Hydrostatic pressure induces expression of interleukin 6 and tumour necrosis factor α mRNAs in a chondrocyte-like cell line

Kenji Takahashi, Toshikazu Kubo, Yuji Arai, Isao Kitajima, Masaharu Takigawa, Jiro Imanishi, Yasusuke Hirasawa

Abstract

Objective—To clarify the effect of pressure on the expressions of proteoglycan core protein and metabolism related cytokines in a chondrocyte-like cell line, HCS-2/8.

Methods—HCS-2/8 cells were exposed to 1, 5, 10, or 50 MPa of hydrostatic pressure (HP) for two hours, and mRNA expressions of interleukin 6 (IL6) and tumour necrosis factor α (TNFα) were examined by using reverse transcription-polymerase chain reaction (RT-PCR) method with specific primer sets; and mRNA of proteoglycan core protein, stromelysin, and tissue inhibitor of metalloproteinase 1 (TIMP1) were measured with northern blotting.

Results—HP exposure caused temporal morphological changes of the cells, but did not affect cellular viability. IL6 and TNFα mRNA expressions were not observed in the control cells under the atmospheric pressure, whereas in the cells treated with HP, pressure dependent enhancement of IL6 mRNA expression was observed between 30 minutes and four hours after the HP release. TNFα mRNA expression also increased 30 minutes after the exposure to 50 MPa of HP and disappeared four hours later. Proteoglycan core protein mRNA levels increased between 30 minutes and four hours after the exposure to 1 or 5 MPa of HP, whereas the levels decreased after 10 or 50 MPa of HP. Stromelysin and TIMP1 mRNA signals did not respond to HP.

Conclusions—HP at excessively high levels induced IL6 and TNFα expression and reduced the expression of proteoglycan core protein, while physiological levels of HP increased the expression of proteoglycan core protein. These findings are important when considering the pathology of osteoarthritis.

Articular cartilage is an important component of the joint, and it is always exposed to pressure produced by weight bearing and muscle contraction. Cartilage matrix mainly consists of proteoglycan and collagen, and this realises a cushioning effect of the matrix against mechanical stresses. However, if the mechanical stresses are increased to a level excessively higher than the physiological levels, cartilage matrix will be impaired and osteoarthritis (OA) could occur. This process of disease progression is confirmed by the fact that (a) clinical deformation in OA starts from the weight bearing area,' and (b) many OA related models are prepared by making mechanical changes on the joints. Chondrocytes play an important part in the metabolism and maintenance of cartilage matrix, therefore, it is quite important to elucidate how chondrocytes respond to pressure.

Recently, as a cause of onset and progression of OA, locally produced humoral factors, for example, cytokines and proteases, have been considered important, in addition to mechanical stresses. Among them, tumour necrosis factor α (TNFα) and interleukin 6 (IL6) were reported to be upregulated in cartilage of an experimental OA model or OA patients. TNFα promote connective tissue degradation through the inhibition of extracellular matrix synthesis, and induces expression of collagenase and stromelysin in chondrocytes, as well as interleukin 1β. IL6 plays a prominent part in the coordinated systemic host defence response to injury. IL6 production in chondrocytes markedly increases when IL1β, TNFα, or transforming growth factor β1(TGFβ1) are present; and IL6 induces tissue inhibitor of metalloproteinase1 (TIMP1).

However, to our knowledge, the pathology of OA has not been investigated in terms of the relation between pressure and cytokine expression. This prompted us to examine whether mechanical stress changed non-matrix chondrocyte expression, such as expression of OA related cytokines.

This study investigated the effect of pressure in mRNA expression of proteoglycan core protein, cytokines such as TNFα and IL6, which are implicated in OA, stromelysin, and TIMP1, by using a chondrocyte-like cell line, HCS-2/8, which can be subcultured and possesses characteristic features of normal chondrocytes.

