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Time-dependent effects of intermittent hydrostatic pressure on articular chondrocyte type II collagen and aggrecan mRNA expression

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Abstract—The normal loading of joints during daily activities causes the articular cartilage to be exposed to high levels of intermittent hydrostatic pressure. This study quantified effects of intermittent hydrostatic pressure on expression of mRNA for important extracellular matrix constituents. Normal adult bovine articular chondrocytes were isolated and tested in primary culture, either as high-density monolayers or formed aggregates. Loaded cells were exposed to 10 MPa of intermittent hydrostatic pressure at a frequency of 1 Hz for periods of 2, 4, 8, 12, and 24 hrs. Other cells were intermittently loaded for a period of 4 hrs per day for 4 days. Semiquantitative reverse transcription polymerase chain reaction assays were used to assess mRNA signal levels for collagen types II and I and aggrecan. The results showed that type II collagen mRNA signal levels exhibited a biphasic pattern, with an initial increase of approximately five-fold at 4 and 8 hrs that subsequently decreased by 24 hrs. In contrast, aggrecan mRNA signal increased progressively up to three-fold throughout the loading period. Changing the loading profile to 4 hrs per day for 4 days increased the mRNA signal levels for type II collagen nine-fold and for aggrecan twenty-fold when compared to unloaded cultures. These data suggest that specific mechanical loading protocols may be required to optimally promote repair and regeneration of diseased joints.

Key words: *aggrecan, articular cartilage, collagen, hydrostatic pressure, mechanical loading.*

INTRODUCTION

Clinical experience in humans and experimental studies with animal models confirm that mechanical loads provide an essential stimulus for maintenance of normal articular cartilage homeostasis (1–7). Alterations in joint loading due to immobilization (8–10), ligamentous laxity (11–13), impact (14), or increased subchondral bone stiffness (15) result in pathological changes in cartilage that are characteristic of osteoarthritis. *In vitro* studies confirm that articular cartilage cells, the chondrocytes, respond to specific loading conditions through anabolic or catabolic reactions induced by the stress and strain imparted to the cells by physical stimulation (16–18).

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The contribution of mechanical loading to the metabolic activity of articular chondrocytes has been defined in part by mathematical analyses of the magnitude and distribution of forces across joint surfaces (19–23). Mechanical tests confirm that chondrocytes in cartilage of diarthrotic joints experience 7 to 10 MPa of hydrostatic pressure with normal activities of daily living (24,25). Numerical analysis of the influence of mechanical loading on tissue differentiation predicts increased cartilage thickness at surface areas exposed to high intermittent hydrostatic pressure (26–30). Thinner cartilage coincides with areas exposed to lower levels of hydrostatic pressure and tensile forces arising tangential to the joint surface (26–30).

Experimental studies confirm that hydrostatic pressure influences articular cartilage matrix metabolism when applied *in vitro*. Hall et al. (31) established that hydrostatic pressure at levels of 5–15 MPa modulates $^{35}SO_4$ and ^{3}H-proline incorporation rates into adult bovine articular cartilage *in vitro*. Organ culture experiments of Klein-Nulend et al. (32) demonstrate that sites of proteoglycan production coincide with regions of pure hydrostatic pressure. Physiological levels of intermittent hydrostatic pressure enhance mRNA signal levels for aggrecan and type II collagen when applied for only 4 hrs (33). In a study of load-controlled compression on bovine cartilage explants, a transient up-regulation of aggrecan mRNA expression was observed after 1 hr of loading (34).

The documented effects of hydrostatic pressure on chondrocytes suggest that specific loading regimens serve as a controllable stimulus for modulation of cartilage macromolecule expression. The purpose of this study was to test the hypothesis that intermittent pressure applied to chondrocytes would enhance type II collagen and aggrecan mRNA levels without altering type I collagen mRNA expression *in vitro*. The experimental approach relied on use of RT-PCR for a semiquantitative determination of mRNA signal levels for collagen and aggrecan relative to the signal levels of an intracellular protein, beta-actin, which provided an internal cellular reference signal.

