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Effects of dynamic compressive loading on chondrocyte biosynthesis in self-assembling peptide scaffolds

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Abstract

Dynamic mechanical loading has been reported to affect chondrocyte biosynthesis in both cartilage explant and chondrocyte-seeded constructs. In this study, the effects of dynamic compression on chondrocyte-seeded peptide hydrogels were analyzed for extracellular matrix synthesis and retention over long-term culture. Initial studies were conducted with chondrocyte-seeded agarose hydrogels to explore the effects of various non-continuous loading protocols on chondrocyte biosynthesis. An optimized alternate day loading protocol was identified that increased proteoglycan (PG) synthesis over control cultures maintained in free-swelling conditions. When applied to chondrocyte-seeded peptide hydrogels, alternate day loading stimulated PG synthesis up to two-fold higher than that in free-swelling cultures. While dynamic compression also increased PG loss to the medium throughout the 39-day time course, total PG accumulation in the scaffold was significantly higher than in controls after 16 and 39 days of loading, resulting in an increase in the equilibrium and dynamic compressive stiffness of the constructs. Viable cell densities of dynamically compressed cultures differed from free-swelling controls by less than 20%, demonstrating that changes in PG synthesis were due to an increase in the average biosynthesis per viable cell. Protein synthesis was not greatly affected by loading, demonstrating that dynamic compression differentially regulated the synthesis of PGs. Taken together, these results demonstrate the potential of dynamic compression for stimulating PG synthesis and accumulation for applications to in vitro culture of tissue engineered constructs prior to implantation.

Keywords: Cartilage; Mechanical loading; Tissue engineering

1. Introduction

Articular cartilage defects may occur as a result of traumatic injury, or from degenerative diseases such as osteoarthritis. Such defects have a limited capacity for repair, and may require novel regenerative approaches to restore biological and mechanical functionality of damaged or diseased tissue. Tissue engineering strategies target the use of cell populations in order to regenerate functional repair tissue. One approach is to deliver isolated chondrocytes (Brittberg et al., 1994) or chondro-progenitor cells to voids created by the removal of dysfunctional tissue. To facilitate delivery, cells may be encapsulated in or attached to a biocompatible scaffold. A variety of materials are under investigation for cartilage tissue engineering, including biologically derived and synthetic polymers and hydrogels (Glowacki, 2000). Such scaffolds provide a three-dimensional template in which cells can initiate and mediate tissue repair.

The ultimate success of cell-seeded constructs for cartilage repair depends on appropriate long-term

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maturation of functional repair tissue in vivo, and integration with surrounding cartilage and bone. To achieve these goals, constructs may benefit from a period of in vitro culture prior to implantation to initiate a cellular repair response (Schaefer et al., 2002; Lee et al., 2003a, b). Culture conditions may be optimized to enhance extracellular matrix (ECM) synthesis, or stimulate proliferation and chondrogenesis of progenitor cells. ECM deposition in vitro may increase the mechanical functionality of the cell-seeded construct to withstand loading forces encountered after implantation (Butler et al., 2000). This could be critical for scaffolds that are significantly weaker than native cartilage (Lee et al., 2001; Rotter et al., 2002). Recent studies have incorporated physical stimulation of cell-seeded constructs during culture. Bioreactors have been designed to convect culture medium around (Vunjak-Novakovic et al., 1999) or perfuse directly through scaffolds (Pazzano et al., 2000), or impart hydrostatic pressure on the constructs (Carver and Heath, 1999; Mizuno et al., 2002; Angele et al., 2003). For each physical factor, conditions have been identified in which ECM synthesis and/or cell division was accelerated over static controls.

The effects of dynamic compression on chondrocyte biosynthesis have been well-characterized in cartilage explants and chondrocyte-seeded scaffolds. In explants, continuously applied dynamic compression (Sah et al., 1989) and dynamic tissue shear (Jin et al., 2001) have been found to increase synthesis of proteins and proteoglycans (PGs) over 24 h of loading. In chondrocyte-seeded agarose hydrogels, continuous dynamic compression increased chondrocyte biosynthesis during short-term loading (Buschmann et al., 1995; Lee and Bader, 1997; Hunter and Levenston, 2002). Intermittent compressive loading increased PG synthesis during short-term loading (Chowdhury et al., 2003) and material properties and GAG content over several weeks of culture (Mauck et al., 2000, 2002). Additional studies showed that dynamic compression (Davisson et al., 2002; Lee et al., 2003a, b) and shear (Waldman et al., 2003) could affect chondrocyte biosynthesis in tissue engineering scaffolds. These studies suggest that loading may enhance the long-term deposition of ECM in cell-seeded constructs during in vitro culture.

In this study, we investigated the effects of long-term dynamic compression on cellular biosynthesis and ECM retention in a chondrocyte-seeded peptide hydrogel, a scaffold actively under investigation for applications to cartilage repair (Kisiday et al., 2002). Initial studies were conducted with chondrocyte-seeded agarose hydrogels to explore the effects of various non-continuous loading protocols on chondrocyte biosynthesis. Thus, our first objective was to identify an appropriate protocol that would increase PG synthesis over control cultures maintained in free-swelling conditions, using a well-established chondrocyte culture scaffold (Benya and Shaffer, 1982; Buschmann et al., 1992). Combinations of periods of loading and free-swelling culture were explored to maximize retention of newly synthesized ECM. Based on these results, an optimized loading protocol was applied to cell-seeded peptide hydrogels with the second objective of quantifying the effects of dynamic compression on synthesis and accumulation of ECM, viable cell density, and mechanical properties for up to 39 days of in vitro loading.

