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Alterations in gene expression in response to compression of nucleus pulposus cells

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Abstract

BACKGROUND CONTEXT—It is clear that mechanical forces are involved in initiating disc degeneration but also have the potential to exert beneficial effects. However, the signaling pathways initiated by mechanical stress and thresholds for these responses have not been elucidated. We have developed a metabolically active compression system with the advantages of having the ability to test cells in vitro as well as within their native matrix and control exposure to environmental factors. We hypothesized that nucleus pulposus cells would respond to compressive stress with different thresholds for alterations in catabolic and anabolic gene expression.

PURPOSE—The purpose of the study was to establish the utility of a novel compression chamber and examine the effects of various magnitudes and durations of compression on nucleus pulposus inflammatory, catabolic, and anabolic gene expression.

STUDY DESIGN—In vitro controlled examination of intervertebral disc cell responses to compression.

METHODS—A chamber capable of imparting 0 to 20 MPa of hydrostatic compression onto nucleus pulposus cells was fabricated. Healthy rabbit nucleus pulposus cells were cultured in alginate beads and exposed to static compression at 0.7, 2, and 4 MPa for 4 or 24 hours. Gene expression analysis (real-time polymerase chain reaction) was performed to compare markers of inflammation (inducible nitric oxide synthase, cyclooxygenase-2), matrix catabolism (matrix

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metalloproteinase-3), and antica-tabolic/anabolic metabolism (tissue inhibitor of metalloproteinase-1, aggrecan) in control and compressed cells.

RESULTS—Compression resulted in magnitude- and duration-dependent changes in gene expression. Increasing magnitudes showed more anticatabolic gene expression changes, whereas increasing duration resulted in increases in procatabolic gene expression.

CONCLUSION—These data demonstrate favorable effects of compression in relation to genes involved in matrix homeostasis and procatabolic gene expression in response to sustained loading levels, consistent with traumatic effects. These data provide an improved understanding of how compression affects cell signaling, which has the potential to be exploited to initiate repair and prevent matrix breakdown.

Keywords

Intervertebral disc; Gene expression; Compression

Introduction

Disc degeneration and its sequelae are among the most common reasons for chronic back pain. Intervertebral disc degeneration increases with age and is present in nearly all spines by the age of 50 years [1–3]. Despite the high prevalence of this disorder, little is known about the factors that predispose the disc to degeneration. The intervertebral disc is the major compression-carrying component of the spine, with the nucleus experiencing compressive stress and the annulus experiencing tensile stress. One factor that has been implicated in disc degeneration is compressive force, with excessive compression implicated in animal models as a causative agent for disc degeneration [4,5]. Clinical evidence is also suggestive of this effect as patients with low back pain experience greater spine compression during lifting tasks than asymptomatic individuals [6]. However, the mechanism through which mechanical compression contributes to disc degeneration is unclear. One plausible mechanism is through the activation of mechanical signals, which regulate catabolic and anabolic pathways of the cells within the matrix. For example, in the nucleus pulposus, high compressive forces result in loss of structural proteoglycans [7] and increased expression of matrix proteases [8].

In addition to the destructive effects of mechanical forces, it is clear that beneficial effects of motion and load exist as well. There appears to be a protective effect of moderate physical loading of the spine [9] and patients with disc degeneration have lower resting pressure measurements than asymptomatic controls [10]. In addition, promising clinical evidence continues to demonstrate the effectiveness of motion-based therapies in the treatment of low back pain [11–13]. Although causality cannot be inferred from these studies, animal studies do suggest a causative relationship. Interestingly, animal models of dynamic loading have been able to demonstrate an anabolic response to compressive force in the production of matrix structural proteins [14,15] both in vitro and in vivo. Similarly, physiologic levels of hydrostatic pressure stimulate production of proteoglycans and tissue inhibitors of metalloproteases, which slow matrix degradation [16]. Clinical experience supports this as there appears to be a relative protective effect of moderate physical loading of the spine [9].

