Selective iNOS inhibitor 1400W enhances anti-catabolic IL-10 and reduces destructive MMP-10 in OA cartilage. Survey of the effects of 1400W on inflammatory mediators produced by OA cartilage as detected by protein antibody array

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Abstract

Objectives

In osteoarthritis (OA), the balance between catabolic and anabolic mediators and their regulators in cartilage is disturbed. Proinflammatory cytokine interleukin-1 (IL-1) plays a central role in cartilage destruction and nitric oxide (NO) mediates many of its destructive effects. In the present study, we investigated the secretion of 40 mediators related to inflammation or cartilage degradation by OA cartilage samples with a protein antibody array. The effects of IL-1 and a selective iNOS-inhibitor 1400W on the mediator release were also studied.

Methods

Cartilage tissue was obtained from the leftover pieces of total knee replacement surgery from OA patients. Protein antibody array was used to measure production of 40 mediators in the culture medium. ELISA was used to confirm the antibody array results.

Results

OA cartilage secreted spontaneously 15 out of the 40 measured mediators. IL-1β enhanced production of 11 of these inflammatory mediators in OA cartilage along with increased NO production. Treatment with a selective iNOS inhibitor 1400W enhanced the production of IL-10, while the levels of MMP-10 were reduced in IL-1 -treated OA cartilage.

Conclusions

OA cartilage produces many of the mediators involved in the pathogenesis of OA. The ability of 1400W to enhance levels of anti-catabolic IL-10 and to reduce levels of destructive MMP-10 points to the anti-inflammatory mechanisms that iNOS-inhibitors may have.

Key words

Nitric oxide, osteoarthritis, cartilage, protein antibody array.

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Introduction

Osteoarthritis (OA) is considered as a slowly progressing inflammatory disease which is characterized by the destruction of the articular cartilage (1). In addition to the cartilage, the pathologic process involves the entire joint, including the subchondral bone, ligaments, capsule, synovial membrane and periarticular muscles (2). In the normal joint, chondrocytes, synovial fibroblasts and macrophages produce cytokines and growth factors at low levels to maintain the homeostasis as the cartilage is continuously remodelled. In OA, the balance between catabolic and anabolic mediators and their regulators is disturbed. The chondrocytes, the only cellular component in articular cartilage, are activated to produce growth factors and inflammatory and destructive mediators in an attempt to restore the normal homeostasis (3).

The destructive and inflammatory process in the osteoarthritic joint is mediated by several cytokines. Interleukin-1 (IL-1) is among the most important cytokines in cartilage destruction (3). It is capable of inducing the production of nitric oxide (NO) through inducible NO-synthase (iNOS) -pathway in human chondrocytes (4-7), which appear to be the major source of NO in the OA joint (8). Accordingly, markers of enhanced NO production have been found in osteoarthritic joints (9-11). NO mediates many of the destructive effects of IL-1 in inflamed joints. NO has been reported to activate matrix metalloproteinases (12, 13), inhibit proteoglycan (14-16) and collagen (17) synthesis and induce chondrocyte cell death (18, 19). NO is also involved in the enhancement of the inflammation by reducing the production of TGF-β (20) and endogenous IL-1 receptor antagonist (IL-1ra) in chondrocytes (21, 22) and by contributing to the resistance against anabolic effects of IGF-1 (23). NO has also been shown to sustain activation of NF-κB providing a persistent transcription of NF- κB dependent genes (24). iNOS-inhibitors have been found to slow down the destruction of the articular cartilage in an experimental model of osteoarthritis (25-28).

Protein antibody array is a novel method enabling simultaneous screening of several proteins in a biological sample. In addition, the method is sensitive and offers a wide detection range (29). In the present study, we investigated the secretion of 40 mediators related to inflammation or cartilage degradation (cytokines, growth factors and destructive enzymes) from human OA cartilage samples by a protein antibody array. The aim of the study was to investigate 1) the spontaneous and IL-1 induced secretome of the OA cartilage and 2) the role of NO in the production of these mediators by using a highly selective iNOS-inhibitor, 1400W.

