

The Expression and Regulation of Nitric Oxide Synthase in Human Osteoarthritis-affected Chondrocytes: Evidence for Up-regulated Neuronal Nitric Oxide Synthase

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Summary

Classically, osteoarthritis (OA) has been considered a noninflammatory disease. However, the detection of selected inflammatory mediators in osteoarthritic fluid, in the absence of significant inflammatory cell infiltrate, is increasingly appreciated. We sought to identify the inflammatory component in human OA-affected cartilage that may be involved in cartilage damage/destruction. Using Western blot analysis and an antibody to the conserved region of nitric oxide synthase (NOS), we have observed up-regulation of NOS, one of the “key players” of inflammation, in chondrocytes of OA-affected patients. Remarkably, none of the cartilage samples examined from normal joints demonstrated detectable amounts of this NOS. Western blot analysis using the same α -NOS antibody indicated that this NOS from OA-affected cartilage (OA-NOS) was larger in size than (and distinct from) transfected human hepatocyte or murine inducible NOS (iNOS) (150 versus 133 kD) and similar in size to neuronal constitutive NOS (ncNOS). Antibodies specific for iNOS showed binding to murine and human iNOS but not to OA-NOS, endothelial constitutive NOS, or ncNOS. Antibodies specific for ncNOS bound to ncNOS and also to OA-NOS, but not to murine or human iNOS or endothelial constitutive NOS. Incubation of OA cartilage in serum-free medium resulted in spontaneous release, for up to 72 h, of substantial amounts of nitrite (up to $\sim 80 \mu\text{M}/100 \text{ mg}$ wet tissue), which could be inhibited by at least 80% with various inhibitors of iNOS, including inhibitors of protein synthesis and transcription factor NF- κ B, but which (unlike murine macrophage iNOS) was not sensitive to hydrocortisone or TGF- β . Exposure of OA-affected cartilage to interleukin 1 β , tumor necrosis factor- α , and lipopolysaccharide resulted in ~ 20 –50% augmentation of nitrite accumulation, which was also sensitive to cycloheximide and pyrrolidine dithiocarbamate. Hence, our data indicate that OA-NOS (based on immunoreactivity and molecular weight) is similar to ncNOS and that it releases nitric oxide, which may contribute to the inflammation and pathogenesis of cartilage destruction in OA.

Osteoarthritis or “osteoarthrosis”: Which term is more appropriate? Classically, osteoarthritis (OA), unlike rheumatoid arthritis (RA), is defined as an inherently non-inflammatory disorder of movable joints characterized by deterioration of articular cartilage and the formation of new bone at the joint surfaces and margins (1). In contrast to RA, the synovial fluid in OA typically contains few neutrophils ($<3,000/\text{mm}^3$), and, except for advanced disease, the synovium does not exhibit significant cellular proliferation or infiltration by inflammatory leukocytes. The molecular pathogenesis of OA is increasingly understood by the elucidation of events within the articular cartilage. For

example, altered dynamic equilibrium between matrix synthesis and degradation by human chondrocytes has recently been implicated as having a primary role in the degeneration of articular cartilage resulting in OA (2, 3). This includes up-regulation of catabolic activities, such as secretion of degradative proteases, and/or down-regulation of anabolic activities such as collagen and proteoglycan synthesis (2, 3). Cytokines such as IL-1 and TNF, which induce nitric oxide (NO) production in human chondrocytes (4), have also been implicated in the destruction of cartilage in OA (3).

In general, NO produced by the constitutive isoform of

nitric oxide synthase (cNOS) is a key regulator of homeostasis, whereas the generation of NO by the inducible NOS (iNOS) plays an important role in inflammation, host defense responses, and tissue repair (5). iNOS has been found in various cell types (5). Exposure of rabbit (6) and human chondrocytes (4, 5) to cytokines and endotoxin leads to accumulation of nitrite in the medium. Studies involving animal arthritis models and analysis of human synovial fluids have implicated NO in the pathogenesis of arthritis (7, 8). Induction of arthritis in rodent models resulted in increased production of nitrite before the onset of clinical symptoms. The appearance of articular symptoms and joint degeneration in these animal models could be inhibited by administration of an NOS inhibitor (7). Neuronal cNOS (ncNOS) has been shown to be present in several cell types (5). Its function outside the vicinity of the neuronal system has recently been recognized, where ncNOS-knockout mice show abnormalities in the gastrointestinal tract similar to pyloric stenosis (9).