The most serious spine pathology relates to the highest and lowest degrees of physical activity. However, the magnitudes and conditions under which mechanical forces initiate trauma versus healing are not well understood. Defining and understanding these thresholds and the mechanisms responsible for the observed effects are critical to facilitate development of exercise-based protocols and surgical interventions designed to optimize beneficial loading patterns. The integrity of the disc matrix depends on the catabolic/anabolic balance within the disc tissue, thus alterations in the metabolism of the resident cells has the potential to shift this balance toward repair.

Similar to the classic studies of Nachemson [17], new in vivo measurements demonstrate that lying prone results in a pressure of 0.1 MPa at the nucleus pulposus, and lifting a 20-kg weight with a round flexed back 2.3 MPa [18]. As 1 MPa has been shown to have effects on matrix gene expression in annulus fibrosus in vivo [19], we investigated gene expression in response to compressive forces above and below this level in an in vitro system of healthy rabbit nucleus pulposus cells to understand how mechanical signaling regulates expression of genes involved in intervertebral disc degeneration. In addition, we chose time points of 4 and 24 hours for analysis to allow for examination of changes in gene expression. We hypothesized that different magnitudes and durations of compression would result in alterations in expression of key genes involved in disc matrix homeostasis.

Material and methods

Design of a compression chamber

A compression chamber has been constructed composed of stainless steel with Teflon fittings capable of imparting 0 to 20 MPa of hydrostatic compressive load. The system uses compressed nitrogen pumped into a lower chamber separated from the sample chamber by a flexible diaphragm of Ultra-Strength Buna-N Rubber Plain Back, 1/16" Thick 50A Durometer (Part no. 8969K191; McMaster-Carr, Cleveland, OH, USA; see Fig. 1). We chose the interface based on a finite element analysis of the proposed system, exploring the effect of various materials and thicknesses of the diaphragm and assuming a 1-in radius. The analysis demonstrated that as the thickness of the diaphragm was decreased, the relationship between pressure in the lower and upper chambers approached a one-to-one relationship. We, therefore, chose the above diaphragm material, which also has the advantage of elastic recoil to facilitate future studies using dynamic compression protocols. The entire system is gas or pressure sterilizable and housed in a tissue culture incubator during experiments to maintain temperature constant at 37°C. The volume of the sample chamber is 80 mL and designed with inlet/outlet ports to eliminate the presence of gas above the media to prevent an air/gas interface, which would alter the hydrostatic pressure environment. Cell viability within the chamber was confirmed by trypan blue exclusion assay after exposure to compressive stress up to 4 MPa, is greater than 80% after both 4 and 24 hours, and is equivalent to that of control cells not housed in the compression chamber.

Isolation of rabbit intervertebral disc cells

Nucleus pulposus cells were isolated from intervertebral discs from skeletally mature New Zealand white rabbits immediately after sacrifice as previously described [20] (Institutional

Animal Care and Use Committee protocol #0310239B-1). Nucleus pulposus cells (Passage 1) were cultured in 10 μ L 1.2% alginate beads (chosen to maintain cell phenotype [21]) at a density of 4×10^6 cells/mL and incubated in F-12, 10% fetal bovine serum, 1% P/S at 37°C, and 5% CO₂ for 4 to 5 days before experiments.

Application of compressive stress

Nucleus pulposus cells in alginate beads (12 beads per experiment) were mounted in the compression system enclosed in dialysis tubing (2,000 molecular weight cut-off) filled with 1-mL sterile media in a standard tissue culture incubator at 37°C and 5% CO₂. Static compression was applied at 0.7, 2, and 4 MPa for 4 or 24 hours. In addition, to examine the persistence of the gene expression effects, cells were exposed to the appropriate regimen for 4 hours and then cultured in the absence of compression for an additional 20 hours before RNA isolation (4+20 hours). Control beads were housed in dialysis tubing and incubated in analogous culture media. All outcome measures were compared with these noncompressed control cells.