Materials and methods

Patients, tissue cultures

Cartilage tissue was obtained from the leftover pieces of total knee replacement surgery. The study was approved by the ethics committee of Tampere University Hospital and the patients gave their written approval. The donor patients, age ranging from 59 to 74 years were all diagnosed to have OA. Full thickness pieces of articular cartilage were removed aseptically from subchondral bone with a scalpel and cut into small pieces (cubes sizing approximately 2 mm). The cartilage samples were incubated at 37°C in humidified 5% carbon dioxide atmosphere in Dulbecco's modified Eagle's medium (DMEM) with glutamax-I containing 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomysin (100 μg/ml) and amphotericin B (250 ng/ml) (all obtained from Gibco BRL, Paisley, UK) for 120 h. Aliquots of the culture media were kept at -20°C until assayed.

NO production

Concentrations of nitrite, a stable product of NO in aqueous solutions, were measured using the Griess reaction (30). Tissue pieces were weighed and the results were expressed as pmol of nitrite / mg of tissue.

Protein antibody array

Custom-made protein antibody array (RayBio Human Cytokine Antibody Array, Raybiotech, Inc., Norcross, GA, USA) was used to detect proteins secreted by cartilage into the culture

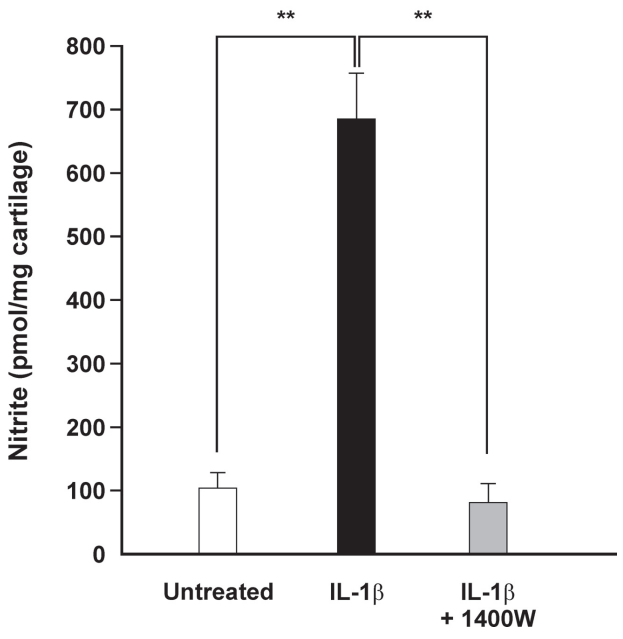

Fig. 1. The effect of a selective iNOS-inhibitor 1400W (1 mM) on NO-production in IL-1β (4 ng/ml) -treated OA cartilage. The inhibitor was added into to culture at the beginning of the 120 h incubation. Nitrite was measured in the culture as an indicator of NO synthesis by Griess reaction. Results are expressed as mean ± SEM, samples from 8 patients (n=8). Statistical significance was calculated with Wilcoxon matched pairs test. ** = $p<0.01$.

medium. The processing protocol provided by the producer was followed. All three array membranes (untreated, IL-1 and IL-1+1400W) for each patient were processed simultaneously. Array membranes were incubated for 30 min in 2 ml of blocking buffer, then incubated for 2 hours in 1 ml of the tissue culture medium diluted 1:1 with blocking buffer, and washed. The array membranes were incubated overnight at 4°C with 1 ml biotin-conjugated secondary antibodies and washed. This was followed by a 2-hour incubation with 2 ml of a peroxidase-labeled streptavidin solution. The arrays were then exposed for 1 min by FluorChemTM 8800 imaging system (Alpha Innotech, San Leandro, CA, USA). Quantification of the chemiluminescent signal was carried out with FluorChemTM software v.3.1.

Normalization of the results

For the comparison between different membranes the results were normalized by using positive and negative controls in each membrane. The relative levels of proteins secreted into the culture medium (relative mediator levels) were determined by comparing these normalized signal intensities. Mean relative mediator levels were calculated (n = 8 OA patient samples). We used the culture medium (containing 10% serum) without cartilage as a control for the untreated OA cartilage.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-10 (Sanquin, PeliPairTM, Amsterdam, The Netherlands), MMP-10 (Raybiotech, Inc., Norcross, GA, USA), leptin and TGF-β2 (R&D Systems, Inc, Minneapolis, USA) in the culture media were also determined by ELISA.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance of the results was calculated by using Mann-Whitney test and Wilcoxon matched pairs test. Differences were considered significant when $p<0.05$.