Increased concentrations of nitrite have been reported in human synovial fluid from RA and OA patients, suggesting that NO may be a mediator of inflammation in both diseases (8). In the present study, we sought to identify the source of NO in OA and to characterize the respective NOS. In this paper, we report the following: (a) OA-NOS expressed under pathological conditions is similar to ncNOS (and distinct from iNOS) and is not detected in normal cartilage; (b) human OA cartilage spontaneously releases micromolar concentrations of NO; and (c) the prolonged (72-h) accumulation of NO by OA cartilage in *ex vivo* experiments is indicative of NO up-regulating factor(s) within the cartilage, and this phenomenon is sensitive to inhibitors of protein synthesis and of the transcription factor NF- κ B, but not to TGF- β or hydrocortisone.

Materials and Methods

Reagents and Cell Lines. A macrophage-like cell line, RAW 264.7, was obtained from American Type Culture Collection (Rockville, MD). Monoclonal α -mouse macrophage iNOS Ab, monoclonal α -human endothelial cNOS (ecNOS) Ab, and polyclonal α -human ncNOS Ab were obtained from Transduction Laboratories (Lexington, KY); specific polyclonal α -mouse macrophage iNOS Ab were from Affinity Bioreagents, Inc. (Neshanic Station, NJ); protease inhibitors, hydrocortisone, cycloheximide, pyrrolidine dithiocarbamate (PDTTC), aminoguanidine and LPS were from Sigma Chemical Co. (St. Louis, MO); human IL-1 β , TGF- β , and TNF- α were from Fisher Scientific Co. (Pittsburgh, PA); and HN^G-monomethyl-L-arginine monoacetate (L-NMMA) from Cyclo,ps BioChemical Corp. (Salt Lake City, UT).

Isolation of Bovine Chondrocytes. Bovine cartilage was washed after removing the perichondrium, cut into small pieces, and digested with pronase (0.1%) in PBS for 30 min, followed by digestion with collagenase P (0.1%) for 12–16 h in F-12 medium. Cells were then washed and resuspended in the respective media for experiments.

Extraction of Human Chondrocyte NOS and Western Blotting. OA cartilage was obtained from OA patients who underwent knee replacement surgery and were free of steroidal/nonsteroidal

antiinflammatory drugs for at least 2 wk before surgery; nonarthritic normal knee cartilage was obtained from the Musculoskeletal Transplant Foundation (Shrewsbury, NJ) within 24 h of death, or from autopsies the same day. Slices from articular cartilage affected by OA (and normal controls) were frozen at -70°C , milled to fine particles in liquid nitrogen, and sequentially extracted (10 ml/g wet weight tissue) with neutral salt buffer (Tris-HCl:saline) containing protease inhibitors (1 mM PMSF, 2 mM *N*-ethylmaleimide, and 0.025 mg/ml leupeptin), followed with the same buffer containing 10 mM EDTA, with 4 M guanidine-HCl, and then with a detergent buffer containing 10% SDS with protease inhibitors. Samples were run on 9% or 4–15% gradient SDS-PAGE gels under nonreducing conditions, transferred to nitrocellulose, and Western blotted with the required α -NOS Ab. Bound antibody was detected by a secondary antibody conjugated with horseradish peroxidase, and developed using the enhanced chemiluminescence Western blotting system (Amersham Corp., Arlington Heights, IL) on x-ray film (Xomatic; Eastman Kodak Co., Rochester, NY).

Isolation of Recombinant Human Hepatocyte iNOS. Human hepatocyte iNOS was obtained from 3T3 fibroblasts (National Institutes of Health, Bethesda, MD) transduced with a retrovirus carrying the human hepatocyte iNOS cDNA. The construction and characterization of the stable expression of iNOS in these cells have been reported by Tzeng et al. (10).

