Effect of NOS2 Gene Deficiency on the Development of Autoantibody Mediated Arthritis and Subsequent Articular Cartilage Degeneration

HISAYOSHI KATO, KEIICHIRO NISHIDA, AKI YOSHIDA, ITSURO TAKADA, CHERIE McCOWN, MASATSUGU MATSUO, TAKURO MURAKAMI, and HAJIME INOUE

ABSTRACT. ***Objective.*** To determine the effect of NOS2 gene deletion on articular cartilage degradation in autoantibody mediated arthritis (AMA).

Methods. Female C57BL/6Ai-[ko] NOS2 N5 (NOS2–/–) mice (7–8 weeks old) and the counterpart C57/Bl6 Crj mice (wild-type, WT) were studied. Arthritis was induced by intraperitoneal injection of 4 mg of an arthritogenic cocktail of 4 monoclonal antibodies raised against type II collagen twice on Day 0 and Day 1 followed by intraperitoneal injection of 50 µg of lipopolysaccharide on Day 2. Individual limbs were scored for arthritis in 4 grades; the total maximum score per mouse was 16. Femoral condyles and tibial plateaus of both knee joints were collected on Day 15 for immunohistological studies on nitrotyrosine and matrix metalloproteinase (MMP)-3 and -9. DNA fragmentation in chondrocytes was detected by the nick-end labeling (TUNEL) method. Blood was also collected on Day 15 to determine serum levels of nitrite/nitrate and interleukin 1ß (IL-1ß).

Results. Both NOS2–/– and WT mice with AMA developed clinically apparent arthritis. In WT mice, the arthritis progressed rapidly and reached the peak score 11.4 ± 2.9 on Day 12, whereas the arthritis in NOS2–/– mice was milder and the peak score was 7.7 ± 2.8 on Day 13 ($p < 0.05$). The serum nitrite/nitrate levels, histological grades of articular cartilage degradation, and numbers of apoptotic chondrocytes and nitrotyrosine positive chondrocytes were significantly lower in NOS2–/– mice with AMA than in WT mice with AMA. Conversely, significant differences were not observed in MMP-3 or -9 expression in chondrocytes, or in serum IL-1ß levels between these 2 groups of mice.

Conclusion. NOS2 gene deletion did not affect the inflammatory responses, but reduced the cartilage degradation. (J Rheumatol 2003;30:247–55)

Key Indexing Terms:
CARTILAGE NITRIC OXIDE NOS2 AUTOANTIBODY MEDIATED ARTHRITIS

Nitric oxide (NO), a highly reactive free radical, has been implicated as an important mediator of immune and inflammatory responses[1]. Increased NO concentrations have been reported in serum or synovial fluid from patients with a variety of rheumatic diseases, including rheumatoid arthritis (RA)[2], osteoarthritis (OA)[3], and Sjögren's syndrome[4], suggesting that activation of the NO pathway might augment the disease activity in humans. It has also been reported that the development of arthritis is accompanied by induction of inducible forms of NO synthase (iNOS/NOS2) in experimental arthritis. The production of NO was observed in synovial cells[5], chondrocytes[6,7], endothelial cells[8], and blood mononuclear cells[9] in response to inflammatory cytokines such as interleukin 1ß (IL-1ß) and tumor necrosis factor (TNF-α). Based on these observations, it appears that NOS2, which catalyzes much larger amounts of NO than constitutive forms of NOS, might amplify inflammatory pathways and contribute to tissue damage [10].

Despite numerous studies of NOS inhibitors in experimentally induced arthritis[11-20], there is little information on the influence of NOS inhibitors on articular cartilage degeneration in arthritic joints. *In vitro* studies have shown that NO secreted excessively by chondrocytes in response to catabolic cytokines, or presumably abnormal mechanical stress[21], plays a variety of regulatory roles in inflammatory reactions, such as (1) inhibiting proteoglycan and type II collagen synthesis[22], (2) increasing susceptibility to oxidant injury[23], (3) stimulating production and activation of matrix metalloproteinase (MMP)[24,25], (4) inactivating tissue inhibitor of metalloproteinases[26], (5) downregulating IL-1 receptor antagonist expression[27], (6) interfering with chondrocyte migration and attachment to fibronectin[28,29], and (7) inducing apoptosis[30-33]

From the Department of Orthopaedic Surgery, and Second Department of Anatomy, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan.

Supported by a Health Science Research Grant from the Ministry of Health and Welfare of Japan.

H. Kato, MD; K. Nishida, MD, PhD, Assistant Professor; A. Yoshida, Laboratory Technician; I. Takada, MD; C. McCown, MA; M. Matsuo, MD; H. Inoue, MD, PhD, Professor, Department of Orthopaedic Surgery; M. Murakami, MD, PhD, Professor, Second Department of Anatomy, University Graduate School of Medicine and Dentistry.

Address reprint requests to Dr. K. Nishida, Department of Orthopaedic Surgery, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama City, Okayama 700-8558, Japan. E-mail knishida@po.harenet.ne.jp

Submitted November 1, 2001; revision accepted July 22, 2002.

of chondrocytes that subsequently trigger articular cartilage degeneration. In an experimental OA model, Pelletier, *et al* demonstrated that L-NIL has a beneficial effect in protecting cartilage destruction by reducing the activity of MMP in cartilage tissue and by inhibiting IL-1ß production of synovium[34]. In addition, van den Berg, *et al*[35] found that NOS2 deficiency prevented the inhibition of chondrocyte proteoglycan synthesis in zymosan induced monoarticular arthritis (ZIA). Their histological studies have shown there were significantly fewer cartilage lesions and osteophyte formations in NOS2–/– mice with ZIA than in NOS2 positive normal controls.

In this study, we used NOS2 gene deficient mice to examine the effects of NO on articular cartilage destruction in an inflammatory arthritis model. We used a combination of subarthritogenic levels of monoclonal antibodies (Mab, a cocktail of 4 clones) raised against type II collagen and bacterial lipopolysaccharide (LPS)[36,37] to induce rapid, destructive arthritis.

MATERIALS AND METHODS

Experimental groups. Female NOS2–/– mice (C57BL/6Ai-[ko] NOS2 N5; Taconic Farms Inc., Germantown, NY, USA), 7 to 8 weeks old, and age matched female WT C57BL/6 Crj mice (Charles River Japan, Inc., Yokohama, Japan) were used. They were fed a standard commercial diet and tap water *ad libitum* at the Laboratory Animal Center for Biochemical Research, Okayama University Graduate School of Medicine and Dentistry, under standard conditions.