Gene expression

Immediately after compression, cells were released from the beads by sodium citrate dissolution and mRNA isolated using a Qiagen mini-prep kit (Qiagen Inc., Valencia, CA, USA), including a DNase step to remove genomic material. Gene expression analysis (real-time reverse-transcriptase polymerase chain reaction) was performed utilizing the SYBR green detection system (VWR, West Chester, PA, USA) using custom designed primers (see Table) that have been validated in our laboratory [22]. Genes were selected to compare markers of inflammation (inducible nitric oxide synthase [iNOS] and cyclooxygenase-2 [COX-2]), matrix catabolism (matrix metalloproteinase-3 [MMP-3]), anticatabolic metabolism (tissue inhibitor of metalloproteinase-1 [TIMP-1]), and structural component (aggrecan) in control and compressed cells. The Δ Ct method was utilized to calculate relative gene expression compared to the appropriate control after normalizing to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as described previously [23] and all PCR measurements from each sample repeated in duplicate. Glyceraldehyde 3-phosphate dehydrogenase was chosen as the housekeeping gene because prior data demonstrated that alternative housekeeping gene beta-actin was significantly downregulated in response to compression compared with GAPDH, and immunofluorescence demonstrated GAPDH detection throughout 28 days of compression, suggesting that GAPDH is a reasonable choice as a housekeeping gene for mechanobiological testing [24]. In comparing two different compression magnitudes, relative gene expression values were expressed by setting the comparison gene expression regimen (0.7 MPa) value at 1 by convention (data presented in Fig. 2). To facilitate comparison of similar regimens at different durations, percent relative gene expression was calculated as $(1 - \text{relative gene expression}) \times 100$, where negative values represent inhibition of gene expression, and positive values represent stimulation of gene expression compared with control cells (data presented in Fig. 3).

Statistical analysis

Values represent the average of three to seven trials $\pm$ standard error, with 95% confidence intervals calculated to determine statistical significance. The confidence intervals were calculated based on the *t* distribution because of the small sample size. Computations were performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and R statistical software (<http://www.r-project.org/>).

Results

After 4 hours, low levels of compression (0.7 MPa) resulted in increased COX-2 gene expression (83%) and modestly decreased expression of iNOS and genes involved in matrix homeostasis MMP-3, TIMP-1, and aggrecan (3–30%) when compared with control cells. Moderate compression (2 MPa) showed a similar pattern, with the exception of an increase (18%) in expression of TIMP-1. High levels of compression (4 MPa) also inhibited iNOS (18%) and stimulated COX-2 (184%) but demonstrated a significant reversal in the expression of genes controlling matrix homeostasis, with increase in both MMP-3 (45%) and TIMP-1 (31%). Because intervertebral disc cells do not experience an absence of compression in vivo, we also examined the change between moderate (2 MPa) or high (4 MPa) levels of compression and low levels of compression, mimicking normal physiologic movements (0.7 MPa), to assess the change in gene expression, which would be expected during load variations in vivo. The relative gene expressions between 0.7 MPa, set at 1, and moderate or high levels of compression are shown in Fig. 2. In examining moderate (2 MPa) compression, gene expression of inflammatory mediators iNOS and COX-2 as well as modulator of matrix turnover MMP-3 were noted to statistically significantly decrease compared with physiologic levels of compression. Aggrecan gene expression demonstrated a trend toward increased expression and TIMP-1 toward decreased expression, but these did not reach statistical significance. In comparing the difference from physiologic to high (4 MPa) compression, gene expression was noted to decrease for all genes tested, including aggrecan. However, only iNOS demonstrated statistical significance.

In addition to differences in gene expression in response to differing magnitudes, duration of compression also influenced gene expression profiles. Twenty-four hours of continuous compression resulted in gene expression profiles distinct to those observed after 4 hours for all magnitudes tested. Interestingly, the same magnitude of compression (0.7 MPa) that caused a decrease in iNOS and MMP-3 after 4 hours demonstrated increase in gene expression of these catabolic mediators after 24 hours. In addition, increase in COX-2 and aggrecan expression was also noted after 24 hours (Fig. 3, Left). At moderate levels of compression (2 MPa), the inhibition of iNOS and MMP-3 that was observed after 4 hours of compression was also lost after 24 hours, with increased gene expression of both iNOS and MMP-3. Aggrecan, TIMP-1, and COX-2 expression remained relatively unchanged (Fig. 3, Middle). Prolonged compression at high magnitude (4 MPa) resulted in increases in iNOS, COX-2, and MMP-3 with smaller increases in TIMP-1 and aggrecan (Fig. 3, Right).