Table 1 Cytokine specific oligonucleotide primers for PCR

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primer</th>
<th>Position</th>
<th>cDNA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6</td>
<td>S</td>
<td>242–261</td>
<td>819</td>
<td>15</td>
</tr>
<tr>
<td>AS</td>
<td>4535–4554</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>S</td>
<td>1521–1540</td>
<td>468</td>
<td>16</td>
</tr>
<tr>
<td>AS</td>
<td>2270–2289</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β actin</td>
<td>S</td>
<td>103–122</td>
<td>540</td>
<td>17</td>
</tr>
<tr>
<td>AS</td>
<td>619–642</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

S: sense. AS: antisense.
Methods

**CELL CULTURE**

HCS-2/8 cells were seeded in 60 mm plastic Petri dishes (Falcon Plastic Corp, Oxnard, CA), at a density of $2.0 \times 10^6$ cells/3 ml of Dulbecco’s modified Eagle’s medium (DMEM: Nissui Pharmaceutical Co, Tokyo, Japan) containing 10% fetal bovine serum (FBS: GIBCO BRL Co, Gaithersburg, MD), 60 µg/ml of kanamycin, and 0.292 mg/ml of L-glutamine (Wako Pure Chemical Industries Ltd, Osaka, Japan). The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. The medium was changed twice a week.

**PRESSURE APPLICATION**

The cells reached confluence after approximately one week of culture, and then they were exposed to hydrostatic pressure (HP) ranging from 1 MPa to 50 MPa as previously described. The Petri dishes were placed in a deformable Teflon pouch, which was filled with serum free DMEM containing the same concentrations of kanamycin and L-glutamine as mentioned above, and packed completely after removing air bubbles. The pouch then was placed in a stainless steel pressurisation vessel, exposed to 1, 5, 10, or 50 MPa of HP for two hours. After depressurisation, 3 ml of fresh serum free DMEM was given to each Petri dish, and the dish was maintained at atmospheric pressure in the air containing 5% CO₂, until analysis. Cells seeded in Petri dishes and placed in the same apparatus under the same conditions but not treated with HP exposure were used as non-pressurised controls.

**ANALYSIS OF CYTOKINES AND PROTEOGLYCAN CORE PROTEIN mRNA IN THE CONTROLS AND HP EXPOSED CELLS**

Total RNA was extracted from the controls and HP exposed cells between 30 minutes and eight hours after HP exposure by using the acid guanidinium thiocyanate-phenol chloroform method. The RNA preparations were analysed with a reverse transcription-polymerase chain reaction (RT-PCR) method for mRNA expressions of IL6 and TNFα; and with northern blotting for mRNA expressions of proteoglycan core protein, stromelysin and TIMP1. β-actin mRNA expression was examined by both RT-PCR and northern blotting, to ensure the quality of the RNA preparation and to prove the reliability of RT-PCR and northern blot protocols used in this study. RT-PCR assay was performed as previously described. Specific primer sets for IL6, TNFα, and β-actin were designed based on the published sequence data. Table 1 summarises their primer positions. Each of the four doses of total RNA (0.2, 1, 2.5, and 5 µg) was reverse transcribed with oligo (dT)16 primer by using MoMuLV RNase H-reverse transcriptase (GIBCO BRL Co, Gaithersburg, MD) in 10 µl reaction mixture under the conditions recommended by the manufacturer. Specific sequences of cytokine mRNAs were amplified by using specific primers for them and Taq DNA polymerase (Wako Pure Chemical Industries), as previously described. RT-PCR products

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Figure 1  Morphological changes of HCS-2/8 after exposure to HP. Phase contrast photomicrograms of cells cultured in DMEM containing 10% FBS after HP exposure. (A) Control cells under atmospheric pressure. (B) Thirty minutes after applying 50 MPa of HP. (C) Twenty four hours after applying 50 MPa of HP. (D) Thirty minutes after applying 5 MPa of HP. Bar = 100 µm.