MATERIALS AND METHODS

Chondrocyte Isolation

Adult bovine articular cartilage was collected as sterile, full-thickness pieces by dissection of radiocarpal joints obtained fresh from a local abattoir. Cartilage cells were released following overnight treatment at 37°C in 5.0 percent CO_2 with collagenase type II and type IV (Worthington, Freehold, NJ) at a final concentration of 0.6 mg/ml each, in 15 ml of Dulbecco's Modified Eagle's Medium/Ham's F12 (DMEM/F12, Gibco BRL, Grand Island, NY) containing 25 μg/ml gentamicin (Sigma, St. Louis, MO). Single cells were filtered through a nylon mesh filter and subsequently collected by repeated centrifugation at 600×g and resuspension in Dulbecco's phosphate-buffered saline (PBS; 3×50 ml). A final cell pellet was suspended in serum-free DMEM/F12 and the cell number determined by counting in a hemacytometer. Viability was assessed by Trypan Blue exclusion and always exceeded 95 percent. The chondrocytes were plated at high density (1×10^5 cells/cm^2) on 60-mm tissue plates and the cultures were maintained at 37°C in a humidified atmosphere of 5.0 percent CO_2. For attachment in serum-free conditions, the individual plates were pretreated overnight with poly-d-lysine (Sigma; 0.1 mg/ml) and washed twice with PBS without calcium or magnesium.

Serum-Containing or Serum-Free Medium

Culture medium was either DMEM/F12 containing dialyzed, heat-inactivated fetal bovine serum (Gibco BRL) at a concentration of 10-percent vol/vol, or a serum-free 1:1 mixture of DMEM/F12 supplemented with selenium and liposomes (35). Liposomes were prepared by dissolving lecithin, cholesterol, sphingomyelin, and vitamin E acetate in 1 ml of 2:1 chloroform/methanol vol/vol which was dried under N_2. One ml of DMEM/F12 was added and the lipid mixture was sonicated 3 times for intervals of 3 minutes each, using a microtip with a 70-percent duty cycle. The liposome stock was prepared at 1,000 times the final concentration, kept under N_2, and stored at 4°C. In some experiments, ascorbate was added to the medium at a concentration of 50 μg/ml.

Mechanical Loading with Intermittent Hydrostatic Pressure

Hydrostatic pressure was cyclically applied at 10 MPa at a frequency of 1 Hz. Intermittent hydrostatic pressure was applied either continuously, with cells removed at periods of 2, 4, 8, 12, and 24 hrs, or at interval loading, with cells removed after a 4-day period during which loading was limited to 4 hrs per day. Each experimental time point was tested in triplicate, and each experiment was carried out for a minimum of three independent trials. Pressure was generated within a commercially available, stainless steel pressure vessel interfaced to a

servo-hydraulic loading frame. The experimental design permitted complete evacuation of air from the system so the application of pressure was purely hydrostatic. In a modification of a previously published approach (33), the culture plates were placed in the loading vessel within sterile, heat-sealed bags containing 45 ml of culture medium (DMEM/F12 containing 30 mm HEPES for pH stability in the absence of continuous infusion of carbon dioxide), as shown in **Figure 1.** Temperature control was achieved by immersion of the pressure vessel in a circulating water bath maintained at 37ºC. No measurable change in temperature occurred over loading periods up to 96 hrs. Control cultures were maintained under identical conditions in heat-sealed bags placed in an identical container in the same water bath as the loaded cultures.

