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OSTEOARTHRITIS and CARTILAGE

The increased synthesis of inducible nitric oxide inhibits IL-1ra synthesis by human articular chondrocytes: possible role in osteoarthritic cartilage degradation

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Summary

The degradation of osteoarthritic (OA) cartilage is likely related to the synthesis and the release of catabolic factors by chondrocytes. Nitric oxide (NO) has recently been suggested as playing a role in cartilage degradation. Since NO production is largely dependent on stimulation by IL-1, its effects on factors regulating the IL-1 biological activity, such as IL-1ra, are of the utmost importance. This study examined and compared the level of NO production by normal and OA cartilage and chondrocytes, as well as studied the effect of IL-1-induced NO production on the synthesis and steady-state mRNA of interleukin-1 receptor antagonist (IL-1ra).

The NO baseline production by normal cartilage explants was undetectable but inducible by rhIL-1β. OA cartilage spontaneously produced NO. About a two-fold increase in NO production was found in OA rhIL-1β-stimulated (0.5–100 units/ml) cartilage as compared with the similarly stimulated normal cartilage. On chondrocytes rhIL-1β-stimulation (0.5–100 units/ml) produced a dose-dependent enhancement of both NO production and IL-1ra synthesis. Treatment with 200 μM N^{g}-monomethyl-L-arginine (L-NMA), a well known NO synthase inhibitor, induced over 70% inhibition of the NO production and a marked increased IL-1ra synthesis (average of 84%) and expression (mRNA level). Inhibition of prostaglandin synthesis by indomethacin had no effect on both the NO production or the IL-1ra level.

In the present study, we demonstrated the capacity of OA cartilage to produce a larger amount of NO than the normal controls, both in spontaneous and IL-1-stimulated conditions. These data support the notion that, *in vivo*, OA chondrocytes are stimulated by factors, possibly IL-1, which in turn may induce the expression of NO synthase, thus the synthesis of NO itself. Importantly, our results showed that the elevation of NO production may be an important factor in the pathophysiology of OA since it can reduce IL-1ra synthesis by chondrocytes. As such, an increased level of IL-1, associated with a decreased IL-1ra level, may be responsible for the stimulation of OA chondrocytes by this cytokine, leading to an enhancement of cartilage matrix degradation.

Key words: Nitric oxide, IL-1ra, Cartilage, Chondrocyte, Osteoarthritis.

Introduction

The erosion of osteoarthritic (OA) cartilage is mediated by the release of catabolic factors by chondrocytes under the influence of proinflammatory cytokines [1]. Both catabolic and antianabolic mechanisms are believed to play a role in the depletion of OA cartilage matrix components. Proinflammatory cytokines, such as IL-1 and TNF-α, are instrumental in inducing both of these mechanisms [1]. These cytokines can induce the synthesis of degradative enzymes such as metalloproteases [2, 3] at the same time that they induce the synthesis of potent enzyme activators such as plasminogen activator and plasmin [4, 5]. Moreover, they may be responsible for reducing the anabolic (repair) process by decreasing the synthesis of cartilage matrix macromolecules such as collagen type II [6] and aggrecan [7]. A relative deficit in one of the major natural inhibitors of the IL-1 effect, interleukin-1 receptor antagonist (IL-1ra), has been shown both in OA and rheumatoid arthritis (RA) [8, 9] to be a possible cause for an increased level of IL-1 activity in arthritic disorders. The exact reason(s) for the discoordinate expression and synthesis of IL-1 and IL-1ra under these pathological conditions remains unknown. However, the possible role of IL-1 in this phenomenon may be important as this cytokine

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was shown to be capable of regulating the synthesis of IL-1ra [10].

Recent studies have demonstrated the presence of an increased amount of nitric oxide (NO) in OA and RA human synovial fluid [11]. Potential intra-articular sources of NO are multiple and include synovial fibroblasts and chondrocytes [12–15]. The chondrocytes possess the inducible form of NO synthase (iNOS) which can be stimulated by inflammatory cytokines such as IL-1 and TNF-α [13–15]. Under IL-1 stimulation, chondrocytes produce NO in amounts comparable to the macrophages [14]. NO appears to mediate the IL-1 proteoglycan synthesis suppression in articular cartilage [16]. These observations bring forth the potential role of NO as a mediator of the pathophysiological changes taking place in arthritis. Moreover, the recent demonstration of the ability of NO synthase inhibitor at reducing arthritic lesions in experimental models [17] suggests the important role that NO may play in arthritic diseases.

