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Dynamic Compression Stimulates Proteoglycan Synthesis by Mesenchymal Stem Cells in the Absence of Chondrogenic Cytokines

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The objective of this study was to evaluate the effect of dynamic compression on mesenchymal stem cell (MSC) chondrogenesis. Dynamic compression was applied to agarose hydrogels seeded with bone marrow-derived adult equine MSCs. In the absence of the chondrogenic cytokine transforming growth factor beta (TGF β), dynamic compression applied for 12 h per day led to significantly greater proteoglycan synthesis than in unloaded TGF β -free cultures, although at a rate that was approximately 20% to 35% of unloaded TGF β cultures. These data suggest that the emergence of aggrecan dominated a chondrogenic response to loading as increases in proteoglycan synthesis. Cross-sectional analyses were conducted to subjectively identify potential spatial distributions of heterogeneous differentiation. In loaded samples, cell viability and metachromatic staining was low near the porous compression platen interface but increased with depth, reaching levels in the lower portion of the hydrogel that resembled unloaded TGF β cultures. These results suggest that the combination of high hydrostatic pressure and low dynamic strain and fluid flow had a stronger effect on chondrogenesis than did low hydrostatic pressure coupled with high dynamic strain and fluid flow. Next, the 12-h per day loading protocol was applied in the presence of TGF β . Biosynthesis in loaded cultures was less than in unloaded TGF β samples. Taken together, these data suggest that the duration of loading necessary to stimulate mechanoinduction of MSCs may not be optimal for neo-tissue accumulation in the presence of chondrogenic cytokines.

Introduction

THE FIELD OF regenerative medicine encompasses novel therapies that seek to regrow damaged tissues that do not spontaneously heal. One approach is to populate the damaged area with cells isolated from biopsied autologous tissue that can stimulate a repair response. Such cell-based therapies have received considerable attention for the repair of articular cartilage,¹⁻³ a tissue that has limited repair capacity because of the lack of vascularity and low cell density. Recently, bone marrow-derived mesenchymal stem cells (MSCs) have received extensive consideration as a cell type for cartilage repair.^{4,5} Laboratory experiments have demonstrated cytokine-induced MSC chondrogenesis in the presence of members of the transforming growth factor beta (TGF β) superfamily.⁶⁻⁸ In addition to gene expression and qualitative protein assessment of chondrogenesis, quantitative extracellular matrix (ECM) synthesis studies have dem-

onstrate that MSCs are capable of synthesizing neo-tissue on the order of that reported for chondrocyte cultures.⁹⁻¹¹ Such laboratory studies have fostered enthusiasm that MSCs may be capable of regenerating a biologically and mechanically functional neo-cartilage *in vivo*.

Although the ability of MSCs to undergo chondrogenesis has been well characterized, translation to a clinical repair strategy has not been realized. It has been proposed that cell-based therapies will encounter restrictions on *ex vivo* manipulation because of economic factors associated with engineering a repair tissue.¹² These considerations increase the likelihood that therapies will be centered on minimally manipulated cell systems, such as joint injections,¹³ or seeding of scaffolds within defect sites.¹⁴ In these cases, the joint environment will have a significant influence on MSC differentiation and ECM synthesis.

Chondrocytes are sensitive to mechanical loading that is associated with joint functioning.¹⁵ These findings suggest

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that loading may affect MSC chondrogenesis *in vivo*. Consequently, laboratory studies have begun to investigate the influence of mechanical loading by culturing MSCs in bioreactors that mimic certain aspects of joint loading. The majority of studies have focused on dynamic compression or dynamic hydrostatic pressure to approximate physiological tissue strains and pressures that can occur with joint usage. One approach has been to apply mechanical loading in the presence of TGF β , a frequently used cytokine for inducing MSC chondrogenesis.⁶ Dynamic compression protocols based on physiological parameters of cartilage strain¹⁵ have been found to increase chondrocyte-like gene expression^{16–19} and stimulate the accumulation of cartilage-like ECM^{16,18,19} more than in unloaded TGF β control cultures. However, a decrease in chondrogenesis with dynamic compression has also been reported.²⁰ Dynamic hydrostatic loading, on the order of what occurs during routine joint functioning,²¹ has been proven to upregulate chondrogenic gene expression^{22,23} and cartilage-like protein synthesis^{22,24} in the presence of TGF β . These studies demonstrated that certain mechanical loading protocols can have an additive effect on TGF β -mediated chondrogenesis.