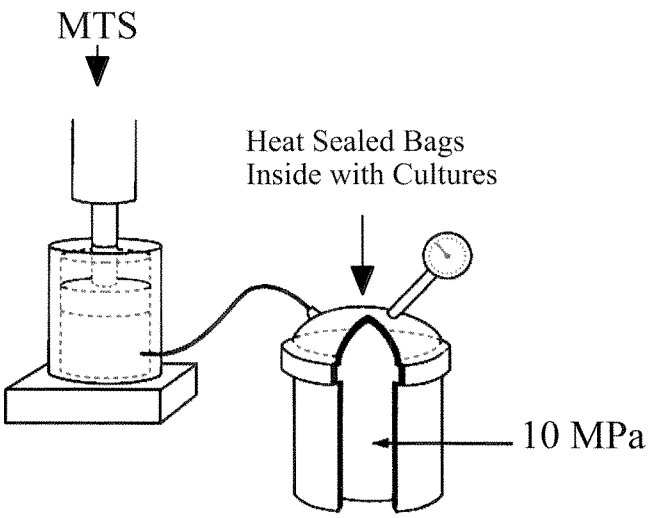

Figure 1.
Diagram of the method for application of intermittent hydrostatic pressure to articular chondrocytes.

Analysis of Aggrecan and Type II Collagen mRNA Signal Levels

To permit multiple samples to be tested for each loading condition, an experimental approach using semiquantitative RT-PCR was used for analysis of aggrecan and type II collagen mRNA signal levels, as described in previous studies of MMP-9 expression (36). After each test period, total RNA was extracted from the loaded and unloaded cells by the guanidinium isothiocyanate method (37) with a commercially available tri-reagent (Sigma, St. Louis, MO). The typical yield of cellular RNA per 60-mm plate was 5 μg. All RNA preparations were routinely screened for integrity of the ribosomal RNA. Total RNA concentration was determined by spectrophotometry and adjusted to 200 ng/μl for reverse transcription using random-hexamer priming. The mRNA sample was converted to single-stranded cDNA using m-MLV reverse transcriptase (Gibco BRL) in the presence of RNase inhibitor (5-Prime, 3-Prime, Inc., Boulder, CO) and in the presence of 500 μM dNTPs (Perkin Elmer Cetus, Norwalk, CT). The reaction was carried out at 37ºC for 15 min, 42ºC for 10 min, 47ºC for 10 min, and finally raised to 99ºC to inactivate the reverse transcriptase. The reaction mixture was diluted 10 times and used for PCR. The target sequences in the reverse-transcripted cDNA samples were amplified by PCR, using sequence-specific oligonucleotide primers designed to yield approximately 200-bp sequences spanning exons within aggrecan (38) and type II collagen (39) genes. DNA size analysis and DNA sequencing of the specific products determined the validity of the products generated using the primer sets. PCR was carried out with 1.0 μl of cDNA in a 0.5-ml reaction tube containing 1.5 μl of PCR master mix; the reaction was initiated at 65ºC to avoid nonspecific annealing. The PCR master mix contained 125 mM Tris HCl, 50 mM ammonium sulfate, 3.75 mM magnesium chloride, 62.5 mM dNTPs, 300 nM of each of the downstream and upstream primers, and 0.625 U/ml Tfl DNA polymerase (Epicentre Technologies, Madison, WI). 32p-α-dCTP at 3,000 Ci/mmol (Amersham NEN) was added to the master mix to yield 0.1 mCi/ml final concentration for random radiolabelling of amplified products. The total reaction volume at the start of PCR was 2.5 ml. For comparison of relative expression, 0.5 ml of a primer solution containing 900 nM of an oligonucleotide primer set for amplification of the 3′-untranslated region of beta-actin, 50 mM Tris HCl, 20 mM ammonium sulfate, and 1.5 mM magnesium chloride was added at the tenth cycle and amplified in the same reaction tube. Beta-actin mRNA signal served as a internal control to monitor for tube-to-tube variations in amplification conditions and differences in the initial concentration or loading of cDNA. The thermocycle program included one cycle of 95ºC for 3 minutes of initial heating, followed by repeated cycles of 95ºC for 1 minute and 65ºC for 1 minute. Final extension was carried out at 72ºC for 5 minutes. The total cycle number employed in this study was 30 cycles for aggrecan and type II collagen and 26 cycles for beta-actin. The amplified products from PCR were separated on 5-percent polyacrylamide gels and these were directly analyzed using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). Relative expression of the mRNA was expressed as specific signal levels and as a ratio of signal of the aggrecan and type II to the beta-actin signal.

