Morphological, immunocytochemical and biochemical studies in human osteoarthritic chondrocytes exposed to IL-1β and cyclical hydrostatic pressure

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Abstract

Objective

To investigate the in vitro *effects of cyclic hydrostatic pressure (HP), of a magnitude and frequency close to those that presumably exist in articular cartilage, on human osteoarthritic chondrocytes cultivated for 48 hrs in the presence or absence of interleukin-1β (IL-1β).*

Methods

Pressurization cycles in the form of sinusoidal waves (minimum pressure 1 MPa, maximum pressure 5 MPa) at a frequency of 0.25 Hz for 3h were assessed on cultured chondrocytes obtained from the femoral heads of osteoarthritic patients. Under these conditions, we evaluated proteoglycan (PG) levels and nitrites production in the culture medium by the immunoenzymatic method and examined the morphology of chondrocytes by transmission electron microscopy (TEM). Moreover, immunocytochemical investigations were performed to localize inducible nitric oxide synthase (iNOS).

Results

The presence of IL-1β led to a very significant decrease in PG levels and to an increase in NO production. When the chondrocytes were cultured in the presence of HP, a statistically significant restoration of PG levels was observed, but pressurization did not significantly increase the PG levels in cells damaged by IL-1β. After pressurization, there was a slight decrease in the concentration of NO under basal conditions and a statistically significant decrease in the IL-1β induced release of NO. The results concerning metabolic production were further confirmed by the morphological findings obtained by TEM and immunocytochemical studies.

Conclusion

This study confirms the protective role of HP which stimulates PG production and counteracts IL-1β induced NO release. These data are supported by morphological and immunocytochemical findings.

Key words

Chondrocytes, hydrostatic cyclical pressure, interleukin-1β, nitric oxide, osteoarthritis.

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Introduction

Osteoarthritis (OA) is one of the most common rheumatic diseases directly involving the articular cartilage. The structural breakdown of proteoglycans (PG) and collagen of the cartilage during OA is believed to be the result of increased catabolic activity by chondrocytes (1). Interleukin 1 (IL-1) β is one of the cytokines involved in the degradation processes of cartilage; in response to IL-1β, chondrocytes secrete neutral metalloproteinases (MMPs) and nitric oxide (NO) (1, 2).

NO is a highly reactive cytotoxic free radical that has been implicated in tissue injury in a variety of diseases (3). NO promotes numerous effects in chondrocytes: inhibition of PG and collagen synthesis (4); activation of MMPs (5); induction of apoptosis (6); reduction of IL-1 R antagonist (7) and reduction of transforming growth factor beta (8). Several *in vitro* studies have shown the importance of mechanical compression or hydrostatic pressure (HP) as a modulator of cartilage metabolism. The biosynthetic response of chondrocytes to HP *in vitro* varies with the magnitude, frequency and duration of loading (9-15).

In this study, we investigated the *in vitro* effects of cyclic HP of a magnitude and frequency close to those presumably existing in articular cartilage (16, 17) on human OA chondrocytes cultivated in the presence or absence of IL-1β during 48 hrs of culture. Under these conditions, we evaluated the PG levels and NO production in the culture medium and the morphology of chondrocytes by transmission electron microscope (TEM). Immunocytochemical investigations were also performed to localize inducible nitric oxide synthase (iNOS).

Methods

Cell culture

Articular cartilage was obtained from the femoral heads of 6 patients with OA defined by ACR criteria (18) who were undergoing surgery for total hip prostheses. The mean age of the patients was 65 years (range 62-70). Written consent was obtained from each participant in the study.

Macroscopically, the cartilage was not altered, but a histological study of representative samples showed typical osteoarthritic changes such as the presence of chondrocyte clusters, loss of methachromasia and fibrillation. Immediately after surgery the cartilage specimens were cut aseptically, minced into 2 mm^2 pieces and sequentially digested by clostridial collagenase (SIGMA, Italy), 1 mg/ml in phosphate buffered saline (PBS) (in mM: NaCl 140, KCl 2.7, NaH_2PO_4 8.1, K_2HPO_4 1.5, pH 7.4) containing 200 U/ml of penicillin, 200 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B. Collagenase digestion was carried out at 37°C for 18 hrs with moderate stirring. The chondrocytes obtained after collagenase digestion were rinsed twice in A saline solution (in mM: 10 HEPES, 140 NaCl, 5 KCl, 5 Glucose, pH 7.4) and centrifuged for 10 min at 700 g. As shown by the Trypan blue viability test, 90-95% of the recovered cells were alive.