In addition, we examined the persistence of gene expression changes after 4 hours of compression through measurement after 20 hours of incubation; the data show a pattern similar to that observed after 24 hours of continuous compression, although the changes

were of lesser magnitude (Fig. 3). A notable exception to this was at low levels of compression (Fig. 3, Left), where COX-2 and TIMP-1 showed inhibition after the 20-hour incubation as opposed to the stimulation seen after 24 hours of continuous compression. Similarly, at 2 MPa (Fig. 3, Middle), aggrecan expression was noted to increase in the 4+24-hour regimen, compared with inhibition after both 4 and 24 hours of continuous compression. However, none of these changes reached statistical significance.

Discussion

This work demonstrates the feasibility of a metabolically active system that allows examination of the pathways involved in mechanical signaling in response to compression in intervertebral disc cells. We have used a hydrostatic compression system as opposed to direct compression because we have the advantage of precise control over the amount of compression experienced in addition to precise ability to alter the local environment of the cells. In addition, the system was designed to facilitate testing of irregularly shaped samples, such as the nucleus pulposus, which will be the topic of future studies. In direct compression systems, the shape of the sample interface can significantly change the amount of compression created. However, in our system, we will have the ability to test intact nucleus pulposus tissue, after controlling osmolar gradients to prevent swelling. We believe that this will allow future extension of this work to include testing of cells within their native matrix, which likely has a profound effect on mechanical signaling. However, it should be noted that this system does not recreate additional factors, which may affect matrix homeostasis, such as fluid flow and tensile forces. Although small animal models, including rat and mouse, of dynamic compression are available, these models do not allow for similar control of mechanical stimuli or environmental conditions as in an in vitro system. Our system allows for facile testing of various magnitudes and frequencies of cell or tissue samples and greater variability in environmental conditions, facilitating investigations into mechanistic pathways involved in mechanical signaling in intervertebral disc cells.

These data demonstrate that the cells of the nucleus pulposus exhibit differential gene expression in response to different loading magnitudes and times. It was observed that on increasing the magnitude of compression after 4 hours, catabolic mediator gene expression was found to decrease, without significant change in anabolic or anticatabolic gene expression (see Fig. 4). This pattern would be expected to produce a beneficial effect in overall matrix integrity. In addition to prior studies demonstrating a potential anabolic effect of favorable magnitudes of compression [14–16], this concept is further supported by in vivo studies of simulated microgravity, which demonstrate associations of decreased loading with decreased glycosaminoglycan content and aggrecan gene expression [25]. The 24-hour data demonstrate that prolonged levels of compression result in loss of the anticatabolic effects observed after 4 hours of loading at lower (0.7 and 2 MPa) magnitudes and exacerbate the catabolic gene expression changes seen at high (4 MPa) magnitudes (see Fig. 4). This would be expected to have a net detrimental effect on matrix homeostasis. This may suggest a fatigability of the signaling system or undesirable feedback effects at the cellular level. Expression of matrix homeostasis genes demonstrates a time dependence in an in vivo rat model of disc compression, underscoring the importance of this variable [26]. In addition, Korecki et al. [27] has recently demonstrated a minimal effect of frequency but a

significant effect of cell maturity, in altering matrix expression. It would, therefore, be of interest to examine the effect of cell maturity on catabolic and inflammatory changes in our system in the future. However, the overall net effect of changes in matrix metalloprotease activity and inhibitor concentrations will influence net matrix homeostasis, and cannot be inferred from gene expression changes alone, warranting further study of protein products.