The aims of the present study were to document and compare the spontaneous level of NO production by normal and OA cartilage, as well as to examine the effect of IL-1β on the NO production by these tissues. Moreover, since IL-1 is a predominant cytokine in OA [1, 8] and that a relative deficit exists in OA between the synthesis of this cytokine and the synthesis of one of its major natural inhibitors, IL-1ra [8, 9], we explored the possible implication of NO in this imbalance.

Materials and Methods

SPECIMEN SELECTION

Articular cartilage was obtained from the knee joints of patients with primary OA (68 $\pm$ 7 years old, mean $\pm$ S.D.) who underwent knee arthroplasty, or from normal controls (56 $\pm$ 12 years old) at post mortem within 12 h of death. The diagnosis of OA was based on clinical and radiological evaluations [18]. For normal specimens, the individuals had no history of joint disease, and each specimen was examined macroscopically to ensure that only normal tissue was used. Immediately after the resection of the knee, the specimens were kept on ice, washed with a sterile cold physiologic saline solution and dissected under aseptic conditions.

CARTILAGE EXPLANTS

In the first series of experiments, cartilage explants were obtained by dissecting full thickness strips of cartilage across the articular cartilage and processed for culture. The cartilage was cut into pieces (approximately 100 mg), rinsed several times in Dulbecco modified Eagle's medium (DMEM, Gibco Canadian Life Technologies, Burlington, Ontario, Canada), and incubated for 3 days at 37°C in a humidified atmosphere of 5% CO_2/95% air in DMEM containing antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml; Gibco) in the presence of an increasing concentration (0.5–100 units/ml) of human recombinant (rh) IL-1β (Roussel Uclaf, Romainville, France). Each experiment was performed in duplicate. At the end of the incubation period, NO production was determined on the culture medium of each sample.

In the second series of experiments, specimens from OA tibial plateaus were excised from two areas: the fibrillated or lesioned and the non-fibrillated areas. The cartilage was cut into pieces and incubated as above in the presence or absence of 25 units/ml of rhIL-1β.

CHONDROCYTES

Articular cartilage specimens were rinsed, dissected and chondrocytes released as previously described [19]. Briefly, sequential enzymatic digestion was performed with 1 mg/ml pronase (Boehringer Mannheim, Indianapolis, IN, USA) for 1 h followed by 6 h with 2 mg/ml collagenase (Type IA, Sigma, St-Louis, MO, USA) at 37°C in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT, USA) and antibiotics, as above. Cells were seeded at high density (2×10^5 cells/well) in 24-well culture plates (#08-757-156, Corning Costar Corporation, Cambridge, MA, USA), and allowed to grow until confluence (about 3–4 days) in DMEM supplemented with 10% FCS and antibiotics. The culture medium was changed every second day and, 24 h prior to the experiment, the cells were incubated in fresh serum-free medium. In order to ensure the chondrocyte phenotype, only high density primary passage cells were used. At confluence, the chondrocytes were incubated in the presence of increasing concentrations (0.5–100 units/ml) of rhIL-1β in the absence or presence of 200 μM of N^g-monomethyl-L-arginine-monoacetate (L-NMA; Calbiochem-Novabiochem Corp., San-Diego, CA, USA), a well known NO synthase inhibitor, for 3 days at 37°C in humidified conditions. Each experiment was performed in duplicate. The NO production, as well as the IL-1ra level, were determined on the culture medium.