Protocol for induction of arthritis. Arthritis was induced by a combination of Mab specific for type II collagen and LPS according to Terato's method[36]. An arthritogenic cocktail of the 4 Mab (Chondrex Inc., Redmond, WA, USA) recognized the independent epitopes clustered within the CB11 fragment of type II collagen, thus are capable of fixing complements and subsequently inducing severe arthritis in variety of strains of mice regardless of their MHC types. NOS2–/– and wild-type (WT) mice were injected intravenously with 4 mg Mab on Day 0 and Day 1, followed by intraperitoneal injection of 50 µg LPS on Day 2.

Clinical evaluation of arthritis. The mice were monitored for development of arthritis every day after the first Mab injection. Arthritis was scored into 4 grades according to the method of Terato, *et al*. Each limb was graded individually, thus the maximum cumulative clinical arthritis score per mouse was 16 points, scored as follows: 0 = normal, 1 = mild but definite redness and swelling of the ankle or wrist or redness and swelling of any severity in any single digit, 2 = moderate to severe redness and swelling of ankle and wrist, 3 = redness and swelling of the entire foot including digit, 4 = maximally inflamed limb with involvement of multiple joints.

Blood and knee joint tissue collection. Blood was collected at necropsy on Day 15 and centrifuged at 3000 rpm for 10 min at 4°C, and the plasma was stored at –20°C until examination. Mice were perfused with 4% paraformaldehyde after blood collection and the knee joints were dissected on Day 15 and fixed in the same solution for 24 h. The samples were divided into 2 blocks in the sagittal plane, and decalcified through 0.3 M EDTA (pH 7.5) for 7–10 days, dehydrated by graded series of ethanol, and embedded in paraffin[38].

Histological analysis of knee joints. Standard sagittal sections of 4.5 µm were prepared and stained with safranin O and counterstained with fast green. Histological examinations for cartilage damage were performed by 2 independent blinded observers, and graded according to Freemont's system[39]; i.e., grade 0 = normal cartilage; 1 = cartilage surface irregularities and loss of metachromasia adjacent to superficial chondrocytes; 2 = fibrillation of cartilage and the formation of some chondrocyte clusters, with only minor loss of surface cartilage; and 3 = gross cartilage abnormalities including loss of superficial cartilage, extension of fissures close to subchondral bone, and a large number of chondrocyte clusters.

TUNEL staining. Deparaffinized sections were pretreated with bovine testicular hyaluronidase (1 mg/ml) dissolved in 0.1% phosphate buffered saline (PBS) for 60 min at 37°C. The endogenous peroxidase was then blocked by immersing the tissue section in 0.3% H_2O_2 in PBS at room temperature for 30 min. DNA fragmentation in chondrocytes was detected by terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine triphosphate (dUTP) biotin nick-end labeling (TUNEL) methods. Sections were counterstained with methyl green. Tissue sections incubated without TdT served as the negative control.

Immunostaining for nitrotyrosine (NT) and MMP-3 and -9. For immunostaining of NT, deparaffinized and dehydrated sections were pretreated in a microwave oven at 650 W for 15 min in 0.01 M citrate buffer (pH 6). For immunostaining of MMP-3 and -9, the tissue sections were treated with 20 µg/ml and 10 µg/ml, respectively, of proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) for 60 min at room temperature. The endogenous peroxidase was blocked by immersing the specimens in 0.3% H_2O_2 in PBS, and then treated with normal goat serum. Rabbit polyclonal anti-NT antibody (6 µg/ml; Argonex, Lake Placid, NY, USA), anti-MMP-3 antibody (10 µg/ml), or anti-MMP-9 antibody (10 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as primary antibodies and incubated with specimens at room temperature for 30 min, and then washed with PBS. Then they were incubated with biotinylated goat anti-rabbit antibody (7.5 µg/ml) at room temperature for 30 min. Tissue sections were visualized by diaminobenzidine (DAB) and counterstained with methyl green. Stained sections incubated with normal rabbit nonimmune serum or those incubated without primary antibodies were used as negative controls.

Semiquantitative analysis of TUNEL, NT, and MMP positive cells. In the TUNEL analysis and NT and MMP-3 and -9 immunostaining, the chondrocytes in which cytoplasm or nuclei stained definitely or diffusely were counted as positive, regardless of the staining pattern. The number of TUNEL, NT, and MMP-3 and -9 positive cells was counted individually in 3 different areas of both femoral condyle and tibial plateau under a microscope (×400) and averaged. The percentage of positive chondrocytes was determined by dividing the number of positive chondrocytes by the total number of chondrocytes in all cartilage layers within the section.

Assay for serum IL-1ß and nitrite/nitrate. The serum levels of IL-1ß and nitrite/nitrate were determined using the Mouse ELISA IL-1ß Kit (Endogen Inc., Woburn, MA, USA) and the Total Nitric Oxide Assay Kit (R&D Systems, Minneapolis, MN, USA), respectively, according to the manufacturer's instructions. The sensitivity of assay was 0.1 pg/ml for IL-1ß and 10 µM for nitrite/nitrate. Fluorescence intensity was measured at wavelength 540 nm using a fluorescence plate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. Data were expressed as mean ± SD. Statistical analyses were performed using the Mann-Whitney U test with Macintosh StatView-J 5.0 (SAS Institute Inc., Cary, NC, USA); p values < 0.05 were considered significant.

RESULTS

Development of autoantibody mediated arthritis. Clinically apparent arthritis, characterized by marked swelling or redness of limb joints, developed in both NOS2–/– mice and WT mice one day after LPS injection. Arthritis in the WT mice with AMA progressed rapidly and markedly compared to NOS2–/– mice with AMA (Figure 1). The clinical symptoms of active arthritis reached a peak on Day 12 in WT mice with AMA, and scored 11.4 ± 2.9 points. The arthritis score in NOS2–/– mice with AMA at peak on Day 13 was 7.7 ± 2.8.