Reagents

IL-1β was purchased from Genzyme, Cambridge, MA, USA. 1400W was kindly given by Dr. Richard G. Knowles, Glaxo SmithKline Research & Development, Stevenage, UK.

Results

IL-1 -induced NO production through iNOS

The proinflammatory cytokine IL-1β (4 ng/ml) stimulated NO production in OA cartilage. A highly selective iNOS inhibitor 1400W (1 mM) inhibited IL-1 -induced NO accumulation indicating that it was synthesized through iNOS pathway (Fig. 1).

Table I. Protein antibody array. A schematic diagram of the Human Cytokine Antibody Array shows the locations of the controls and the duplicate spots of proteins. BMP: bone morphogenetic protein; FGF: fibroblast growth factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; ICAM: intercellular adhesion molecule; IFN-γ: interferon-γ; IGF-1: insulin growth factor; IL: interleukin; IL-1ra: interleukin-1 receptor antagonist; IL-1sR: interleukin-1 soluble receptor; LIF: leukocyte inhibitory factor; MMP: matrix metalloproteinase; NEG: negative control; OSM: oncostatin M; POS: positive control; sTNFR: soluble tumor necrosis factor receptors; TIMP: tissue inhibitor of metalloproteinase; TGF-β: transforming growth factor-β.

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	BMP-4	BMP-6	BMP-7	FGF-4	FGF-6	FGF-7	FGF-9	GM-CSF
2	POS	POS	NEG	NEG	BMP-4	BMP-6	BMP-7	FGF-4	FGF-6	FGF-7	FGF-9	GM-CSF
3	ICAM-1	IFN-γ	IGF-1	IL-1sR1	IL-1sR2	IL-10	IL-13	IL-17	IL-18	IL-1ra	IL-4	IL-6
4	ICAM-1	IFN-γ	IGF-1	IL-1sR1	IL-1sR2	IL-10	IL-13	IL-17	IL-18	IL-1ra	IL-4	IL-6
5	IL-8	LAP (TGF-β1)	Leptin	LIF	MMP-1	MMP-10	MMP-13	MMP-2	MMP-3	MMP-9	OSM	sTNFR1
6	IL-8	LAP (TGF-β1)	Leptin	LIF	MMP-1	MMP-10	MMP-13	MMP-2	MMP-3	MMP-9	OSM	sTNFR1
7	sTNFR2	TGF-β1	TGF-β2	TGF-β3	TIMP-1	TIMP-2	TIMP-4	TNF-α	BLANK	BLANK	BLANK	POS
8	sTNFR2	TGF-β1	TGF-β2	TGF-β3	TIMP-1	TIMP-2	TIMP-4	TNF-α	BLANK	BLANK	BLANK	POS

Inflammatory mediators produced by OA cartilage and the effect of IL-1

The antibody array contained 40 mediators related to inflammation or cartilage destruction, *i.e.*, cytokines, growth factors and destructive enzymes, as shown in Table I. OA cartilage produced spontaneously 15 out of the 40 mediators as listed in Table II. Proinflammatory cytokine IL-1β (4 ng/ml) enhanced the production of 11 mediators, most pronouncedly that of interleukin-6 (IL-6), interleukin-8 (IL-8) and matrix metalloproteinase-1 (MMP-1) as shown in Table III. Representative antibody array membranes are shown in Figure 2.

The effect of iNOS-inhibitor 1400W on IL-1-stimulated protein production by OA cartilage

Inhibition of NO production with a selective iNOS inhibitor 1400W (1 mM) enhanced the production of anti-catabolic IL-10 and decreased the production of catabolic leptin, anabolic TGF-β2 as well as MMP-10 in IL-1β (4 ng/ml) -treated cartilage as detected by protein antibody array (Fig. 2D and Table IV).

In order to confirm statistically significant changes in mediator levels detected by protein antibody array, IL-10, leptin, TGF-β2 and MMP-10 levels in culture medium were measured also with ELISA. IL-1β (4 ng/ml) stimulated significantly IL-10 production and this was further enhanced with 1400W (1 mM) - treatment. The increase in IL-10 production after 1400W-treatment was 140% as detected by protein antibody array and 190% by ELISA (Fig. 3A and Table IV). Leptin and TGF-β2 results could not be confirmed with ELISA due to their low concentrations in the samples. Similarly to the protein antibody array results, IL-1β induced MMP-10 production and this was inhibited with 1400W. The decrease in MMP-10 production after 1400W-treatment was about 30% as detected by protein antibody array and about 55% as measured by ELISA (Fig. 3B and Table IV).