Results and Discussion

We initiated our studies by standardizing conditions to extract NOS directly from OA-affected articular cartilage before cell isolation. OA cartilage was sequentially extracted with various buffers and Western blotted using a monoclonal α -mouse macrophage iNOS Ab that cross-reacts with human hepatocyte iNOS. Approximately 90% of NOS from OA-affected cartilage (OA-NOS) was present in the neutral salt fraction, \sim 10% was seen in the EDTA-containing buffer, and no OA-NOS was detected in either the guanidine or detergent extracts (Fig. 1 *a*).

To further evaluate the expression of OA-NOS, equal amounts of extracts from OA-affected and nonarthritic articular cartilage were subjected to Western blot analysis using the same α -mouse macrophage iNOS mAb. All 12 patients with OA demonstrated expression of a 150 kD NOS, whereas no NOS was detected from nonarthritic control cartilage (Fig. 1 *b*). The OA-NOS detected in all 12 patients had a M_r of 150 kD, which was distinct from mouse macrophage iNOS. This experiment demonstrates that, under pathological conditions, the OA-affected cartilage expresses an isoform of NOS distinct in size from iNOS. The absence of NOS activity (nitrite release) in normal resting human chondrocytes has also been reported by other investigators (4), in accord with our own observation that expression of iNOS or OA-NOS is not detectable in normal cartilage or chondrocytes.

The M_r of the human OA-NOS was further compared with other known isoforms of NOS by Western blot analysis (Fig. 2). At the same time, various antibodies were tested for their cross-reactivity with OA-NOS and other known isoforms of NOS. As expected, a monoclonal α -mouse macrophage iNOS Ab (raised against the peptide

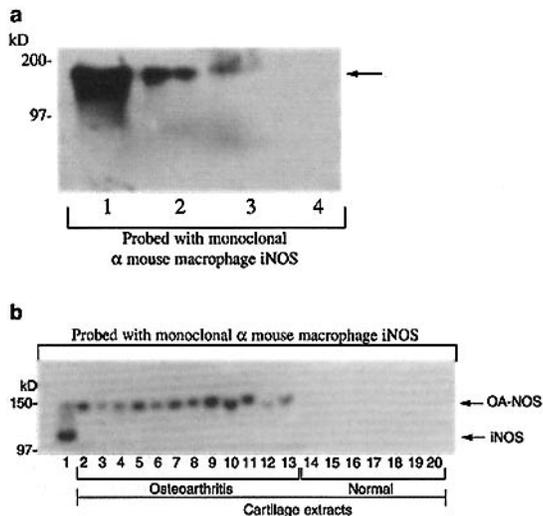


Figure 1. (a) Western blot analysis of NOS on 9% SDS-PAGE. Various extracts from OA cartilage were prepared sequentially as described in Materials and Methods. Lane 1, neutral salt extract; lane 2, EDTA extract; lane 3, 4 M guanidine-HCl extract; lane 4, detergent extract. (b) Neutral salt extracts from knee articular cartilage of 12 OA patients (lanes 2–13) and 7 nonarthritic (normal) individuals (lanes 14–20). Lane 1 shows iNOS from murine macrophages (RAW 264.7) stimulated with LPS for 24 h. iNOS was detected with an α -murine macrophage iNOS mAb. Extracts were prepared from equal wet weight of cartilage samples, and equal amounts were loaded in each lane.

sequence 961–1144), showed reactivity with 133-kD human hepatocyte iNOS and murine macrophage iNOS. This monoclonal α -mouse macrophage iNOS Ab also bound to 150-kD human OA-NOS and rat ncNOS (Fig. 2 a), thus indicating that this α -iNOS mAb also cross-reacts with ncNOS. However, a polyclonal Ab raised against the amino acid sequence 1095–1289 from human ncNOS bound to 150-kD human OA-NOS (and also, as expected to 150-kD rat ncNOS), but did not detect the 133 kD iNOS (Fig. 2 b). A polyclonal α -mouse macrophage iNOS Ab (raised from the peptide sequence 1131–1144) bound to 133-kD mouse macrophage and human hepatocyte iNOS, but not to 150-kD human OA-NOS or rat ncNOS (Fig. 2 c). None of these antibodies showed any reactivity with extracts obtained from human umbilical vein endothelial cells (HUVEC). This HUVEC extract was tested by probing it with an α -ecNOS antibody, which showed a 133-kD ecNOS band (data not shown). The monoclonal α -mouse macrophage iNOS Ab also cross-reacts with 133-kD bovine and rodent chondrocyte iNOS (unpublished data). These results show that the 150-kD human OA-NOS and ncNOS, which are similar in size (Fig. 2), are distinct from iNOS from human hepatocytes (native and transfected), human B cells, bovine and rodent chondrocytes, and murine macrophages (10–12), which are 133 kD (Fig. 2). We conclude that the OA-NOS is similar to ncNOS, based on their similar size, their shared reactivity to antibodies specific for 150 kD ncNOS, and their lack of reactivity to polyclonal α -iNOS antibodies specific for the 133-kD iNOS.

