21 days of culture.

B. Loading Protocol:

Three consecutive studies are reported in this manuscript. Study 1 was performed without TGF- β3 with loading

initiated on day 0. Studies 2 and 3 were performed with TGF-β3 supplementation (14 or 21 days) with loading initiated at either day 0 (Study 2) or delayed until the applicatio β3.

In all cases the loading protocol consisted of a 10% peak-to-peak deformation in unconfined compression with impermeable platens, 1-Hz frequency, for 3 hrs/day, 5 days/week. The duty cycle consisted of of 3hrs of continuous loading followed by 21 hrs of rest. Deformational loading was carried out at 37°C and 5% CO_2 in a humidified incubator. FS controls were positioned adjacent to the loading device.

C. Material Testing:

Cylindrical constructs were tested in unconfined compression using a custom computer-controlled testing system [25]. Stress-relaxation tests were conducted to 10% strain at a strain rate of 0.05% strain/sec after an initial 0.02 N tare load. The Young's modulus (E_Y) of the construct was calculated from the equilibrium stress and initial cross-sectional area.n of growth factor was discontinued (Study 3). Dynamic loading applied at day 0 is abbreviated CDL, and termed DDL when initiated after the discontinuation of TGF-

D. Biochemical Content

The biochemical content of each sample was assessed by first measuring the sample wet weight, lyophilizing overnight, and measuring dry weight. Gross water content was determined from the difference. Once dry, the samples were digested in proteinase-K overnight at 56°C, as described previously [17, 18]. Aliquots of digest were analyzed for glycosaminoglycan (GAG) content using the 1,9-dimethylmethylene blue dye-binding assay [6, 7]. A further aliquot was acid hydrolyzed in 12 N HCl at 110°C for 16 hours, dried over NaOH, and resuspended in assay buffer (24 mM citric acid monohydrate, 0.012 v/v glacial acetic acid, 85 mM sodium acetate trihydrate, 85 mM sodium hydroxide, pH 6.0). Ortho-hydroxyproline (OHP) content was then determined via a colorimetric assay by reaction with chloramine T and dimethylaminobenzaldehyde [26], scaled for microplates. OHP content was converted to total collagen content using the conversion of 1:10 ratio of OHP:Collagen [27]. Each biochemical constituent (GAG, and collagen) was normalized to tissue wet weight.

III. Results

All constructs matured with time in *in vitro* culture, developing significantly different mechanical properties and biochemical composition depending on their culture conditions. Transient exposure to TGF-β3 strongly enhanced tissue elaboration in FS controls (E_Y=43±4 kPa (Study 1, no TGF-β3) vs. E_Y=311±95 kPa (Study 2, with TGF-β3) on day 35 (Fig 1, 2). The effectiveness of dynamic loading was also strongly influenced by the presence or absence of TGF-β3: DL groups achieved a 33% increase in E_Y over the corresponding FS group when no TGF-β3 was present in the culture medium (Fig 1a). Conversely, an 87% decrease in E_Y was observed compared to FS controls when loading took place from the outset, concurrent with TGF-β3 supplementation (Fig 2a). Finally, a 25% increase was observed when loading was introduced after discontinuation of TGF-β3 application (Fig 3a). GAG values were similar to or exceeded those of native cartilage in FS TGF-β3 discontinuation groups and in DDL groups (Fig 1c-3c), however, for all groups, collagen content was significantly lower than that of native tissue at all time points (Fig 1c-3c). For comparison, the mechanical and biochemical properties of juvenile articular cartilage were measured (n=5): E_Y=994±280kPa, GAG=5.9±0.9 (% w.w.), Collagen=21±0.7 (% w.w.)

IV. Discussion

The sequential application of growth factors (e.g., TGF-*β*1/FGF-2 followed by IGF-1) has previously been described for cartilage tissue engineering [23]. Important to these sequential protocols is the discontinuation step in which the growth factor is either removed or replaced with another factor. For the protocol adopted in this paper, cultures supplemented transiently with TGF-*β*3 consistently yielded cartilage-like tissue with higher mechanical properties than those derived from cultures with continuous (or no) growth factor supplementation [4]. In this study, we report that that sequential application of physiologic deformational loading after culturing with the growth factor TGF-*β*3 (for 2-3 weeks) yields significantly stiffer chondrocyte-seeded agarose constructs than cultures in which deformational loading was applied during the initial 2-3 week TGF-*β*3 exposure period. Using this culture protocol, engineered constructs were found to reach Young's modulus and GAG levels similar to that of native (parent) articular cartilage after only 42 days of culture. Collagen levels remained similar to levels achieved previously with serum-supplemented media [16]. While the underlying mechanisms leading to these findings remain to be elucidated, we speculate that there may be a role of enhanced convective transport of growth factor due to mechanical loading during the early culture period that adversely influences the chondrocyte response to application of deformational loading during subsequent culture without TGF-*β*3 [5, 15]. Interestingly, in cultures in which TGF-*β*3 is maintained without discontinuation (data not shown), the application of dynamic loading resulted in constructs with improved properties compared to free-swelling constructs, although these were less than are reported here with our protocol of applying loading after the discontinuation of TGF-*β*3. We have previously reported synergistic interactions between TGF-*β*1/loading as well as IGF-1/loading [16]. Together, our findings suggest that an optimal strategy for the functional tissue engineering of articular cartilage may incorporate sequential application of different growth factors

