Regulation of gene expression in intervertebral disc cells by low and high hydrostatic pressure

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Abstract Intervertebral disc structures are exposed to wide ranges of intradiscal hydrostatic pressure during different loading exercises and are at their minimum during lying or relaxed sitting and at maximum during lifting weights with a round back. We hypothesize that these different loading magnitudes influence the intervertebral disc (IVD) by alteration of disc matrix turnover depending on their magnitudes. Therefore the aim of this study was to assess changes in gene expression of human nucleus cells after the application of low hydrostatic pressure (0.25 MPa) and high hydrostatic pressure (2.5 MPa). IVD cells isolated from the nucleus of human ($n = 18$) and bovine ($n = 24$ from four animals) disc biopsies were seeded into three-dimensional collagen type-I matrices and exposed to the different loading magnitudes by specially developed pressure chambers. The lower pressure range (0.25 MPa, 30 min, 0.1 Hz) was applied with a recently published device by using an external com-

pression cylinder. For the application of higher loads (2.5 MPa, 30 min, 0.1 Hz) the cell-loaded collagen gels were sealed into sterile bags with culture medium and stimulated in a newly developed water-filled compression cylinder by using a loading frame. These methods allowed the comparison of loading regimes in a wide physiological range under an equal three-dimensional culture conditions. Cells were harvested 24 h after the end of stimulation and changes in the expression of genes known to influence IVD matrix turnover (collagen-I, collagen-II, aggrecan, MMP1, MMP2, MMP3, MMP13) were analyzed by real-time RT-PCR. A Wilcoxon signed-rank test¹ and a Wilcoxon 2-sample test² were performed to detect differences between the stimulated and control samples¹ and differences between low and high hydrostatic pressure². Multiple testing was considered by adjusting the p value appropriately. Both regimes of hydrostatic pressure influenced gene expression in nucleus cells with opposite tendencies for the matrix forming proteins aggrecan and collagen type-I in response to the two different pressure magnitudes: Low hydrostatic-pressure (0.25 MPa) tended to increase collagen-I and aggrecan expression of human nucleus cells ($P < 0.05$) but only to a small degree. High hydrostatic pressure (2.5 MPa) tended to decrease gene expression of all anabolic proteins with significant effects on aggrecan expression of nucleus cells ($P = 0.004$). Low hydrostatic pressure had no influence on the expression of matrix metalloproteinases (MMP1, MMP2, MMP3 and MMP13). In contrast, high hydrostatic pressure tended to increase the expression of MMP1, MMP3 and MMP13 of human nucleus cells with high individual-variations. The decreased expression of aggrecan ($P = 0.008$) and collagen type II ($P = 0.023$)

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and the increased MMP3 expression ($P = 0.008$) in response to high hydrostatic pressure could be confirmed in additional experiments with bovine nucleus cells. These results suggest that hydrostatic pressure as one of the physiological stimuli of the IVD may influence matrix turnover in a magnitude dependent way. Low hydrostatic pressure (0.25 MPa) has quite small influences with a tendency to anabolic effects, whereas high hydrostatic pressure (2.5 MPa) tends to decrease the matrix protein expression with a tendency to increase some matrix-turnover enzymes. Therefore, hydrostatic pressure may regulate disc matrix turnover in a dose-dependent way.

Keywords Human intervertebral disc cells · Low and high hydrostatic pressure · Gene expression · Annulus · Nucleus

Introduction

Disc degeneration is influenced by multiple factors like age [4], nutrition [2, 17], mechanical factors [16] and genetics [19, 18]. In the present study we focus on the influence of mechanical loading on intervertebral disc (IVD) cells as both overload as well as immobilisation are supposed to accelerate the disc degeneration. A recently published review of the literature clearly pointed out that in animal models at least disc degeneration can be provoked by inappropriate mechanical signals [16, 19]. Multiple mechanical factors affect the IVD in vivo [1, 14], as this structure has to provide flexibility, stabilization and shock absorbance in its function as the joint of the spine. Although, mechanical loading appears to influence tissue turnover, there is currently little information on how the load intensities affect cellular responses.

In vivo, wide ranges of mechanical load occur in the IVD. In vivo measurements of intradiscal pressure have shown that hydrostatic pressure in the nucleus reaches different values with exercises such as flexion, extension, lateral bending, weight lifting or lying down [20]. Whereas pressure magnitudes range between 0.1 and 0.3 MPa under low loading conditions, single exercises can result in intradiscal pressure of up to 2.5 MPa or even more [20]. Disc tissue is adapted to these wide loading ranges by a highly organized morphological and macromolecular matrix composition adapted to the customary loading regime. Although, the disc is a tissue of low cellularity, cellular activity regulates matrix turnover, resulting either in matrix maintenance or matrix degradation [13]. Mechanical loading may regulate disc matrix structure and composition by altering

the disc cell behavior affecting proliferation, gene expression and ultimately biosynthesis.