Table II. Mediators produced spontaneusly by OA cartilage as detected by protein antibody array during 120 h incubation. Mean relative mediator levels in culture media. The relative mediator levels were calculated for each sample by using the positive and negative controls included in every antibody array membrane. Statistical significance was calculated with Mann-Whitney test and statistically significant results are presented. OA cartilage from 8 donor patients (n=8), *= $p<0.05$. For abbreviations, see Table I.

	Mean relative mediator levels in culture medium	
	Background (*i.e.*, medium without OA cartilage)	Mediators produced by OA cartilage
Catabolic		
Leptin (OB)	2.7	6.7*
Anti-catabolic		
IL-10	2.1	3.7*
sTNFR1	3.0	5.5*
Anabolic		
LAP(TGF-β1)	3.0	12.5*
Modulatory		
IL-6	2.0	445.1*
IL-8	11.1	747.4*
MMPs		
MMP-1	2.1	18.8*
MMP-3	6.1	372.5*
MMP-9	3.4	7.2*
MMP-10	15.8	229.7*
MMP-13	3.6	49.7*
TIMP-1	24.8	150.6*
TIMP-2	4.8	337.8*
TIMP-4	4.5	11.6*
Others		
ICAM-1	2.4	6.2*

Table III. Mediators produced by IL-1β (4 ng/ml) -treated OA cartilage as detected by protein antibody array during 120 h incubation. Fold increase/decrease is shown as compared to untreated OA cartilage. Statistical significance was calculated with Wilcoxon matched pairs test from normalized signal intensities and statistically significant results are presented. OA cartilage from 8 donor patients (n=8), *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$. For abbreviations, see Table I.

	IL-1β -treated cartilage
	Fold increase/ decrease[#]
Catabolic	
Leptin (OB)	3.1***
Anti-catabolic	
IL-10	3.5**
sTNFR2	2**
Anabolic	
BMP-7	1.8**
FGF-4	1.3*
Modulatory	
IL-6	53**
IL-8	5.3**
MMPs	
MMP-1	6.4*
MMP-2	1.7**
MMP-3	0.7**
MMP-10	2.4**
TIMP-2	0.7*
Others	
ICAM-1	2**

[#]Fold increase/decrease compared to untreated OA cartilage.

Discussion

Osteoarthritis is a chronic disease characterised by gradual loss of the articular cartilage due to changes in metabolism towards inflammatory and cartilage degrading direction. According to current understanding, no single cytokine or growth factor can be defined to be responsible for this process. Instead, it is believed that there are several anabolic, catabolic and regulatory mediators involved. To study this cytokine network we chose 40 mediators related to OA on custom-made protein antibody array membrane and investigated their production in eight human OA cartilage samples. OA cartilage was found to take actively part in the cartilage metabolism by producing spontaneously *e.g.*, catabolic leptin, anti-catabolic IL-10 and sTNFR1, anabolic