as well as *temporal* superposition of applied deformational loading.

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V. FIGURES

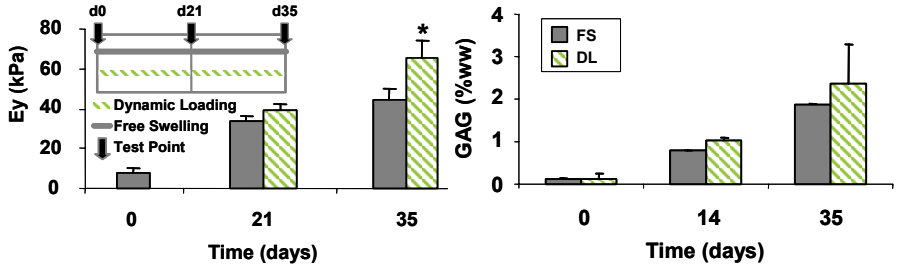

Figure 1: (Study 1) The effect of dynamic deformational loading in the absence of TGF-β3: E_Y (left) and GAG (middle). *=p<0.005 for FS vs. DL (n=4).

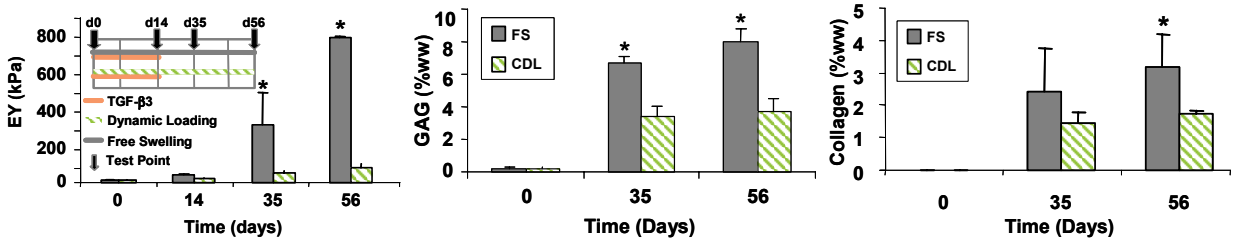

Figure 2: (Study 2) The effect of dynamic deformational loading applied concurrently with exposure to TGF-β3: E_Y (left), GAG (middle), collagen (right). *=p<0.005 for FS vs. CDL (n=4-6).

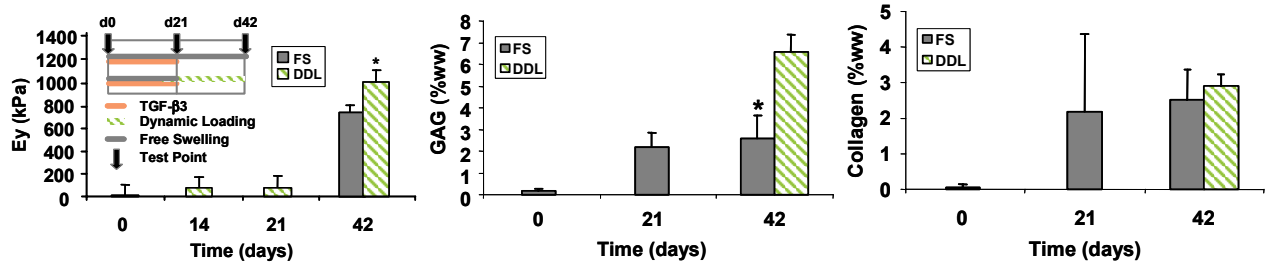

Figure 3: (Study 3) The effect of dynamic deformational loading initiated after the discontinuation of TGF-β3: E_Y (left), GAG (middle), and collagen (right). *=p<0.005 for FS vs. DDL (n=4-6).