Statistical Methods

Significance of differences between loaded and unloaded samples were examined using the general linear method for one-way analysis of variance (ANOVA) with the addition of Tukey's correction for multiple comparison testing (SAS, Cary, NC). Determination of the mRNA signal levels by semiquantitative RT-PCR techniques provided sufficient differences between treated and untreated samples so that a power level was achieved to determine significance at $p<0.05$ with five independent trials. In the case of the mRNA quantification, the hypothesis being tested was that a change in matrix gene expression occurred relative to the expression of beta-actin. Beta-actin expression was determined not to change in response to intermittent hydrostatic pressure. This then permitted paired t-tests to be used to test for significance from the different culture samples.

RESULTS

Chondrocytes Cultured as High-Density Monolayers

Preliminary experiments demonstrated that the primer sets developed for bovine collagen types I and II and aggrecan yielded predicted reaction products that were subsequently validated by DNA sequence analysis. The PCR conditions were optimized to ensure linearity of product production based on initial cDNA concentration and number of amplification cycles. Under the conditions of this study, the mRNA signal levels attributable to type I collagen were detectable but were significantly lower than signal levels for type II collagen. The relative ratio of type II collagen signal following 34 cycles of amplification to type I collagen signal at 40 cycles was 4 to 1.

Articular chondrocytes plated and maintained as high-density monolayer cultures at atmospheric pressure (unloaded control cultures) did not exhibit significant variation with respect to signal levels for type II collagen mRNA or beta-actin mRNA. These observations remained true over a time course of 2, 4, 8, 12, and 24 hrs (**Figure 2**). Similar results were observed in the unloaded cultures with respect to the type I collagen mRNA signal.

Exposure of chondrocytes to intermittent hydrostatic pressure did not alter beta-actin mRNA signal levels over a time course of 2, 4, 8, 12, and 24 hrs when compared to the unloaded control cells (**Figure 3**). However, application of intermittent hydrostatic pressure increased type II collagen mRNA expression approximately five-fold following a 4-hr exposure when compared to unloaded cells ($p<0.05$, ANOVA with Tukey's multiple comparison correction). Type II collagen mRNA levels remained elevated after 8 hrs of exposure to intermittent hydrostatic pressure, but continued loading of the cells for 12 and 24 hrs decreased the type II collagen mRNA signal levels (**Figure 4**).

In contrast to type II collagen, intermittent hydrostatic pressure increased aggrecan expression in a time-dependent manner. Aggrecan expression increased approximately three-fold over the control signal levels, following loading periods of 12 and 24 hrs (**Figure 5**).

Type I collagen mRNA signal levels remained detectable but unchanged after treatment of the cells with intermittent hydrostatic pressure. Adding ascorbate at 50

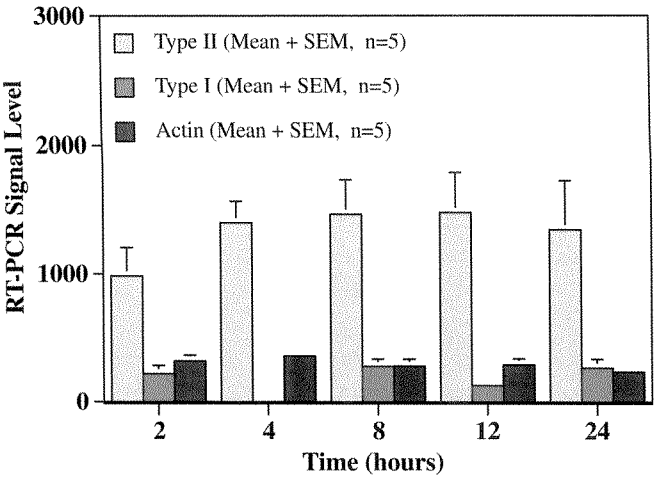

Figure 2.
RT-PCR signal levels for type II and type I collagen and beta-actin expression in unloaded control cells.