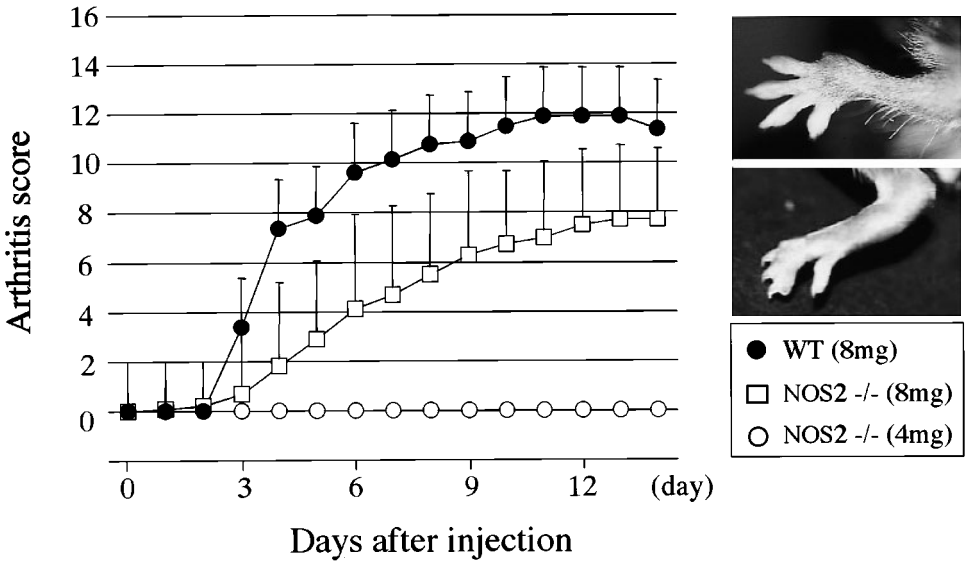

Figure 1. Clinical scores for AMA in wild-type (WT) and NOS2–/– mice over time. Eight WT mice and 10 NOS2–/– mice were then injected intraperitoneally with 8 mg of Mab to type II collagen on Day 0 and Day 1 (4 mg each) and with 50 µg of LPS on Day 2. Clinical arthritis was not observed in NOS2–/– mice that received injection of 4 mg Mab and 50 µg LPS. The clinical symptoms from Day 4 to Day 14 were significantly lower in the NOS2–/– mice (8 mg Mab) compared with WT mice ($p < 0.05$). Inset: photos from WT mice with arthritis on Day 12 (arthritis score 4); upper panel, a forepaw, lower panel, a hind paw.

The clinical symptoms from Day 4 to Day 14 were significantly lower in the NOS2–/– mice with AMA (8 mg Mab) compared with the WT mice with AMA ($p < 0.05$).

Microscopic findings. Most of the knee joint tissues from the WT mice with AMA showed marked pathological changes, including synovial hyperplasia with a large number of infiltrated inflammatory cells, and pannus formation at the cartilage–bone junction. However, these histological changes were relatively mild in NOS2–/– mice with AMA (Figure 2). The number and percentage of samples in each grade are summarized in Table 1. Mann-Whitney U test showed that the histological grade of articular cartilage of NOS2–/– mice with AMA was significantly lower than that of WT mice with AMA, both in femoral condyles and tibial plateaus ($p < 0.001$).

TUNEL staining. TUNEL positive chondrocytes were localized in the superficial and deep layers of articular cartilage in both WT and NOS2–/– mice with AMA. The number of TUNEL positive chondrocytes from the femoral condyle and tibial plateau in NOS2–/– mice with AMA was significantly lower than that of WT mice ($p < 0.01$, $p < 0.001$, respectively) (Table 2, Figure 3A, 3C). However, the percentage of TUNEL positive chondrocytes in the cartilage from control mice without arthritis was 7.2 ± 1.5% within the femoral condyle and 8.8 ± 0.6% within the tibial plateau, values that were significantly lower than in the cartilage from WT and NOS2–/– mice with AMA (Figure 3E).

Nitrotyrosine staining. NO production of chondrocytes was evaluated indirectly by determining NT by the immunohistochemical method using rabbit polyclonal anti-NT antibodies. The NT positive chondrocytes and TUNEL positive chondrocytes were localized from the superficial to the deep layers of articular cartilage in the cartilage sections from both WT and NOS2–/– mice with AMA. The number of NT positive chondrocytes from the femoral condyle and tibial plateau in the NOS2–/– mice was statistically lower than that in the WT mice ($p < 0.01$) (Table 2, Figures 3B, 3D).

MMP-3 and MMP-9. There was a marked increase in MMP-3 and MMP-9 positive chondrocytes in the superficial and deep layers of cartilage sections from both WT and NOS2–/– mice (Table 2, Figure 4). Conversely, no significant difference was observed in the number of MMP-3 and -9 positive cells between WT and NOS2–/– mice, even though the arthritis score in WT mice was higher than that in NOS2–/–.

Serum IL-1ß and nitrite/nitrate concentrations. The serum IL-1ß levels on Day 15 in WT and NOS2–/– mice with AMA were 65.3 ± 10.0 and 62.2 ± 5.2 pg/ml, respectively, and no significant difference was observed between the 2 groups. However, these values were much higher than those in controls without arthritis ($p < 0.001$), indicating that IL-1ß concentrations may reflect the inflammatory reactions at joints, and partially contribute in upregulating MMP expression by chondrocytes (Figure 5A). Similarly, serum levels of nitrite/nitrate were significantly elevated to 133.4 ± 24.9 µM in the WT mice with AMA compared to 60.1 ± 25.7 µM in controls ($p < 0.02$). Serum nitrite/nitrate levels in the