Further experiments were carried out in which

the chondrocytes were seeded as above but in four-well culture plates (#76-037-03, Flow Laboratories, ICN Biomedicals, Mississauga, Ontario, Canada). They were divided into the following groups: (1) no additive (control), (2) 25 units/ml rhIL-1β, (3) 25 units/ml rhIL-1β plus 200 μM L-NMA, (4) 25 units/ml rhIL-1β, 200 μM L-NMA plus 10 mM L-arginine (Sigma), (5) 25 units/ml rhIL-1β plus 1.5 μg/ml indomethacin (Sigma), and (6) 25 units/ml rhIL-1β plus 10 μg/ml cycloheximide (Sigma). The doses corresponded to the final concentration used for incubation. Each drug except indomethacin was dissolved in DMEM. Indomethacin was dissolved in DMEM containing absolute ethanol; the final concentration of ethanol in each well plate culture was 0.5%. For each experiment a control having 0.5% ethanol was done. Results showed no difference between the control with DMEM or ethanol. Media with additives were sterilized by pressure filtration (millex filters-gv 0.22 μ; Millipore Ltd, Mississauga, Ontario, Canada). The incubation period was 24 h at 37°C in humidified conditions. Each experiment was performed in duplicate. The NO production, IL-1ra and prostaglandin E_2 (PGE_2) levels were determined on the culture medium, and the IL-1ra gene expression on the chondrocytes.

NO PRODUCTION IL-1ra AND PGE_2 DETERMINATIONS

NO production was determined by the measurement of the nitrite release using the Griess reaction [20]. Measurement of IL-1ra was accomplished using a specific ELISA from Research and Diagnostics Systems (Minneapolis, MN, USA); the sensitivity was 6.5 pg/ml. PGE_2 was measured using the Amersham scintillation proximity assay (SPA; Amersham Corp., Oakville, Ontario, Canada) system using $|^{125}I|$-PGE_2, according to the manufacturer's protocol. The sensitivity of the assay was 8–10 pg/ml. Assays under each condition were performed in triplicate, following the manufacturer's instructions.

IL-1ra mRNA EXPRESSION: NORTHERN BLOTTING

Total RNA was isolated as previously described [21]. Briefly, chondrocytes were lysed in a preheated buffer (60°C) containing 20 mM sodium acetate, pH 5, 0.5% sodium dodecyl sulfate (SDS) and 1 mM EDTA. The lysate was then extracted twice with preheated phenol (60°C) in 20 mM sodium acetate, pH 5. The aqueous phase was precipitated overnight with three volumes of absolute ethanol at −20°C. After being centrifuged (20 min, 13,000 ***g***, 4°C), the RNA pellet was solubilized in sterile water and the RNA quantitated spectrophotometrically.

Five micrograms of the total RNA were resolved on 1.2% agarose-formaldehyde gel. Twenty-eight S and 18 S ribosomal RNA bands were visualized using ethidium bromide staining. Following transfer to a nylon membrane (Hybond N, Amersham Corp.) in 10 mM sodium acetate buffer, pH 7.8 containing 20 mM Tris and 0.5 mM EDTA, overnight at 4°C, the RNA was cross-linked to the membrane by exposure to U.V. light.

Human IL-1ra probes were constructed by cloning the reverse transcriptase polymerase chain reaction (RT-PCR) amplified fragments from synovial fibroblasts into the *Eco*RV site of pBluescript SK + vector. The RT-PCR was performed according to the instructions from Perkin-Elmer Cetus (Norwalk, CT, USA) and carried out with GENE ATAQ controller (Pharmacia LKB, Mississauga, Ontario, Canada) as previously described [21]. The primer sequences for the sense was 5'-CTCTGGGAGAAAATCCAGCAAG-3' and 5'-CTTCGTCAGGCATATTGGTGAG-3' for the antisense corresponding to positions 149–174 bp and 540–561 bp respectively, of the published sequence [22]. The size of the amplified fragment was 413 bp. The identity of the fragment was verified by treating the PCR product with *Kpn* I. By analyzing the resultant fragments by agarose gel electrophoresis, we obtained the expected (according to the published sequence, 22) bands at 143 and 270 bp.