Figure 2  Pressure dependent enhancement of IL6 mRNA expression. (A) IL6 RT-PCR products from the cells at 30 minutes (a) and (b) and four hours (c) and (d) after HP exposure. Two point five microgram (a) and (c) and 1 µg (b) and (d) of total RNA were reverse transcribed in 10 µl reaction mixture. (B) β-actin RT-PCR products from the cells at 30 minutes (a) and two hours (b), which confirm cDNA isolation. One microgram of total RNA was reverse transcribed in 10 µl reaction mixture. C: control cells treated under the atmospheric pressure.
were analysed in ethidium bromide containing agarose gels and evaluated for the expression of each cytokine’s mRNA.

Northern blotting was performed as described elsewhere. The total RNA (7 µg) was fractionated in a formaldehyde containing agarose gel, transferred to a nylon membrane, and prehybridised. The prehybridisation mixture was replaced with fresh solution containing 200 ng/ml of probes. Hybridisation was performed overnight at 65°C. Chemiluminescent detection by using DIG luminescent detection kits (Boehringer-Mannheim Biochemica, Mannheim, Germany) and Renaissance nucleic acid chemiluminescence reagent kit (DuPont NEN) was performed according to the manufacturer’s specifications. Kodak

Figure 3  Induction of TNFα mRNA expression after HP exposure. The cells at 30 minutes after the exposure to 50 MPa of HP expressed TNFα mRNA. Two point five microgram (a) and 1 µg (b) of total RNA were reverse transcribed in 10 µl reaction mixture. No TNFα mRNA expression was detected in the cells between four and eight hours after HP exposure. C: control cells treated under the atmospheric pressure.

Figure 4  Effect of HP exposure on proteoglycan core protein mRNA expression. Cells were exposed to 1, 5, 10 or 50 MPa of HP for two hours, and total RNA was extracted at 30 minutes (A) and four hours (B) after the release of HP. Equal amounts of total RNA were fractionated by gel electrophoresis, transferred to a nylon membrane, and hybridised to RNA probes. For quantification, the signals were measured by using a densitometer. A photograph of gel staining (right side) shows equal amounts of RNA were applied. β actin mRNA expression is also shown. C: control cells treated under the atmospheric pressure.
Results

In all duplicated or triplicated experiments for each test item, almost identical results were obtained. Figures are those of the representative case of the repeated experiments.

MORPHOLOGY OF HCS-2/8 CELLS AFTER HP EXPOSURE

After HP exposure, the cells were observed under a phase contrast light microscope. The HCS-2/8 cells have a polygonal shape under atmospheric pressure (fig 1A). This shape changed to almost spherical after the exposure to 50 MPa of HP for two hours (fig 1B). After the pressure was released, the cells regained their original polygonal shape within 24 hours (fig 1C). The cells that were exposed to lower magnitudes of HP (5 to about 10 MPa), demonstrated milder morphological changes (fig 1D), and returned to the polygonal shape soon after the pressure release. Exposure to 1 MPa of HP resulted in less morphological changes on the cells. Examination by using trypan blue dye exclusion assay showed that cellular viability was not affected by these HP levels.

EFFECTS OF HP ON CYTOKINE mRNA EXPRESSIONS IN HCS-2/8 CELLS

All PCR products having an expected size are evaluable in the agarose gel. mRNAs for β actin were clearly detected at a constant level in all samples. After the exposure to 1, 5, 10, or 50 MPa of HP for two hours, total RNAs were isolated from the cells and analysed identically by using PCR. IL6 mRNA and TNFα mRNA expressions were not observed in the control cells treated under the atmospheric pressure. In the PCR assays using various RNA concentrations, pressure dependent enhancement of IL6 mRNA expression on the cells was observed between 30 minutes and four hours after HP exposure (fig 2). No IL6 mRNA expression was observed eight hours after HP. TNFα mRNA expression was also increased at 30 minutes after the exposure to 50 MPa of HP and disappeared four hours after the release of HP (fig 3).