Laboratory studies have also evaluated load-induced chondrogenesis in the absence of chondrogenic cytokines.^{19,23,25–27} These experiments, conducted under conditions in which cartilage-like differentiation was minimal at best, represent the ability of loading to independently induce MSC chondrogenesis from the undifferentiated state in which the MSCs are culture-expanded. Dynamic compression^{19,25,26} and hydrostatic pressure²³ have both been found to stimulate gene expression markers of chondrogenesis more than unloaded TGF β -free culture conditions. Less is known about the effect of loading on cartilage-like ECM synthesis, an important outcome given that cartilage-like gene expression may not directly correlate with protein synthesis.^{9,28} In MSC-seeded agarose hydrogel, dynamic compression applied over the first 5 days of a 28-day experiment stimulated an approximate doubling in glycosaminoglycan (GAG) content by the end of the timecourse.²⁷ Taken together, these studies demonstrate a positive effect of loading on chondrogenesis of undifferentiated MSCs.

The cited results represent compelling examples of MSC chondrogenesis in response to loading, especially in the identification of cartilage-like gene expression. However, these studies contain a high degree of variability, such as differences in loading modality, parameters, and duration, that does not allow for general conclusions to be drawn as to the potential of mechanical loading to assist MSC-mediated cartilage repair. In this study, our primary focus was the effect of loading duration on dynamic compression-induced ECM synthesis in the absence of chondrogenic cytokines. Bone marrow-derived MSCs were isolated from adult horses, an animal source that is used as a model for human therapies.²⁹ Culture-expanded MSCs were seeded into agarose hydrogels and then subjected to two intermittent loading protocols in the absence of chondrogenic cytokines. The first protocol was based on an alternate-day duty cycle, defined as 3 h of intermittent loading every other day, which was previously found to stimulate chondrocyte biosynthesis.³⁰ For the second protocol, the duration of loading was increased to 12 h each day to evaluate the effect of a more-sustained mechanical input. In both cases, loading was

applied for nearly 3 weeks as a conservative evaluation of chondrogenesis, because multiweek stimulation has been shown to be necessary to maximize cartilage-like ECM synthesis.^{23,31} In addition to quantitative measures of ECM synthesis, cell viability and histological staining was conducted to identify potential spatial distributions of heterogeneous differentiation in the hydrogel. Finally, the 12-h-per-day loading protocol was applied to MSC-seeded agarose in the presence of TGF β for 15 days to explore potential synergistic or antagonistic relationships between this dynamic compression protocol and a chondrogenic cytokine.

Methods

Bone marrow harvest and MSC culture

Bone marrow was harvested from the ileum of healthy, skeletally mature (2- to 5-year-old) mixed-breed horses euthanized for reasons unrelated to this project. The nucleated cells were separated from the red blood cells using centrifugation, resuspended in low-glucose Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, and seeded in tissue culture flasks at a concentration of 0.66×10^6 nucleated cells/cm². Confluent MSC colonies developed over 10 to 12 days, at which point the cells were reseeded at 12×10^3 MSCs/cm² and expanded in growth medium containing 1 ng/mL fibroblast growth factor-2.³² MSC cultures were passaged at a split ratio of 1:3 twice before seeding in hydrogel scaffolds.