Prehybridization was performed for 18 h at 68°C in a 5 × SSC buffer (20×: 200 mM sodium citrate, 3 M NaCl, pH 7) containing 50% formamide, 7% SDS, 0.1% N-lauroyl-sarcosine, 2% blocking reagent (purified casein fraction in 100 mM maleic acid and 150 mM NaCl, pH 7.5), 10 μg/ml polyadenylic acid and 50 μg/ml denatured salmon tested DNA. Hybridization was carried out in the same buffer containing 50 ng/ml antisense RNA IL-1ra labeled with digoxigenin-11 uridyl triphosphate (DIG-11-UTP) (Boehringer Mannheim, Laval, Quebec, Canada) for 18 h at 68°C. Stringent serial post-hybridization washes were carried out with a final one in a 0.1 × SSC buffer, followed by a brief washing in a maleate buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) containing 0.3% Tween-20 (Sigma). The detection was done with Lumigen PPD [4-methoxy-4-(3-phosphatephenyl) spiro-(1,2-dioxetane-3,2'-adamantane) disodium salt] as substrate for alkaline phosphatase conjugated to anti-DIG antibody Fab-fragments, as described in the Boehringer Mannheim user's guide (Boehringer Mannheim GM 6H, Germany, 1993).

The membranes were then subjected to autoradiography using Kodak XAR5 films (Eastman

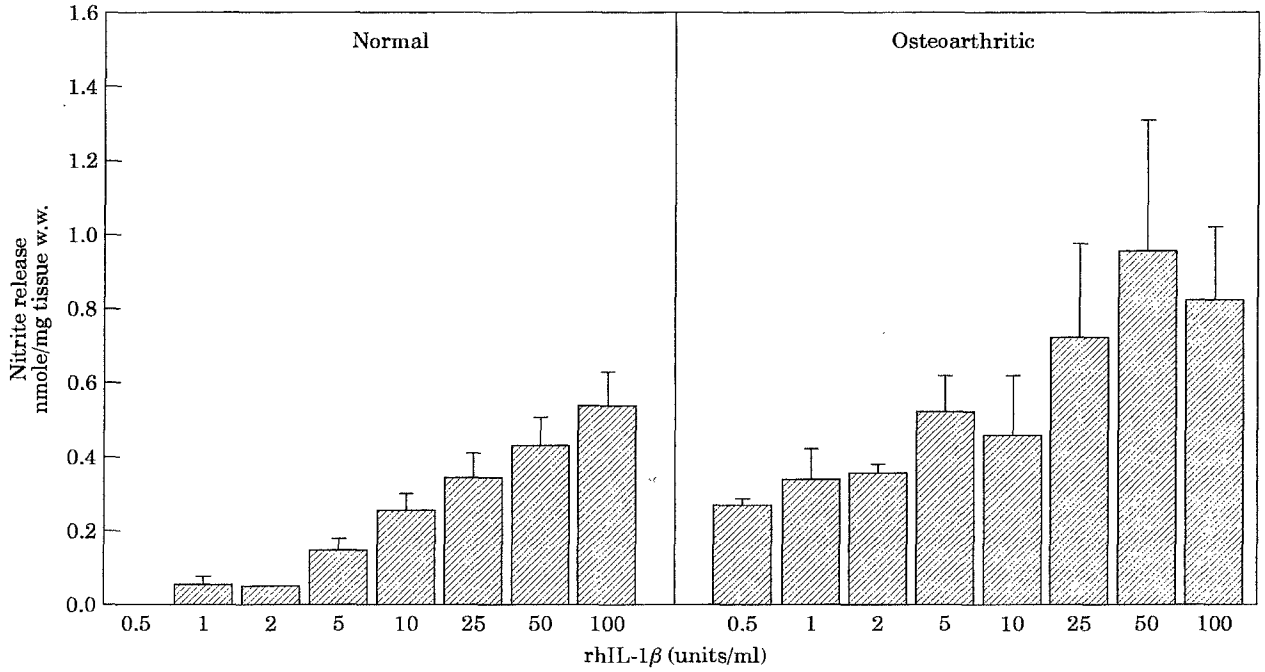

FIG. 1. Human normal ($N = 3$) and OA ($N = 3$) knee cartilage explants were cultured in medium for 3 days in the presence of an increasing amount of rhIL-1β (0.5–100 units/ml). The level of NO production was measured via the amount of nitrite release in the medium, following the Greiss reaction. Values are expressed per mg tissue wet weight, and data represented as the mean $\pm$ S.E.M.