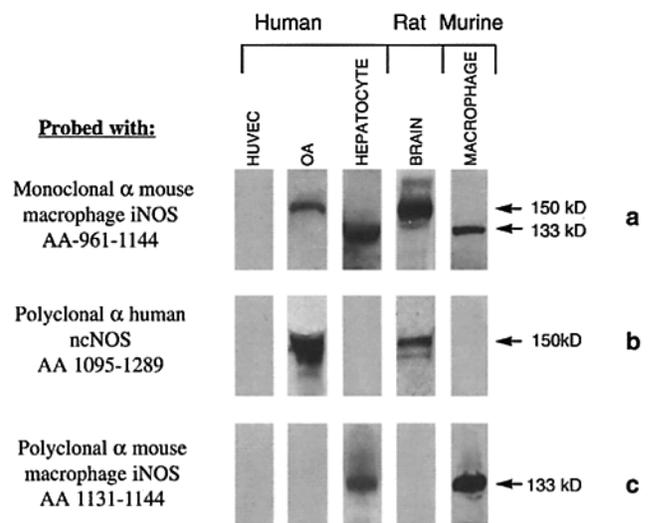


Figure 2. Western blotting of various cell extracts with antibodies to NOS. Cell extracts from HUVEC, human OA cartilage (OA), hepatocyte iNOS transfected into 3T3 cells (HEPATOCYTE), rat brain (BRAIN), and murine macrophages stimulated with 100 ng/ml LPS for 16 h (MACROPHAGE) were prepared and run on 5–15% SDS-PAGE gel, and Western blotted with various antibodies as described in Materials and Methods. Panels A, B, and C were probed with various α -NOS Ab as described. The amino acid region from which each of these antibodies was raised is designated in the figure as AA. The data represent one of two identical representative experiments.

These observations pose an obvious question: What is the role of human chondrocyte iNOS in OA? It should be noted that two groups (13, 14) have cloned the human chondrocyte iNOS from cDNA libraries prepared from (in vitro) IL-1-stimulated human chondrocytes. These libraries were screened with a degenerative oligonucleotide or murine iNOS cDNA. The OA-NOS seems to be distinct from the human chondrocyte iNOS. Apparently, human chondrocytes express at least two isoforms of NOS that are differentially regulated, namely iNOS (13, 14) and OA-NOS. Therefore, we propose that the known NO-mediated chondrocyte dysfunction that could lead to OA is the result of up-regulation of OA-NOS.

To evaluate the biological activity of OA-NOS, we standardized parameters to set up organ culture of OA-affected cartilage in ex vivo conditions. We observed that ~50–100 mg of OA cartilage from various patients released ~10–80 μ M of nitrite after 48 h in serum-free medium, which could be inhibited by >90% with 500 μ M L-NMMA (Fig. 3 a) or aminoguanidine (not shown). Interestingly, in a single case in which cartilage was obtained from both knees, similar levels of nitrite were observed (Fig. 3 a, patient samples Nos. 3a and 3b). These experiments suggest that OA-affected chondrocytes generate micromolar concentrations of NO that have been associated with degradation of articular cartilage (8). Exposure of the OA cartilage in vitro to pharmacological concentrations of IL-1 β , TNF- α , and endotoxin resulted in a consistent increase in NO production at 24 h (Fig. 3 b) and a more pronounced effect at