Agarose hydrogel seeding and culture

Culture-expanded MSCs were encapsulated in 2% (w/v) low-melting-temperature agarose (Invitrogen, Chicago, IL) at a concentration of 10×10^6 cells/mL in a 3-mm-thick, 12-mm-diameter plug geometry, as previously described.³³ The MSC-seeded hydrogels were cultured in high-glucose DMEM supplemented with 1% insulin, transferrin, selenium (Sigma Chemical, St. Louis, MO), 0.1 μ M dexamethasone (Sigma Chemical), and 37.5 μ g/mL ascorbate-2-phosphate (Wako Chemicals, Richmond, VA). Unloaded positive (TGF β +) and negative (TGF β -) controls of chondrogenesis were cultured in the presence or absence of 10 ng/mL recombinant human TGF β -1 (R&D Systems, Minneapolis, MN),⁶ respectively. Dynamic compression was conducted in the absence or presence of 10 ng/mL TGF β . For each experiment, a single 12-mm plug was cast, cultured, and analyzed for each condition.

Application of dynamic compression

Dynamic compression was applied using a specialized loading chamber designed to allow for periods of fully unloaded, free-swelling culture interspersed with periods of dynamic compression.³⁰ For each experiment, one 12-mm cell-seeded hydrogel disk was loaded in uniaxial unconfined compression. Compression was applied using 13-mm-diameter polyethylene porous platens (40% void, 120- μ m pore size) that were attached to the lid of the chamber and aligned coaxially with each sample. In addition, the lid contained a center-mounted spring aligned with a non-culture well in the base such that the spring created an approximately 800- μ m gap between the platens and samples when the lid was unloaded. Displacement of the lid, and

subsequent construct deformation, was controlled using an incubator-housed loading apparatus.³⁴ Experiments used a sinusoidal dynamic compression protocol of 2.5% strain amplitude superimposed on a 7.5% static offset strain at a frequency of 0.3 Hz in displacement control; these loading parameters are within the physiological range of moderate, low-amplitude strain when applied to intact cartilage explants.¹⁵ At the initiation of loading, these parameters created maximum stress response of approximately 2 kPa.

For loading in the absence of TGF β , two dynamic compression duty cycles were explored. The first protocol was defined by 6-h cycles consisting of 45-min periods of compression followed by 5 h and 15 min of free-swelling culture. Each 6-h cycle was applied four times, followed by 24 h of free-swelling culture such that loading was applied on alternate days. In the second protocol, 45 min of dynamic compression was followed by 45 min of free-swelling culture throughout the loading period, resulting in 12 h of loading per day. In the presence of TGF β , MSC-seeded agarose samples were loaded using the second, 12-h/d dynamic compression protocol.

Cellular biosynthesis of ECM macromolecules

Twenty-four hours before the termination of each experiment, dynamic compression samples were removed from the loading chamber. Ten- to 20-mg samples were cut from dynamic compression and unloaded cultures by sectioning perpendicular to the circular face of the plugs. Each sample was cut from the edge of the plug to approximately the midpoint, creating a full-thickness cross-section coaxial to the applied load. Samples were transferred to medium supplemented with 5 μ Ci/mL ³⁵S-sulfate and 10 μ Ci/mL ³H-proline and then cultured for 24 h to measure the rate of synthesis of sulfated proteoglycans and total protein, respectively.³⁵ Labeled samples were rinsed of free label and digested in proteinase K (Roche)-Tris hydrochloric acid solution overnight at 60°C. From the digests, radiolabel incorporation and the total accumulated sulfated GAG content (from the DMMB dye binding assay)³⁶ were measured. These data were normalized to the wet weight of the samples.

Cell viability

A viable cell kit (Promega, Madison, WI) consisting of the fluorescent dyes calcein and ethidium bromide were used to identify viable and dead cells, respectively, by visual inspection. Full-thickness sections were subjectively evaluated at the start and end of each culture.