Cells were cultured in 24-well microplates at a density of 5 x 10^5 cells/well and overlaid with 1 ml of medium containing 10% foetal calf serum (FCS), 200 U/ml penicillin, 200 μg/ml streptomycin and 2 mM glutamine in Dulbecco's minimum essential medium (DMEM). Cells were maintained in an atmosphere of 5% CO_2 in air at 37°C for 48 hrs and immediately utilised for the study.

Pressurization system

The pressurization system that we use has already been described in detail (19). In this study, the chondrocytes covered in culture medium were pressurized by applying sinusoidal waves with a minimum pressure of 1 MPa, a maximum pressure of 5 MPa, and a frequency of 0.25 Hz for 3 hrs. Dishes cultivated in the loading chamber, but without undergoing pressurization served as controls. After pressurization, the culture medium was collected and stored at –70°C for PG and NO determination and the chondrocytes were immediately fixed for TEM and processed for immunocytochemistry.

Biochemical assays

The quantity of PG in the culture medium was measured by the immunoenzymatic method (20) on microplates

for the quantitative measurement of human PG (Medgenix Diagnostics, Belgium). The microplate was read at 450 nm and the quantity of substratum was determined colorimetrically by measuring the absorbance, which is proportional to the PG concentration. The assay sensitivity was 0.9 ng/ml. The DNA content in the culture was analysed according to Labarca and Paigen's fluorimetric method (21).

Nitrite assay

The quantity of nitrites in the culture medium was measured by the Griess method (22). Equal volumes (100 µl) of supernatant and Griess reagent (1% sulfanilamide, 0.1% N-1 naphthylethylenediamide dihydrochloride in 5% H_3PO_4) were incubated on microplates at room temperature for 15 min. Absorbance was measured with a spectrophotometer at 550 nm. The concentration of nitrites was calculated using a standard curve made by successive dilutions of a solution of sodium nitrite in water.

TEM

Cultures of human OA chondrocytes were fixed for 2 hrs at 4°C in cold Karnovsky fixative, rinsed overnight in 0.1 M pH 7.2 cacodylate buffer and post-fixed for 1 hr at 4°C in 1% buffered OsO_4, dehydrated in a graded series of ethanol, and embedded in Epon-Araldite. Ultra-thin sections cut with an LKB III ultramicrotome were collected on copper grids, stained with uranyl acetate and lead citrate and then photographed using a Philips CM10 electron microscope. We examined at least 100 cells from each group.

Immunocytochemistry

OA chondrocytes that had or had not been incubated with IL-1β were mechanically detached from Petri dishes, washed twice in PBS (4°C) and centrifuged at 700 g for 10 min at 4°C. The pellets were resuspended in 1 ml cold PBS. Cells were smeared on glasses slides, air dried, and fixed for 5 min in acetone at 4°C. The specimens were rehydrated in PBS, and treated with 1.5% normal horse serum (NHS) for 20 min in a humid chamber to avoid non-specific staining. Slides were incubated with avidin (15 min R/T) and biotin (15 min R/T) in a humid chamber (Blocking kit, Vector Laboratories, Burlingame, CA). After washing several times in PBS, chondrocytes were incubated in a murine monoclonal antibody against human iNOS (Santa Cruz Biotechnology, Santa Cruz, CA) used at a concentration of 1.2 µg/ml in PBS for 30 min. After thorough rinsing in PBS, slides were incubated for 1 hr at R/T in biotin-conjugated rabbit anti-mouse IgG used at a concentration of 0.5% in 1% NHS. The slides were immersed in a solution of 0.3% H_2O_2 in methanol for 10 min to block endogenous peroxidases. After rinsing, the chondrocytes were treated with avidin-biotin peroxidase complex for 30 min at R/T. iNOS was detected by incubation with 2% diaminobenzidine (DAB) in PBS for 5 min. Ematossiline was used to stain the cells.

