Influence of polysulphated polysaccharides and hydrocortisone on the extracellular matrix metabolism of human articular chondrocytes *in vitro*

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Abstract

Objective

To evaluate the influence of hydrocortisone and two polysulphated polysaccharides (xylosan polysulphate and chondroitin polysulphate) on the extracellular matrix metabolism of chondrocytes cultured in gelled agarose.

Methods

Isolated chondrocytes from normal femoral cartilage of the knee joints of 7 donors were cultured in gelled agarose to maintain their differentiated phenotype. After two weeks of culture, hydrocortisone (0.2 µg/ml), xylosan polysulphate (10 µg/ml) and chondroitin polysulphate (10 µg/ml) were added to the culture media supplemented with or without interleukin (IL)-1β. After one week of incubation, the cells were liberated from the agarose with agarase. Isolated cells were labelled with antibodies against aggrecan and type II collagen, as well as biotinylated hyaluronic acid binding protein to analyse the extracellular matrix (ECM) molecules in the cell-associated matrix (CAM). The levels of matrix metalloproteinase (MMP)-1, -3, and -13, as well as tissue inhibitor of metalloproteinase (TIMP)-1 and -3 were determined after the cells had been permeabilised and stained with the appropriate antibodies. Triplicate samples were analysed with flow cytometry.

Results

IL-1β decreased the accumulation of aggrecan, hyaluronan and type II collagen in the CAM and increased intracellular MMP-1, -3 and -13 at a concentration of 100 pg/ml. Xylosan polysulphate and chondroitin polysulphate restored the expression of these CAM molecules in these IL-1β-treated cultures. Hydrocortisone stimulated the accumulation of CAM aggrecan and hyaluronan whether or not under the exposure to IL-1β. Intracellular MMP-1, -3, -13 and TIMP-1 and -3 of IL-1β-treated cells was downregulated after treatment with hydrocortisone.

Conclusion

Both hydrocortisone and the two polysulphated polysaccharides could stimulate the accumulation of CAM macromolecules of IL-1β-treated chondrocytes. This effect probably resulted in part from the downregulation of MMPs. These agents showed cartilage structure modifying effects in vitro.

Key words

Chondrocytes, extracellular matrix, polysulphated polysaccharide, hydrocortisone, IL-1β, flow cytometry.

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Abbreviations: CAM: cell-associated matrix; CPS: chondroitin polysulphate; ECM: extracellular matrix; HA: hyaluronan; IL: interleukin; MFI: mean fluorescence intensity; MMP: matrix metalloproteinase; OA: osteoarthritis; TIMP: tissue inhibitor of metalloproteinase; XPS: xylosan polysulphate.

Introduction

Hyaline articular cartilage is a viscoelastic tissue covering the bony ends of synovial joints and cushioning subchondral bone (1). Osteoarthritis (OA) is a disease characterised by cartilage destruction. A damaged articular cartilage can affect the normal function of the joint and this ultimately results in clinical symptoms in a proportion of the patients (2). Current treatments with analgesic and anti-inflammatory drugs are partially effective in relieving the symptoms, e.g. pain and stiffness, of OA, but are generally not thought to slow down the process of cartilage destruction (3). Structure/Disease Modifying OA Drugs (DMOADs) are expected to prevent structural damage in normal joints at risk for development of OA, or to retard the progression of structural damage in joints already affected by OA (4).

The fundamental event resulting in the destruction of articular cartilage in OA arises from an imbalance in anabolic and catabolic pathways in this tissue. Matrix metalloproteinases (MMPs) are a group of enzymes involved in the degradation of cartilage matrix (5, 6). Amongst them, MMP-1 (collagenase-1) and MMP-3 (stromelysin-1) have been studied extensively and have been shown to be capable of degrading collagen and aggrecan. Recently, the pivotal role of MMP-13 (collagenase-3) in the degradation of type II collagen has been emphasised (7, 8). Activity of MMPs can be controlled by their endogenous inhibitors, such as tissue inhibitors of metalloproteinase (TIMPs) (9). TIMP-1 and TIMP-3 have been found in cartilage tissue. Normally, the levels of MMPs and TIMPs are in equilibrium to achieve a well-controlled turnover of the extracellular matrix (ECM). In OA cartilage, there is an imbalance between the synthesis of TIMPs and MMPs, favouring an increased active MMPs and matrix degradation (10-17). Inhibition of the MMPs appears to be a target for drug development in the treatment of OA (18, 19).