2. Materials and methods

2.1. Isolation of chondrocytes and casting of cell-seeded peptide and agarose hydrogels

Chondrocytes were isolated from femoral condyles of 1–2-week-old bovine calves as previously described (Ragan et al., 2000). Chondrocytes were seeded in a self-assembling peptide hydrogel of sequence AcN-KLDLKLDLKLDL-CNH_2 at 0.4% (w/v) or 2% low melting temperature agarose (Gibco) hydrogels in 1.6-mm-thick slab structures as previously described (Kisiday et al., 2002). Cells were seeded at a concentration of $15–30 \times 10^6$ cells ml, similar to seeding densities previously found to be responsive to mechanical loading (Buschmann et al., 1995; Mauck et al., 2002). Diameter disks of 12-mm were cored from the hydrogel slabs immediately after casting. All disks were cultured in high glucose DMEM plus 0.1 mM non-essential amino acids, 10 mM HEPES, 0.4 mM proline, 20 μg/ml ascorbate, 100 U/ml penicillin, and 100 μg/ml streptomycin, and further supplemented with 1% ITS (final medium concentration: 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml sodium selenite, Sigma Chemical) and 0.2% FBS (Hyclone). Medium was changed every 1–2 days.

2.2. Dynamic compression

In the base component of a specialized chamber for application of dynamic compression to constructs (Fig. 1A), six wells each hold one 12 mm-diameter disk for loading in uniaxial unconfined compression. Polyethylene porous platens (40% void, 120 μm pore size) of 13 mm diameter were attached to the lid, aligned coaxially with the center of each well. Axial displacement of the lid produced a corresponding deformation of the hydrogel sample. In addition, the lid contained a center-mounted spring aligned with a non-culture well in the base; the spring created ~800 μm gap between the platens and samples when the lid was unloaded. Displacement of the lid, and subsequent construct deformation, was controlled by an incubator-housed loading apparatus (Fig. 1B; Frank et al., 2000). Experiments used sinusoidal dynamic compression protocols with 2.5% strain amplitude superimposed on 5% static offset

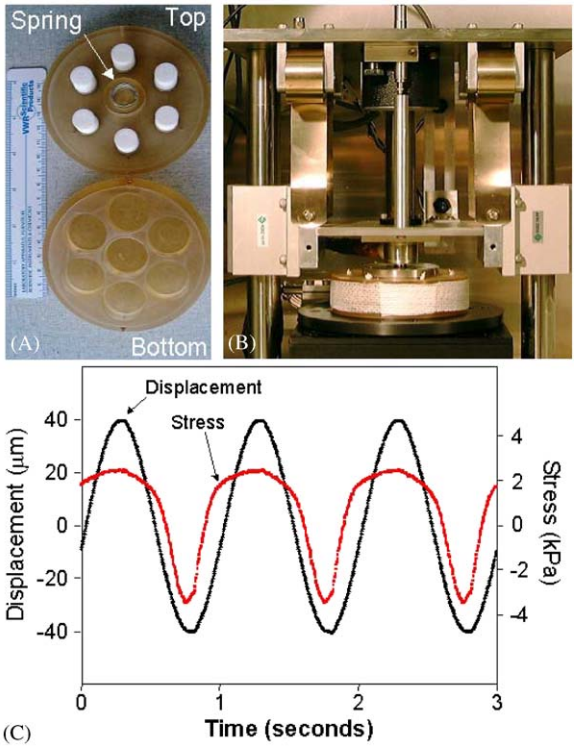

Fig. 1. (A) Culture chamber for application of dynamic compression to chondrocyte-seeded hydrogels. The chamber top contains platens for dynamic compression of hydrogel samples in the bottom chamber, as well as a center-mounted spring to displace platens above hydrogel surfaces for free-swelling culture. (B) Incubator-housed loading apparatus for applying dynamic compression to chamber (see Frank et al., 2000, for details). (C) Applied sinusoidal displacement (2.5% strain amplitude) superimposed on a 5% static offset compression (80 μm displacement), and the resulting cyclic component of the measured stress from days 2–3 of loading using peptide hydrogel disks, which is superimposed on a corresponding ~2 kPa static offset stress. The spring contributed less that 10% to the total measured stress amplitude.

strain at a frequency of 1.0 Hz (Fig. 1C), in displacement control. Loading was performed non-continuously using cycles consisting of 30 min–1h periods of compression followed by 30 min–7 h periods of free swelling culture, the latter achieved by backing the platens 600–800 μm off the hydrogels. The application of sinusoidal displacement resulted in an oscillatory stress (e.g., Fig. 1C). Total harmonic distortion was typically ~20% for both chondrocyte-seeded peptide and agarose hydrogels. (To obtain a more sinusoidal stress and avoid near-lift-off at 1 Hz, a larger offset strain could be applied; however, the current approach was used to minimize deformation during long term loading while still providing a stimulatory physical signal.)

