

Pressure and Shear Differentially Alter Human Articular Chondrocyte Metabolism

A Review

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Homeostasis of articular cartilage depends in part on mechanical loads generated during daily activity whereas inappropriate joint loads result in focal degeneration of cartilage, as occurs in osteoarthritis. We will review results of a series of questions regarding the effects of two types of mechanical loads—intermittent hydrostatic pressure and shear stress—on adult human articular chondrocytes in high-density monolayer culture. Intermittent hydrostatic pressure increased aggrecan and Type II collagen gene expression in normal chondrocytes and induced changes in the cell-associated proteins of normal and osteoarthritic chondrocytes. Hydrostatic pressure also counteracted inhibitory effects of bacterial lipopolysaccharide on matrix protein expression by cultured chondrocytes. Application of shear stress to osteoarthritic chondrocytes increased the release of the proinflammatory mediator, nitric oxide, decreased aggrecan and Type II collagen expression, and induced molecular changes associated with apoptosis whereas hydrostatic pressure increased matrix macromolecule expression. The findings show that the types of load comprising the mechanical loading environment of articular cartilage considerably alter chondrocyte metabolism and suggest that mechanical stimulation may be used for in vitro or in vivo approaches for cartilage engineering.

Osteoarthritis (OA) is a disabling disease because of loss of cartilage and the associated painful joint motion.¹⁴ Intraarticular examination shows that fraying and fibrillation of the cartilage matrix accompany onset of OA,⁴¹ and

continued loading of damaged cartilage leads to progressive focal loss of extracellular matrix²⁰, ultimately requiring total joint arthroplasty.¹⁹ In the future, improved arthroscopic and diagnostic imaging techniques may permit early detection of cartilage damage in OA.

Although outcomes remain questionable, cell-based approaches^{3,6,10} for cartilage repair in OA hold promise for restoring joint function, possibly prolonging or preventing the need for joint replacement.^{18,19,33} With autologous repair, chondrocytes are derived from normal cartilage, expanded in culture and transferred to damaged surfaces using periosteal tissue to hold them in place.^{7,28} With mosaicplasty, multiple osteochondral grafts are moved from one area of the joint surface to another to facilitate a return to weightbearing.^{1,12} With time, osteochondral grafts may exhibit fibrillation and degradation¹⁵ and the overall efficacy of repair depends in part on surface regularity.⁶ Other variables important for these procedures include the state of the donor cartilage, effects of culture or storage and processes involved in implantation.

To achieve an optimal level of cartilage repair, understanding all factors contributing to production and maintenance of a load bearing cartilage matrix will be essential. Buckwalter and Mankin⁵ defined cartilage repair as a restoration of the structural as well as the functional properties of the original tissue. In articular cartilage, aggrecan and Type II collagen are critical components of the extracellular matrix that, together with other accessory proteins, originate from the chondrocytes⁸ and as an assembly impart mechanical properties to the tissue.^{4,34} Therefore, regulation of matrix macromolecule production by articular chondrocytes represents a primary control point for long-term joint function.

We propose that successful cartilage repair depends on how joint loads influence protein expression and matrix production in chondrocytes and cartilage explants. Appropriate joint loads maintain healthy cartilage⁴³ whereas inappropriate loads alter the compositional properties of car-

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tilage observed in OA.^{21,40,44} Two types of load, hydrostatic pressure and shear stress, are differentially localized within regions of the tissue.⁴⁶ In vitro studies confirm that chondrocytes respond to both types of load.^{2,11,31} Because compressive hydrostatic pressure correlates with regions of cartilage with increased matrix thickness,⁴⁶ pressure might be expected to serve as a stimulus for extracellular matrix maintenance.