Glucocorticoids are widely used in rheumatic diseases to alleviate inflammatory symptoms. Their effect on cartilage matrix metabolism remains controversial. Whereas several literature reports pointed out the negative effect of glucocorticoids on the cartilage matrix synthesis (20-22), a number of studies showed the chondroprotective function of this type of agents (23-26). The polysulphated polysaccharides, such as xylosan polysulphate (XPS) and chondroitin polysulphate (CPS) have been shown to possess pharmacological activities, which could stimulate cartilage matrix synthesis (27-30). In this pilot study, we used flow cytometry to assess the influence of these agents on cell-associated matrix (CAM) metabolism of chondrocytes cultured in gelled agarose, with the focus on their regulatory function of the MMP/TIMP system.

Materials and methods

Isolation of chondrocytes

Human articular chondrocytes were isolated as described elsewhere (31, 32), with a few modifications (33). Articular cartilage was obtained at autopsy from seven donors (D1-7: 4 males, 3 females; aged between 31 and 75 years) within 24 hours post mortem. They had died after a short illness and had not been receiving corticosteroids or cytostatic drugs.

Visually intact cartilage was sampled from the femoral condyles, diced in small fragments and digested in a spinner bottle with a series of enzymatic solutions in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Island, NY) with 0.002 M/ml L-glutamine, antibiotics and antimycotics (GIBCO BRL). Cartilage was first treated with 0.25% (w/v) of sheep testes hyaluronidase (Sigma, St. Louis, MO, USA) for 120 min and 0.25% of Pronase (Streptomyces griseus Pronase E; Sigma) for 90 min at 37°C. After an overnight period in DMEM supplemented with 10% foetal calf serum (FCS, GIBCO BRL), a 3 – 6 hr period of incubation with 0.25% collagenase (Clostridium histolyticum; Sigma) in DMEM containing 10% FCS at 37°C resulted in the liberation of isolated cartilage cells. Usually, 150×10^6 chondrocytes could be obtained from femoral condyles of one individual and

over 95% of the cells were viable (Trypan Blue exclusion test) after isolation.

Chondrocytes in agarose culture

Chondrocytes were cultured in gelled agarose as previously described (34) with some modifications (33, 35). Chondrocyte suspension cultures were established in 1.5% agarose (ultralow gelling temperature agarose, Sigma) in 3.8 ml cryotubes (Nunc). Coated cryotubes were filled with 300 µl of chondrocytes/agarose suspension and kept at 4-8°C for 15 min to allow the agarose to gel. The final cell density was approximately 1.0×10^6 chondrocytes per culture. The culture tubes were filled with 3 ml DMEM containing 10% FCS and 50 µg/ml of freshly dissolved ascorbate, and placed in an incubator at 37°C under 5% CO_2.

Treatment with the agents

Chondroitin polysulphate (Arteparon®) was obtained from Luitpold Werk (Munich, Germany). Xylosan polysulfate (Cartrophen®) was a gift from Arthropharm (Bondi Junction, Australia). Hydrocortisone (Solucortef®) was from Upjohn (Brussels, Belgium). Interleukin-1β (recombinant human IL-1β) was purchased from R&D Systems (Abingdon, UK). After two weeks of culture, hydrocortisone (0.2µg/ml), xylosan polysulphate (10 µg/ml) and chondroitin polysulphate (10µg/ml) were added to the culture medium supplemented with or without IL-1β (100 pg/ml) for a one-week incubation period. Nutrient media with the biological agents were changed every three days.