Histological analysis

Samples were fixed in 10% neutral buffered formalin overnight at 4°C and then embedded in paraffin. Consistent with the section technique for ECM synthesis and cell viability assays, the hydrogels were embedded so that sectioning produced a cross-section perpendicular to the plug surfaces. Five- μ m-thick sections were cut and spread on charged slides (Fisher Scientific, Waltham, MA), deparaffinized, and then rehydrated before staining. Sections were incubated in an aqueous, 0.04% toluidine blue O solution (American Master*Tech Scientific, Lodi, CA) for 5 min, then rinsed briefly in de-ionized water and left to air dry.

Statistical analysis

A mixed-model analysis of variance, with a fixed treatment effect consisting of the dynamic compression group and unloaded TGF β - or TGF β + cultures, was analyzed using Proc Glimmix (Version 9.1, SAS Institute, Inc., Cary, NC). The donor horse was used as a random effect because each data set was compiled from replicate experiments using MSCs from separate animals. Individual comparisons were made using the least square means procedure. In all evaluations, $p < 0.05$ was considered significant.

Results

ECM synthesis in response to dynamic compression in the absence of TGF β

Experiments conducted in the absence of TGF β were analyzed after 21 days of culture.

Alternate day loading. ECM synthesis in response to the alternate-day loading protocol was evaluated for MSCs from two donor horses (data not shown). ³H-proline and ³⁵S-sulfate incorporation in dynamic compression cultures averaged 2% and 14% more than unloaded TGF β - cultures, respectively, whereas the average GAG accumulation in loaded cultures was 67% of controls. Given the low levels of biosynthesis in adult equine MSC cultures in the absence of TGF β ,⁹ these effects of dynamic compression were considered negligible.

12h/d loading. ECM synthesis was evaluated for MSCs from three donor horses (Fig. 1). ³H-proline incorporation was similar to that in TGF β - controls ($p = 0.09$). ³⁵S-sulfate incorporation and GAG accumulation in dynamic compression cultures were 5.9 and 2.8 times as high, respectively, as in TGF β - cultures ($p < 0.05$). Biosynthesis in dynamic

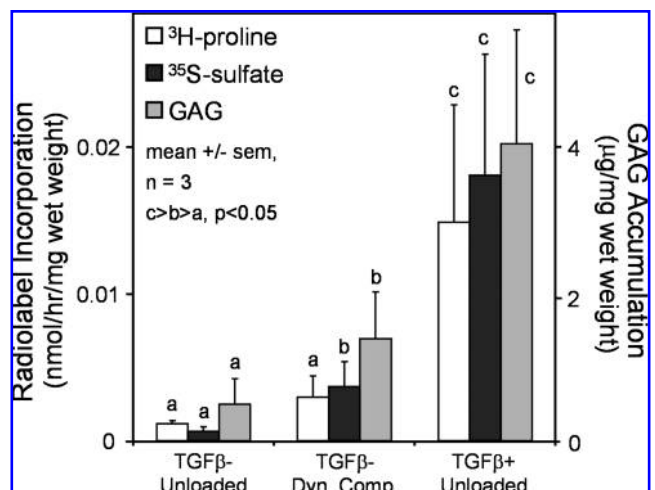


FIG. 1. Extracellular matrix synthesis in response to dynamic compression in the absence of transforming growth factor beta (TGF β) after 21 days of culture. Data for each loading protocol were calculated from cultures established from three donor animals. For each assay, significant differences between conditions were denoted by the labels 'a,' 'b,' and 'c.'

compression cultures was lower than in unloaded TGF β + cultures (Fig. 1). ^3H -proline and ^{35}S -sulfate incorporation in dynamic compression samples were 20% of those of TGF β + samples ($p < 0.05$), whereas GAG accumulation in loaded cultures was 34% of that in TGF β + samples ($p < 0.05$).