Northern blotting was performed to analyse the expressions of proteoglycan core protein, stromelysin, and TIMP1 in the cells exposed to 1, 5, 10, or 50 MPa of HP. Expression of β actin mRNA was found at an almost constant level in all samples. Proteoglycan core protein was detected in the cells grown under the atmospheric pressure, and its mRNA levels were increased 30 minutes and four hours after the exposure to 1 MPa or 5 MPa of HP. In particular, the signal level for this mRNA in the cells exposed to 1 MPa was increased sixfold four hours after the release of HP. On the other hand, in the cells exposed to 10 MPa or 50 MPa of HP, proteoglycan core protein mRNA levels decreased 30 minutes and four hours after the release of HP (fig 4). At eight hours, the mRNA levels returned to almost the same as those under atmospheric pressure. Stromelysin and TIMP1 mRNA signals did not show major response to HP during eight hours after the release of HP (fig 5).

Discussion

The effect of some types of pressure, for example, tension,20 shear force,4 and HP,21–23 has been studied using tissue sections21–22 and cultured cells.23 HP is considered to have the most influential effect on the metabolism of cartilage.24 A recent study25 demonstrated that chondrocytes and extracellular matrix in different zones of a tissue react differently to mechanical loading. Although tissue culture better represents in vivo conditions, direct compressive force given to cartilage tissue will result in such phenomena as deformation of tissues and cells,26 effusion of tissue fluid,27 and increase of proteoglycan levels followed by the increase of cation.28 These factors will affect experimental results, and make the analysis of pressure effect quite difficult. Therefore, we used cultured cells, instead of tissue sections, in the investigations of the effect of HP on the expression of metabolism related cytokines.

HCS-2/8 is a chondrosarcoma cell line, established in 1989 by Takigawa et al.19 HCS-2/8 produces Type II, IX, and XI collagens, as well as cartilage type proteoglycan (aggrecan), as seen in normal chondrocytes. In addition, the reaction of HCS-2/8 to various vitamins and growth factors is similar to those of normal chondrocytes.11 This cell line can maintain these characteristics of normal chondrocytes for a long culture period, and can be a useful model of human chondrocytes. Our study, for the first time, demonstrated that HP can induce expression of IL6 and TNFα mRNAs in the cultured cells that possess characteristic features of normal chondrocytes. In the measurements using RT-PCR, IL6 and TNFα mRNA expressions in the control cells without giving exposure to HP were all negative, while the expressions became positive after exposure to HP regardless of the RNA concentrations and this change was reproducible. Therefore, our RT-PCR findings were evaluated to be valid.

During walking, pressure on a human hip joint ranges between 3 MPa and 10 MPa.25 It
Pressure effects on chondrocyte-like cells

...and the level varies continuously. In a pathological condition, for example, OA, much higher HP could occur. We previously reported that constant 50 MPa of HP induced heat shock protein in HCS-2/8, the expression of which is increased in OA cartilage, and this magnitude of HP adversely affects the metabolism of these cells. Therefore, constant 50 MPa was considered to represent an abnormally high and pathological level of stimulus. IL6 and TNFα have been considered as cytokines that are induced by inflammation or immune reactions. In this study, these cytokines were also induced after exposure to a unphysiologically high pressure level, 50 MPa. This indicates that IL6 and TNFα, which are increased in OA chondrocytes, can be induced by unphysiologically high, excessive pressure. This is important when considering the mechanism of OA development because of an extremely high pressure level.

In this study, cell morphology changed to spherical after giving 50 MPa, while this change was mild under low pressure levels. On the other hand, IL6 mRNA expression was most noticeable at 50 MPa. Our findings on morphological changes after applying HP agree with those of previous studies, and show cytoskeletons, such as actin filament, changed under the HP. Mohtai et al reported that 1.6 Pa of fluid-induced shear induced the expression of IL6 mRNA and IL6 protein, and chondrocytes exposed to fluid induced shear for 48 hours appeared to be elongated and elliptical and their major axes aligned primarily circumferentially. On the other hand, cytoskeletons, for example, actin filament, are believed to be involved in the mRNA expression and play quite an important part in the local expression and function of mRNA.