Preparation of chondrocytes for flow cytometry

Samples of isolated chondrocytes with their CAM were obtained from cultures after agarase digestion of the gelled agarose as previously described (35). Aggrecan and type II collagen in the CAM was tested directly after incubation with the appropriate antibodies for 30 min in the dark at 4°C as described (36,37). 20µl of 50µg/ml FITC-labelled antibodies were used to react with 2×10^5 cells resuspended in 100µl PBS. To test hyaluronan, 2×10^5 cells in 100 µl PBS were first incubated with 20µl of 50 µg/ml biotinylated hyaluronic acid binding protein (bHABP) for 30 min. After washing the cells with PBS, 4 µl of 250 µg/ml avidin-FITC (Becton Dickinson, San Jose, CA, USA) was added for another 30 min incubation at 4°C in the dark.

In order to evaluate the expression of MMP-1, MMP-3, MMP-13, TIMP-1 and TIMP-3 inside the cells, chondrocytes were permeabilized using Cytofix/Cytoperm Plus™ Kit (PharMingen, San Diego, CA, USA) according to the manufacturer's instruction. Briefly, cells in culture were incubated with monensin (GolgiStop™, 4 µl/6ml medium) for 5 hours to block the protein transport from Golgi apparatus. Cells isolated from agarose were then permeabilised using Cytofix/Cytoperm™ solution for 15 min. After a wash in 1x Perm/Wash™ solution, the procedure was followed by the incubation with monoclonal antibodies.

Antibodies used for flow cytometry

Monoclonal antibodies (Mabs) against MMP-1 (clone 36665.111) were bought from R&D systems (Abingdon, United Kingdom). Mabs against MMP-3 (clone 55-2A4), MMP-13 (clone 181-15A12), TIMP-1 (clone 7-6C1) and TIMP-3 (clone 136-13H4) were obtained from Oncogene Research Products (Boston, MA, USA). MMP-1 antibody was against both pro- and active form of MMP-1. MMP-3 antibody recognised latent and active MMP-3 and reacted with MMP-3/ TIMP-1 complexes. MMP-13 Mab recognised both latent and active MMP-13. TIMP-1 antibody reacted with free TIMP-1 and MMP/TIMP-1 complexes. The anti-TIMP-3 recognised both glycosylated and unglycosylated TIMP-3. Mouse anti-human chondrocyte-specific aggrecan Mab (clone 4D11-2A9, Biosource Europe, Nivelles, Belgium) was shown to react specifically with the G1-domain of the invariable hyaluronan-binding region of the human aggrecan molecule, and was used to detect the aggrecan in the chondrocyte CAM. Mouse anti-human type II collagen Mab (clone II-4C11, ICN Biochemicals, Ohio, USA) was chosen to detect type II collagen.

All the antibodies were conjugated with FITC (Fluorescein isothiocyanate, Isomer I, Sigma) as previously described (36) and used in a direct immunofluorescent staining protocol for flow cytometry. FITC-labelled isotype matched mouse IgG_1 (Becton Dickinson) was used as negative controls. Biotinylated hyaluronic acid binding protein (bHABP) was a kind gift from Dr J. Melrose (Raymond Purves Research Laboratories, University of Sidney, Australia) and was used to trace hyaluronan in the CAM.

Flow cytometric analysis

Stained cells were analysed on a flow cytometer (FACSort, Becton Dickinson) with CELLQuest software. From each sample, 15,000 events were analysed. Cells were gated on forward and side scatter to exclude dead cells, debris and aggregates. Propidium iodide was additionally used to exclude dead cells when the epitopes outside the cells, i.e. ECM molecules were analysed (36,37). For comparison between experiments, Quantum Simply Cellular Microbead Kit (Sigma) was used to calibrate the fluorescence scale of the flow cytometer. The mean fluorescence intensity (MFI) of the positive cell population, which is due to the binding of the FITC-labelled antibodies to the specific antigen, was used to quantify the presence of the detected epitopes.

Statistical analysis

Mean values and standard deviations (SD) of the MFI values were calculated from triplicate cell cultures. The one-sided paired Student's t-test was used to analyse the changes in the MFI values of the different variables after treatment of the chondrocyte samples obtained from the 7 donors. Significance levels were set at $p = 0.05$.