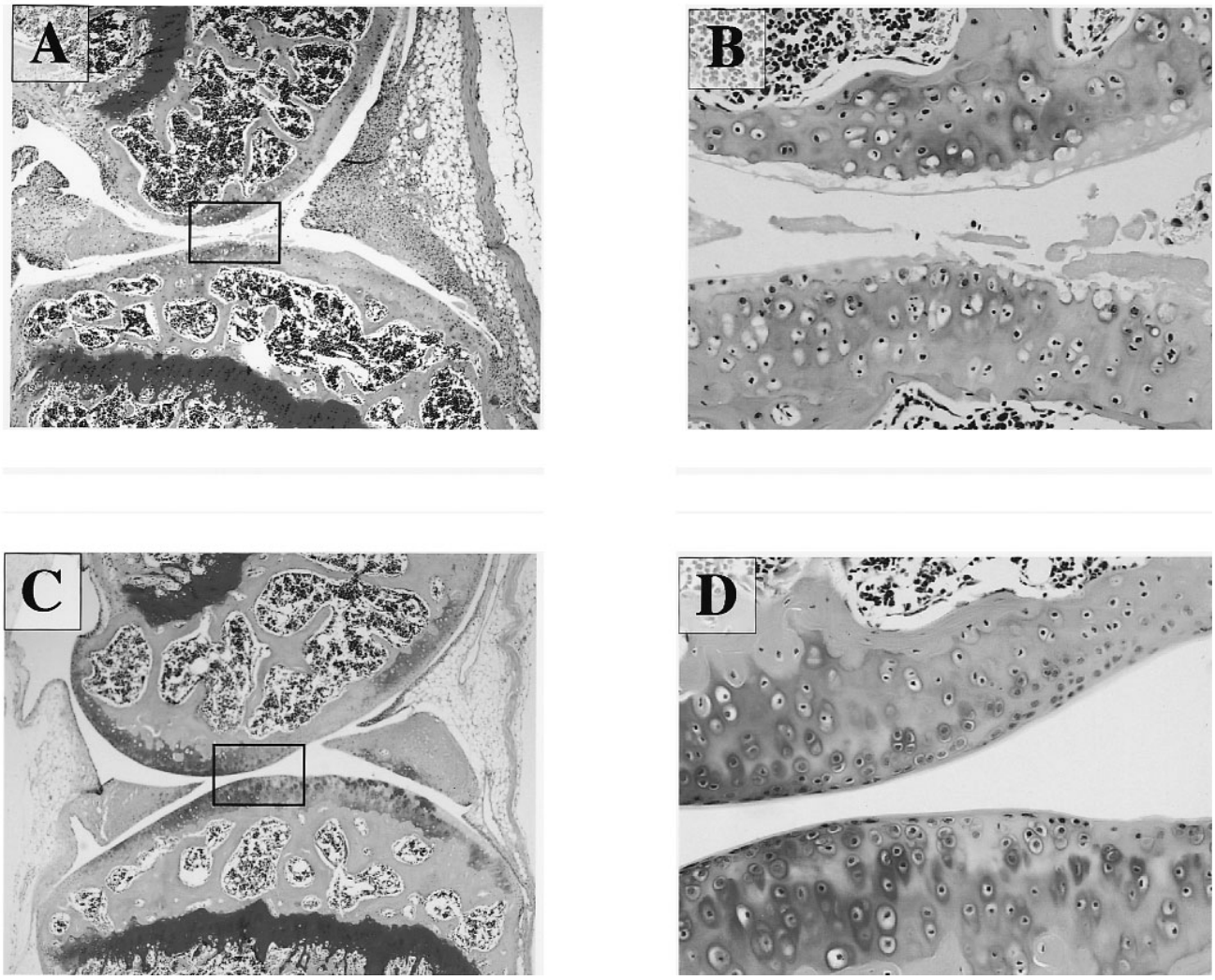

Figure 2. Histology of the knee joint (safranin O staining) on Day 15 after injection of Mab. A. Sections of arthritic knee joint from WT mice showing severe synovitis, including pannus invasion with inflammatory cells and cartilage destruction. B. Higher magnification of panel A inset shows grade 3 histological appearance of articular cartilge, with marked loss of the superficial and middle layers, cluster formation of chondrocytes and loss of safranin O stainability. C. Sections from NOS2–/– mice (8 mg Mab). Synovial inflammation is markedly reduced and cartilage structure is well maintained. D. Higher magnification of panel C inset shows grade 1 histology, with slight loss of safranin O stainability. Original magnification: A and C ×40, B and D ×200.

NOS2–/– mice with AMA remained low at 64.0 ± 14.2 µM, equivalent to the controls without arthritis (Figure 5B).

DISCUSSION

This is the first investigation showing the preventive effects of NOS2 gene deletion on autoantibody mediated inflammatory arthritis and subsequent cartilage degeneration. We used NOS2–/– mice instead of using NO inhibitors in order to avoid the various side effects of NO inhibitors. It was observed that clinical arthritis was ameliorated by NOS2 deletion, even in the presence of unabated joint inflammation. Although the histological grade of cartilage destruction in NOS2–/– mice with AMA was significantly lower than in WT mice, the cartilage of NOS2–/– mice still showed various degrees of destruction, including fibrillation on the cartilage surface, decrease of proteoglycans, and chondrocyte cell death. These results indicated that NO catalyzed by NOS2 is an important mediator of amplification of the inflammatory responses in articular cartilage, but is not completely indispensable for the progression of cartilage destruction in this murine model.

The Mab plus LPS induced arthritis used in this study is a novel model established by Terato, *et al*[36]. LPS bypasses the epitope specificity of autoantibodies to type II collagen and drastically reduces the threshold values of arthrogenic antibody concentrations necessary to induce arthritis. LPS has been used to enhance the severity of arthritis and to synchronize the development of arthritis. The acceleration of arthritis

Table 1. Number (%) of cartilage samples from femoral condyles and tibial plateaus of wild-type and NOS2–/– mice with AMA in each histological grade.

	Femoral Condyle						Tibial Plateau					
Histological Grade	0	1	2	3	Total	p*	0	1	2	3	Total	p*
WT mice	0	1	7	8	16		0	0	5	11	16	
(%)	(0)	(6)	(44)	(50)	(100)		(0)	(0)	(31)	(69)	(100)	
NOS2–/– mice	2	9	7	2	20	0.0008,	1	6	10	3	20	0.0004,
(%)	(10)	(45)	(35)	(10)	(100)	z = –3.358	(5)	(30)	(50)	(15)	(100)	z = –3.295

* Mann-Whitney U test, 2 sided test.

Table 2. Distribution and populations of TUNEL, NT, and MMP-3 and -9 positive chondrocytes in knee joints of WT and NOS2–/– mice with AMA. Values are expressed as mean ± SD.