2.3. Cellular biosynthesis of ECM macromolecules

Culture medium was supplemented with 5 μCi/ml ^{35}S-sulfate and 10 μCi/ml ^{3}H-proline to measure the rate of synthesis of sulfated PGs and total proteins, respectively (Hascall et al., 1983), for 20-h periods. Following digestion in proteinase K (Roche)-Tris HCl solution, radiolabel incorporation and total accumulated sulfated glycosaminoglycan (GAG) content via DMMB dye binding were measured as described previously (Sah et al., 1989). Additional hydroxyproline analyses were performed on selected peptide samples via *p*-dimethylaminobenzaldehyde binding (Stegemann and Stalder, 1967). Radiolabel incorporation and GAG and hydroxyproline content were normalized by plug wet weight (peptide) or DNA content (agarose). (Interference of peptide digest with the DNA spectrofluorometric measurement prevented normalization of peptide samples to DNA content; Kisiday et al., 2002.)

2.4. Quantification of ECM macromolecules lost to the culture medium

In selected cultures, total GAG and hydroxyproline loss to the medium was measured via DMMB dye and *p*-dimethylaminobenzaldehyde-binding analysis, respectively. The percentage of newly synthesized molecules retained in the hydrogel was calculated from the measured content of radiolabeled macromolecules retained in the scaffold and that lost to the medium. Medium fractions were separated into macromolecular and low molecular weight components on a PD10 gel filtration column of Sephadex G-25 (molecular weight cutoff of 1–5000 kDa; Pharmacia, Piscataway, NJ) (Benya and Shaffer, 1982).

2.5. Viable cell density and DNA content

A viable cell kit based on the compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega Corp., Madison, WI) was used to determine viable cell density in peptide hydrogel. At each time point, 4–8 mg sections were cut from the center region of the 12 mm disk. Each section was incubated in MTS medium followed by spectrophotometric quantification of absorbance (Kisiday et al., 2002) Viable cell densities were determined by dividing the MTS absorbance by the wet weight of the sample, assuming a linear relationship between MTS absorbance and viable cell density as seen previously for (Kisiday et al., 2002). Agarose hydrogel digests were analyzed for DNA content via Hoechst spectrofluorometric measurement (Kim et al., 1988).

2.6. Mechanical properties of cell-seeded hydrogels

The equilibrium modulus and dynamic stiffness of 3 mm peptide plugs cored from disks were measured in radially confined uniaxial compression using a Dynastat mechanical spectrometer (IMASS, Hingham, MA)

(Frank and Grodzinsky, 1987a). A porous platen was used to apply five sequential 2% ramp-and-hold compressions to each plug from 10% to 18% strain. Each 2% compression was applied over 60 s, followed by 3 min of hold, resulting in an initial increase and subsequent relaxation of compressive stress. The ratio of the relaxed equilibrium stress to the engineering strain was used to compute the equilibrium modulus. At 14% compressive offset strain, a 0.8–1% amplitude sinusoidal strain was applied at 1.0, 0.5, and 0.1 Hz. The dynamic compressive stiffness was calculated as the ratio of the fundamental amplitudes of stress to strain at each frequency. The equilibrium modulus and dynamic stiffness data were used in combination with the method of Frank and Grodzinsky, 1987b, to calculate the effective hydraulic permeability.

2.7. Histological examination of ECM accumulation

Peptide specimens were fixed and analyzed for PG content via toluidine blue staining (Kisiday et al., 2002). Collagen immunohistochemistry: sections were treated with pepsin for 30 min, washed with TBS buffer, treated with 0.6% H_2O_2 in methanol, rinsed again with TBS, and treated with mouse anti-collagen-type II IgG (Clone CII C1, DSHB, 1:1000 in TBS) for 60 min, as previously described (Domm et al., 2002). After incubation with rabbit anti-mouse IgG (HRP-conjugated, Dako P-0260, Hamburg; 1:200 in TBS containing 1% bovine serum) for 30 min, the sections were rinsed, incubated for 30 min with goat anti-rabbit IgG (HRP-conjugated, Dako P-0448; 1:100 in TBS containing 1% bovine serum), and stained with diaminobenzidine (DAB-Kit, Vector Laboratories). Cell nuclei were counterstained using Meyer's hemalum, and samples were embedded on microscopic slides using Aquatex (Merck).

3. Results

3.1. Normalization of radiolabel incorporation and retention in the scaffold

In our previous studies, incorporation of ^{35}S-sulfate and ^{3}H-proline in free-swelling chondrocyte-seeded peptide and agarose hydrogels reached stable levels from days 15 to 28 after casting (Kisiday et al., 2002). Given that radiolabel incorporation in this study was performed between 15 and 30 days after casting, steady-state radiolabel incorporation was expected for each free-swelling time course. This was, indeed, found to occur in all experiments (data not shown): for any given time course, incorporation in free-swelling cultures at each time point varied by no more than 15% of the average value over the entire time course. Thus, the normalized data for dynamically compressed cultures in Figs. 2 and 7 reflect changes in biosynthesis due to loading, and are not the result of inconsistent behavior of the free-swelling controls.