MSC viability in unloaded cultures

MSC viability immediately after encapsulation in agarose was greater than 95% for all cultures (data not shown). After 21 days of culture, subjectively significant cell death was noted in TGF β - and TGF β + hydrogels (data not shown). The viable cell population appeared to be evenly distributed across the hydrogel cross-section, with TGF β + and TGF β - cultures retaining approximately 50% and 25% viability, respectively.

MSC viability and proteoglycan spatial deposition profile in dynamic compression cultures in the absence of TGF β

Cell viability and histological analyses were performed for the three samples that were subjected to the second, 12-h/d loading protocol. For these figures, only the calcein stain is shown in the interest of clarity. However, ethidium bromine staining verified that non-viable cells were present throughout the samples, especially in areas of low viability. In all cases, MSC viability and proteoglycan deposition showed a heterogeneous spatial profile over the depth of the plug coaxially to the applied compression. Figure 2 shows a full-thickness cross-section from a sample that showed the strongest gradient of metachromatic staining with depth. Cell viability and metachromatic staining near the porous platen interface was low and resembled the pattern observed throughout unloaded TGF β - cultures (insets). Subjectively, cell viability and metachromatic staining in loaded samples increased with depth and reached levels in the lower portion of the hydrogel (adjacent to the chamber base) that were similar to those observed throughout TGF β + cultures (insets). A similar spatial pattern of cell viability and metachromatic staining was observed for the two additional cultures (Fig. 3). In these samples, the maximum viable cell density and metachromatic staining was largely constrained to the lower 500 μm of the sample adjacent to the base.

ECM synthesis, MSC viability, and proteoglycan spatial deposition profile in response to dynamic compression in the presence of TGF β

Dynamic compression in the presence of TGF β was evaluated using the 12-h/d loading protocol. ECM synthesis, viability, and toluidine blue staining was compared with those in unloaded TGF β + cultures for MSCs from three donor horses after 15 days of culture. ^3H -proline and ^{35}S -sulfate incorporation in dynamic compression cultures were 74% and 55%, respectively, of those of unloaded cultures ($p < 0.05$, Fig. 4). Similarly, GAG accumulation in dynamic compression cultures were 64% of those of unloaded samples ($p < 0.05$, Fig. 4). Subjective assessment of cell viability did not show a difference between dynamic compression and unloaded cultures, with many viable cells evenly distributed along the depth of each sample (data not shown). Toluidine blue staining in dynamic compression cultures was homo-

geneously distributed as a function of depth, with a lower intensity of staining than in unloaded control cultures (Fig. 5, representative sections).

Discussion

In this study, the ability of dynamic compression to induce chondrogenesis was evaluated based on ECM synthesis assays that have previously been characterized for TGF β -mediated chondrogenesis of equine MSCs in hydrogel culture.⁹ In the case of the alternate-day loading protocol, the low volume of ECM synthesis and accumulation in loaded cultures suggested a negligible effect of dynamic compression on chondrogenesis. Adjustment of the frequency of the applied loading from alternate day to daily loading, applied over 12 h/d, resulted in significantly greater proteoglycan synthesis than in TGF β - cultures. These data suggest a chondrogenic response to loading because the emergence of aggrecan production,⁹ the major proteoglycan in cartilage, probably dominates ^{35}S -sulfate incorporation and GAG accumulation. Therefore, the duration of the application of loading appeared to be a critical factor in stimulating proteoglycan synthesis, as occurs during chondrogenesis.

Unlike the case of proteoglycan synthesis, the 12-hou/d loading protocol did not significantly increase total protein synthesis in the absence of TGF β . ^3H -proline is a less-specific marker of chondrogenesis in that it is incorporated into several different collagen types, as well as many other proteins. Thus, although the ^3H -proline incorporation data alone do not indicate a change in cartilage-like protein synthesis with loading, quantification of a potential redistribution of newly synthesized proteins, as previously observed for types I and II collagen,³⁷ would better illustrate potential changes in cartilage-like protein accumulation.