Interestingly, the acute effects on gene expression after 4 hours differ from those experienced after a period of rest (ie, after the 20-hour incubation) for low magnitudes (0.7 and 2 MPa), suggesting that the late gene expression responses differ than those seen early after the initiation of mechanical loading. However, at higher magnitudes (4 MPa), gene expression changes after 4 hours paralleled those seen after a period of rest, suggesting that the duration of loading has a greater effect at these higher magnitudes than at lower magnitudes. At 4 MPa, higher duration of loading, not simply higher duration of incubation, was the key to altering gene expression responses. This may relate to an adaptive response at the cellular level at higher magnitudes and underscores the need for further studies examining end products. This effect of delayed changes in gene expression in response to loading have been examined previously in articular chondrocytes after exposure to tensile forces [28], which demonstrated persistence of the observed anti-inflammatory effect after 4 hours. However, our data differ from these findings, and in fact demonstrated a reversal in key genes involved in the catabolic process. This may be the result of differences in loading paradigms (compression vs. tension) or cell types. In addition, the Madhavan study also demonstrated that the duration of exposure to mechanical stimulation affected the persistence of the gene expression changes. It is, therefore, possible that different durations of compression may result in different long-term effects and underscore the importance of examining key end points to determine net effects on tissues. In addition, the relatively short time points examined in our study, chosen to highlight changes in gene expression, do not account for changes in cell proliferation, which may affect net matrix homeostasis after longer durations of compression.

This raises a limitation of our present study, which relates to the examination of gene expression changes. Future studies will be required to establish how these gene expression changes are reflected in net matrix homeostasis of intervertebral disc cells. No consensus exists for the magnitude of gene expression changes expected to result in net effects on the cell metabolism, and in fact, it is likely that this differs depending on the gene. For example, large changes in genes, which are expressed at high levels at baseline, such as TIMP, are likely to be less significant than small changes in inducible mediators, such as iNOS, which are expressed at negligible amounts under conditions of homeostasis. Thus, although many of the observed gene expression changes were small and in some cases do not reach statistical significance, the net significance of these changes on matrix homeostasis warrants further study. In particular, future studies examining the protein changes corresponding to these observed gene expression changes would add to the understanding of the effects on net matrix homeostasis.

A perceived limitation of our present study is evaluation of intervertebral disc cells in an in vitro environment. However, we have used a three-dimensional bead culture system, which maintains nucleus pulposus cell phenotype. An advantage of the in vitro environment is that

it provides us with a powerful experimental platform allowing responses to compression in the presence of inflammatory stimulus, pharmacologic interventions, or gene therapy to be examined. Although in vitro models represent simplification of the complex loading schemes experienced by the cells in vivo, modeling has been performed to demonstrate that hydrostatic pressurization, like that applied in the current system, dominates in the nucleus pulposus [29]. Thus, these data will provide the essential building blocks for understanding cellular thresholds to mechanical stimuli under carefully controlled isolated loading schemes and examination of the interactions between compressive and inflammatory signaling. In addition, the magnitudes examined in this study were primarily chosen based on human in vivo data [17,18]. However, in a comparison of animal discs used in research with human discs, it was concluded that disc tissue material properties are largely conserved across animal species and that disc axial mechanics are very similar across animal species [30], justifying comparisons between experimental animal models and human discs. Similarly, intradiscal pressure measurements in New Zealand white rabbits under general anesthesia have been shown to be 0.25 to 0.45 MPa [31]. Although these values are slightly lower than the 0.7 MPa control magnitude used in our studies, actual in vivo pressures in an anesthetized animal are likely lower than those measured during baseline activity. Nevertheless, it must be mentioned that cell culture does not entirely mimic the in vivo environment, and in fact, cell matrix interactions may also contribute to in vivo signaling changes. It is likely that the relevant durations and magnitudes in vivo may actually differ from those examined in this study. The purpose of these studies, however, was to determine that thresholds for these variables exist and allow mechanistic examination of the pathways involved in these thresholds. We feel that in vivo models remain the optimal models for probing the physiologically relevant loading paradigms, whereas models such as ours are better suited for probing mechanistic questions related to mechanical signaling pathways.