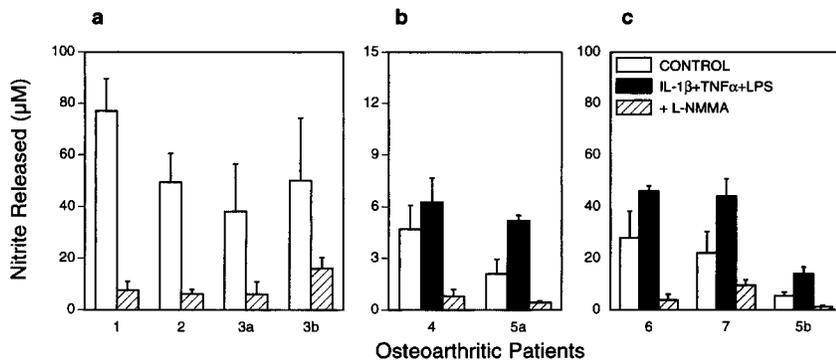


Figure 3. (a) Release of nitrite by OA cartilage in ex vivo conditions after 48 h. Knee articular cartilage from three OA patients was cut into 3-mm discs; 4–6 discs were placed in organ culture in 2 ml medium in the presence and absence (CONTROL) of 500 μM L-NMMA. 3a and 3b were samples obtained from left and right knees of the same patient. Data are expressed as μM nitrite released \pm SD ($n = 4$). The P values between Control and L-NMMA-treated cultures were: 1 < 0.013 ; 2 < 0.002 ; 3a < 0.066 ; 3b < 0.048 . (b) Release of nitrite by OA cartilage in ex vivo conditions after 24 h in the presence and absence of LPS (100 $\mu\text{g}/\text{ml}$), IL-1 β (1 ng/ml), and TNF- α (1 ng/ml). Knee articular cartilage from one OA patient was cut into 3-mm discs; 3–4 discs were

placed in organ culture in 2 ml medium in the presence and absence (CONTROL) of 500 μM L-NMMA. One set of cultures was incubated with LPS, IL-1 β , and TNF- α . Data are expressed as micromoles of nitrite released \pm SD ($n = 7$). The P values between Control and L-NMMA-treated cultures were: 4 < 0.002 ; 5a < 0.001 . The P values between Control versus LPS-, IL-1 β -, and TNF- α -treated cultures were: 4 < 0.0585 ; 5a < 0.001 . (c) Release of nitrite by OA cartilage in ex vivo conditions after 48 h in the presence and absence of cytokines and endotoxin. The experiments were set up as described in Fig. 3 b. Data are expressed as micromoles of nitrite released \pm SD ($n = 4$ –7). The P values between Control and L-NMMA-treated cultures were: 6 < 0.056 ; 7 < 0.099 ; 5b < 0.001 . The P values between Control versus LPS-, IL-1 β -, and TNF- α -treated cultures were: 6 < 0.088 ; 7 < 0.013 ; 5b < 0.001 . Data obtained in 5a and 5b are from the same patient from whom the nitrite was sampled at 24 and 48 h, respectively. Samples obtained at various time intervals were assayed for nitrite by modified Griess reaction (15) using an ELISA reader. The statistics were derived using unpaired Student's t test.