Kodak LTD, Rochester, NY, USA) at room temperature. Autoradiography was subjected to laser scanning densitometry (GS-300 Hoefer Scientific Instruments, San Francisco, CA, USA) for mRNA measurement, and the results calculated as the relative expression normalized to 28 S RNA.

Results

Under unstimulated conditions, NO was produced only in trace amounts in normal cartilage explants. Upon rhIL-1β stimulation, the NO production slowly increased, starting at a concentration of 1 unit/ml and in a dose-dependent manner (Fig. 1). In contrast, OA cartilage explants spontaneously produced NO at a level (0.27 ± 0.02 nmole/mg tissue wet weight of nitrite release) similar to the one found in normal controls upon stimulation with 10 units/ml of rhIL-1β. As for normal cartilage, rhIL-1β induced a dose-dependent elevation of NO production. Upon maximal stimulation (IL-1$\beta \sim 50$ units/ml) OA cartilage produced about twice as much NO as normal explants (Fig. 1). Under these experimental conditions L-NMA, at a concentration of 200 μM, induced over 80% inhibition of the NO production

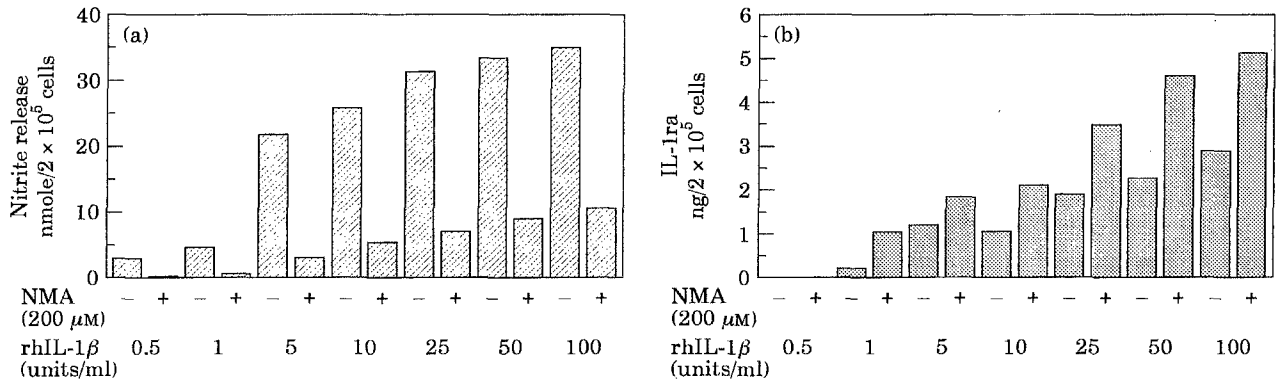

FIG. 2. Human articular cartilage chondrocytes ($N = 2$) in primary monolayer were cultured for 3 days in the presence of inceasing concentrations of rhIL-1β (0.5–100 units/ml) and in the absence or presence of L-NMA (200 μM). Levels of (a) NO production and (b) IL-1ra were measured in the culture medium. Values are expressed per 2×10^5 cells, and data represented as the mean.

(data not illustrated). Knowing that in OA cartilage, the chondrocytes from different location sites may behave differently, OA tibial plateaus were dissected according to the fibrillated or lesional area and non-fibrillated area. The cartilage NO baseline production from the fibrillated area (0.14 ± 0.01 nmole/mg tissue wet weight) tended to be slightly higher than the one from the non-fibrillated sites (0.09 ± 0.03 nmole/mg tissue wet weight). However, upon rhIl-1β stimulation, (25 units/ml) a similar level of nitrite was found for both cartilage sites (fibrillated, 0.54 ± 0.22; non-fibrillated, 0.49 ± 0.14 nmole/mg tissue wet weight).