These data demonstrate anticatabolic effects of short-term static compression on gene expression and increases in catabolic gene expression of prolonged loading at the same magnitude. This suggests that although high levels of loading promote catabolic activity in the disc cells, lower levels of loading that promote anticatabolic activity exist, which have the potential to be exploited therapeutically. However, it is likely that examination of dynamic loading protocols, which may relate more closely to the compression experienced in vivo, may demonstrate differences from the static protocols examined in this study. Although the data presented do not account for all of the pathways regulated by the complex mechanical signaling processes in the intervertebral disc, these studies do demonstrate thresholds for the effect of compression on gene expression. Future studies will examine the net effect of these gene expression changes on matrix homeostasis and the relationship to in vivo levels and frequencies of compression. In addition, future studies will need to be performed on human cells as the degree of degeneration will likely alter the observed responses. These data support the idea that understanding of the effects of mechanical loading, both reparative and traumatic, will require examination of key factors including both magnitude and time. As we begin to consider “dosing” of therapeutic exercise and that adequate time intervals will need to be selected to titrate the desired outcome, these will be key considerations. This work will lead to the establishment of the role of compressive stress in regulating anabolic and catabolic pathways. It is expected that this will provide insight

into the mechanisms behind the positive effects of therapeutic exercise and provide the ability to predict what types of forces may initiate degeneration.

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Fig. 1.
Schematic and photograph of completed custom-built compression chamber.

Fig. 2. Relative gene expression comparing 0.7 MPa compression (set at 1) with 2 and 4 MPa compression. Data are reported relative to 0.7 MPa gene expression averages $\pm$ standard error. *Statistical significance, based on the *t* score.

Fig. 3.

Percent gene expression compared with control cells after exposure to 4 hours of compression, 24 hours of compression, and 4 hours compression followed by 20 hours of incubation at (Top) 0.7 MPa, (Middle) 2 MPa, and (Bottom) 4 MPa. Data are reported relative to control (uncompressed) cell gene expression averages $\pm$ standard error.

*Statistical significance, based on the *t* score.

Hypothesized Effects		Gene Categories	Observed Effects	
Favorable	Detrimental		Increasing Magnitude	Increasing Duration
↓	↑	Inflammatory (iNOS, COX2)	↓	↑
↓	↑	Catabolic (MMP3)	↓	↑
↑	↓	Anti-catabolic/anabolic (TIMP-1, Agg)	↔	↔

Fig. 4.

Summary schematic of general trends observed with increasing magnitudes and durations, and corresponding effects that are hypothesized to be favorable or detrimental to overall matrix homeostasis.

Table**Primer sequences used for RT-PCR**

Gene	Sequence
Aggrecan	Rev: 5'-CGTAAAAGACCTCACCTCCAT-3' For: 5'-GCTACGGAGACAAGGATGAGTTC-3'
COX-2	Rev: 5'-CAGGCACCAGACCAAAACACTT-3' For: 5'-CACGCAGGTGGAGATGATCTAC-3'
GAPDH	Rev: 5'-GCTGAGATGATGACCCCTTTTGG-3' For: 5'-GATGCTGGTGCCGAGTAC-3'
iNOS	Rev: 5'-TCTGTGACGGCCTGATCTTTC-3' For: 5'-CCCCTTCAACGGCTGGTA-3'
MMP-3	Rev: 5'-CCAGTGGATAGGCTGAGCAAA-3' For: 5'-AGCCAATGGAAATGAAAACCTTTC-3'
TIMP-1	Rev: 5'-CCACAAACTTGGCCCTGATG-3' For: 5'-AGCAGAGCCTGCACCTGTGT-3'

RT-PCR, reverse-transcriptase polymerase chain reaction; COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; MMP-3, matrix metalloprotease-3; TIMP-1, tissue inhibitor of metalloproteinase-1; Rev, reverse; For, forward.