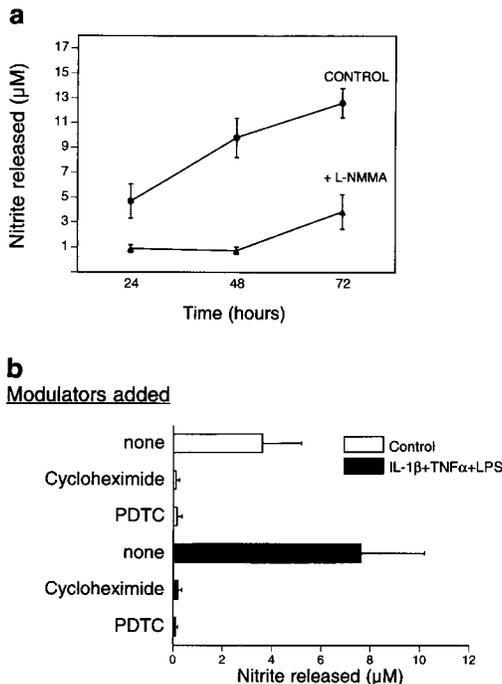


Figure 4. (a) Release of nitrite by OA-affected articular cartilage in ex vivo conditions at different time intervals. Knee articular cartilage from OA patients was cut into 3-mm discs after surgery and placed in Hams-F-12 media, with or without 1 mg/ml BSA, 10 mM HEPES, pH 7.4, together with gentamicin and fungizone (GIBCO BRL, Gaithersburg, MD). Two discs were placed in 1 ml of medium in the presence and absence (CONTROL) of 500 μM L-NMMA. Data are expressed as micromoles of nitrite released \pm SD ($n = 4$ –7). The P values between the Control and L-NMMA-treated cultures for 24, 48, and 72 h were all < 0.002 . (b) Release of nitrite by OA cartilage in ex vivo conditions after 24 h in the presence and absence (Control) of cycloheximide (35 μM) and PDTC (30 μM). OA cartilage was also incubated with LPS (100 $\mu\text{g}/\text{ml}$), IL-1 β (1 ng/ml), and TNF- α (1 ng/ml) in the presence and absence of equivalent amounts of cycloheximide or PDTC. Data are expressed as micromoles of nitrite released \pm SD ($n = 4$). The P value between the Control versus cycloheximide- and PDTC-treated cultures was < 0.001 ; the P

value between cytokine plus endotoxin-treated cultures versus cycloheximide/PDTC was < 0.013 . Samples obtained at various time intervals were assayed for nitrite by modified Griess reaction (15) using an ELISA reader.

48 h (Fig. 3 c). The spontaneous release of NO could be inhibited by L-NMMA as in Fig. 3, a and b. Snyder and co-workers (16) have reported that neuronal NOS is induced in spinal neurons by traumatic injury. Furthermore, human chondrocytes exposed to exogenous NO, or cytokines and endotoxin (at concentrations used in this study), have demonstrated inhibition of growth, chemotactic responses to growth factors, proteoglycan synthesis, and induction of apoptosis (17–19, and unpublished data). Therefore, it is quite conceivable that the up-regulation of OA-NOS is triggered under pathological conditions such as OA, because nitrite levels in the serum of RA (0.44 $\mu\text{mol}/\text{liter}$) and OA (0.21 $\mu\text{mol}/\text{liter}$) patients were significantly elevated in comparison to normal controls (0.14 $\mu\text{mol}/\text{liter}$) (8), whereas synovial fluids from these RA and OA patients demonstrated significant mean nitrite levels of 0.91 and 0.35 $\mu\text{mol}/\text{liter}$, respectively. It should be noted that, unlike murine macrophages (11), human chondrocytes (10⁶) stimulated with cytokines and endotoxin in vitro release nanomolar concentrations of nitrite (4, 20).

To further investigate the regulation of OA-NOS in OA cartilage, a time course release of nitrite was performed. OA cartilage incubated with serum-free medium demonstrated a steady accumulation of nitrite up to 72 h (Fig. 4 a), indicating that production of nitrite is “spontaneously” released and may be constantly stimulated in OA cartilage. This nitrite production was inhibited by L-NMMA (Fig. 4 a). The spontaneous accumulation of nitrite by OA-affected cartilage was evaluated for its sensitivity to cycloheximide and NF- κB inhibitor PDTC. Addition of 3.5 and 17.5 μM

of cycloheximide to OA cartilage showed >90% inhibition of nitrite accumulation as compared with controls within 24 h. The data in Fig. 4 *b* show a representative experiment in which addition of cytokines and endotoxin augmented the release of nitrite in the medium. Nitrite accumulation was significantly blocked by >90% in both the cytokine- and endotoxin-induced cartilage and control cartilage in the presence of cycloheximide or PDTC, thus indicating that the OA-affected chondrocyte NOS activity is sensitive to cycloheximide and PDTC with respect to production of nitrite in ex vivo cultures. The inhibition by PDTC and cycloheximide of nitrite accumulation in OA cartilage may be due to inhibition of de novo protein synthesis of OA-NOS itself, cofactors involved in the regulation of OA-NOS, or both—a hypothesis that must be tested. However, it should be noted that the human nNOS gene has two NF- κ B-like sequences in the 5' untranslated region (21). Incubation of OA-affected articular cartilage, either in basal medium alone or supplemented with BSA, had insignificant impact on the release of nitrite (data not shown). In similar and parallel experiments using normal adult bovine articular cartilage or murine RAW 264.7 cells, we did not observe any detectable amounts (>1 μ M) of nitrite after 48 h (data not shown), indicating that the medium used in these experiments was devoid of any stimulating agent that may have contributed to the up-regulation of NOS in OA cartilage on days 2 and 3 in the ex vivo experiments. As expected, addition of LPS (100 ng/ml and 100 μ g/ml) showed an accumulation of nitrite in the medium after 20 or 48 h in both murine RAW 264.7 cells and bovine articular cartilage, respectively (data not shown).