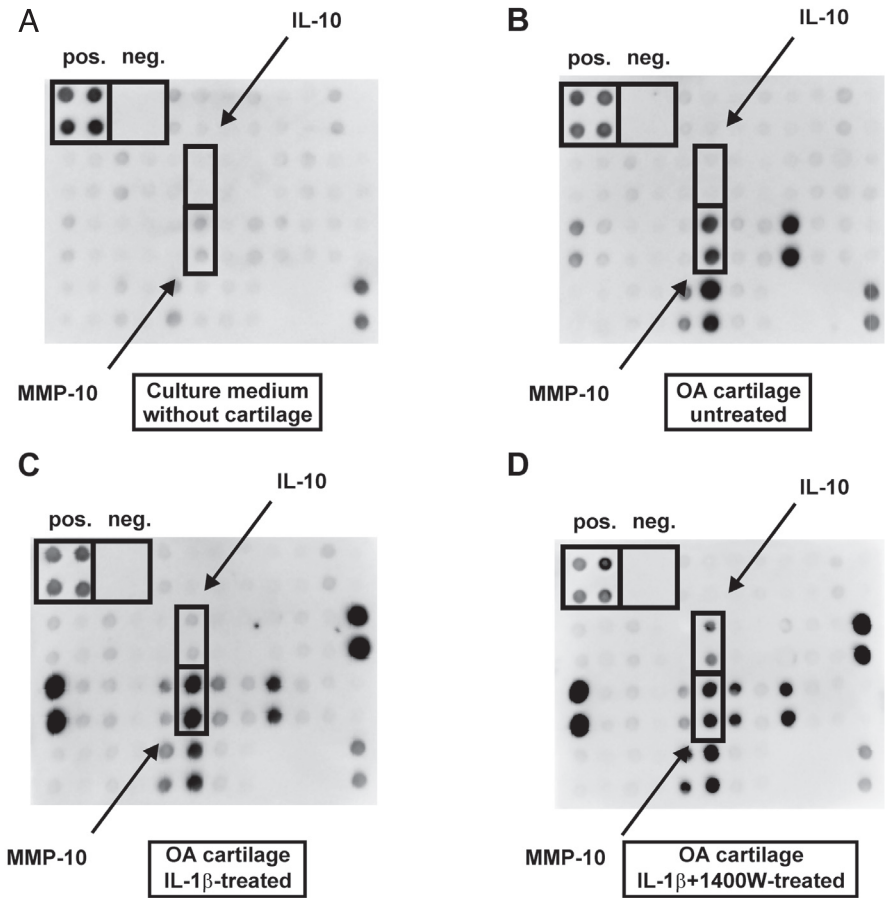

Fig. 2. The effect of a selective iNOS-inhibitor 1400W (1 mM) on mediators of cartilage metabolism in IL-1β (4 ng/ml) -treated OA cartilage. The proteins of the culture medium without OA cartilage (*i.e.*, background) containing 10% serum (A), and untreated (B), IL-1β -treated (C) and IL-1β+1400W -treated OA cartilage (D) after 120 h incubation were detected by protein antibody array. A representative membranes are shown, one OA patient sample of 8 with similar results. IL-10 and MMP-10 are marked.

Table IV. Mediators changed by iNOS-inhibitor 1400W (1 mM) in IL-1β -treated OA cartilage as detected by protein antibody array during 120 h incubation. Fold increase/decrease is shown as compared to IL-1β (4 ng/ml) -treated OA cartilage. Statistical significance was calculated with Wilcoxon matched pairs test from normalized signal intensities and statistically significant results are presented. OA cartilage from 8 donor patients (n=8), *= $p< 0.05$, **=$p<0.01$. For abbreviations, see Table I.

IL-1β and 1400W -treated cartilage	Fold increase/ decrease[#]
Catabolic	
Leptin (OB)	0.8*
Anti-catabolic	
IL-10	2.4*
Anabolic	
TGF-β2	0.7*
MMPs	
MMP-10	0.7**

[#]Fold increase/decrease compared to IL-1β-treated OA cartilage.

LAP (TGF-β1), modulatory IL-6 and 8, several MMPs and their inhibitors. Proinflammatory cytokine IL-1 plays a central role in cartilage destruction (3) and in support was found to further enhance the production 11 mediators in the present study. IL-1 caused the most pronounced change, a 50-fold increase, in interleukin-6 (IL-6) production, and there was also a 5-fold increase in interleukin-8 (IL-8) and a 6-fold increase in MMP-1 production. Also MMP-2 and MMP-10 were significantly increased by IL-1 treatment.

Nitric oxide (NO) mediates many of the destructive effects of IL-1 in articular cartilage (12-24). In support, Pelletier et al. reported reduced destruction of the articular cartilage by using iNOS-inhibitor L-NIL in instability-induced OA in dogs (25). In further studies with this model L-NIL was shown to reduce the levels of matrix metalloproteinase-1 and -3 (MMP-1 and MMP-3) (13), to inhibit chondrocyte apoptosis (27) and to reduce the interleukin-1 converting enzyme (ICE) levels (28). Van den Berg *et al*. studied the development of experimental osteoarthritis induced with intra-articular collagenase injection in iNOS knock-out mice. In this model, iNOS deficiency prevented the degree of cartilage destruction and osteophyte formation (31). NO has several catabolic and anti-anabolic actions in cartilage and thus it is identified as a possible target of treatment in osteoarthritis. Therefore we decided to investigate the effects of selective iNOS inhibitor 1400W (32) on the cytokine production of OA cartilage and used protein antibody array method to investigate the effects of NO on cartilage metabolism. 1400W significantly changed the production of leptin, IL-10, TGF-β2 and MMP-10.