We review results obtained after application of hydrostatic pressure and shear stress on protein expression by isolated adult human articular chondrocytes.^{17,22–25} Four primary questions regarding chondrocyte responses to mechanical loading are examined here. First, does hydrostatic pressure alter aggrecan and Type II collagen in normal human chondrocytes? Second, could hydrostatic pressure counteract effects of proinflammatory factors, such as bacterial polysaccharide, on chondrocyte metabolism? Third, does shear stress considerably alter human OA chondrocyte metabolism and could hydrostatic pressure modulate chondrocyte metabolism after exposure to shear stress? Fourth, can two-dimensional gel analysis detect changes in chondrocyte-associated proteins after application of hydrostatic pressure? The hypothesis underlying these questions was that chondrocyte metabolism and protein expression depend critically on the type of load present.^{32,37,38,39} The data confirm the hypothesis and suggest that recognition of how different types of mechanical loads modulate chondrocyte metabolism may improve treatment of OA.

MATERIALS AND METHODS

In all studies described, osteoarthritic cartilage specimens were collected after total joint arthroplasty under institutional review. Normal cartilage samples were obtained as pathologic specimens at autopsy for osteonecrosis and trauma. Osteoarthritic cartilage samples were collected from regions of the joint surface without visible softening and fraying, indicative of moderate to severe fibrillation. Chondrocytes were freed of matrix by treatment with collagenase (1 mg/mL of Type II and Type IV bacterial collagenase; Worthington Chemical, Freehold, NJ) overnight at 37°C in 15 mL of Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FBS) and 25 µg/mL gentamicin. Dissociated chondrocytes were filtered through a nylon mesh (50 µm) and collected by centrifugation (450 times gravity for 15 minutes). The cells were resuspended in DMEM/F12 (3 times at 50 mL each) and collected by repeated centrifugation steps to remove collagenase. Isolated chondrocytes were counted in a hemacytometer and viability was assessed by trypan blue exclusion. Although cell yields varied with cartilage samples, chondrocyte viability was consistently more than 95%. The cells were then plated in 60 or 100 mm tissue culture dishes (Nunc, Naperville, IL) at a density of 5×10^5 cells per cm² in DMEM/F12 supplemented with 10% FBS, 25 µg/mL ascorbate, and 25 µg/mL gentamicin. Serum containing medium was exchanged before exposure to mechani-

cal loads with serum-free medium containing 25 µg/mL gentamicin, 25 µg/mL ascorbic acid, 1 mmol/L selenium, and a liposome supplement.

Intermittent hydrostatic pressure was applied to chondrocytes cultured on 60 mm plates; the culture plates were immersed in heat-sealed bags filled with fresh serum-free medium with all air evacuated. The bags then were placed in a water-filled pressure vessel connected to a servo-hydraulic testing machine (MTS Systems, Minneapolis, MN), as described previously.³⁸ Intermittent hydrostatic pressure was applied at a level of 10 MPa at a frequency of 1 Hz for 4 hours. All specimens were harvested after another 24 hours of incubation to assess postloading cellular processing.

Shear stress was applied using a cone viscometer as previously described.³² Briefly, the cultures were exposed to shear stress levels of 0.16, 0.41, 0.82, and 1.64 Pa based on rotating velocities of 20, 50, 100, or 200 rpm. A servo-electronic feedback circuit controlled speed. These loading conditions establish concentric flow that is accompanied by a radial secondary flow without turbulence. Control cultures were maintained in identical culture conditions except for exposure to shear stress. Application of shear stress was limited to 2 hours, with the chondrocytes being maintained in culture for a postloading period of 24 hours to provide time for metabolic changes to occur. Each experiment was done in triplicate and repeated with individual cell preparations isolated from three to five cartilage specimens.

In experiments examining effects of shear stress on chondrocytes, levels of nitrite, a relatively stable end product of nitric oxide oxidation, served as the indicator of nitric oxide synthesis. Nitrite levels in the culture medium were determined spectrophotometrically using the Griess reaction. Briefly, an aliquot (100 µL) of collected culture medium was incubated with 50 µL of a 0.1% solution of sulfanilamide in 5% phosphoric acid and 50 µL of a 0.1% solution of N-1-naphthyl-ethylenediamine dihydrochloride (Sigma Chemical Co., St Louis, MO) for 10 minutes for measurement of absorbance at 550 nm. To inhibit nitric oxide production, the nitric oxide synthase inhibitors, N ω -nitro-L-arginine methyl ester (L-NAME, 1 mmol/L) and L-N⁵-(1-iminoethyl) ornithine (NIO, 1 mmol/L) (Sigma Chemical Co.) were added to the cultures. To model inflammatory stimulation, chondrocytes were activated by adding lipopolysaccharide (LPS; Escherichia coli Serotype 0111:B4, Sigma Chemical Co.) to the cultures for 18 hours before exposure to mechanical loading.