	Femoral Condyle		Tibial Plateau	
	WT, n = 16	NOS2–/–, n = 20	WT, n = 16	NOS2–/–, n = 20
TUNEL, %	34.1 ± 12.1 *	18.8 ± 9.7	38.8 ± 13.7	20.6 ± 6.8 *
NT, %	37.2 ± 8.9 *	24.9 ± 8.9	38.6 ± 7.7 *	28.2 ± 7.8
MMP-3, %	29.1 ± 6.0	25.7 ± 6.7	28.7 ± 5.6	26.5 ± 5.8
MMP-9, %	33.4 ± 9.8	30.5 ± 11.3	35.6 ± 7.9	34.6 ± 7.8

* $p < 0.01$, ** $p < 0.001$.

with LPS could be also associated with the enhancement of the deposition of antibodies onto the cartilage surface. AMA also has an advantage in inducing arthritis in mice resistant to collagen induced arthritis (CIA), such as C57/BL6, Balb/c, SCID, and other strains[40]. Regardless of the susceptibility to CIA, a high dose of Mab to type II collagen alone is capable of inducing severe and chronic arthritis associated with cartilage destruction. It is important to note that LPS (B cell mitogen) can be replaced with MAM and SEB, which are T cell mitogens produced by *Mycoplasma arthritidis* and *Staphylococcus aureus*, respectively, to trigger arthritis in the presence of subarthritogenic levels of autoantibodies to type II collagen (Dr. K. Terato, personal communication). From this observation, we assume that the synergistic effect of LPS with autoantibodies in inducing arthritis is mediated by common mediators released by stimulation with these toxins rather than particular cell types such as CD4 or CD8 of the T cell family. More important, these bacterial toxins stimulate NO production through the nuclear factor-κB activation pathway.

Chondrocyte apoptosis appears to have a critical role in cartilage matrix breakdown, because chondrocytes are the only specific cell type in cartilage responsible for the production and maintenance of the extracellular matrix of articular cartilage. Apoptotic chondrocytes have been described in OA[41,42] and RA[43,44] cartilage, but were rarely seen in normal cartilage. Because NO is considered to be one of the primary inducers of chondrocyte apoptosis[45], our study was designed to determine whether NOS2 deletion could affect chondrocyte apoptosis in an AMA model. The histological grade and the population of TUNEL positive chondrocytes were significantly lower in the cartilage from NOS2–/– mice with AMA than that of WT mice with AMA. These results might lend qualified support to the interpretation of harmful effects of NO on chondrocytes. However, the inflammatory reaction at joint tissues in NOS2–/– mice with AMA was relatively lower than that of WT mice, indicating that the lower degree of cartilage damage might depend on the lower inflammatory reactions at joint tissues. Apoptotic changes and TUNEL positive chondrocytes were still frequently observed in NOS2–/– mice with AMA, suggesting that NOS2 induced NO production is not the only stimulant for chondrocyte apoptosis.

Since it is known that NO activates MMP[24,25], while it inactivates the tissue inhibitor of metalloproteinase[26] and promotes IL-1ß induced cartilage destruction through the activation of MMP-3 and -9[27], we then examined the effect of NOS2 gene deletion on MMP production by chondrocytes. Interestingly, the numbers of MMP-3 and -9 positive chondrocytes did not differ significantly between WT mice with AMA and NOS2–/– mice with AMA. Horton, *et al*[46] reported that both IL-1ß and TNF-α upregulated the MMP-3 and