TGF β + cultures were maintained as an example of MSC chondrogenesis that results in a high rate of repair tissue synthesis. Despite the stimulation of proteoglycan synthesis in dynamic compression cultures, load-induced ECM synthesis was less than that due to TGF β treatment. From these data, the average GAG content for loading at 0.3 Hz was approximately 34% of that of TGF β + samples. In comparison, agarose hydrogels seeded with immature bovine MSC cultures and subject to near-physiological dynamic compression for the first 5 days of a 28-day period reported GAG accumulation that was approximately 15% of that of unloaded TGF β + samples.²⁷ These bulk data, averaged over full-thickness samples, suggest that dynamic compression may have only a modest overall additive effect on chondrogenesis with time in culture.

The stimulation of chondrogenesis in dynamic compression samples at a level that was inferior to that in TGF β + cultures suggests two potential interpretations. First, dynamic compression may have induced widespread differentiation of a chondrocyte-like phenotype with a poorer ability to synthesize ECM, or load-induced differentiation may have occurred in only a subset of cells, which would likewise result in an overall decrease in bulk ECM synthesis. Similar to cartilage,^{38,39} agarose is a porous, hydrated scaffold that responds in a non-homogeneous, time-dependent manner to dynamic compression.^{40,41} In this study, the hydrogel dimensions (3-mm thick, 12-mm in diameter) and porous platen design ensured that the mechanical environ-

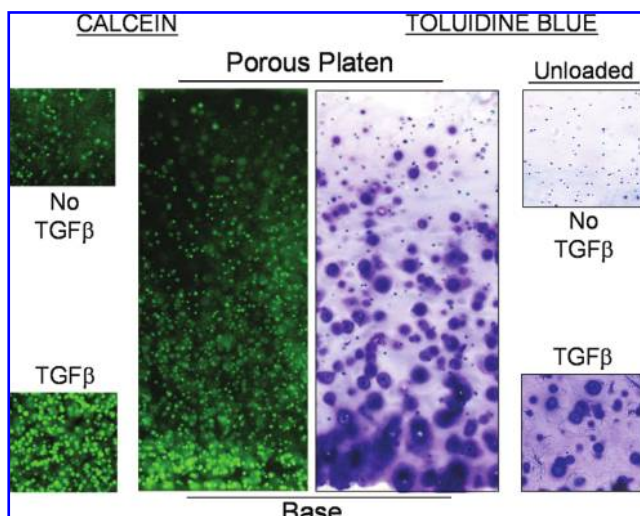


FIG. 2. Viable cell distribution and proteoglycan deposition over full-thickness (3 mm) sections of samples loaded using the 12-h/d protocol in the absence of TGF β (Day 21). Insets show representative sections from unloaded TGF β - and TGF β + controls. Color images available online at www.liebertonline.com/ten.

ment varied as a function of depth between the porous platen and impermeable chamber base.^{38,39} Specifically, zones near the porous platen would be expected to experience the highest fluid flow in response to the dynamic loading, resulting in the greatest local dynamic strain. In deeper zones, there would be relatively less fluid flow and compressive strain but much higher levels of hydrostatic pressure. Therefore, cross-sectional analysis was performed to identify potential heterogeneous responses in these zones of interest.

It is well established that MSC chondrogenesis over a period of weeks leads to the accumulation of a proteoglycan-