In order to clarify the mechanisms of load-induced changes on matrix turnover, several studies have investigated loading effects on disc tissues and cells, using widely differing experimental models, either in situ or in vitro. Handa et al. [8] reported that different levels of hydrostatic pressure (HP) may act as an anabolic or catabolic factor on human disc metabolism depending on the pressure level. Ishihara et al. [9] found that HP has magnitude-dependent effects in the different regions of the IVD. Using a rabbit cell culture model in monolayer and alginate culture, Kasra et al. [10] found that high frequency and high amplitude hydrostatic stress stimulated collagen synthesis in outer annulus cells, whereas the lower amplitude and frequency had little effect. Also, MacLean et al. [11] observed in their study based on a rat-tail model that cellular responses depended on pressure magnitude and frequency. They were able to define a loading threshold of 0.2 MPa, reflecting homeostasis, which preserved tissue composition and found changes in the gene expression in case of under- or overshooting this load. In our own pilot study on human cells exposed to mechanical stimulation in a 3D collagen type-I gel, we have found nucleus and annulus cells to respond to cyclically applied HP at 0.25 MPa [12]. Changes in the gene expression showed anabolic tendencies, but the effects were relatively minor.

Most studies to date have reported results from cells of different animal species; the effects of different pressure magnitudes on human disc cells are however poorly investigated. We assume that the wide ranges of HP that can occur in the nucleus of the human IVD contribute to the regulation of matrix turnover. In this study, we have chosen two different pressure magnitudes within the physiological range (0.25–2.5 MPa) [20], assuming that these two widely differing loading parameters will result in varying effects on nucleus cells from intervertebral discs. We hypothesized that application of HP results in changes in the expression of anabolic (aggrecan, collagen-I, collagen-II) and catabolic (MMP1, MMP2, MMP3, MMP13) genes with differences between the two pressure magnitudes (0.25 MPa, 2.5 MPa).

Materials and methods

Disc cell isolation and preparation of cell loaded collagen matrices

Cell isolation and cell culture was conducted as previously described [12]. Briefly, cells were isolated by

collagenase–pronase digestion from the nucleus pulposus of human disc biopsies from 18 donors (average age 39 ± 17) who underwent surgery because of disc herniation (for details of all patients see Table 1). To confirm the results with disc cells from disc tissue without any degeneration, we have used caudal bovine discs as a model for young and healthy subjects for some selected experiments. Bovine disc cells were isolated from the nucleus of each six caudal discs from four animals (age < 24 months) according to the same protocol as for human nucleus cells. The primary cells were expanded in monolayer for up to three subcultures. Cell loaded collagen type-I gels with a cell density of 0.3×10^6 cells/ml were prepared according to the manufacturers protocols (Ars Arthro AG, Esslingen, Germany) and poured into round standard culture dishes (3 ml each). The final concentration of type I collagen was 3 mg/ml. Depending on the cell number available, gels for low and/or high-pressure magnitudes were produced and covered with 2 ml of serum-free culture medium [12]. Because of the partly low cell numbers, not all donors were included into each experiment (details see Table 1). For each magnitude, culture groups of eight gels were equally divided into two subgroups and were exposed either to the respective HP or were maintained under the same conditions without mechanical stimulation.

Hydrostatic pressure experiments

Application of low HP (0.25 MPa) was conducted as described in our pilot study [12]: cell-seeded cultures were stimulated inside a custom-made pressure chamber by air compression. Loading was applied once for

30 min with a frequency of 0.1 Hz. Control dishes were placed in an identical apparatus but no pressure was applied. Following mechanical stimulation, both subgroups were cultured for further 24 h in an incubator before examining cellular effects. For technical reasons, this method was limited to low pressure magnitudes, therefore, a novel compression cylinder (see Fig. 1) was developed for the application of high HP (2.5 MPa). There was no significant difference on gene expression with the two different stimulation devices as pre-experiments performed with bovine disc cells at equal low pressure conditions have shown. For the high-pressure experiments, cell loaded collagen gels were sealed into sterile infusion bags that were filled with each 20 ml of serum-free culture medium with careful removal of air bubbles. For mechanical stimulation one part of these bags was transferred into the cylindrical tube of the compression device that was filled completely with 37°C tempered water and closed with a specially developed cover top that also allowed a complete removal of any residual air in the chamber by water injection into the system. The closed cylinder was placed into a hydraulic loading frame that was calibrated for application of defined loading conditions. Intermittent HP was applied with a computer-based loading program and monitored by a pressure sensor situated inside the chamber. Pressure of 2.5 MPa was applied during 30 min with a frequency of 0.1 Hz, resulting in 180 loading cycles. The control group samples were incubated at 37° without any mechanical stimulation at equal culture conditions. After cessation of stimulation, both stimulated samples and controls were maintained for furthermore 24 h in an incubator before examining cellular effects. For