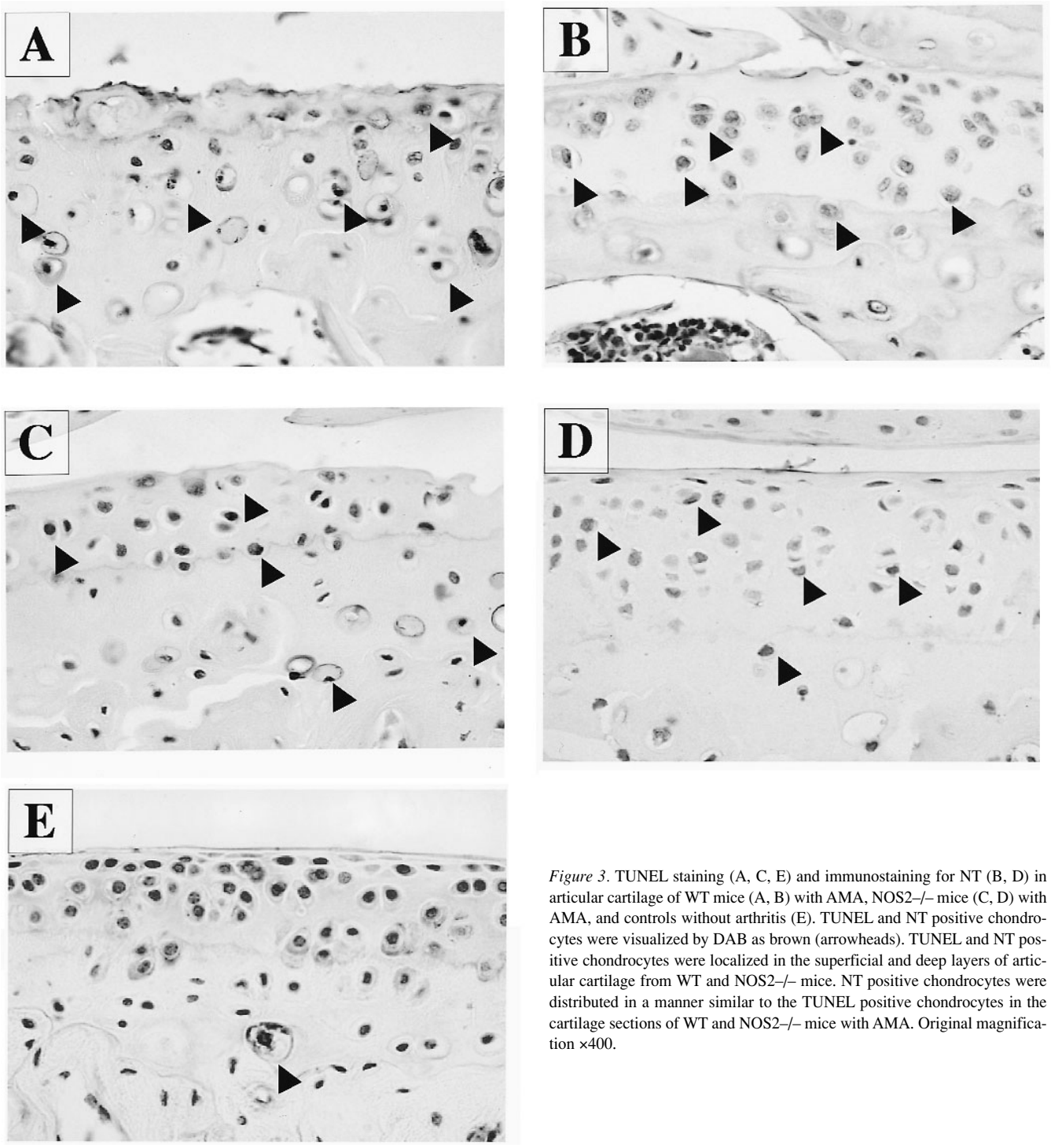

Figure 3. TUNEL staining (A, C, E) and immunostaining for NT (B, D) in articular cartilage of WT mice (A, B) with AMA, NOS2–/– mice (C, D) with AMA, and controls without arthritis (E). TUNEL and NT positive chondrocytes were visualized by DAB as brown (arrowheads). TUNEL and NT positive chondrocytes were localized in the superficial and deep layers of articular cartilage from WT and NOS2–/– mice. NT positive chondrocytes were distributed in a manner similar to the TUNEL positive chondrocytes in the cartilage sections of WT and NOS2–/– mice with AMA. Original magnification ×400.

MMP-9 mRNA expression in immortalized rat chondrocytes; however, the NOS inhibitor *N*-methyl arginine did not prevent expression of these cytokine induced MMP. The unexpected increase in serum concentrations of IL-1ß in our study in both WT and NOS2–/– mice with AMA raises the possibility that IL-1ß can directly upregulate MMP expression to promote cartilage destruction. Serum levels of IL-1ß in NOS2–/– mice with AMA did not differ from those in WT mice, suggesting that NOS2 deficiency does not involve feedback regulation of IL-1ß production.

The Griess reaction and immunohistochemistry findings for NT confirmed the production of NO in serum and joint tissues, respectively. Serum concentrations of nitrite/nitrate in NOS2–/– mice with AMA were significantly lower than in

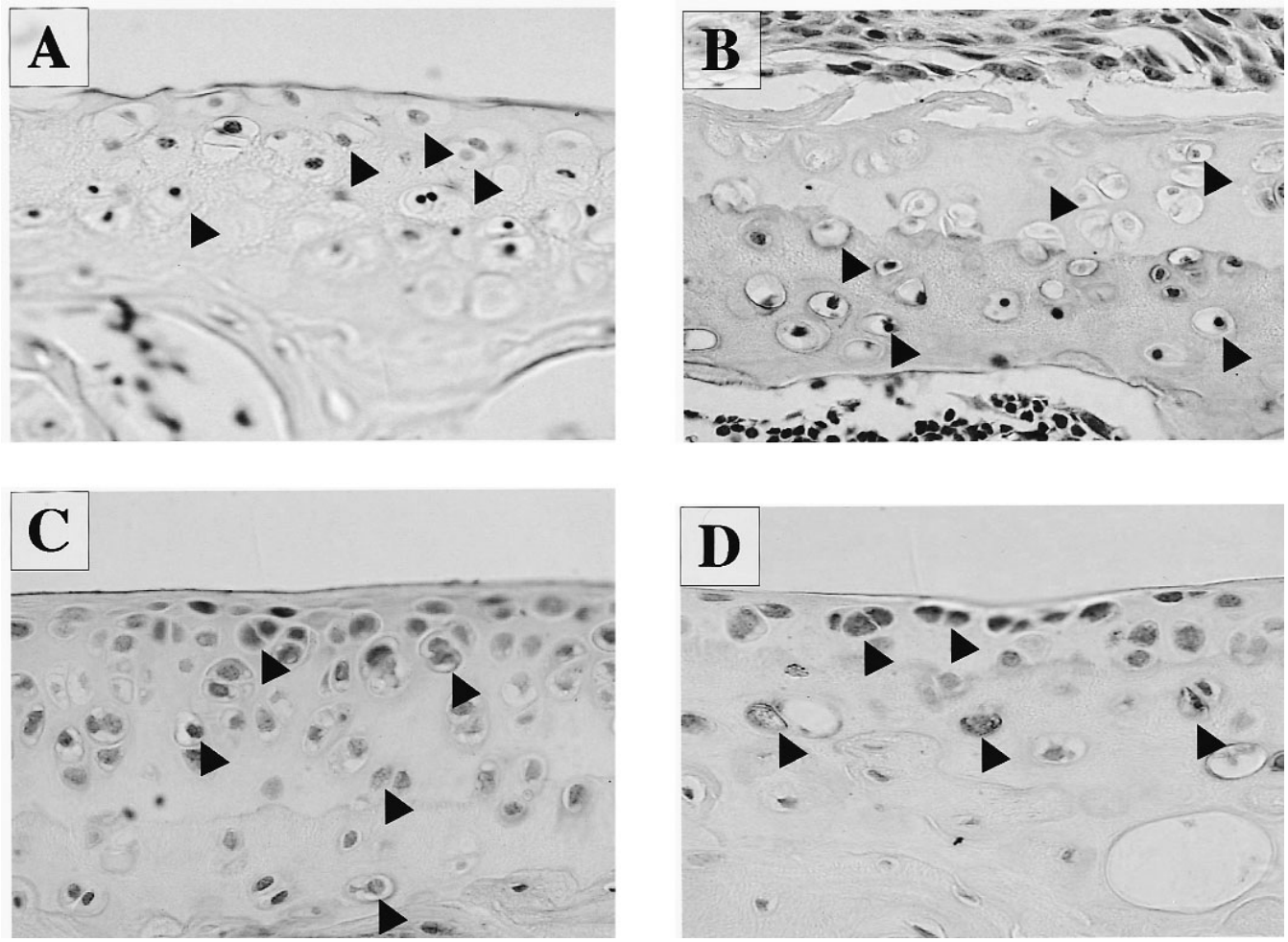

Figure 4. Immunohistochemical localization of MMP-3 (A, C) and MMP-9 (B, D) in articular cartilage of WT (A, B) and NOS2–/– mice with AMA (8 mg Mab) (C, D). MMP-3 and -9 positive chondrocytes were visualized by DAB as brown (arrowheads). MMP-3 and -9 positive chondrocytes were localized in the superficial and deep layers of articular cartilage from WT and NOS2–/– mice with AMA. Magnification ×400.