These studies also indicate that a PDTC/cycloheximide-sensitive “NO-up-regulating factor(s)” may be present within the OA cartilage. Potential up-regulator(s) of OA-NOS may be one or a combination of the following manifestations: (a) autocrine cytokines/growth factors produced by chondrocytes in OA cartilage; (b) interaction with cell surface receptor (glutamate receptors/CD53) or matrix components (fibronectin and collagen) that can modulate NOS (22, 23); (c) diffusion of soluble (paracrine) factors into the cartilage in vivo from other cellular sources of the intra-articular region (e.g., endothelial cells, lining of the synovial capillaries, local inflammatory cells, and/or synovial fibro-

blasts); (d) abnormal mechanical forces seen by the chondrocytes in the three-dimensional architecture, because shear stress is reported to release nitrite from articular chondrocytes (24).

We further evaluated the effect of hydrocortisone and TGF- β , known modulators of iNOS (5, 25), on OA-NOS expression in ex vivo conditions. Unlike the effects of cycloheximide and PDTC, there was no significant effect of 10 μ M of hydrocortisone (10.3 ± 4.9) or 2.5 ng/ml of TGF- β (8.7 ± 2.3) on the spontaneous release of nitrite ($9.6 \pm 1.5 \mu$ M) from OA cartilage in organ cultures, whereas LPS-stimulated RAW 264.7 cells showed a significant decrease (6.4 ± 0.5 and $2.8 \pm 0.7 \mu$ M, $n = 3$; $P < 0.001$) in nitrite released in the presence of equivalent amounts of hydrocortisone and TGF- β , respectively (5, 25). These experiments show that some of the regulatory properties of OA-NOS are distinct from iNOS. This may be because the rodent chondrosarcomas express a 133 kD iNOS (Amin et al., unpublished data). Although TGF- β and hydrocortisone may not have a direct effect on OA-NOS expression, they are known to contribute to chondrogenesis and to repair and healing mechanisms in the cartilage (26). Furthermore, previous studies by Thorbecke and co-workers (27) have demonstrated that injecting mice with TGF- β suppresses collagen-induced arthritis, thus indicating that the role of TGF- β (other than its ability to suppress iNOS activity in the rodent system) may also be to suppress inflammatory processes such as infiltrating T cells. However, targeting of 133-kD iNOS for pharmacological intervention in animal models of arthritis in vivo, or bovine chondrocytes in vitro, should proceed with caution, keeping in mind the characteristic features of human OA-NOS.

In summary, articular chondrocytes are a source of increased levels of intraarticular NO that have been speculated in OA patients (8). Our data further support the notion that NO, a known inflammatory mediator, is released in sufficient quantities (4, 8) to cause chondrocyte dysfunction and to damage cartilage integrity, and thus may be one of the key mediators in the pathogenesis of OA. Further study of the mechanisms governing NOS expression and of the consequences of increased NO production by OA-affected cartilage will help to define potential targets for pharmacological intervention and gene therapy.

We thank the Musculoskeletal Transplant Foundation for providing normal human cartilage, and Dr. Anthony Recupero, Ms. Susan Pesce, and Dr. Frank McNeil for kindly providing antibodies, cytokines, and L-NMMA, respectively. We thank Ms. Ann Rupel for typing and editing the manuscript.

This study was supported by institutional funds from the Hospital for Joint Diseases and, in part, by the Joseph and Sophia Abeles Foundation.

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Received for publication 30 November 1994 and in revised form 16 June 1995.

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