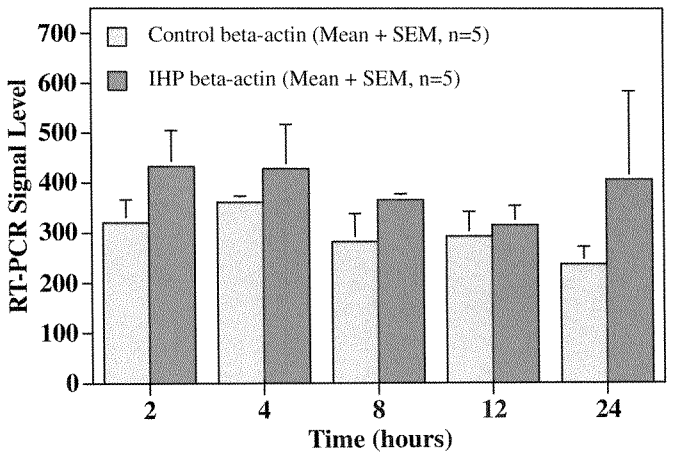

Figure 3.
RT-PCR signal levels for beta-actin expression in cells exposed to intermittent hydrostatic pressure and in unloaded control cells.

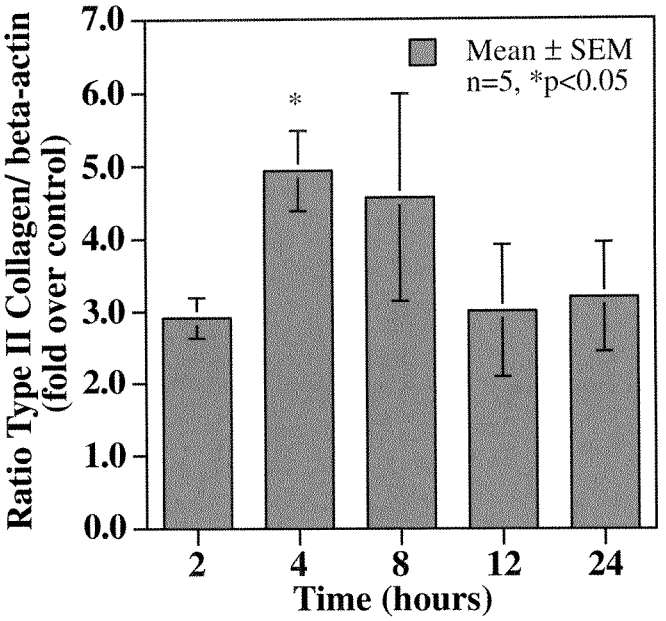

Figure 4.
RT-PCR signal levels for type II collagen expression following exposure of high-density monolayer cultures to intermittent hydrostatic pressure. The data are expressed as a ratio of type II collagen signal to beta-actin expression as a function of levels present in unloaded control cultures. * denotes p<0.05 relative to the signal levels at 2 hrs.