Controls for secondary antibodies were carried out by omitting the primary antibodies.

Slides were examined under a Leitz Aristoplan microscope with Olympus BH-2 optics. The same procedures were carried out on the cultivated OA chondrocytes exposed to cyclical HP. At least 100 chondrocytes from each group were evaluated.

Morphometric and statistical analysis

The data were expressed as the mean ± SD of PG released into the culture medium per microgram of DNA and NO (ng/10^6 cells) released into the culture medium in the 6 tested cultures. The Mann-Whitney U test was used for the statistical analysis; $p < 0.05$ was considered significant.

For the morphometric studies, we analyzed sections of three different blocks from each group. For the standardization and comparison of the different groups, only medially sectioned chondrocytes were investigated; 100 chondrocytes were selected using the nucleous/cytoplasm ratio as the selection criterion. Our analysis was based on an established method for ultrastructural quantitative evaluation of changes in chondrocyte function (23). Mitochondria and Golgi bodies were counted and recorded as the mean ± SD. Because the distribution was not normal in all cases, statistical analysis for differences between groups was performed using distribution-free non-parametric tests. Morphological para-meters were evaluated by the Mann-Whitney U test. Values of $p < 0.05$ were considered significantly different.

Immunocytochemistry staining intensity was scored by the same researcher as either absent or from limited to intense (14, 15). In each group, the scores were expressed as percentages of the total number of cells studied.

Results

The total PG concentration in the culture medium during the 48 hrs under baseline conditions and in the presence of IL-1β at a concentration of 5 ng/ml with or without HP was evaluated. The presence of IL-1β resulted in a very significant decrease ($p < 0.001$) in PG levels.

In the presence of HP, there was a highly significant increase ($p < 0.001$) in the level of PG in the culture medium under basal conditions, while pressurization did not significantly increase the PG levels in the cells damaged by IL-1β.

NO (ng/10^6 cells) production in the culture medium under baseline conditions in the presence of IL-1β with or without pressurization was also evaluated. After pressurization there was a slight, but not significant decrease in the concentration of NO under basal conditions.

The presence of IL-1β was associated with a very significant increase ($p < 0.001$) in NO production, but when the cells were cultured in the presence of IL-1β and HP, a statistically significant decrease ($p < 0.05$) in NO production was observed.

These results were confirmed by the morphological findings obtained by TEM, which indicated the effects of IL-1β and HP on the metabolic functions of the chondrocytes. Figure 1a shows cultured OA chondrocytes under baseline conditions: the nucleus appears euchromatic and the cytoplasm contains rough endoplasmic reticulum, Golgi bodies, and mitochondria. An OA chondrocyte cultured under baseline conditions in the presence of IL-1β shows (Fig. 1b) several vacuoles in the cytoplasm and a reduced number of mitochondria and Golgi bodies (Table I).

HP partially restored many of the characteristic cytoplasmic structures in the chondrocytes under baseline conditions (Fig. 1c): the nucleus is euchromatic, the cytoplasm contains a reduced number of vacuoles and rough endoplasmic reticulum, and lipid droplets and mitochondria are present but limited to a part of cytoplasm that also contains a number of vacuoles.

The number of mitochondria after pressurization increased significantly ($p < 0.01$) (Table I).