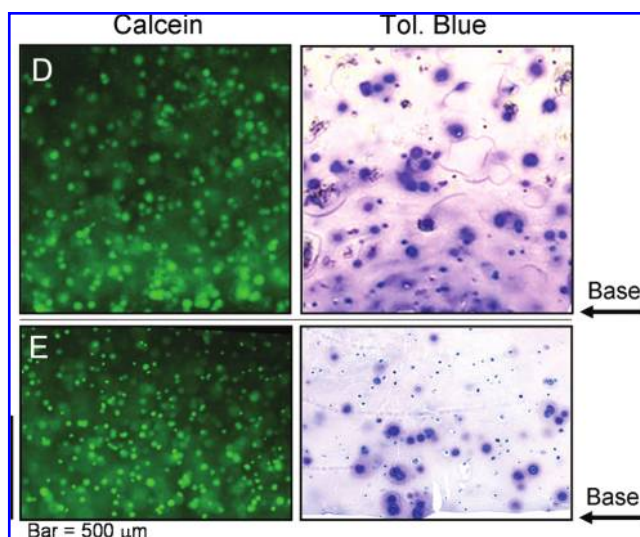


FIG. 3. Viable cell distribution and proteoglycan deposition adjacent to the impermeable base after 21 days of the 12-h/d loading protocol in the absence of TGF β . Color images available online at www.liebertonline.com/ten.

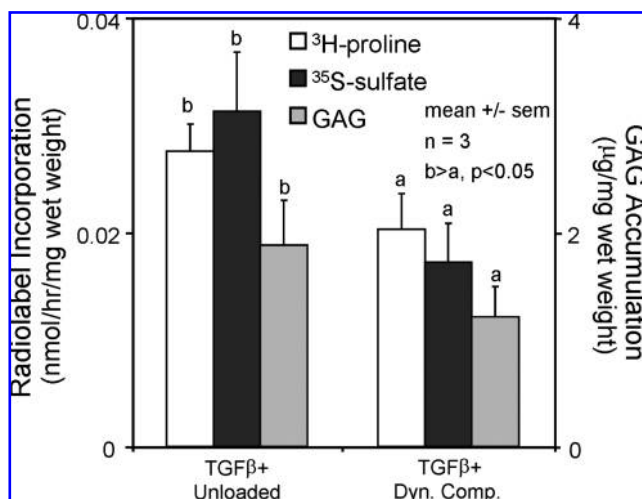


FIG. 4. Extracellular matrix synthesis in response to dynamic compression in the presence of TGF β after 15 days of culture. Dynamic compression samples were loaded using the 12-h/day protocol. Data were calculated from cultures established from three donor animals. For each assay, significant differences among conditions were denoted by the labels 'a' and 'b'.

rich ECM that can be identified using histological staining.⁶ Furthermore, it has been reported that MSC viability in hydrogel culture is better maintained in medium conditions that promote chondrogenesis.¹⁸ Therefore, cell viability and

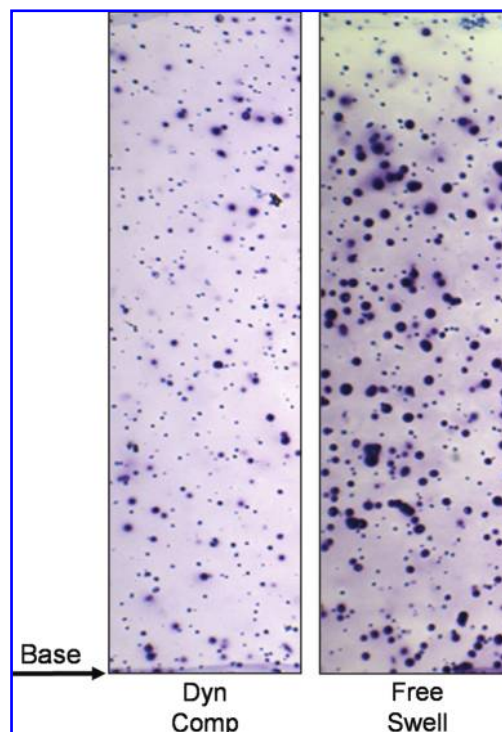


FIG. 5. Proteoglycan deposition over full-thickness (3 mm) sections of samples maintained in medium containing TGF β (Day 15). Dynamic compression samples were loaded using the 12-h/d protocol. Color images available online at www.liebertonline.com/ten.