3.2. Dynamic compression of chondrocyte-seeded agarose hydrogels

Chondrocyte-seeded agarose was used to study the effect of various non-continuous loading cycles on chondrocyte biosynthesis. Agarose samples were seeded at 15×10^6 cells/ml, and dynamic compression initiated at multiple time points between 0 and 22 days after casting. Loading was applied for 3–5 days, with radiolabel incorporation performed during the last 20 h of the loading period. Loading protocols were defined by cycles consisting of a period of dynamic compression followed by a period of free-swelling culture, with (loading/free-swelling) = (30 min/30 min) (1 h/1, 3, 5, or 7 h). For all loading cycles, ^{35}S-sulfate incorporation was 20–80% of free-swelling controls, with p-values typically less than 0.001 (e.g., Fig. 2A). Next, a compression protocol was designed in which loading was applied on alternate days. Each loading cycle consisted of 45 min of compression followed by 5 h 15 min of free-swelling culture; this 6 h cycle was repeated four times, and was followed by 24 h of free-swelling culture to complete the alternate day loading cycle. ^{35}S-sulfate incorporation was 25% ($p < 0.001$) and 12% ($p < 0.05$) higher than controls on days 4 and 12 (Fig. 2B). Based on these data, the effects of dynamic compression on chondrocyte-seeded peptide hydrogels were explored for both continuous and alternate day application of the defined 6 h loading cycle.

3.3. Dynamic compression of chondrocyte-seeded peptide hydrogels

3.3.1. Continuous loading

A fourteen-day loading experiments was performed in which chondrocyte-seeded peptide hydrogels were subjected to continuous application of the 6 h loading cycle defined in the alternate day loading protocol above. Peptide hydrogels (30×10^6 cells/ml) were cultured free-swelling for 14 days prior to loading. In dynamically compressed samples, ^{35}S-sulfate incorporation on day 5 was 60% of controls ($p < 0.001$), but increased with time in culture to 122% of controls on day 14 ($p < 0.005$) (Fig. 2C). Total GAG accumulation was not significantly different from free swelling controls at either time point ($p = 0.84, 0.92$, data not shown).

3.3.2. Alternate day loading

The alternate day loading protocol defined in the agarose study was applied to peptide hydrogels (15×10^6 cells/ml) over an 11-day time course after an initial 22 days of free-swelling culture. ^{35}S-sulfate

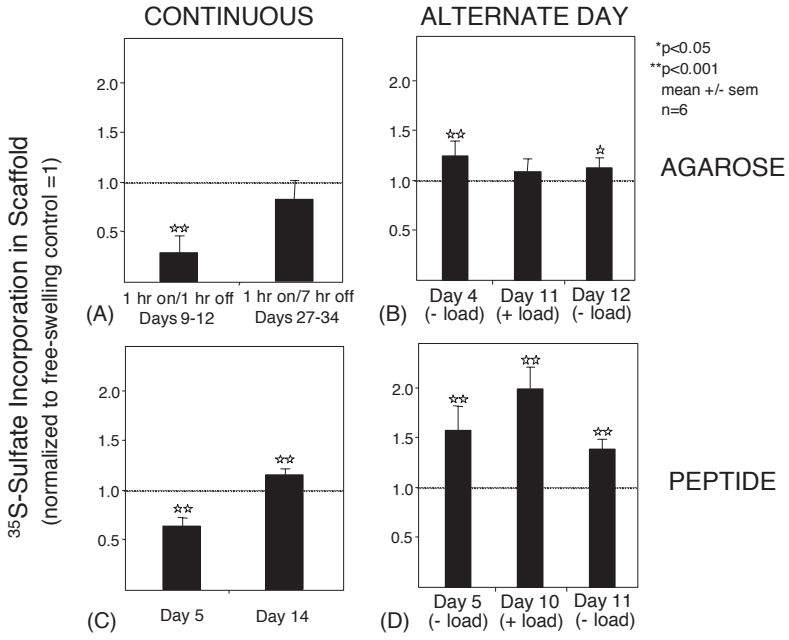

Fig. 2. Dynamic compression of chondrocyte-seeded peptide and agarose hydrogels—Proteoglycan synthesis. Radiolabel incorporation of ^{35}S-sulfate for quantification of newly synthesized proteoglycans retained in the scaffold. Free-swelling controls are equal to 1 for all figures, p values show significant differences vs. pairwise controls—AGAROSE: (A) Representative data for continuous repetition of defined loading cycles. Similar data were obtained for a variety of loading cycles in which dynamic compression was applied for 12.5–50% of the length of the cycle. (B) Alternate day loading. Dynamic compression was applied for 45 min over a 6 h cycle. This loading cycle was repeated four times, followed by 24 h without loading to complete the alternate day loading protocol. PEPTIDE HYDROGEL: (C) Continuous application of the 6 h loading cycle defined in the agarose alternate day loading protocol. (D) Alternate day loading as performed for (B).

incorporation in dynamically compressed samples was significantly higher than that in controls at each time point (Fig. 2D), with the greatest increase on the day of loading (day 10, two-fold increase over controls, $p<0.001$) relative to the days of free swelling culture (day 5, 60% increase, $p<0.005$; day 11, 40% increase, $p<0.001$). The total GAG content of compressed samples was 9% higher than free-swelling controls on days 10 and 11 ($p<0.05$, data not shown).