Levels of apoptosis were determined based on phosphatidylserine (PS) expression in chondrocytes cultured on coverslips and subjected to shear stress as described above. Phosphatidylserine was detected by annexin fluorescein isothiocyanate (FITC) conjugated annexin V (V-FITC) labeling (Annexin V-FITC Apoptosis Detection Kit, Oncogene, Cambridge, MA) using confocal microscopy (Nikon Eclipse TE300, MRC-1024 Laser Scanning Confocal Imaging System, Bio-Rad Laboratories, Hercules, CA). Apoptosis was also quantified by detection of nucleosomal cleavage of DNA in cell lysates added to a commercially available ELISA (Oncogene). Assays for bcl-2 proto-oncogene and Fas antigen were done using commercially available kits (bcl-2 ELISA and Fas/APO-1 ELISA, Oncogene) according to the manufacturer's instructions.

Matrix macromolecule gene expression after mechanical loading was determined by RT-PCR analysis of mRNA. After mechanical loading, the cells were lysed and total RNA was isolated with Tri-Reagent (Sigma Chemical, St. Louis, MO). Total RNA (800 ng) was reverse-transcribed using Omniscript reverse transcriptase (Qiagen, Valencia, CA) in a reaction buffer containing 1 μ mol random primer (GIBCO), 0.5 mmol each dNTP, and 10 units ribonuclease inhibitor (GIBCO). After incubation at 37°C for 60 minutes, the reaction was stopped by heating at 93°C for 5 minutes. Multiplex PCR amplification was done in a thermocycler (PTC-100, MJ Research Inc, Watertown, MA) using HotStarTaq DNA polymerase (Qiagen). Primer sequences used for transcript detection were as follows: Type II collagen (sense), 5'-CTGGCTCCCAACTGCCA-ACGTC-3', and Type II collagen (antisense), 5'-TCCTTTGGGTTTGCACGGATTGT-3'; aggrecan (sense), 5'-CACTGTTACCGCCACTTCCC-3', and aggrecan (antisense), 5'-GAGAT-CGTTCCACTCGCCCT-3'; actin (sense), 5'-CAGGTCATCACYATYGGCAATGAGC-3', and actin (antisense), 5'-CGGATGTCMACGTC-ACACTTCATGA-3'. The PCR was done using initial denaturation at 95°C for 15 minutes, followed by 25 cycles of denaturation at 94°C (for 1 minute), subsequent annealing at 58°C (for 40 seconds), and extension at 72°C (for 40 seconds). The final cycle (at 72°C) included 10 minutes for extension; the PCR products were observed on ethidium bromide-stained 1.2% agarose gels. The signals were quantified by imaging analysis software (ImageQuant, V1.2, Molecular Dynamics, Sunnyvale, CA) and were normalized to the expression of a constitutively expressed gene, beta-actin.

For analysis of proteins released from the chondrocytes by ELISA, medium samples from cultures exposed to mechanical loading or unloaded controls were concentrated using ultrafiltration membrane concentrators that retain molecules greater than 5 kd (Amicon Inc, Beverly, MA). Cytokine levels were quantified using capture-based ELISA plates prepared with monospecific antibodies to IL-6 and MCP-1 according to the manufacturer's directions (R&D Systems, Minneapolis, MN).