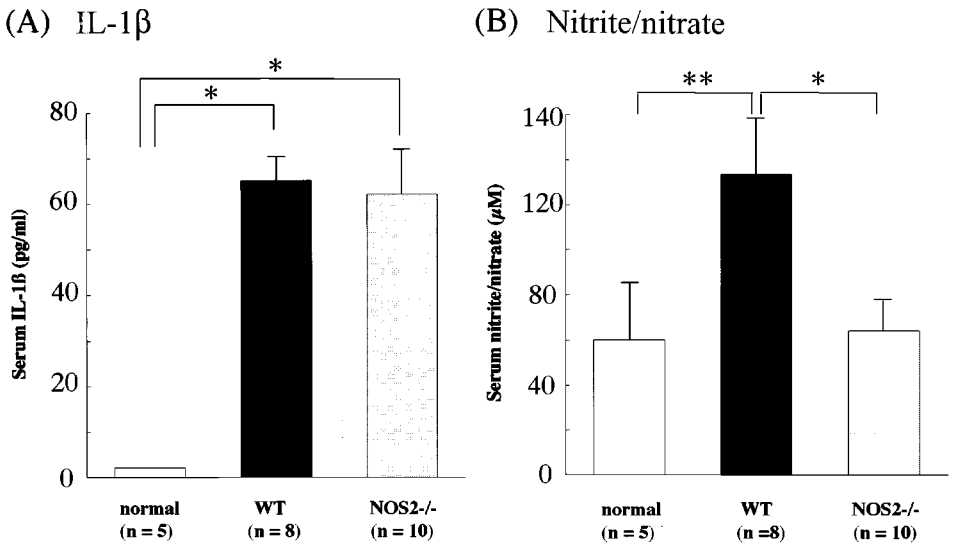

Figure 5. Serum levels of IL-1ß (by ELISA) (A) and nitrite/nitrate (Griess reaction) (B) of normal mice without arthritis and WT and NOS2–/– mice with AMA at 15 days after injection of 8 mg Mab. Values are expressed as mean ± SD (*p < 0.001, **p < 0.02).

WT mice, but higher than in the normal mice without arthritis. The percentage of NT positive chondrocytes in NOS2–/– mice with AMA was about 24–28%, significantly lower than the NT positive cells (39%) in WT mice. These findings were similar to those described for cartilage in a canine OA model using a selective NOS2 inhibitor[34,47]. NO is probably produced through the other isoforms of NOS, such as endothelial NOS (eNOS/NOS3). Although the expression of NOS3 by chondrocytes was not measured in this study, positive staining for NOS3 was observed in chondrocytes from NOS2–/– mice (data not shown). Thus the contribution of the NOS3 isoform in NO formation and the stimulation mechanism of NOS3 in chondrocytes remain to be defined.

NOS2 gene deletion reduced cartilage destruction following partial suppression of inflammatory reactions in this autoantibody mediated arthritis model. Our results suggest that the activation of MMP by IL-1ß can accelerate cartilage destruction without the contribution of NOS2.

ACKNOWLEDGMENT

The authors thank Dr. K. Terato (Chondrex Inc.) for invaluable advice on the manuscript.

REFERENCES

1. Xie QW, Cho HJ, Calaycay J, et al. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 1992;256:225-8.
2. St. Clair EW, Wilkinson WE, Lang T, et al. Increased expression of blood mononuclear cell nitric oxide synthase type 2 in rheumatic arthritic patients. J Exp Med 1996;184:1173-8.
3. Amin AR, Di Cesare PE, Vyas P, et al. The expression and regulation of nitric oxide synthase in human osteoarthritis-affected chondrocytes: evidence for upregulated neuronal nitric oxide synthase. J Exp Med 1995;182:2097-102.
4. Konttinen YT, Platts LA, Tuominen S, et al. Role of nitric oxide in Sjögren's syndrome. Arthritis Rheum 1997;40:875-83.
5. Sakurai H, Kohsaka H, Liu MF, et al. Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. J Clin Invest 1995;96:2357-63.
6. Hayashi T, Abe E, Yamate T, Taguchi Y, Jasin HE. Nitric oxide production by superficial and deep articular chondrocytes. Arthritis Rheum 1997;40:261-9.
7. Grabowski PS, Wright PK, Van 't Hof RJ, Helfrich MH, Ohshima H, Ralston SH. Immunolocalization of inducible nitric oxide synthase in synovium and cartilage in rheumatoid arthritis and osteoarthritis. Br J Rheumatol 1997;36:651-5.
8. Kilbourn RG, Belloni P. Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with tumor necrosis factor, interleukin-1, or endotoxin. J Natl Cancer Inst 1990;82:772-6.
9. Weinberg JB, Misukonis MA, Shami PJ, et al. Human mononuclear phagocyte inducible nitric oxide synthase (iNOS): analysis of iNOS mRNA, iNOS protein, biopterin, and nitric oxide production by blood monocytes and peritoneal macrophages. Blood 1995;86:1184-95.
10. Farrell AJ, Blake DR. Nitric oxide. Ann Rheum Dis 1996;55:7-20.
11. McCartney-Francis N, Allen JB, Mizel SB, et al. Suppression of arthritis by an inhibitor of nitric oxide synthase. J Exp Med 1993;178:749-54.
12. Stefanovic-Racic M, Morales TI, Taskiran D, McIntyre LA, Evans CH. The role of nitric oxide in proteoglycan turnover by bovine articular organ cultures. J Immunol 1996;156:1213-20.
13. Conner JR, Manning PT, Settle S, et al. Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. Eur J Pharmacol 1995;273:15-24.
14. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43:109-42.
15. Christopherson KS, Bredt DS. Nitric oxide in excitable tissues: physiological roles and disease. J Clin Invest 1997;100:2424-9.
16. Tanaka S, Matsui T, Murakami T, et al. Immunological abnormality associated with the augmented nitric oxide synthesis in adjuvant-induced arthritis. Int J Immunopharmacol 1998;20:71-84.
17. Fletcher DS, Widmer WR, Luell S, et al. Therapeutic administration of a selective inhibitor of nitric oxide synthase does not ameliorate the chronic inflammation and tissue damage associated with adjuvant-induced arthritis in rats. J Pharmacol Exp Ther 1998;284:714-21.
18. Vermeire K, Thielemans L, Matthys P, Billiau A. The effects of NO synthase inhibitors on murine collagen-induced arthritis do not support a role of NO in the protective effect of IFN-gamma. J Leukoc Biol 2000;68:119-24.
19. Veihelmann A, Landes J, Hofbauer A, et al. Exacerbation of antigen-induced arthritis in inducible nitric oxide synthase-deficient mice. Arthritis Rheum 2001;44:1420-7.
20. McInnes IB, Leung B, Wei XQ, Gemmell CC, Liew FY. Septic arthritis following staphylococcus aureus infection in mice lacking inducible nitric oxide synthase. J Immunol 1998;160:308-15.
21. Das P, Schurman DJ, Smith RL. Nitric oxide and G proteins mediate the response of bovine articular chondrocytes to fluid-induced shear. J Orthop Res 1997;15:87-93.
22. Taskiran D, Stefanovic-Racic M, Georgescu H, Evans C. Nitric oxide mediates suppression of cartilage proteoglycan synthesis by interleukin-1. Biochem Biophys Res Commun 1994;200:142-8.
23. Clancy RM, Abramson SB, Kohne C, Rediske J. Nitric oxide attenuates cellular hexose monophosphate shunt response to oxidants in articular chondrocytes and acts to promote oxidant injury. J Cell Physiol 1997;172:183-91.
24. Murrell GA, Jang D, Williams RJ. Nitric oxide activates metalloprotease enzymes in articular cartilage. Biochem Biophys Res Commun 1995;206:15-21.
25. Sasaki K, Hattori T, Fujisawa T, Takahashi K, Inoue H, Takigawa M. Nitric oxide mediates interleukin-1-induced gene expression of matrix metalloproteinases and basic fibroblast growth factor in cultured rabbit articular chondrocyte. J Biochem (Tokyo) 1998;123:431-9.
26. Frears ER, Zhang Z, Blake DR, O'Connell JP, Winyard PG. Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite. FEBS Lett 1996;381:21-4.
27. Pelletier JP, Mineau F, Ranger P, Tardif G, Martel-Pelletier J. The increased synthesis of inducible nitric oxide inhibits IL-1ra synthesis by human articular chondrocytes: possible role in osteoarthritic cartilage degradation. Osteoarthritis Cartilage 1996;4:77-84.
28. Frankel SR, Clancy RM, Ricci JL, DiCesare PE, Rediske JJ, Abramson SB. Effects of nitric oxide on chondrocyte migration, adhesion, and cytoskeletal assembly. Arthritis Rheum 1996;39:1905-12.
29. Millward-Sadler SJ, Wright MO, Lee H, et al. Integrin-regulated secretion of interleukin 4: a novel pathway of mechanotransduction in human articular chondrocytes. J Cell Biol 1999;145:183-9.
30. Blanco FJ, Ochs RL, Schwarz H, Lot M. Chondrocyte apoptosis induced by nitric oxide. Am J Pathol 1995;146:75-85.
31. Amin AR, Abramson SB. The role of nitric oxide in articular cartilage breakdown in osteoarthritis. Curr Opin Rheumatol