Total PG synthesis was determined on days 10 and 11 by quantifying newly synthesized ^{35}S-sulfate macromolecules lost to the culture medium together with that retained in the scaffold. Free-swelling control hydrogels retained 97–99% of ^{35}S-macromolecules. During loading on day 10, compressed samples retained 98% of ^{35}S-macromolecules. During the subsequent 20 h without loading on day 11, scaffold-retention of ^{35}S-macromolecules was similar to that during the period of loading (98%).

3.3.3. Extended 39-day alternate day loading study

Peptide hydrogels were seeded with chondrocytes at 30×10^6 cells/ml and maintained in free-swelling conditions for 14 days prior to loading. Samples were analyzed for total GAG and viable cell density on days 0, 8, 16, and 39. ^{35}S-sulfate incorporation was evaluated on day 8 only. GAG loss to the medium was evaluated at each medium change (every 1–2 days) throughout the 39-day time course. Mechanical testing and histological evaluations were performed on day 39. GAG accumulation in compressed samples was not significantly different from controls after 8 days of loading ($p=0.47$, Fig. 3A), despite a two-fold increase in ^{35}S-sulfate incorporation on day 8 ($p<0.001$, data not shown). By day 16, GAG accumulation in compressed samples was 5.8 μg/mg wet weight, ~38% higher ($p=0.01$) than in the controls. Over 39 days of loading, GAG accumulation in compressed samples was 22% higher than controls (15.5 vs. 12.7 μg/mg ww, Fig. 3A). This translated into total GAG contents (including GAG accumulation over the 14 days prior to loading) that were 13% higher in compressed samples, containing significantly more GAG matrix (24.6 μg/mg ww) than controls (21.8 μg/mg ww) ($p<0.05$).

3.3.4. Histological analyses

Toluidine blue staining of compressed samples showed PG deposition that was reasonably uniform throughout the cross-section thickness, with slightly higher staining in cell-associated (pericellular) regions (Fig. 3B). PG staining was also present throughout the free-swelling hydrogel (Fig 3C), though not distributed as uniformly, and only in certain areas was staining as intense as that in dynamically compressed samples. Type II collagen was distributed throughout compressed and

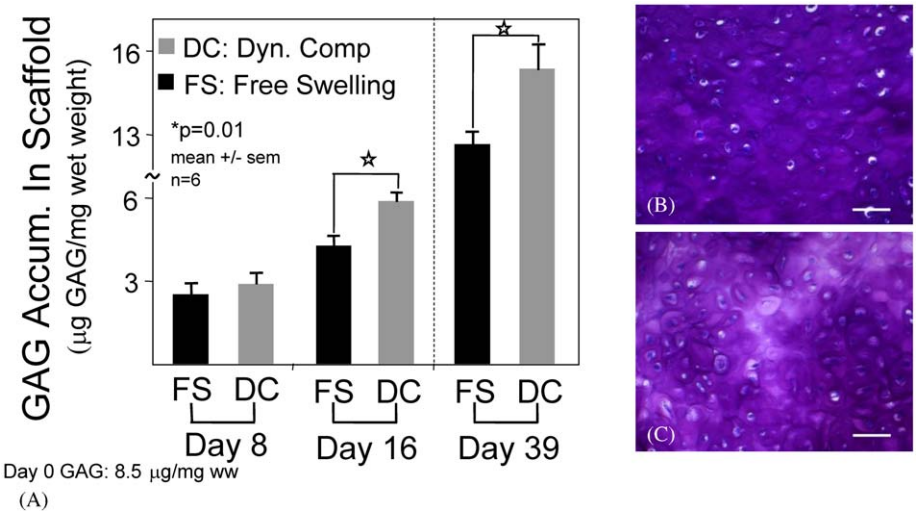

Fig. 3. (A) GAG accumulation in chondrocyte-seeded peptide hydrogels subjected to alternate day loading or maintained in free-swelling conditions. (B) Toluidine blue staining of chondrocyte-seeded peptide hydrogel after 39 days of alternate day dynamic compression, bar = 100 μm. (C) Toluidine blue staining of matched 39-day free-swelling control sample, bar = 100 μm.

free-swelling hydrogels (immunohistochemical data not shown).

3.3.5. Compressive mechanical properties

The day 39 confined compression equilibrium modulus of dynamically compressed samples was 97 kPa, an increase of 18% over controls (82 kPa) (Fig. 4). The dynamic stiffness of both dynamically loaded and free-swelling controls samples increased with increasing frequency, characteristic of poroelastic tissues such as cartilage (Frank and Grodzinsky, 1987a). The dynamic stiffness of compressed samples was ~60–70% higher than that of controls at each frequency tested. The hydraulic permeability computed from the dynamic stiffness decreased from $1.1 \times 10^{-13}\,m^4/(N\,s)$ in controls to $1.9 \times 10^{-14}\,m^4/(N\,s)$ in loaded samples.