Table 1 Patients data and associated experiments

Donor no.	Age (years)	Gender	Location	Low HP (0.25 MPa)	High HP (2.5 MPa)
1	70	M	L4/5	Nucleus	–
2	41	F	L4/5	Nucleus	–
3	15	M	C5/6	Nucleus	Nucleus
4	29	M	L1/2	Nucleus	Nucleus
5	64	M	L4/5	Nucleus	–
6	44	M	L4/5	Nucleus	Nucleus
7	51	M	L4/5	–	Nucleus
8	36	M	L4/5	–	Nucleus
9	58	M	L3/4	Nucleus	–
10	–	F	L4/5	–	Nucleus
11	18	F	L1/L2	Nucleus	Nucleus
12	43	F	L5/S1	–	Nucleus
13	14	F	T6-L2	Nucleus	Nucleus
14	55	M	L5/S1	Nucleus	–
15	35	M	L4/5	Nucleus	–
16	60	M	L4/4	Nucleus	–
17	45	M	L4/5	Nucleus	–
18	41	M	L5/S1	Nucleus	–

The mean age $\pm$ SD of all donors was 39 ± 17

Fig. 1 Scheme of the stimulation device for the application of high hydrostatic pressure (2.5 MPa) and photo of a sterile bag with cell-seeded collagen gels

studying a possible influence of the applied mechanical loads on cell viability, 24 h after the end of mechanical stimulation by hydrostatic pressure each one cell loaded collagen gel from two loading experiments were transferred into microdishes with the highest optical quality bottom (Ibidi GmbH, Munich, Germany) and stained with fluorescein diacetate (120 nM) (FDA, Sigma-Aldrich, Germany) to visualize living cells and with propidium iodide (60 nM) (PI, Sigma-Aldrich, Germany) to stain dead cells as recently described [5]. After 10 min of incubation, both simulated cultures and controls were analyzed by a laser scanning microscopy at 488 and 543 nm excitation.

Analysis of gene expression

Cellular effects were examined as previously described [12]. Briefly, gels were dissolved enzymatically, cells were recovered, total RNA was prepared, measured and transcribed into cDNA. Specific primers were designed for aggrecan, collagen-I, collagen-II, MMP1, MMP2, MMP3 and MMP13 [12], using published gene sequences (NCBI Entrez search system), cloned and used as standards for real-time RT-PCR on the ICycler system (Biorad, Muenchen, Germany). The amount of the respective amplification product was determined relative to the house-keeping gene GAPDH in each subgroup. Gene expression of the stimulated subgroups was related to the associated control group.

Statistics

For both pressure magnitudes, statistical analysis was performed on ΔC_t values according to a modified method described by MacLean et al. [11] The average

C_t value of each duplicate measurement of each sample was normalized to the house-keeping gene GAPDH in each sample ($\Delta C_t = C_{t(\text{aggrecan})} - C_{t(\text{GAPDH})}$). The ΔC_t of each stimulated sample was related to the respective ΔC_t of each control sample. A Wilcoxon signed-rank test was performed to detect differences between the stimulated and associated control group (null hypothesis = 1.0 in case of no difference between both groups). A nonparametric Wilcoxon 2-sample test was performed in order to determine differences between stimulatory effects seen after low HP (0.25 MPa) and high HP (2.5 MPa). Multiple testing was considered by adjustment of the P value appropriately ($P = 0.007$ for the analysis of seven target genes).

Results

The results of this study showed that hydrostatic pressure influenced gene expression of nucleus cells from both human and bovine discs depending on the magnitude of the applied load (Fig. 2):

Both pressure magnitudes caused only small alterations of gene expression in the stimulated cultures compared to the respective unstimulated controls that were maintained at identical culture conditions as the respective loaded samples (each for the low and high pressure experimental setup) but without any application of mechanical stimulation. Though the total effects on gene expression were quite small, there was an opposite tendency in the effects of low (0.25 MPa) and high (2.5 MPa) hydrostatic pressure for the matrix forming proteins aggrecan and collagen type-I. Human nucleus cells from 14 donors tended to increase aggrecan and collagen type-I expression in response to 0.25 MPa and decreased mRNA expression of both target genes in response to 2.5 MPa ($n = 9$). These differences between low HP and high HP were significant for aggrecan ($P = 0.0028$) and collagen-I ($P = 0.0062$). Collagen type-II expression tended to be decreased in response to both pressure magnitudes and did not show significant differences in response to the two loading conditions.