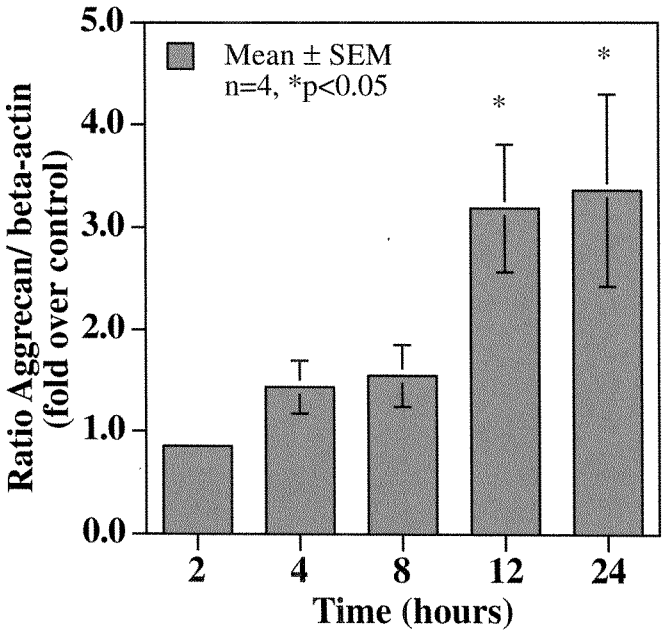

Figure 5.
RT-PCR signal levels for aggrecan expression following exposure of high-density monolayer cultures to intermittent hydrostatic pressure. The data are expressed as a ratio of aggrecan signal to beta-actin expression as a function of levels present in unloaded control cultures. * denotes p<0.05 relative to the signal levels at 2 hrs.

μg/ml or adding ascorbate with fetal bovine serum at a concentration of 1 percent vol/vol did not alter collagen or aggrecan mRNA expression from the pattern described above for either unloaded or loaded cultures.

Chondrocytes in Culture as Aggregates

To determine whether the distribution of the cells as a monolayer was influencing the expression of matrix macromolecules, chondrocytes were maintained in serum-free medium in the absence of polylysine to generate cellular aggregates. The aggregates were exposed to intermittent hydrostatic pressure using continuous loading over a 24-hr period. In the absence of loading, the pattern of type II collagen mRNA expression in the aggregated cells was similar to that observed for cells in high-density monolayer culture, in that no changes were evident. In the presence of intermittent hydrostatic pressure, a similar biphasic pattern for signals representing type II collagen mRNA was observed. The ratio of type II collagen to beta-actin mRNA signal was increased following loading for 4 and 8 hrs, with decreased signal levels at 12 and 24 hrs (**Figure 6**). Type I collagen mRNA signal was detectable but was not changed by hydrostatic pressure. Aggrecan signal levels were representative of those observed in the plates.

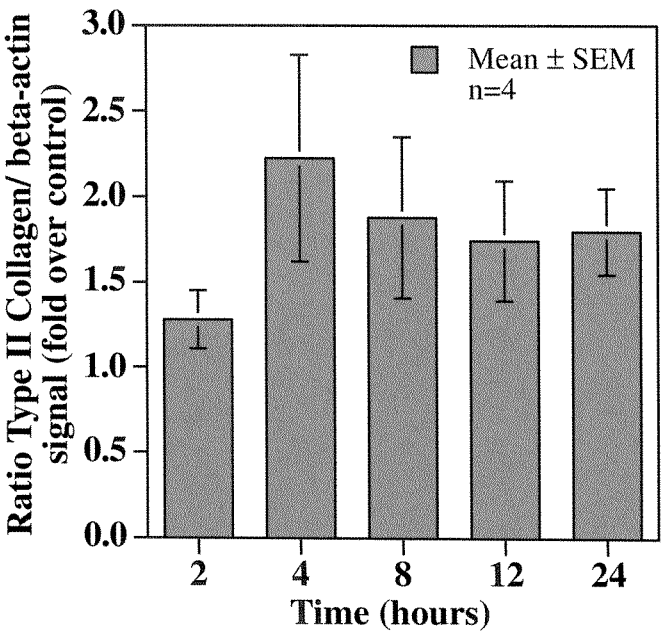

Figure 6.
RT-PCR signal levels for type II collagen expression following exposure of aggregate cultures to intermittent hydrostatic pressure. The data are expressed as a ratio of type II collagen signal to beta-actin expression as a function of levels present in unloaded control cultures.