Preliminary experiments assessing various time periods (0–7 days) as well as L-NMA concentrations (0–1000 μM) revealed that NO production from IL-1β-stimulated chondrocytes increased slowly, reaching a plateau at 3 days of incubation which remained stable until at least the seventh day. L-NMA induced a rapid NO production decline in which 25 μM showed a 60% inhibition and proceeded slowly with a 90% inhibition at 200 μM and 98% at 1000 μM. Therefore, all subsequent experiments were performed with a 3-day incubation period (unless otherwise stated), and using, when appropriate, 200 μM L-NMA.

The production of NO by human chondrocytes under stimulation with IL-1β also demonstrated a dose–response curve which reached a plateau at around 25 units/ml [Fig. 2(A)]. The addition of 200 μM of L-NMA induced a 93% to 70% decrease in NO production from 0.5–100 units/ml of IL-1β-stimulated cells, respectively. Interleukin-1β also induced an increased synthesis of IL-1ra [Fig. 2(B)]. However, when the production of NO was blocked by L-NMA (200 μM), an enhancement of the IL-1ra synthesis was found over the IL-1 control value [Fig. 2(B)]; an average increase of 84% (starting at 5 units/ml IL-1β) in the IL-1ra level was found in the presence of L-NMA, which appeared to correspond to the decrease in the production of NO [Fig. 2(A)].

Furthermore, additional experiments were carried out for which the effect of L-NMA, L-arginine, indomethacin and cycloheximide were evaluated for their effect on NO production, PGE_2 and IL-1ra synthesis [Fig. 3(A)] as well as on IL-1ra gene expression [Fig. 3(B)]. Results showed that L-arginine partially reversed the effect of L-NMA for both NO production and IL-1ra. Indomethacin inhibited over 75% of the PGE_2 synthesis (data not illustrated) but had no effect on the IL-1 -induced nitrite level nor on the IL-1ra synthesis [Fig. 3(A)]. Cycloheximide, on the other hand, suppressed NO production by 60% and IL-1ra synthesis by 94%. As illustrated in Fig. 3(B)] rhIL-1β produced a significant enhancement of the steady state level of IL-1ra mRNA, which was further enhanced when NO production was blocked by L-NMA. As for the protein, the effect of L-NMA on the IL-1ra gene expression was also reversed by the addition of L-arginine. Interestingly, cycloheximide exhibited a 67% inhibition of the IL-1β-induced level of IL-1ra mRNA.

Discussion

In the present study, we demonstrated that OA cartilage has the capacity to spontaneously produce NO, and in a larger amount than in normal cartilage especially when stimulated with IL-1β. These data, support the notion, that *in vivo*, OA chondrocytes are stimulated by factors which may, in turn, induce the expression of NO synthase, thus the synthesis of NO. The factors which may stimulate the NO production by the chondrocytes include several proinflammatory cytokines such as IL-1, TNF-α and LIF (13, 14, 23, 24). At this time, it is tempting to speculate whether IL-1 is the likely candidate involved in this process, as this cytokine is the most abundant and predominant in OA tissues [1]. The *in situ* presence, in OA cartilage [25, 26] of IL-1 may in fact explain why, when the OA explants were stimulated by IL-1, that very little increase of NO production was observed at concentration of IL-1β less than 5 units/ml. Moreover, OA chondrocytes appeared to be hyper-responsive to IL-1 stimulation as twice as much nitrite was found when OA cells were compared to normal. A similar phenomenon has previously been reported by our group with respect to metalloprotease synthesis [19] and seems related to an increased level of IL-1 receptor in OA chondrocytes [19].

Interestingly, the production of NO appeared to be uniform throughout the OA cartilage and only a slight increase was found in fibrillated cartilage. This observation may reflect the fact that the stimuli responsible for the increased production of NO, an upregulation of the inducible NO synthase (iNOS), is a factor which can diffuse within the articular cartilage. In addition, this could also result from a diffusion of an inflammatory cytokine such as IL-1 from the synovium. The fact that chondrocytes from both OA cartilage sites were equally responsive to the IL-1 treatment is strongly indicative that the cells from either area conserved equal capacity to express the iNOS gene. The increased level of NO detected in the synovial fluid of OA and RA patients [27] predominately originates from an increased level of NO production by the cartilage chondrocytes as human

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