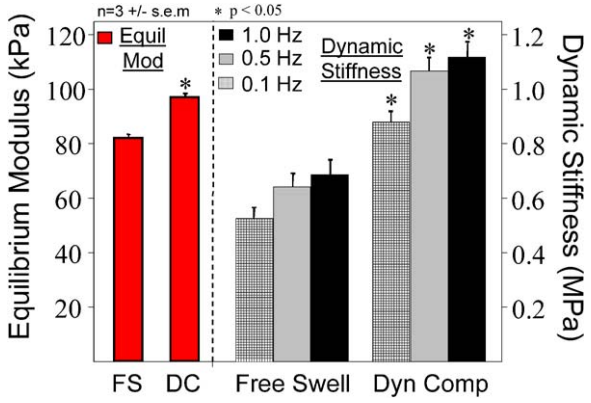

Fig. 4. Compressive mechanical properties of dynamically loaded chondrocyte-seeded peptide gels. Uniaxial confined compression equilibrium modulus and dynamic stiffness of chondrocyte-seeded peptide hydrogel subjected to alternate day loading versus that maintained in free-swelling conditions for 39 days.

3.3.6. Cumulative GAG loss to the medium

GAG loss was analyzed together with scaffold accumulation of GAG to determine total GAG biosynthesis (Fig 5A). For free-swelling controls, cumulative GAG loss to the medium represented 32%, 36%, and 22% of total GAG synthesis for samples terminated on days 8, 16, and 39, respectively. (Previous studies have also shown GAG loss to the medium for chondrocyte-seeded agarose hydrogels (~40%) to be greater than the loss of ^{35}S-sulfate labeled macromolecules (<5%), Buschmann et al., 1992.) In dynamically compressed samples, cumulative GAG loss to the medium represented a higher percentage of total GAG biosynthesis, with 47%, 39%, and 37% of total GAG lost to the medium by days 8, 16, and 39, respectively. Total GAG synthesis was 51%, 46%, and 53% higher for loaded samples than paired control hydrogels on days 8, 16, and 39, respectively. The rate of GAG loss (per 24 h) in free-swelling control cultures was ~0.1 μg GAG/ww/day (Fig. 5B). These values represented no more than loss of 1.5% of the GAG content in the scaffold per day, with calculated values of 1.1%, 1.4%, 1.1%, and 0.6% on days 0, 8, 16, and 39, respectively. GAG loss to the medium was higher for loaded cultures at all time points, and was initially highest between days 3–9, with a maximum loss of ~0.36 μg GAG/ww/day for medium collected on day 5. GAG loss decreased to ~0.15–0.2 μg GAG/ww/day from days 9 to 23, then increased with time in culture, reaching rates of GAG loss similar to that of peak values within the first 9 days. GAG loss in loaded samples represented 2.2%, 1.5%, and 1.3% of the GAG content in the scaffold on days 8,

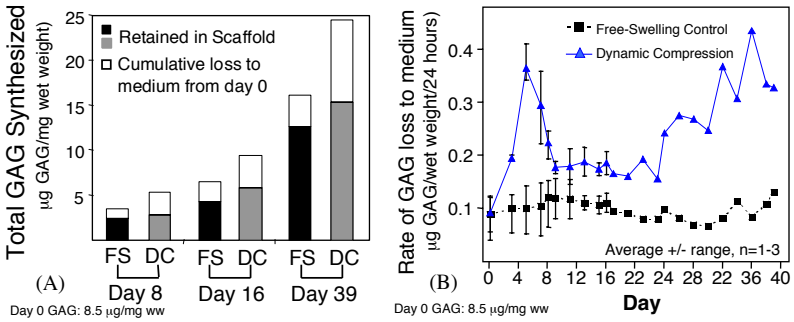

Fig. 5. Effects of dynamic compression on total GAG synthesis in chondrocyte-seeded peptide gels. (A) Total GAG synthesis in peptide hydrogel samples subjected to alternate day loading or maintained in free-swelling culture. GAG loss to the medium is cumulative from day 0 until the samples were removed from culture on specified day. (B) Rate of GAG loss to the medium over 39-day loading period. For each medium change, the amount of GAG loss per scaffold wet weight was normalized to the time in culture in order to calculate the rate of GAG loss per day.

16, and 39, respectively. However, these time points do not include peak GAG loss within the first 8 days of culture during which GAG loss was estimated to be ~4% of the scaffold content/day.

3.3.7. *Viable cell densities*

The viable cell densities of free-swelling controls did not change significantly with time in culture (Fig. 6). Relative to day 0, control MTS absorbance values varied by less than 6% at subsequent time points, with no significant effect of culture duration (one-way ANOVA, $p = 0.8$). Dynamic compression caused a modest but significant decrease in viable cell density by 15–20% relative to paired control values at days 8, 16, and 39 ($p < 0.01, 0.05, 0.01$, respectively).