Gene expression of the matrix turnover enzymes MMP1, MMP2, MMP3 and MMP13 was not influenced by the low-pressure magnitude of 0.25 MPa (Fig. 3). In response to the high-pressure magnitude of 2.5 MPa, MMP1, MMP3 and MMP13 expression tended to be increased with a stronger tendency for MMP3 expression ($P = 0.0128$). For all observed results there were quite big individual variations between nucleus cell responses of the different human donors. Therefore,

Fig. 2 Comparison of hydrostatic pressure effects on matrix protein expression of human nucleus cells induced by different pressure magnitudes. Relative changes of mRNA expression normalized to the endogenous control (GAPDH) of stimulated cell-collagen constructs (0.25 and 2.5 MPa) compared to the respective unstimulated controls ($n = 14$ for 0.25 MPa, $n = 9$ for 2.5 MPa) are shown. (*) indicates significant pressure-induced differences ($P < 0.007$, Wilcoxon signed-rank test) and (*) indicates significant differences between the two loading groups (Aggrecan: $P = 0.0028$, Collagen-I: $P = 0.0062$, nonparametric Wilcoxon 2-sample test)

we repeated some selected experiments with nucleus cells isolated from each six bovine caudal discs of four animals. With this smaller number of experiments, we could confirm the results of a decreased aggrecan ($P = 0.008$) and collagen type-II expression ($P = 0.023$) and an increased MMP3 expression ($P = 0.008$) in response to 2.5 MPa with strong tendencies (Fig. 4). No significant influence of low hydrostatic pressure could be found on gene expression of the matrix forming proteins collagen type-I, collagen

Fig. 4 Influence of high hydrostatic pressure (2.5 MPa) on gene expression of bovine nucleus cells. Relative changes of mRNA expression normalized to the endogenous control (GAPDH) of stimulated cell-collagen constructs (2.5 MPa) compared to the respective unstimulated controls from four bovine subjects are shown. (*) indicates pressure-induced differences with $P < 0.02$ (Aggrecan: $P = 0.008$, Collagen-II: $P = 0.023$, MMP3: $P = 0.008$, Wilcoxon signed-rank test)

type-II, aggrecan and the MMPs with bovine nucleus cells (not shown).

The investigation of cell viability could not show any influences of the applied mechanical load neither in response to the low nor to the high magnitude of hydrostatic pressure. In both stimulated cultures and unstimulated controls the percentage of dead cells was smaller than 5% (red fluorescence) and there was no difference between stimulated cultures and the respective unstimulated controls.

Fig. 3 Comparison of hydrostatic pressure effects on matrix metalloproteinase expression of nucleus cells induced by different pressure magnitudes. Relative changes of mRNA expression normalized to the endogenous control (GAPDH) of stimulated cell-collagen constructs (0.25 and 2.5 MPa) compared to the respective unstimulated controls ($n = 14$ for 0.25 MPa, $n = 9$ for 2.5 MPa) are shown

Discussion

Our present study supports the hypothesis that HP as one of the physiological stimuli of the IVD could influence matrix turnover by altering expression of genes for matrix forming and degrading proteins in a magnitude-dependent way. Low HP (0.25 MPa) had little influence on gene expression of human nucleus cells showing a tendency to increase aggrecan and collagen type-I expression but had no effect on gene expression of the MMPs examined. The effects of high hydrostatic pressure (2.5 MPa) on gene expression were also relatively small but were the opposite to those seen at low pressure: expression of the matrix forming proteins aggrecan, collagen type-I and collagen type-II tended to fall (at the level of significance for aggrecan) while expression of enzymes involved in

matrix turnover, MMP1, MMP3 and MMP13 tended to be stimulated. Similar responses were seen in bovine nucleus cells, which also showed a significant decrease of aggrecan and collagen type-II expression and a significant increase of MMP3 expression under high pressure. From these results it appears that hydrostatic pressure had similar affects on cells from different mammalian species; as it is one of the principal mechanical signals seen by the disc cells it could influence disc matrix turnover by regulating gene expression of matrix forming proteins and matrix turnover enzymes.