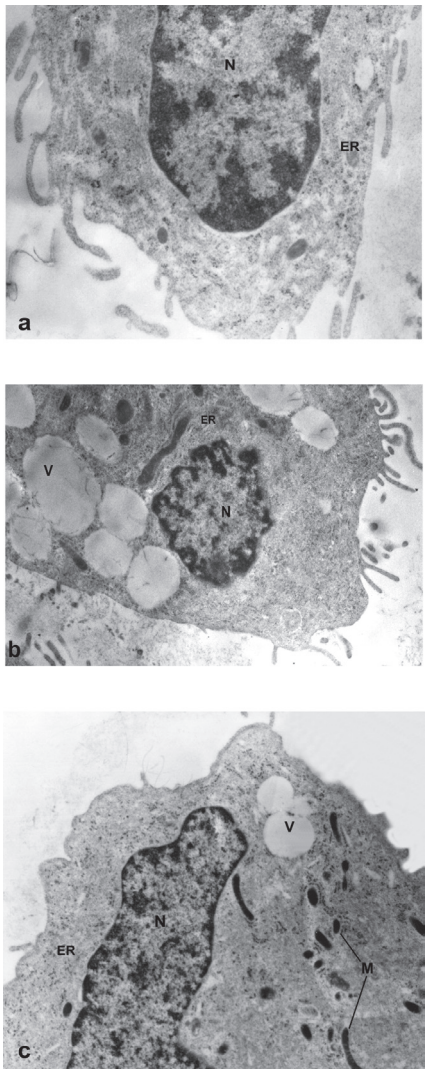

Fig. 1. TEM sections of cultured OA chondrocytes. (**a**) Baseline conditions. The cell shows an euchromatic nucleus (**N**) and abundant cytoplasm in the rough endoplasmic reticulum (**ER**). (**b**) With IL-1β. The cell presents a highly vacuolate cytoplasm (**V**), nucleus (**N**) and rough endoplasmic reticulum (**ER**). (**c**) With IL-1β + HP the cells recover their normal status. The cytoplasm contains rough endoplasmic reticulum (**ER**); nucleus (**N**), mitochondria (**M**), and vacuole (**V**) (all images x10,000).

Table I. Number of organelles in OA chondrocytes under basal conditions and after pressurization.

	Basal conditions		After pressurization	
	OA	OA + IL1β	OA	OA + IL1β
Mitochondria (no.)	3.1 ± 1.5	2.7 ± 0.8	6.5 ± 2.3*	4.0 ± 1.7
Golgi bodies (no.)	1.9 ± 1.2	0.9 ± 2.5	2.5 ± 1.6	1.8 ± 2.1

*$p < 0.01$ (Mann-Whitney test) OA pressure versus OA basal conditions.

In Figure 2, cytochemical examination of chondrocytes showed that the distribution of iNOS had a clear localization of the signal inside the cytoplasm, and the label was intense in 70% of the OA cells under baseline conditions (Fig. 2a). In OA cells treated with IL-1β, the signal appeared to be strongest in the cytoplasm in 85% of the examined chondrocytes (Fig. 2b).

After pressurization, the localization of iNOS in OA chondrocytes (Fig. 2c) was reduced to 58% whereas in the presence of IL-1β the percentage of cells showing an intense localization (73%) was the same as that in OA cells under baseline conditions (70%). These values are reported in Table II.

Discussion

Articular cartilage tissue is constantly subject to loading, and HP is one of the many factors that operate in the articular cartilage subjected to loading (9-11). In our study, the applied pressure was within the physiological range of the human joint. In fact, pressure levels of 5 MPa are most often encountered in the knee joint during normal walking (17). We applied HP for as short a time as possible (3 hrs) to approximate physiological conditions.

The chondrocytes subjected to HP by us presented greater metabolic activity, which was expressed by a very significant increase in PG levels in the culture medium under basal conditions. Increased anabolic activity was confirmed by TEM analysis; chondrocytes presented a significant improvement in cellular features, confirming this shift towards more anabolic activity.

In our opinion, the increase in PG in the culture medium, which was also observed under basal conditions, could be caused by a stimulation of cellular activity induced by cyclical HP, as demonstrated by other authors (10, 13, 24). HP appears to modulate aggrecan biosynthesis through membrane-mediated pathways, such as the transport of cations, aminoacids and macromolecules. It has also been suggested that HP may alter the action of the membrane Na+/K+ pump, thus altering intracellular K+ concentrations (17).

Furthermore, cyclic AMP (cAMP), which has been identified as an important mediator of PG synthesis and cartilage growth, seems to play a direct role in the mechanical stimulation of matrix biosynthesis. Constant HP that inhibits PG synthesis also inhibits cAMP accumulation through a calcium-mediated process (25). Conversely, intermittent HP in chondrocyte cell culture systems results in concurrent increases in both the cAMP and PG synthesis rates (26).