In the present study, protein antibody array showed significant decrease by 1400W in the production of leptin and TGF-β2 which are both related to OA pathogenesis. Leptin, an obesity related hormone, has been found in synovial fluid (SF) from OA patients in concentrations that are similar or higher than those measured in serum (33, 34). Furthermore, articular cartilage has been reported to release leptin (34) and to express functional leptin receptor Ob-R (35) pointing to a pathophysiological role for leptin in OA. *In vitro*, leptin has been shown to potentiate IL-1 and IFNγ -induced NO production in human chondrocytes (36, 37). Also TGF-β levels are elevated in the synovial fluid from OA patients and in the OA process the anabolic factor TGF-β is regarded mainly as a repair factor, but it may also lead to osteophyte formation and fibrosis. TGF-β enhances production of extracellular matrix proteins, stimulates chondrocyte proliferation, suppresses IL-1-induced NO production in chondrocytes, and has been suggested as a therapy for OA (38-40). 1400W decreased the production of

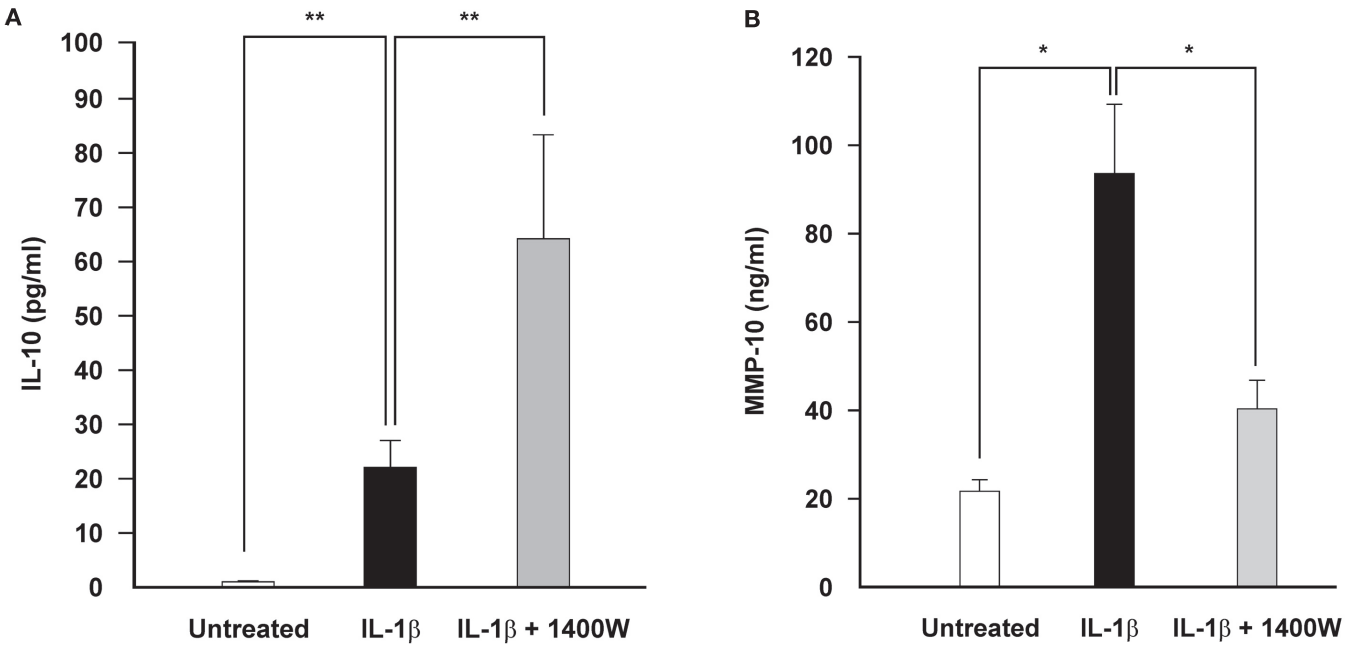

Fig. 3. The effects of a selective iNOS-inhibitor 1400W (1 mM) on IL-10 (A) and MMP-10 (B) production in IL-1β (4 ng/ml) -treated OA cartilage during 120 h incubation. Mediators were measured in the culture medium by ELISA. Results are expressed as mean ± SEM, samples from 7-8 patients (n=7-8). Statistical significance was calculated with Wilcoxon matched pairs test. *= $p<0.05$, **= $p<0.01$.

catabolic leptin and anabolic TGF-β2 as detected by the protein antibody array. However, these results could not be confirmed by ELISA due to their low concentrations in the samples and therefore need to be confirmed in future studies.