Total cellular protein was isolated from the organic phase of the Tri-Reagent sample after the extraction of RNA according to the manufacturer's directions (Sigma Chemical Co.). For Western blotting, the proteins were solubilized in a 1% solution of sodium dodecyl sulfate (SDS), quantified by a detergent resistant protein assay and electrophoresed on a 4% to 20% gradient SDS-polyacrylamide gel. Separated proteins were transferred electrophoretically to Immobilon P membranes (Millipore, Bedford, MA). All procedures for the Western blot were done using the Western Breeze immunodetection kit (Invitrogen, Carlsbad, CA). A mouse antihuman monoclonal Type II collagen antibody was obtained from Chemicon International (Temecula, CA). Aggrecan antibody was prepared from purified aggrecan core protein as described previously.³⁹ For two-dimensional gel analysis, the proteins were solubilized in 8 M urea and separated by isoelectric focusing using IPG strips (pH range, 3–10; Amersham Biosciences, Piscataway, NJ). The proteins were reduced, alkylated and separated in a second dimension using Novex gradient 4 to 40% Zoom SDS-gels (Invitrogen, Carlsbad, CA). The proteins were stained overnight in SYPRO Ruby (Molecular Probes,

Eugene, OR) and observed by laser scanning instrumentation (Typhoon, Amersham Biosciences, Piscataway, NJ).

In the studies being reviewed, one-way ANOVA with post-hoc Newman-Keuls or Tukey's correction for multiple comparison was used for statistical comparisons with p values < 0.05 considered significant.

RESULTS

A primary question regarding effects of mechanical loading on cartilage was whether hydrostatic pressure could serve as a stimulus to alter aggrecan and Type II collagen expression. This question was answered in a study with normal human chondrocytes exposed to intermittent hydrostatic pressure applied at different magnitudes and times at a frequency of 1 Hz (Figs 1 and 2).¹⁷ Levels of 5 and 10 MPa but not 1 MPa of intermittent hydrostatic pressure applied for 4 hours (1 day only) increased aggrecan mRNA signal relative to beta-actin by 1.3-fold and 1.5-fold, respectively, when compared with unloaded control cultures (Fig 1). Extending the loading period to 4 days increased aggrecan mRNA signal levels relative to beta-actin by 1.4-fold, 1.8-fold, and 1.9-fold for all levels of pressure, when compared with unloaded cultures (Fig 1). Unlike aggrecan, increased Type II collagen mRNA signal only occurred when pressures of 1, 5, and 10 MPa were applied for 4 hours per day for 4 days. The mean increases of Type II collagen signal relative to beta-actin mRNA signal were 1.2-fold (1 MPa), 1.6-fold (5 MPa),

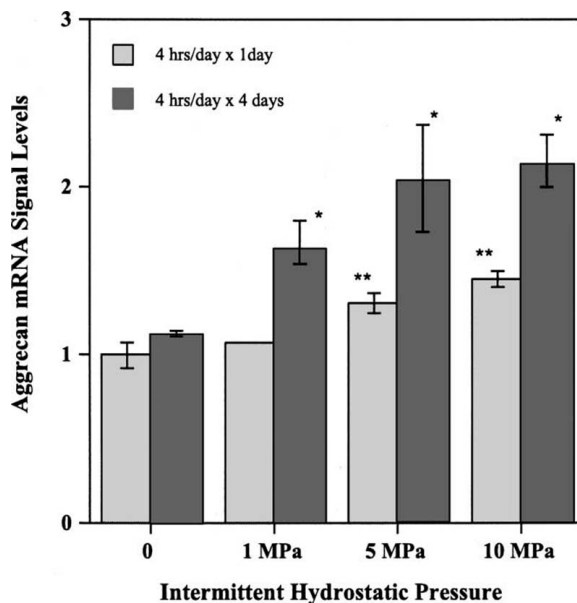


Fig 1. This figure shows that intermittent hydrostatic pressure increases aggrecan gene expression in normal human chondrocytes in a dose-dependent manner. The asterisk denotes that the changes in the mRNA expression levels were significant at a level of p < 0.05.