Results

As an illustration, the percentage changes in the expression of the different variables in one of the chondrocyte samples were presented in Table I. The MFI values of the CAM compounds and intracellular MMPs/TIMPs after treatment in the seven chondrocyte samples were illustrated in Figures 1-3.

Table I. Effects of polysulphated polysaccharides and of hydrocortisone on cell-associated matrix molecules and on intracellular MMP/TIMP in chondrocytes. Percentage changes of the variables after treatment.

		Aggrecan	Collagen II	Hyaluronan	MMP-1	MMP-3	MMP-13	TIMP-1	TIMP-3
A.	XPS	101.9 ± 18.5	107.1 ± 18.2	107.0 ± 23.2	106.8 ± 12.9	99.4 ± 15.1	92.2 ± 11.2	86.5 ± 6.4**	88.2 ± 6.5**
	CPS	115.9 ± 20.9	105.1 ± 13.7	102.0 ± 17.6	104.3 ± 13.3	94.3 ± 19.7	97.8 ± 4.7	99.5 ± 17.6	101.5 ± 15.7
	Hydrocortisone	169.0 ± 43.3**	99.8 ± 6.3	132.9 ± 17.6**	72.6 ± 3.7**	73.0 ± 21.6*	81.6 ± 22.2	85.3 ± 16.3	78.4 ± 14.2*
	IL-1β	54.4 ± 13.1**	82.5 ± 12.5*	80.6 ± 2.6**	121.1 ± 17.6*	159.1 ± 40.2*	134.4 ± 21.4**	136.8 ± 32.4	124.1 ± 25.5
B.	XPS	137.9 ± 15.4**	112.3 ± 15.7	122.3 ± 20.3	92.5 ± 12.0	100.1 ± 20.4	86.5 ± 13.6	83.0 ± 1.7**	82.9 ± 5.3**
	CPS	148.3 ± 17.0**	111.9 ± 19.4	136.6 ± 31.1*	88.6 ± 4.7**	98.2 ± 10.8	88.7 ± 10.4	78.7 ± 7.0**	81.3 ± 10.0**
	Hydrocortisone	185.6 ± 23.2**	110.2 ± 15.0	175.4 ± 19.5**	71.7 ± 14.5**	67.9 ± 16.0**	76.2 ± 9.8**	81.2 ± 17.0	70.2 ± 14.6**

A. Native chondrocytes. Individual values of mean fluorescence intensity (MFI) in the control cultures was normalised to 100, and the percentage changes for MFI in treated chondrocytes were then calculated; B. IL-1β-treated chondrocytes. IL-1β-treated chondrocytes were used as controls in series B. **: $p < 0.05$; * : $0.05 < p < 0.1$.

The baseline values of these parameters in native chondrocytes were normalized to 100 to illustrate the percentage changes after exposure to the respective agents. To test the changes induced in IL-1β-treated cells, the values obtained after exposure to IL-1β were normalized.

In native chondrocytes, apart from a significantly improved aggrecan synthesis by CPS (Fig. 1). No pertinent changes in CAM molecule content or in intracellular MMP/TIMP levels were observed after treatment with the polysulphated polysaccharides (Figs. 2 and 3).

Hydrocortisone was shown to increase aggrecan and hyaluronan accumulation in the CAM with 125.7% and 32.9%, respectively, and to decrease the intracellular levels of MMP-1, MMP-13 and TIMP-3 to 72.3%, 81.1% and 84.7% of the control value (Figs.1-3).

IL-1β at a concentration of 100 pg/ml significantly decreased the expression of aggrecan and hyaluronan in the CAM to 54.1% and 80.5% of the control values, respectively, and there was a downregulation of the expression of type II collagen to 69.6% of the control value. Intracellular MMP-1, MMP-3, MMP-13 levels significantly increased with 17.8%, 17.7%, and 31.2%, respectively. TIMP-1 and -3 inside the cells increased with 61.0% and 30.0%.