3.3.8. *Protein synthesis in peptide hydrogels*

^{3}H-proline incorporation was measured simultaneously with all ^{35}S-sulfate data of Fig. 2. Continuous application of the 45 min on/5 h 15 min off loading cycle decreased ^{3}H-proline incorporation to 40% and 60% that of free-swelling controls on day 5 and 14 of loading, respectively ($p < 0.001$, data not shown). ^{3}H-proline incorporation in alternate-day loaded samples was similar to controls on day 5 ($p = 0.72$), and ~20% less than controls on days 10 and 11 ($p = 0.034$, 0.001, Fig. 7B). The quantification of ^{3}H-proline labeled macromolecules in the medium showed that for loaded (vs. free swelling) samples on days 10 and 11, ~93% (vs. ~87%) of newly synthesized proteins were retained in the scaffold. Similar results were obtained for agarose hydrogels (Fig. 7A). *Hydroxyproline content* in both loaded and free-swelling samples increased with time in culture at a similar rate, with no statistically significant difference ($p = 0.09-0.6$) at each time point (Fig. 7C). Cumulative hydroxyproline loss to the medium (quantified for the first 8 days of loading only) was 0.30 μg/mg wet weight for both loaded and free swelling conditions.

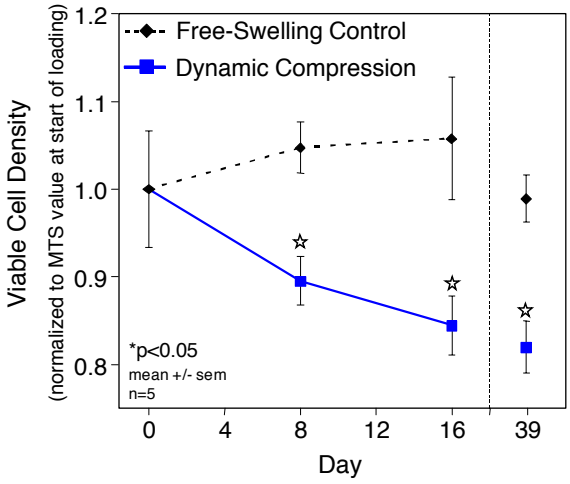

Fig. 6. Dynamic compression of chondrocyte-seeded peptide—viable cell density. MTS viable cell density of chondrocyte-seeded peptide hydrogel. Dynamic compression samples were loaded using the alternate day loading protocol. p values show significant differences vs. pairwise controls.

4. Discussion

In this study, we found that application of dynamic compression on alternate days was optimal for long-term stimulation of PG synthesis in hydrogel cultures. The extent to which periods of free-swelling culture were necessary to intersperse between periods of loading was unexpected, given that continuous loading over short-term culture (24–48 h) was previously found to stimulate PG synthesis (Buschmann et al., 1995; Lee and Bader, 1997). In the present study, the significant inhibition of PG synthesis in agarose culture observed for a range of continuous loading protocols applied over 3–5 days suggested that uninterrupted loading would not increase PG synthesis over the longer term. Thus, incorporating periods of free-swelling culture may be critical in the design of dynamic loading stimuli for use with increasing loading duration.

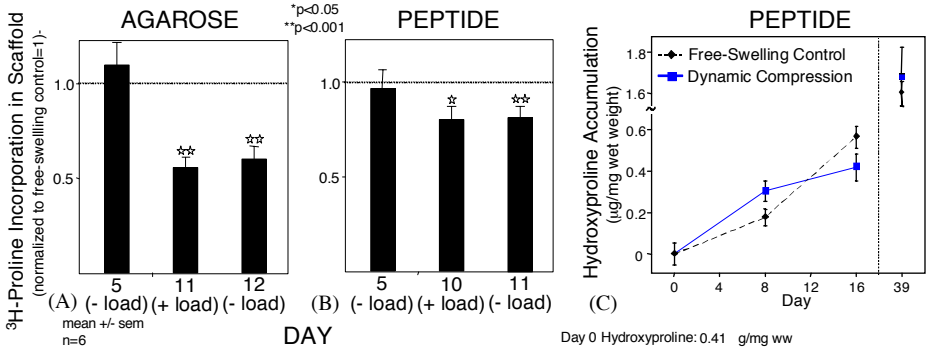

Fig. 7. Dynamic compression of chondrocyte-seeded peptide and agarose hydrogels—protein synthesis. Protein synthesis resulting from alternate day loading: ^{3}H-proline incorporation and retention in chondrocyte-seeded (A) agarose and (B) peptide hydrogels. Free-swelling controls are equal to 1, p values show significant differences vs. pairwise controls. (C) Hydroxyproline accumulation for chondrocyte-seeded peptide hydrogels subjected to alternate day loading and free-swelling controls. Assuming a type II collagen composition of one hydroxyproline per 7.3 amino acids (Herbage et al., 1977), collagen consisted of ~1.3% of the hydrogel wet weight on day 39 for both loaded and free-swelling cultures.