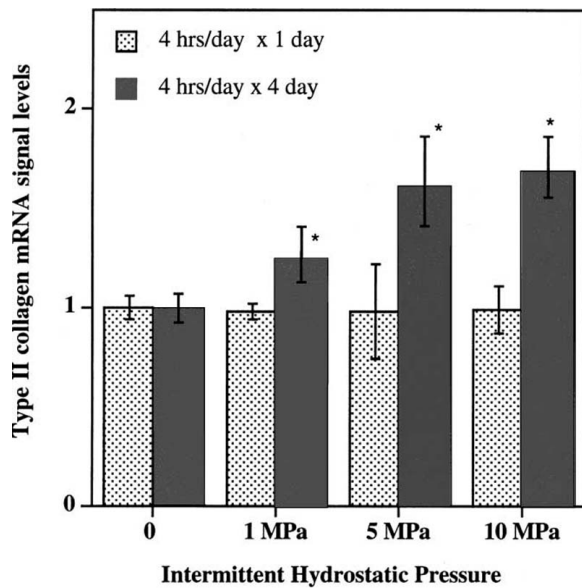


Fig 2. This figure shows that intermittent hydrostatic pressure increases Type II collagen gene expression in normal human chondrocytes in a dose-dependent manner. The asterisk denotes that the changes in the mRNA expression levels were significant at a level of $p < 0.05$.

and 1.7-fold (10 MPa), all of which were statistically different from control cultures (Fig 2). Western blotting confirmed an increase in the presence of aggrecan and Type II collagen after application of intermittent hydrostatic pressure at 5 and 10 MPa for 4 days, when cell-associated protein extracts were compared between loaded and unloaded cultures.

A second question was whether intermittent hydrostatic pressure could counteract effects of a proinflammatory mediator, such as bacterial lipopolysaccharide (LPS), on chondrocyte metabolism. Exposure of chondrocytes to LPS increased the nitrite levels to $15.3 \pm 1.4 \mu\text{mol}$ whereas control chondrocytes had levels of $4.9 \pm 0.2 \mu\text{mol}$.²⁴ Application of intermittent hydrostatic pressure to LPS-activated cells decreased ($p < 0.001$) nitrite levels to $5.1 \pm 0.2 \mu\text{mol}$ (Fig 3). Lipopolysaccharide also increased mRNA signal levels for inducible nitric oxide synthase (iNOS). Exposure to intermittent hydrostatic pressure decreased LPS-induced iNOS mRNA.

Lipopolysaccharide pretreatment for 18 hours also down-regulated ($p < 0.001$) aggrecan and Type II collagen mRNA signal levels by 56% and 67%, respectively, when compared with chondrocytes without LPS pretreatment and maintained in the absence of load. Application of intermittent hydrostatic pressure to LPS-activated chondrocytes resulted in a 1.7-fold increase ($p < 0.001$) in Aggrecan and Type II collagen mRNA signal levels, relative to chondrocytes exposed to LPS but not treated with pres-

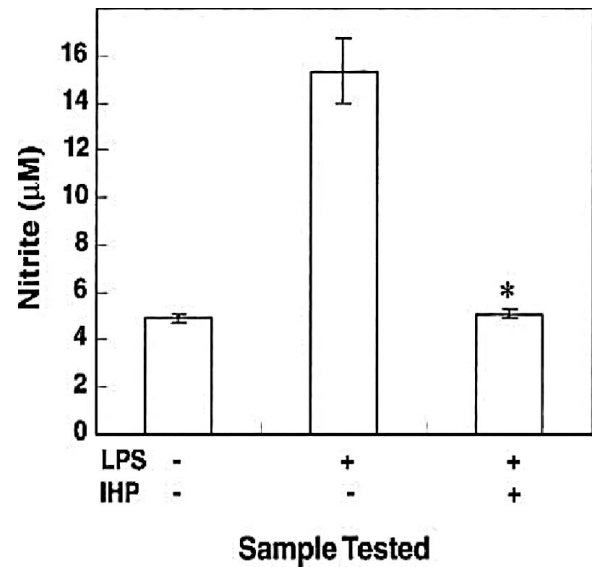


Fig 3. This figure shows that shear stress down-regulates matrix protein expression and intermittent hydrostatic pressure on nitric oxide release by human OA chondrocytes. The asterisk denotes that the changes in the mRNA expression levels were significant at a level of $p < 0.05$.

sure. In addition to effects on aggrecan and Type II collagen expression, lipopolysaccharide pretreatment also increased MCP-1 and MMP-2 mRNA expression by 6.5-fold ($p < 0.01$) and 1.3-fold ($p < 0.01$), respectively. Applying intermittent hydrostatic pressure to LPS-activated chondrocytes decreased ($p < 0.05$) MCP-1 mRNA signal levels by 45% and MMP-2 mRNA signal levels by 15%.