1998;10:263-8.
32. Horton WE Jr, Feng L, Adams C. Chondrocyte apoptosis in development, aging and disease. Matrix Biol 1998;17:107-15.
33. Carson DA, Tan EM. Apoptosis in rheumatic diseases. Bull Rheum Dis 1995;44:1-3.
34. Pelletier JP, Lascau-Coman V, Jovanovic D, et al. Selective inhibition of inducible nitric oxide synthase in experimental osteoarthritis is associated with reduction in tissue levels of catabolic factors. J Rheumatol 1999;26:2002-14.
35. van den Berg WB, van de Loo F, Joosten LA, Arntz OJ. Animal model of arthritis in NOS2-deficient mice. Osteoarthritis Cartilage 1999;7:413-5.
36. Terato K, Hasty KA, Reife RA, Cremer MA, Kang AH, Stuart JM. Induction of arthritis by monoclonal antibodies. J Immunol 1992;148:2103-8.
37. Terato K, Harper DS, Griffiths MM, et al. Collagen induced arthritis in mice: synergistic effect of E. coli lipopolysaccharide bypasses epitope specificity in the induction of arthritis with monoclonal antibodies to type II collagen. Autoimmunity 1995;22:137-47.
38. Nishida K, Doi T, Matsuo M, et al. Involvement of nitric oxide in chondrocyte cell death in chondro-osteophyte formation. Osteoarthritis Cartilage 2001;9:232-7.
39. Freemont AJ, Hampson V, Tilman R, Goupille P, Taiwo Y, Hoyland JA. Gene expression of matrix metalloproteinases 1, 3, and 9 by chondrocytes in osteoarthritic human knee articular cartilage is zone and grade specific. Ann Rheum Dis 1997;56:542-9.
40. Barthold SW, Beck DS, Hansen GM, Terwilliger GA, Moody KD. Lyme borreliosis in selected strains and ages of laboratory mice. J Infect Dis 1990;162:133-8.
41. Blanco FJ, Guitian R, Vazquez-Martul E, de Toro FJ, Galdo F. Osteoarthritis chondrocytes die by apoptosis. A possible pathway for osteoarthritis pathology. Arthritis Rheum 1998;41:284-9.
42. Heraud F, Heraud A, Harmand MF. Apoptosis in normal and osteoarthritic human articular cartilage. Ann Rheum Dis 2000;59:959-65.
43. Kim HA, Song YW. Apoptotic chondrocyte death in rheumatoid arthritis. Arthritis Rheum 1999;42:1528-37.
44. van 't Hof RJ, Hocking L, Wright PK, Ralston SH. Nitric oxide is a mediator of apoptosis in the rheumatoid joint. Rheumatology 2000;39:1004-8.
45. Hashimoto S, Takahashi K, Amiel D, Coutts RD, Lotz M. Chondrocyte apoptosis and nitric oxide production during experimentally induced osteoarthritis. Arthritis Rheum 1998;41:1266-74.
46. Horton WE Jr, Udo I, Precht P, Balakir R, Hasty K. Cytokine inducible matrix metalloproteinase expression in immortalized rat chondrocytes is independent of nitric oxide stimulation. In Vivo Cell Dev Biol Anim 1998;34:378-84.
47. Pelletier JP, Jovanovic D, Fernandes JC, et al. Reduced progression of experimental osteoarthritis in vivo by selective inhibition of inducible nitric oxide synthase. Arthritis Rheum 1998;41:1275-86.