Interval Loading of Chondrocytes Cultured as High-Density Monolayers

The consistent increase in type II collagen mRNA observed after periods of 4 and 8 hrs suggested that a different loading protocol might produce a more stable change in matrix macromolecule mRNA signal levels. Therefore, the loading pattern was altered to a regimen that included application of intermittent hydrostatic pressure for 4 hrs per day, with this loading pattern continued for 4 days. Beta-actin mRNA signal levels remained unchanged by this interval-loading pattern. However, the change in loading protocol resulted in a nine-fold increase in the type II collagen mRNA signal relative to unloaded controls (**Figure 7**). Under these loading conditions, signal level of the aggrecan mRNA increased approximately twenty-fold relative to the unloaded controls (**Figure 8**).

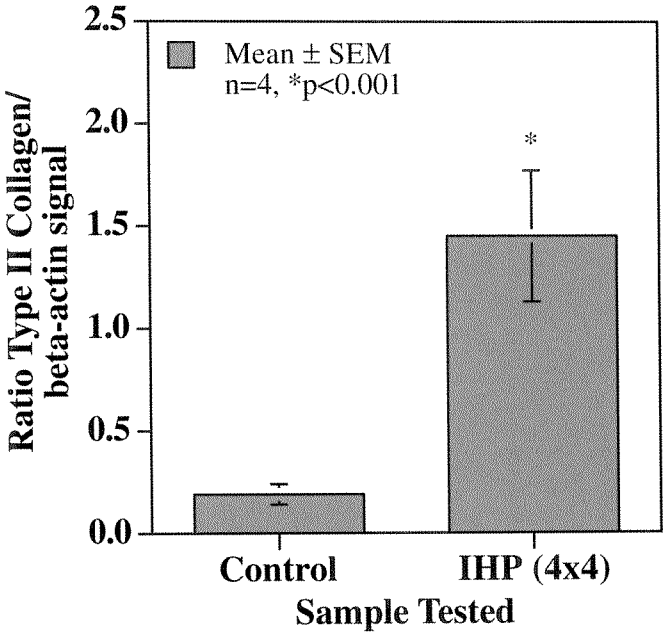

Figure 7.
RT-PCR signal levels for type II collagen following exposure of high-density monolayer cultures to intermittent hydrostatic pressure using interval loading (4 hrs per day for 4 days). * denotes p<0.001 relative to the signal levels in the unloaded control cultures.

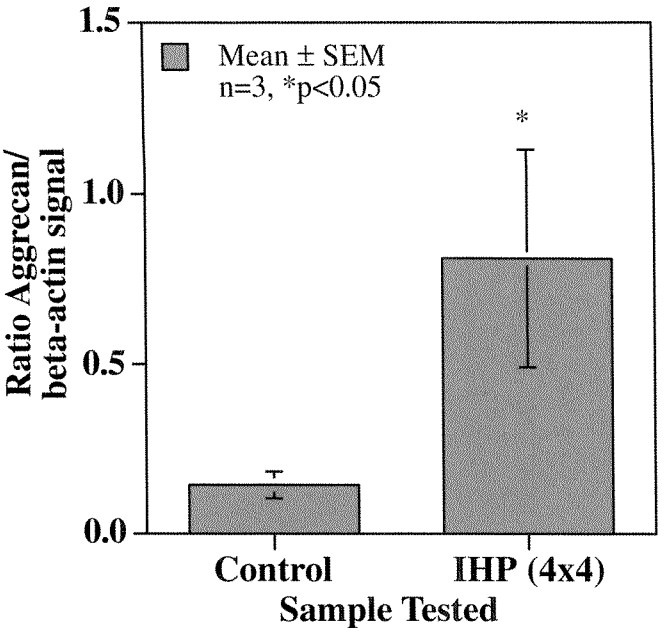

Figure 8.
RT-PCR signal levels for aggrecan following exposure of high-density monolayer cultures to intermittent hydrostatic pressure using interval loading (4 hrs per day for 4 days). * denotes p<0.05 relative to the signal levels in the unloaded control cultures.