A third question of importance with respect to how types of load alter chondrocyte metabolism was whether shear stress could alter matrix macromolecule expression and cell viability. In fact the different types of mechanical loads, shear stress versus hydrostatic pressure, exhibited opposite effects on matrix mRNA expression. Osteoarthritis chondrocytes exposed to shear stress down-regulated ($p < 0.05$) mRNA for Type II collagen from 18% to 25% and aggrecan from 30% to 40% when compared with chondrocytes maintained in the absence of shear stress.²² In contrast, intermittent hydrostatic pressure up-regulated ($p < 0.05$) Type II collagen mRNA by 24%, 53%, and 112% with 1, 2, and 4 days of interval-based mechanical loading, respectively. Expression of aggrecan mRNA was also up-regulated ($p < 0.05$) by 35%, 59%, and 126% by application of intermittent hydrostatic pressure for 1, 2, or 4 days, respectively.

With respect to effects on chondrocyte viability, exposure of the cells to shear stress was accompanied by a dose-dependent increase in the release of nitric oxide that correlated with increased evidence of apoptosis and down-

regulation of bcl-2 expression (Fig 4). Externalization of phosphatidylserine (PS) to the outer plasma membrane occurred after exposure to shear stress and the quantity of nucleosomal DNA fragments were increased ($p < 0.001$) to 0.7, 5.7, 9.6, and 38.7 units (U)/mL as the levels of shear stress increased from 0.2, 0.4, 0.8, and 1.6 Pa, respectively (Fig 4). Nucleosomal degradation was not observed in chondrocytes in the absence of shear stress. Expression of the proto-oncogene bcl-2, an inhibitor of apoptosis, decreased ($p < 0.001$) from 44 U/mL in control samples to 40, 28, 26, and 10 U/mL as the shear stress increased from 0.2 to 1.6 Pa. Addition of inhibitors of nitric oxide production decreased nucleosomal degradation by 62% and 74% ($p < 0.001$) by NIO and L-NAME, respectively, when compared with chondrocytes exposed to shear stress in the absence of inhibitors. Addition of L-NAME to chondrocytes exposed to shear stress increased levels of the antiapoptotic oncoprotein bcl-2 by 2.7-fold when compared with chondrocytes exposed to shear stress without the inhibitor.

Shear stress induces a significant increase in the release of nitric oxide from human OA chondrocytes that is load and time dependent. As the level of shear stress increases from 0.2 rpm to 1.6 Pa, nitrite levels increase ($p < 0.001$) from 8 to 15 μmol in a dose-dependent manner at 24 hours of culture whereas in the absence of shear stress, the mean nitrite concentration in the chondrocyte culture medium was 5 μmol .²³ Applying shear stress (1.6 Pa) for 2, 6 and 24 hours increased ($p < 0.05$) the concentration of nitrite to 14, 18, and 30 μmol , respectively.

In contrast to shear stress, nitrite levels remain unchanged by application of intermittent hydrostatic pressure at 10 MPa for 4 hours a day for 1, 2, or 4 days. However, chondrocytes with hydrostatic pressure (10 MPa, 1 Hz for 4 hours) after exposure to shear stress (1.6 Pa for 2 hours) decreased ($p < 0.05$) nitric oxide release by 35% in chondrocytes when compared with cells exposed to shear stress alone.

A final question regarding effects of hydrostatic pressure on chondrocytes was whether only matrix macromolecule proteins or the multiple proteins associated with chondrocytes would be altered by loading. To address this question, a method was developed for analysis of cell-associated proteins using two-dimensional gel chromatography and fluorescent quantification by laser image detection (Typhoon, Amersham Biosciences, Piscataway, NJ). Analysis of chondrocyte-associated proteins revealed different patterns for chondrocytes (Fig 5A) not loaded when compared with extracts of chondrocytes exposed to intermittent hydrostatic pressure (Fig 5B). These data show that multiple proteins encoding by chondrocyte genome are subject to regulation by hydrostatic pressure, either as