The low-pressure magnitude that we have chosen in this study corresponds to the lowest physiological load experienced, which occurs in the nucleus of IVDs during lying or sitting in a very relaxed position as shown by *in vivo* intradiscal pressure measurements [20]. The frequency of 0.1 Hz (ten-fold lower than the physiological walking frequency which is about 1 Hz) is low and may correspond to occasional movements loading the disc in these postures. At these low mechanical loads, disc matrix turnover might be in a steady-state with no or only little alterations of cellular activity; these low loads may support the maintenance of disc matrix. This could explain that no or only little influences on gene expression found in response to these low pressures. In contrast, the chosen magnitude of high HP (2.5 MPa) corresponds to a quite high physiological load, occurring when lifting a load with a round back [20]. These *in vitro* results, suggest that a repetition of this loading situation for 30 min at 0.1 Hz (180 loading cycles) might stimulate matrix turnover processes and decrease matrix formation via down-regulation of matrix forming and up-regulation of matrix degrading enzymes.

Differential effects of mechanical loading on expression of matrix forming and degrading enzymes, in agreement with the findings of the present study have also been found in other investigations, indicating that disc matrix turnover might be regulated in a magnitude and frequency dependent way: Handa et al. [8] observed for human IVD cells that physiologic levels of HP act as an anabolic factor for stimulation of proteoglycan synthesis whereas abnormal high (above 3 MPa) or abnormal low (below 0.1 MPa) pressure levels caused catabolic effects. Also in bovine discs hyper-physiologic pressure magnitudes inhibited proteoglycan biosynthesis [9]. Similar findings were obtained in studies with rodent disc models. MacLean et al. [11] have found in an *in vivo* rat-tail compression study an increase of anabolic and catabolic protein expression of nucleus cells in response to 1 MPa compression depending on the frequency of the ap-

plied stimulus. At low-pressure magnitudes (0.2 MPa) the authors observed no or only little effects on gene expression. Immobilization, however, also influenced gene expression significantly. Kasra et al. [10] found in a study with rabbit IVD cells that HP effects on collagen type-I expression of nucleus cells depended on both loading magnitude and frequency and also observed differences between annulus and nucleus cells. The findings of our recent study with human disc cells showed that applications of low magnitudes of HP had only small influence on gene expression and that the effects were quite low [12]. At the higher-pressure magnitude of 2.5 MPa the observed effects on human nucleus cells were more evident for some target genes and could be confirmed with bovine nucleus cells.

The relatively low effects of pressure on gene expression that we have found in the present study might be due to our sample processing procedure: the collagenase digestion step of the cell-collagen constructs before mRNA isolation might diminish differences between stimulated samples and controls, as gene expression in the cells could be further influenced during this 30 min incubation period. The variability of our results for human nucleus cells might be influenced by several factors. First of all, most human disc biopsies were obtained from tissue recovered during surgical removal of herniated discs. The tissue was separated intra-operatively into the annulus and nucleus region. As an exact separation is often very difficult, there might be heterogeneous cell populations. Findings in the literature indicate that annulus and nucleus respond differently to hydrostatic pressure [9]. Therefore, a mixture of both cell types with various portions of the respective cell type in the tested cell populations might lead to nonuniformity of the results. Moreover, different degrees of degeneration of the disc biopsy samples could also influence the high variations of human disc cells. Also the culture period to get enough cells for the experiments was not the same for all patients, as disc cells from the different donors varied in their mitogenic activity. All these findings could have an influence on the observed donor–donor variation in the human cell samples. The bovine disc cell culture system allows a better separation of the different zones of the disc. In this system, all subjects were in the same age group (18–24 months) and had no degenerative alterations of the disc tissue. For these reasons, the results with bovine disc cells showed a more consistent response.

From these results it can be assumed that also in the IVD—comparable to other connective tissues like bone [3, 6, 15]—a window of normal loading range might exist, within which the effects on gene expression

are quite low. However, loadings that are below or above this “steady state window” might regulate gene expression of IVD cells in an anabolic or catabolic manner, depending on the extent. Additional loading parameters, such as frequency may enhance these effects on gene expression. As isolated disc cells embedded in a collagen matrix (present study) and cells in their normal environment (= complete discs or explants) [8, 9, 11] respond similar to the load application, one might conclude that the effects are not or only partially mediated via cell-matrix interactions. Mechanotransduction might occur via rapid changes in ion-transport across cell membranes with the consequence of altered intracellular ionic composition as proven for chondrocytes [7]. These changes could stimulate gene expression or affect enzyme activity. Possibly, similar mechanisms that still have to be investigated also exist in disc cells. The observed differential responses of different genes as well as magnitude variations and donor variations underline the complexity of the cellular responses to mechanical signalling.

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