DISCUSSION

The results presented here demonstrate that intermittent hydrostatic pressure provided a controllable stimulus for modulation of articular chondrocyte extracellular matrix macromolecule expression. The data confirm that the cellular response varied with the duration of the signal. For the signal representing type II collagen mRNA, a short period of loading consistently resulted in increased expression, whereas a continuously applied load did not maintain increased type II collagen mRNA signal levels. In contrast to collagen, the aggrecan signal expression continued to increase throughout the duration of load. The shift to interval loading of 4 hrs per day for 4 days provided the most prominent increase in signal levels for both type II collagen and aggrecan mRNA. Further study will be required to ascertain the extent to which variation in time of load and duration of loading determines matrix formation at the protein level.

A number of experimental approaches have demonstrated that cartilage cells react to confined and unconfined compressive loading under *in vitro* testing conditions depending on time, magnitude, and frequency of loading (40–43). Depending on the geometry of loading, static compression inhibits glycosaminoglycan synthesis and decreases amino acid uptake (44). Repetitive loading of cartilage has produced variable responses depending on the range of forces applied, the technique for applying the forces, and the frequency of loading (45,46). In closely controlled companion trials comparing static and dynamic compression, the tissue biosynthetic response correlated with threshold-dependent effects that

depended on amplitude and frequency of loading (47). Intermittent compressive forces also changed the nuclear size of the cells and the size of cell aggregates formed in the presence of serum. In these experiments, intermittent compressive forces were 130 mbars above ambient pressure applied at a frequency of 0.3 Hz by compressing the gas phase in test tubes.

As shown in this study, the response of the chondrocyte to hydrostatic pressure depends on whether the applied load is within physiological levels or at levels that exceed normal ranges (48–50). The beneficial effects of joint motion and loading on cartilage are apparent from animal studies on free autogenous periosteal grafts as a repair process for full-thickness cartilage defects (51). Neochondrogenesis was enhanced by the use of continuous passive motion, which would increase levels of intermittent compressive hydrostatic pressure in the joints. In other systems, cartilage samples exposed to 1 to 5 MPa of hydrostatic pressure expressed elevated levels of transforming growth factor beta, whereas cartilage samples exposed to 50 MPa increased heat shock protein 70 expression (52). Short-term exposure of chondrocytes to hydrostatic pressure influences the intracellular stress fibers (53). With excessively high levels of hydrostatic pressure, the molecular mechanisms underlying the chondrocyte heat shock protein response to load involved stabilization of mRNA rather than just transcriptional activation (54).

In a study of load-controlled compression of chondrocytes, a transient up-regulation of aggrecan mRNA was observed after 1 hr of loading (55). Longer loading periods were associated with a return of the signal to levels that matched control explants. The aggrecan mRNA response was blocked by inhibitors of cyclic AMP, suggesting that classical signal transduction pathways were involved. A persistence of aggrecan mRNA signal led the authors to suggest that inhibition of proteoglycan synthesis by prolonged periods of static compression may be mediated through pathways other than suppression of mRNA levels. Other systems of loading show that chondrocytes react to specific loading regimens by changes in cell shape and volume that influence metabolic activity (56,57). These experiments demonstrate that the cells within the explants respond either through inhibition or stimulation of biosynthetic activity, depending on the loading conditions.

The data presented here suggest that repair and regeneration of articular cartilage will be facilitated by defined loading conditions. Our previous studies with hydrostatic pressure (33) and shear stress (58–60) amply demonstrate the plasticity of the chondrocyte with respect to modulation of gene expression. Recent results showing that the chondrocyte can react to hydrostatic pressure though membrane channel events further define how physical events perturb cartilage cells (61,62). Defining the precise protocols that result in an optimal chondrocyte response to mechanical stimulation will require comparison of loading regimens in concert with studies on the geometry of the cells in culture. The expected outcome of this work is that mechanical stimulation may serve as an adjunctive therapy to facilitate cartilage repair and regeneration in diseased diarthrotic joints (63).

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