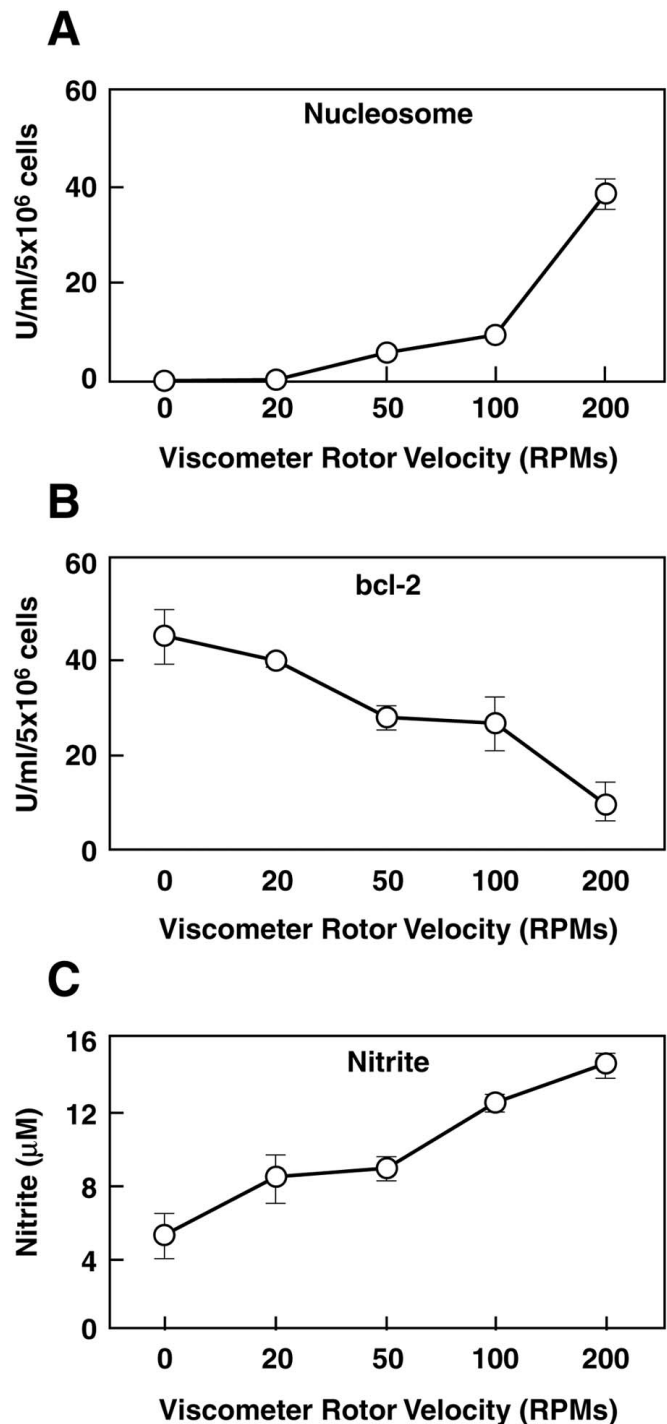


Fig 4A–C. This figure shows the effects of shear stress on induction of nitric oxide and the associated pattern of nucleosome degradation in human OA chondrocytes. (A) The dose-dependent release of nitric oxide is shown. (B) The decrease in bcl-2, which is associated with increased susceptibility of cells to apoptosis, is shown. (C) The increase in Fas, which is associated with onset of cell death through the apoptotic process, is shown.