In IL-1β-depressed cells, XPS and CPS increased IL-1β-depressed CAM aggrecan with 49.9% and 63.5%, and hyaluronan with 22.2% ($p = 0.058$) and 36.6%, respectively (Fig. 1). A significant increase in CAM type II collagen could also be observed (XPS: +33.2%;

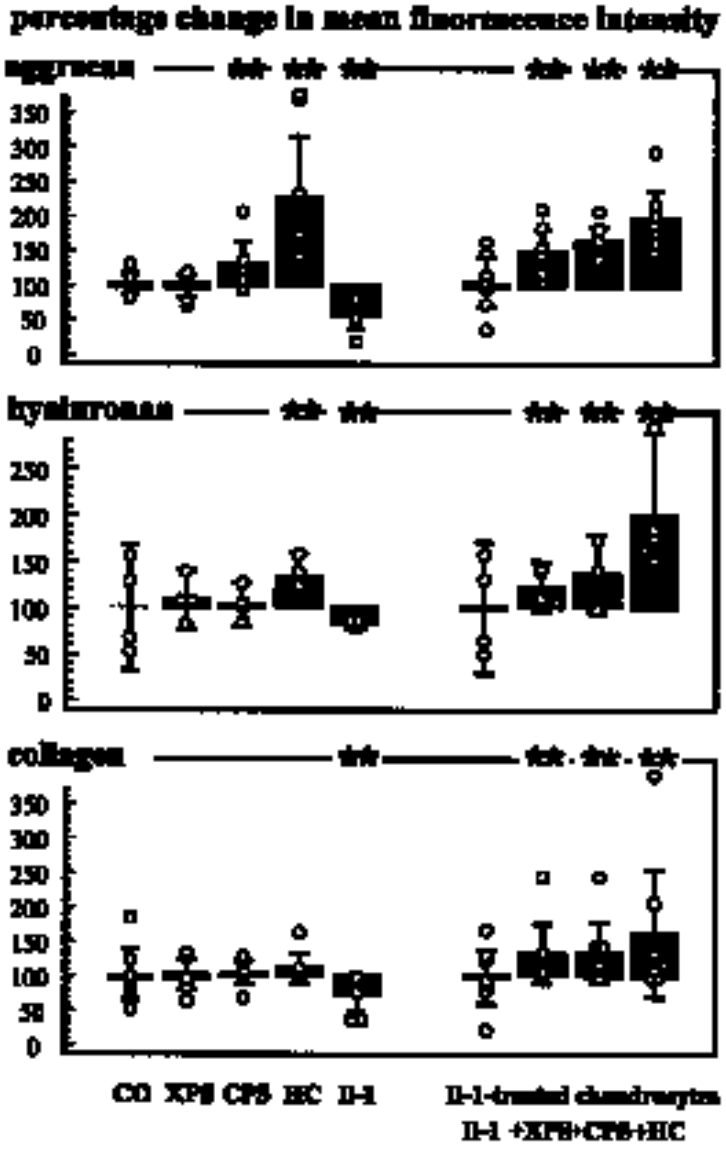

Fig. 1. Percentage increases of chondrocyte mean fluorescence intensities (MFI) for cell-associated matrix aggrecan, hyaluronan and type II collagen. CO:control cultures: baseline values for each of the 7 donor chondrocyte cultures were normalized to 100 and are represented. XPS:xylosan polysulphate, CPS: chondroitin polysulphate, HC: hydrocortisone, IL-1:interleukin-1: values for each of the 7 IL-1β-treated chondrocyte cultures were normalized to 100 and used as baseline values for the XPS, CPS and HC induced changes. Mean values (bar) ± 2 SEM (ç—ç) are given. ** $p < 0.05$; * $0.05 < p < 0.10$.