histological staining in loaded samples were evaluated as an indication of a potential heterogeneous chondrogenic response. In the three dynamic compression samples, the zones near the chamber base contained the highest density of viable cells, suggesting that chondrogenesis was preferentially stimulated in deep zones of maximal hydrostatic pressure. Histological analysis demonstrated that metachromatic staining closely resembled the patterns of cell viability, further supporting the potential for heterogeneously distributed chondrogenesis. This metachromatic staining pattern generally resembles that of articular cartilage,⁴² a compositional profile that correlates with *in vivo* loading,⁴³ although the modest level of proteoglycan accumulation here would suggest that *in vivo* development was not replicated. Although additional measures would be necessary to fully characterize the extent of chondrogenesis as a function of location in the hydrogel, including potential regional differentiation into the zones of adult cartilage, these data suggest that a heterogeneous response to loading was at least partially responsible for the reduced ECM synthesis relative to TGF β + cultures and that zones of relatively high hydrostatic pressure and low dynamic strain and fluid flow had a stronger effect on chondrogenesis than did low hydrostatic pressure coupled with high dynamic strain and fluid flow. These conclusions potentially support previous findings for immature bovine MSCs, which showed that the highest load-induced aggrecan gene expression coincided with maximal hydrostatic pressure and dynamic strain and minimal fluid flow.²⁷

Although the functional assays in this study demonstrated the net effect of dynamic compression protocols, complex mechanical interactions may have contributed to the outcomes. Specifically, the lack of observed chondrogenesis near the platen surface in plugs loaded for 12 h/d was unlikely to have occurred merely from an absence of mechanical stimulation, as seen in unloaded TGF β - cultures. Instead, strain-induced apoptosis,⁴⁴ or the physical or enzymatic disruption of secreted ECM, may have overcome any chondrogenic mechanical factors present in these areas. Such details merit future consideration for the optimization of dynamic compression protocols for MSC chondrogenesis.

Based on the stimulatory effect of the 12-h/d loading in the absence of TGF β , this protocol was tested in the presence of TGF β to explore potential synergistic or antagonistic relationships between the two stimuli. The decrease in ECM synthesis and accumulation demonstrated an inhibitory effect of loading in TGF β . Although loading may have prevented the induction of chondrogenesis of the initially undifferentiated MSCs or simply decreased the metabolic activities of the cells, the similar levels of cell viability between loaded and control cultures suggest that a reduction of biosynthesis within the strongly chondrogenic cytokine environment was at least partially responsible for these data. Taken together with the results from TGF β -free loading, these data suggest that the mechanical factors necessary to independently induce chondrogenesis may be detrimental to differentiation and subsequent biosynthesis in the presence of a chondrogenic cytokine, especially when loading is applied before the accumulation of ECM.¹⁸ Given that loading for 12 h/d is higher than in previous studies that report enhanced chondrogenesis with loading in TGF β ,^{16,18,19} it seems

possible that the duration of loading is an important factor, as has been reported for chondrocyte cultures.³⁰

In this study, we focused on the effect of dynamic compression in the absence of chondrogenic cytokines in support of treatment strategies targeting the implanting or injecting of undifferentiated MSCs. Therefore, any mechanical loading experienced by the cells would result from joint functioning associated with movement or rehabilitation. The finding that an extended duration of loading is necessary to stimulate chondrogenesis may challenge the physical abilities of the recipient, although given that compression-induced hydrostatic pressure proves to be the major influence on MSC chondrogenesis, it is possible that therapeutic pressures can be applied using passive means. Previously, finite element analyses have predicted compression-induced hydrostatic pressures of less than 1 kPa in agarose constructs compressed using near-physiological deformation levels.^{25,27} Although this value is significantly less than that measured in joints during normal activities, it is closer to the 0 to 3 kPa reported for continuous passive motion.⁴⁵ Therefore, it appears possible that dynamic compression of MSC-seeded agarose may serve as a model for exploring the influence of passive rehabilitation activities.

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Disclosure Statement

No competing financial interests exist.

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