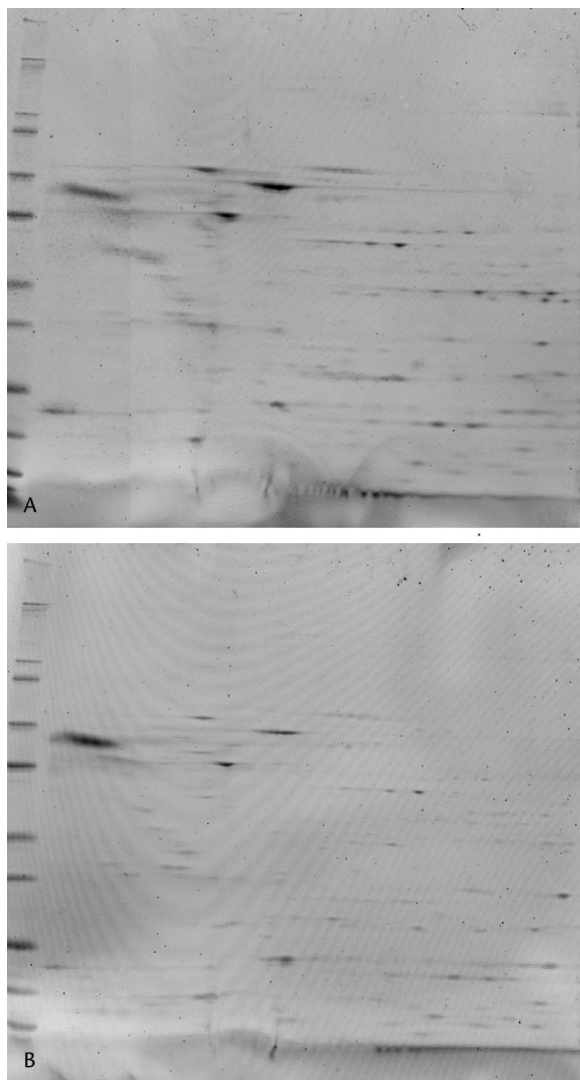


Fig 5A–B. This figure depicts the proteins associated with human OA chondrocytes in the high-density monolayer cultures and the changes that take place after exposure of the cells to intermittent hydrostatic pressure. (A) The distribution of protein in unloaded chondrocyte cultures is shown. (B) The distribution of proteins in cultures exposed to intermittent hydrostatic pressure is shown.

a consequence of transcriptional events, translational control, or posttranslational processing.

DISCUSSION

The results reviewed here confirm that exposure of chondrocytes to different types of mechanical loads results in a shift in the types of protein expressed. Intermittent hydrostatic pressure increased expression of aggrecan and Type II collagen at pressures of 5 to 10 MPa, levels that fall within the range normally occurring in the hip during daily activity.¹³ Applying shear stress to OA chondrocytes de-

creased expression of cartilage matrix proteins whereas intermittent hydrostatic pressure increased matrix protein expression. Shear stress also increased release of nitric oxide, a reactive oxygen metabolite implicated in joint pathogenesis.²² The increase in nitric oxide was associated with an increase in chondrocyte apoptosis, and shear stress effects on nitric oxide release were decreased by application of hydrostatic pressure.

The precise molecular mechanisms by which mechanical stress alters chondrocyte metabolism require further study. Questions remain regarding the nature of chondrocyte signaling pathways for transduction of mechanical stimuli from the extracellular matrix to the nucleus.^{36,45,47} The presumption is that the chondrocyte plasma membrane serves as a focal point for recognition of the mechanical stimulus, whether it is hydrostatic pressure or shear stress. If true, protein-dependent signaling, analogous to growth factor receptors, ion channels, or cytoskeletal components, may be involved. A number of studies implicate integrins and other integral membrane proteins with changes in chondrocyte metabolism in response to shear stress and pressure.^{26,29,30,35} Intracellular pathways likely depend on multiple levels of kinases⁹ for activation of specific transcription factors that integrate with promoters and enhancers to regulate gene expression.^{16,27,42,48}

As reviewed here, articular chondrocytes exhibit diverse metabolic states depending the type of mechanical load present. Although the precise mechanisms remain to be established, the potential is high that modulation of chondrocyte protein expression can be achieved by specific loading conditions. Methods to express individual proteins within the cartilage proteome may be instrumental in advancing methods for diagnosis and treatment of the OA through cartilage repair. Regulation of cartilage metabolism also might enable rehabilitation protocols where microprocessor feedback devices control immediate post-surgical joint loading for optimal cartilage repair. Finally, information gained regarding mechanisms underlying hydrostatic pressure and shear stress modulation of chondrocyte metabolism may expand the potential for treatment of OA by cartilage engineering.

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