As detected by protein antibody array and confirmed by ELISA, iNOS inhibitor 1400W was shown to increase the levels of anti-catabolic IL-10 and to decrease the levels of destructive MMP-10 supporting the anti-inflammatory role that iNOS inhibitors may have in the treatment of OA. The expression level of both IL-10 mRNA and protein has been reported to be higher in human OA chondrocytes than in cells from healthy controls (41). IL-1 has been shown to upregulate IL-10 production in human OA chondrocytes (42) and this was confirmed in the present study. To our knowledge, the effect of NO on IL-10 production has not been studied in cartilage or chondrocytes before. Our results showed that 1400W increased the production of IL-10 in OA cartilage. These data are supported by previous findings showing that an iNOS inhibitor was able to enhance the production of IL-10 in spleen cells (43). IL-10 is classified as an anti-catabolic cytokine since it is able to resist the actions of catabolic cytokines. IL-10 has been shown to inhibit IL-1, IL-6 and TNF-α production in human OA chondrocytes (42), in LPS-treated macrophages (44) and in addition to previous cytokines, IL-8 production in activated human monocytes (45). IL-10 is also reported to reduce TNF-α-induced prostaglandin E_2-production in OA synovial fibroblasts (46) and to reverse bacterial antigen stimulated inhibition of cartilage proteoglycan synthesis by human mononuclear cells (47). The present results on enhancing effect of iNOS inhibitor 1400W on IL-10 levels in IL-1 -treated OA cartilage suggests that NO suppresses anti-catabolic IL-10 production in OA cartilage.

Local imbalance in proteinases and their endogenous inhibitors is suspected to cause the increased degradation of cartilage matrix in OA (48). NO has been suggested to increase the production of MMP-1, MMP-2, MMP-3, MMP-9 in chondrocytes and MMP-13 in bovine aortic endothelial cells (12, 13, 49, 50) and it may regulate metalloproteinase activation via S-nitrosylation (51). It has been reported that MMP-10 (stromelysin-2) is expressed in chondrocytes within the growth plate of human neonatal rib bone (52) as well as in synovial tissue from rheumatoid joint (53, 54). MMP-10 has one of the widest substrate spectra of the MMPs, it is known to degrade collagens III, IV and V, gelatin, casein, aggrecan, elastin, proteoglycan link protein, fibronectin, proMMP-1 and proMMP-8 (54). MMP-10 has recently been implicated in the cartilage degradation in arthritis (55). To our knowledge, this was the first time the inhibitory effect of iNOS inhibitor was studied on MMP-10 production. In the present study, MMP-10 was shown to be produced by human OA chondrocytes. The expression was upregulated by IL-1 and downregulated by iNOS-inhibitor 1400W. Therefore, the results of the effects of 1400W on IL-10 and MMP-10 production in OA cartilage support the favourable effect that iNOS-inhibitors may have in the treatment of OA.

Protein antibody array is a useful tool *e.g.*, when investigating the effects of therapeutically potential molecules (29), especially in studies with limited amount of tissue, as OA cartilage. The method has advantages as compared to ELISA: the simultaneous detection of multiple cytokines, higher sensitivity and greater detection range. However, the method

is not quantitative as is ELISA and in the present study, the results were confirmed by ELISA. The results show that protein antibody array is suitable for screening purposes but further development and optimization are needed to increase the usefulness of this new assay.

In our study, we used protein antibody array method to investigate the effects of iNOS inhibitor 1400W on IL-1-induced protein production in human OA cartilage. ELISA confirmed results showed that iNOS-inhibitor 1400W enhanced the production of anti-catabolic IL-10 and reduced destructive MMP-10 levels. These data support the favourable anti-inflammatory effects that iNOS-inhibitors may have in arthritis.

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