Alternate day dynamic compression of peptide hydrogels significantly increased GAG accumulation after 16 and 39 days of loading compared to free-swelling controls (Fig. 3A). The concomitant significant increase in equilibrium modulus with GAG fixed charge density by day 39 (Fig. 4) is consistent with trends reported for certain cartilages (Rivers et al., 2000), though such correlations are not always observed (Froimson et al., 1997). Interestingly, the relative increase in the dynamic stiffness of mechanically stimulated constructs compared to controls (~60% higher over a range of strain frequencies, Fig. 4) was even higher than that of the equilibrium modulus, with a five-fold reduction in permeability. In previous bioreactor studies involving chondrocyte-seeded PGA constructs, a nearly two-fold increase in GAG content between static flasks and rotating vessels after 6 weeks of culture translated into approx. a four-fold increase in equilibrium modulus (~200 vs. 50 kPa) and approx. five-fold decrease in hydraulic permeability (~10 vs. 50 $m^4/(N\,s)$) (Vunjak-Novakovic et al., 1999). These latter permeability data are on the order of those observed in our loaded peptide hydrogel samples; therefore, the decrease in permeability may be attributed to the more even distribution of GAG as seen in toluidine blue stained sections (Fig. 3B and C), or possibly a more appropriate assembly and organization in response to alternate day dynamic compression. Such factors may further illustrate how mechanical stimulation may affect the functionality of a chondrocyte-seeded hydrogel by means other than bulk ECM deposition (Mauck et al., 2000).

It is interesting to note that there was no difference in GAG accumulation between loaded and control cultures by day 8 (Fig. 3A), while ^{35}S-sulfate incorporation was significantly higher by two-fold in loaded samples on day 8 and, in a separate study, on day 5 (Fig. 2D). The compression-induced loss of GAG to the medium during the first 8 days (Fig. 5A and B) appears to have offset the increase in GAG synthesis during this first week of loading, resulting in little or no change in GAG accumulation. Thus, dynamic compression appeared to first induce a period of increased PG turnover after which both PG synthesis and net accumulation surpassed free-swelling values.

After the initial peak GAG loss within the first 8 days, GAG loss decreased to values ~50–100% higher than controls for the next 16 days. This decrease in GAG loss starting on day 8 was not due to a decrease in GAG synthesis, as total GAG synthesis on day 16 was ~50% higher than that in controls (Fig 5A). Therefore, increasing GAG accumulation in loaded peptide scaffold by day 16 coincided with a reduction in GAG loss to the medium. From days 23–39, GAG loss to the medium increased over time compared to controls (Fig. 5B). With no intermediate time point of GAG accumulation between days 16 and 39, it is difficult to project an exact relationship between GAG loss to the medium and retention in the scaffold. However, given the constant percentage increase in total GAG synthesis caused by dynamic compression on days 8, 16, and 39 (Fig. 5A), the higher rates of GAG loss to the medium after day 24 may be consistent with a decrease in the rate of GAG accumulation. Further experiments are necessary to identify the mechanism(s) of GAG loss, and to better understand the role of enhanced efflux of normal aggrecan versus proteolytic degradation in the mechanical regulation of PG synthesis and turnover.

During the initial alternate day loading peptide time course, the loss of ^{35}S-sulfate-labeled macromolecules from dynamically compressed cultures to the medium was minimal and occurred as a similar percentage of the total newly synthesized macromolecules as in free-swelling cultures. This observation is also consistent

with the hypothesis that the increase in accumulation of newly synthesized PGs caused by dynamic compression in this system is largely due to the difference in total biosynthesis caused by compressive stimulation, and not a result of dynamic loading affecting the efficiency in which molecules are retained in the scaffold. Over the subsequent 39-day alternate day loading peptide time course, loading tended to slightly lower viable cell densities. Taken together, these data strongly suggest that increases in total PG synthesis and accumulation in the scaffold were primarily due to increases in PG synthesis on a per-cell basis caused by loading throughout each peptide time course.

In this study, increases in synthesis of PGs in peptide hydrogels were accompanied by a slight reduction in ^{3}H-proline incorporation. Hydroxyproline analyses further demonstrated that alternate day compression did not greatly affect collagen synthesis. Thus, the present loading protocol appears to have differentially stimulated the synthesis and accumulation of PGs. It is possible that dynamic compression preferentially stimulated chondrocyte synthesis of macromolecules that resist compression, i.e., PGs. Interestingly, such differential stimulation has also been observed in cartilage explants (Jin et al., 2001), in which dynamic tissue shear increased ^{3}H-proline incorporation (primarily associated with collagen synthesis in this bovine calf system; Sah et al., 1991) almost two-fold greater than ^{35}S-sulfate incorporation. Thus, the design of specific loading/deformation regimes may have a major impact on the molecular composition of accumulated ECM in tissue engineered scaffolds (Waldman et al., 2003).

As a model for application to cartilage repair, bovine chondrocytes seeded in self-assembling peptide hydrogels demonstrated accelerated maturation of a PG-rich ECM when stimulated by long-term alternate day intermittent dynamic compression. Increased GAG content was consistent with an increase in both static and dynamic compressive stiffness of the constructs. While enhanced material properties of constructs targeted for implantation may serve to reduce the potential of mechanical failure, the optimal quantity and composition of ECM deposition that leads to a complete repair response in vivo is not known. Therefore, mechanical compression applied in vitro may be used to influence matrix deposition and molecular composition to provide unique constructs for use in vivo.

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