The stimulating effect of physiological HP reported here is in agreement with that found by other authors who also utilized human osteoarthritic cartilage (27, 28). Some studies have shown that OA chondrocytes are more sensitive to physiological HP than normal chondrocytes (14, 15, 27). In our study, however, the stimulating effect of pressure was not enough to counterbalance the negative effect created by the addition of IL-1β, which does indeed induce a serious metabolic and morphological imbalance. Recently Fan *et al.* (29) demonstrated that OA chondrocytes are less responsive to IL-1 in comparison to normal chondrocytes. However our data only focused on OA chondrocytes. Some studies have shown that NO is a potent mediator of cartilage damage in OA (1, 4-8).

Here we have demonstrated that, as reported previously (30, 31), IL-1β significantly increases the production of NO. When cells were cultivated under pressure, reduced production

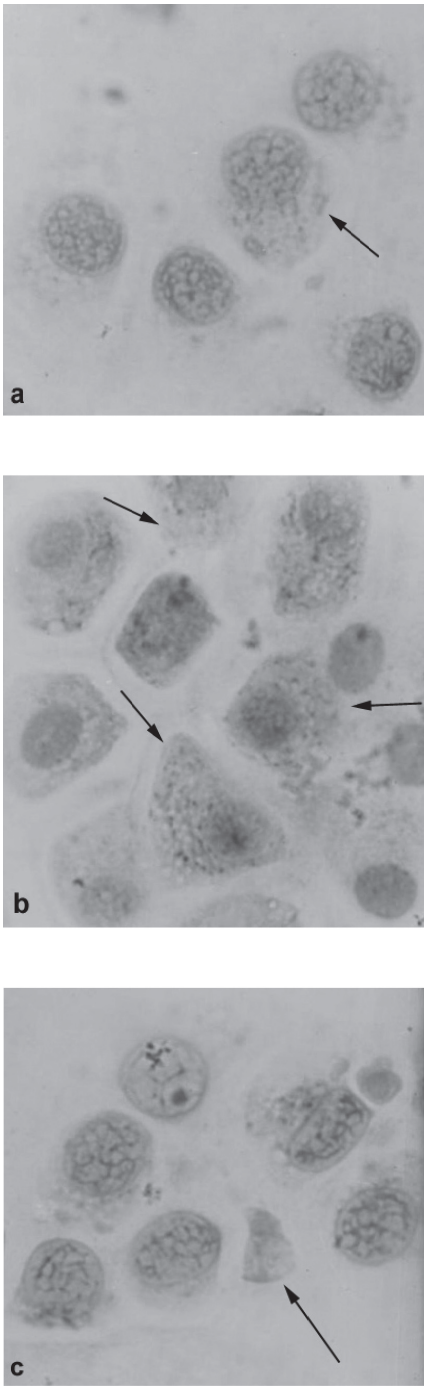

Fig. 2. Micrographs of cultured OA chondrocytes labelled with iNOS antibody. (**a**) Baseline conditions; (**b**) with IL-1β; (**c**) with IL-1β + HP. The cells show a different immunolabelling pattern (**arrows**) (all images x1,000).

was recorded under basal conditions; moreover, physiological HP significantly decreased Il-1β-induced NO production. These data were confirmed by immunocytochemical studies of the expression of iNOS. Our results confirm the protective effect of cyclical HP on the release of NO by chondrocytes when stimulated by various factors (32, 33).

Table II. Percentage of OA chondrocytes showing the iNOS signal after immunocytochemistry under different experimental conditions (14).

	Basal conditions		After pressurization	
Immunolocalization	OA	OA + IL-1β	OA	OA + IL-1β
Absent	10%	2%	14%	7%
Limited	20%	13%	28%	20%
Intense	70%	85%	58%	73%

In conclusion, our study confirms the importance of HP in chondrocyte metabolism. The use of isolated chondrocytes in the study of the metabolic response to loading must be adopted with caution because of the important role of the natural extracellular matrix in signal transduction and in transmitting the external load to the cell and intracellular components. However, chondrocyte cultures are the most suitable experimental models for understanding both the etiopathogenetic mechanisms of OA and which type of physical activity may be best suited for the prevention and treatment of OA. Furthermore, the present results confirm that mechanical stimulation may be used for *in vitro* and *in vivo* approaches to cartilage engineering.

More sophisticated experiments will be necessary to analyse all the effects of pressure on chondrocyte metabolism to clarify its mechanisms of action and the role of mechanical factors on the etiopathogenesis of OA.

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