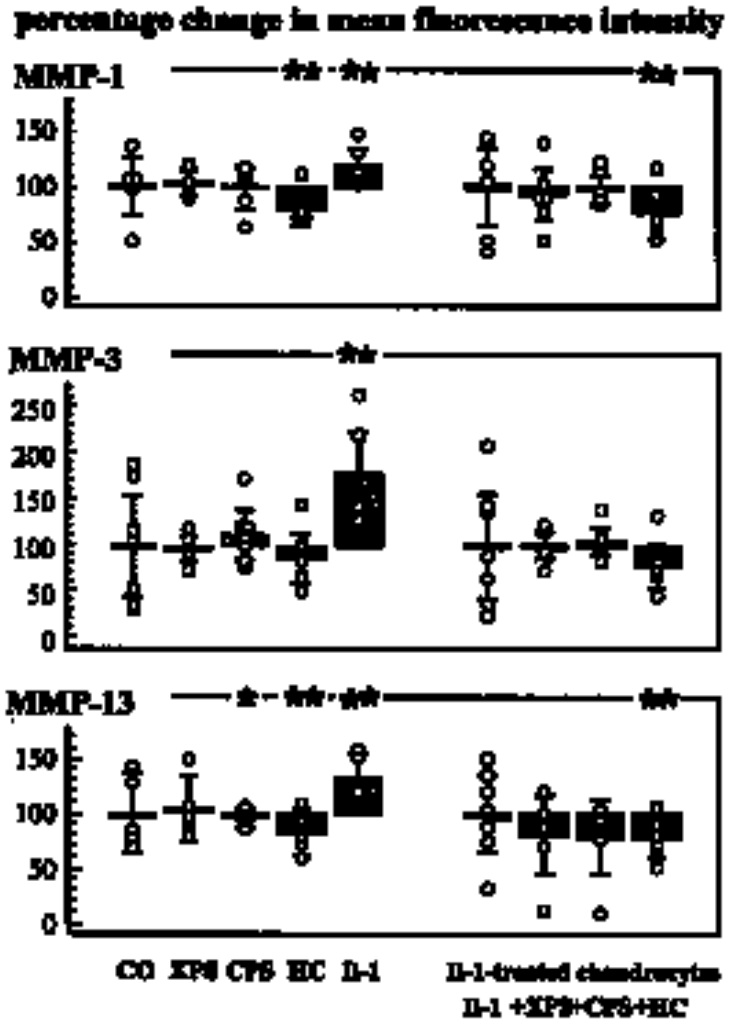

Fig. 2. Percentage increases of chondrocyte mean fluorescence intensities (MFI) for intracellular MMP-1,-3 and –13. CO:control cultures:baseline values for each of the 7 donor chondrocyte cultures were normalized to 100 and are represented. XPS: xylosan polysulphate, CPS: chondroitin polysulphate, HC: hydrocortisone, IL-1: interleukin-1: values for each of the 7 IL-1β-treated chondrocyte cultures were normalized to 100 and used as baseline values for the XPS, CPS and HC induced changes. Mean values (bar) ± 2 SEM (ç—ç) are given. ** $p < 0.05$; * $0.05 < p < 0.10$.

CPS: +34.4%). None of the sulphated polysaccharides did affect intracellular MMP levels (Fig. 2). Similarly, the effects of the polysaccharides on cellular TIMP were not that obvious. XPS depressed IL-1β-upregulated TIMP-3 to 81.7% of the control value, and a significant decrease of merely TIMP-3 to 85.9% of the baseline value was seen under CPS (Fig. 3).

Hydrocortisone upregulated IL-1β-depressed CAM aggrecan, hyaluronan and type II collagen (Fig. 1) and significant percentage changes were obtained (aggrecan: +97.5%; hyaluronan: +100.4%; collagen: +61.7%). Intracellular MMP-1 and -13 levels were downregulated to 75.7% and 76.4% of the control values, respectively (Fig. 2). Intracellular TIMP-1 and -3 decreased to 83.6% and 80.6% of the control values, respectively (Fig. 3). Hydrocortisone restored IL-1β-induced changes of all variables to the baseline levels seen in native chondrocytes.