These findings suggest that changes in actin filament could be involved closely with IL6 induction by mechanical stress. In the same way, the changes in actin filament could also be involved in TNFα induction under 50 MPa of HP. Mechanotransduction of cells has been reported by many researchers, and it is thought that ion channels take an important part in the initial response to mechanical stress, and changes in intracellular calcium ion concentrations and cAMP production are largely involved in the regulation of gene expression caused by mechanical stress. It has been hypothesized that mechanical stress is transferred from extracellular matrix to nuclei via cytoskeleton, which is connected to integrins on cellular surface. In OA cartilage tissues, the number of chondrocytes that are in the dynamic process of polymerisation and de-polymerisation of cytoskeleton elements, is reported to be increased. Buschmann et al demonstrated the importance of cell matrix interaction in the cellular reaction against pressure by using the primary chondrocyte/agarose culture. HCS-2/8 in plate culture and at a confluent level is reported to express integrin and to generate large volume of chondrocyte-like matrix, and this matrix is accumulated around the cell. In this study, we loaded HP to HCS-2/8 at a confluent level, but this cell at a sparse or subconfluent level reacted less apparently to HP (unpublished data). Therefore, we consider that integrin and extracellular matrix may also take an important place in the present findings.

Cytoskeleton may also be involved in the regulation of proteoglycan synthesis in chondrocytes. We previously reported that 1 or 5 MPa of HP induced an increase of S sulphate uptake into chondrocyte-like cells, and 10 or 50 MPa of HP induced a decrease. In this study, expression of proteoglycan core protein mRNA increased after giving 1 or 5 MPa of HP, and it decreased after giving 10 or 50 MPa. This change in the expression volume caused by HP was larger than the changes in S sulphate uptake. On this point, CS-Szabo et al reported that changes in the levels of proteoglycan core protein mRNA were several fold greater than expected. Therefore, our findings on proteoglycan core protein mRNA are thought to be consistent to the previous findings, and as shown in another study, this shows that HP affects extracellular matrix production on transcriptional level. TNFα is reported to induce IL6, but in the present study, expression of TNFα mRNA occurred transiently immediately after the exposure to HP. Therefore, we cannot conclude whether TNFα directly induced IL6. Our chronological findings also suggest that these cytokines would not affect proteoglycan expression.

In this study, HP did not affect the expression of stromelysin and TIMP1 mRNAs, and this was in contrast with our expectation. In OA, imbalance between proteases and inhibitors is thought to be implicated, but constant HP for two hours did not influence the imbalance at transcriptional level. We also reported that HP increased the expression of TGFβ1, which can induce TIMP1, and shear stress to chondrocytes is reported to increase TIMP1 mRNA expression. TNFα increases stromelysin expression, and IL6 increases TIMP1 expression. The difference between our findings and expectation could be attributable to the constant HP used in this study, and to the monitoring of mRNAs after the exposure to HP. We applied constant pressure instead of cyclic pressure, because our purpose was to isolate the specific regulatory signals after giving abnormal pressure. Because Smith et al reported that cells react to cyclic pressure more noticeably than to constant pressure, future studies using cyclic HP and that monitor gene and protein expressions would be of interest. In addition, HCS-2/8 is not only a chondrocyte-like cell line but also a transformed cell line, and transformed cell lines are known to be resistant to HP in comparison to primary cultures. To better understand our findings, mRNA studies should be repeated in primary chondrocyte cultures. Monitoring the IL6 and TNF protein productions in addition to mRNA, and comparison between IL6 induction by HP and that by such an inflammatory cytokine as IL1, merit future studies.
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