Discussion

Extracellular matrix turnover in normal and pathological conditions is thought in part to result from the balance in the expression of MMPs and TIMPs. IL-1β is considered to be the main cytokine inducing catabolic processes in cartilage. IL-1β suppresses the expression of type II collagen (38) and the synthesis of aggrecan (39, 40). It stimulates the expression of MMPs (41).

Flow cytometry was used to assess the influence of IL-1β on human articular chondrocytes cultured in agarose, and to explore the effects of two polysulphated polysaccharides and of hydrocortisone on native and IL-1β-treated cells. The technique allowed the accumulation of extracellular matrix compounds to be measured in the cell-associated matrix of the cells after their isolation from the agarose gel (36, 37). Intracellular MMP and TIMP levels were analysed after permeabilisation of the cells.

The intracellular MMP and TIMP levels directly indicate their production by the chondrocytes, but do not reflect either the secretion capacity of cells or the extracellular accumulation. However, changes in intracellular MMP/TIMP levels reflect the capability of the chondrocytes to respond to exogenous biological agents in this *in vitro* culture system.

The results obtained from this study showed that after exposure to 100 pg/ml of IL-1β, chondrocyte CAM aggrecan, hyaluronan and type II collagen decreased. Intracellular MMP-1, MMP-3 and MMP-13, as well as TIMP-1 and -3 levels of the donor chondrocytes were increased. The increased intracellular TIMP levels were regarded as the physiological response to an increase in MMPs, leading to a new equilibrium. Recently, chondrocytes obtained from normal cartilage have been shown to produce closely balanced quantities of MMPs and TIMPs (42).

In IL-1β-untreated chondrocytes, hydrocortisone stimulated the accumulation of aggrecan and hyaluronan and decreased the levels of MMP-1, -13 and TIMP-3. When the accumulation of CAM compounds was considered, the IL-1β-untreated cells did not obviously respond to XPS or CPS.

In IL-1β-treated chondrocytes, however, hydrocortisone and both polysulphated polysaccharides successfully neutralized the cytokine-induced catabolic effects. IL-1β-induced loss of aggrecan, hyaluronan and type II collagen in the CAM was restored after exposure to XPS and CPS. A some-

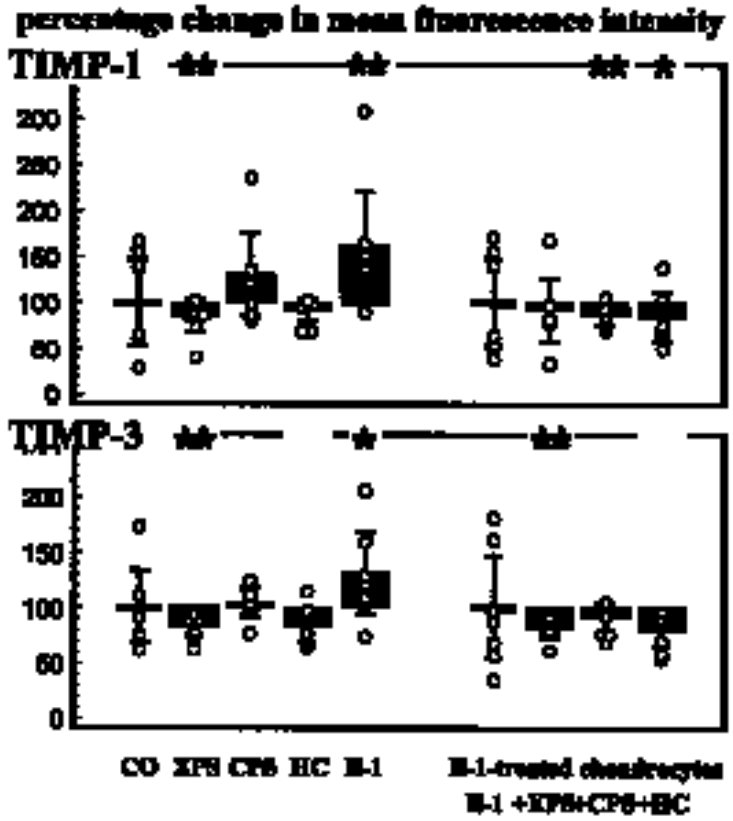

Fig. 3. Percentage increases of chondrocyte mean fluorescence intensities (MFI) for intracellular TIMP-1 and –3. CO: control cultures: baseline values for each of the 7 donor chondrocyte cultures were normalized to 100 and are represented. XPS: xylosan polysulphate, CPS: chondroitin polysulphate, HC: hydrocortisone, IL-1: interleukin-1: values for each of the 7 IL-1β-treated chondrocyte cultures were normalized to 100 and used as baseline values for the XPS, CPS and HC induced changes. Mean values (bar) ± 2 SEM (ç—ç) are given. ** $p < 0.05$; * $0.05 < p < 0.10$.

what more pronounced effect was observed in the hydrocortisone-treated cells. Possibly as a result of its much lower turnover rate (43), the expression of type II collagen in the CAM under exposure to the different agents did not change as much as that of aggrecan or hyaluronan. In the same experimental conditions, hydrocortisone suppressed the upregulated intracellular MMP-1 and -13, and the cellular TIMP levels. Obviously, this downregulation of metalloprotease activities resulted in an increased accumulation of ECM molecules in the chondrocyte CAM. The effects of the two polysulphated polysaccharides on intracellular MMP and TIMP levels were less evident. Since MMP activity was not affected by these agents, it is supposed that the increase in CAM aggrecan may have resulted from the regulation of other proteinases, such as aggrecanase (44).

Since IL-1β-upregulated chondrocyte extracellular matrix catabolism closely mimics the degradation of articular cartilage seen in OA (45), the effects of hydrocortisone and of both polysulphated polysaccharides on IL-1β treated chondrocytes *in vitro* may support their cartilage structure modifying or chondroprotective function *in vivo*. The finding of some structure modifying properties of hydrocortisone on human articular cartilage cells concurs with our previous observations (26). It has to be mentioned that the effect size of hydrocortisone on chondrocyte metabolism was more pronounced that that of the oversulphated polysaccharides and that the ECM structure modifying properties of these dissimilar types of agents may result from a different mechanisms of action.

Degradation of the cartilage matrix is thought to be the consequence of increased activity of several enzymes, which can be produced by chondrocytes. Characteristically, MMP-1 and MMP-3 have been found increased in osteoarthritic cartilage (46, 47). More recently, MMP-8 and MMP-13 have been described as two of the main collagen degrading activities in OA cartilage (7, 17, 48). Aggrecanase is the most prominent aggrecan degrading activity when pathological (OA) cartilage samples were investigated (16). Naturally occurring endogenous inhibitors of MMPs, TIMPs, have been found over-expressed in degenerative joints and are considered as important controlling factors in the actions of MMPs. TIMP-1 and -3 have been shown to inhibit aggrecanase activity in IL-1-treated bovine nasal cartilage explants (49, 50).

Previous studies have shown that a functional chondrocyte TGF-βRII/TGF-β1 pathway correlated with the production of ECM structural macromolecules and the intracellular levels of some of the MMPs and their natural inhibitors. TGF-β has been shown to downregulate the receptor for IL-1 and thereby to antagonize IL-1-induced synthesis of collagenase and stromelysin by chondrocytes (51,52). In human articular cartilage high MMP-1 levels correlated with a more functional TGF-β1/TGF-βRII pathway and upregulated TIMP-1 and -3 levels (42). It was postulated that the contents of the ECM are controlled through the TIMP system and any situation inducing ECM degradation, e.g. inflammatory events or mechanical damage, will activate local autocrine/paracrine responses resulting in a new equilibrium. Hydrocortisone abrogated the IL-1-depressed production of ECM macromolecules and downregulated IL-1-enhanced coexpressed MMP and TIMP activities. It is likely that the concurrent attenuation of MMP and TIMP following hydrocortisone occurred through the activation of some of the locally operational growth factors. The mechanism of action of the polysulphated polysaccharides requires further investigation.

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