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Chondrocyte Apoptosis Induced by Nitric Oxide

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Chondrocytes stimulated with IL-1 produce high levels of nitric oxide (NO), which inhibits proliferation induced by transforming growth factor-β or serum. This study analyzes the role of NO and IL-1 in the induction of chondrocyte cell death. NO generated from sodium nitroprusside induced apoptosis in cultured chondrocytes as demonstrated by electron microscopy, 4',6-dianidino-2-phenylindole dihydrochloride staining, FACS analysis, and histochemical detection of DNA fragmentation. Similar results were obtained with two other NO donors, 3-morpholinosynonimide-hydrochloride and s-nitroso-N-acetyl-D-L-penicillamine. In contrast, oxygen radicals generated by hypoxanthine/xanthine oxidase caused necrosis but did not induce chondrocyte apoptosis. To analyze whether endogenously generated NO induces apoptosis, chondrocytes were stimulated with IL-1, but there was no evidence for apoptotic changes. Combinations of NO inducers such as IL-1, lipopolysaccharide, tumor necrosis factor, and interferon-γ also failed to trigger apoptosis. IL-1-stimulated chondrocytes are known to produce oxygen radicals that react with NO to form products that can induce cell death in other systems. We thus tested IL-1 in combination with the oxygen radical scavengers N-acetyl cysteine, dimethyl sulfoxide, or 5,5'-dimetylpyrroline 1-oxide. Under these conditions IL-1 was able to induce apoptosis, which was inhibited in a dose-dependent manner by the NO synthase inhibitor N-monomethyl L-arginine. Conversely, endogenous oxygen radicals induced by inflammatory mediators caused necrosis under conditions in which the simultaneous production of NO was reduced. These results suggest that NO, but not oxygen radicals, is the primary inducer of apoptosis in human articular chondrocytes. (Am J Pathol 1995, 146:75–85)

Cartilage is one of the tissues with the highest frequency of age-related pathology. Osteoarthritis not only represents the most frequent musculoskeletal disorder but also the most common disease in the aging population.[1] One very remarkable change that occurs with aging of human articular cartilage is a profound loss in tissue cellularity.[2–6] This corresponds to an age-related decline in growth factor responsiveness of human articular chondrocytes.[7,8]

Interleukin 1 (IL-1)-induced catabolic responses in chondrocytes represent central pathogenic events in rheumatoid arthritis and osteoarthritis.[9] IL-1 induces matrix metalloproteinases and other proinflammatory cytokines and inhibits extracellular matrix synthesis.[9] IL-1 also inhibits chondrocyte proliferation.[10] Some of these effects have been associated with the IL-1 induction of oxygen radical formation. Chondrocytes also produce high levels of nitric oxide (NO),[11,12] which is part of the IL-1-induced catabolic program.[13] We recently showed that NO is a mediator of the IL-1 antiproliferative effects in chondrocytes.[14] In other cell types, in which NO inhibits cell proliferation, it has been shown to cause cell death, in some cases by inducing apoptosis.[15–18]

This information provided the basis for the present study, which examined the role of NO in the induction of cell death in chondrocytes. As there were no published data available on apoptosis in chondrocytes, we first provided a morphological and biochemical characterization of apoptosis in this cell type and then defined the effects of NO and other radicals. The results show that NO, but not oxygen radicals, when administered from exogenous sources, induces apoptosis, and IL-1 causes NO-dependent apoptosis in the presence of oxygen radical scavengers.

Materials and Methods

Chondrocyte Isolation and Culture

Cartilage was obtained at autopsy or from the University of California, San Diego, tissue bank from

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donors without history of joint disease. All donors were adults aged 19 to 72 (mean 54) years. Chondrocytes were isolated as described.[19] In brief, cartilage slices were removed from the femoral condyles and washed in Dulbecco's minimal essential medium (DMEM). Tissues were then minced with a scalpel, transferred into a digestion buffer containing DMEM, 5% fetal bovine serum, L-glutamine, antibiotics, and 2 mg/ml clostridial collagenase (Sigma Chemical Co., St. Louis, MO) and incubated on a gyratory shaker at 37 C until the fragments were digested. Residual multicellular aggregates were removed by sedimentation ($1 \times g$) and the cells were washed three times in DMEM with 5% fetal bovine serum before use.

The cells were plated and the various reagents (IL-1, transforming growth factor-β, nitric oxide synthase (NOS) inhibitors, and oxygen radical scavengers) were added at the same time at initiation of culture.

Analysis of DNA Fragmentation

The Klenow labeling procedure was used as described.[20] Cells were harvested by a 5-minute centrifugation at 2000 rpm and resuspended in 10 mmol/L Tris, 1 mmol/L EDTA, and 0.5% Triton X-100, pH 8.0 (10^6 cells per 50 µl of buffer). Extracts were incubated on ice for 20 minutes and briefly vortexed every 5 minutes. Cell debris and high molecular weight DNA were removed by a 10-minute centrifugation at 14,000 rpm at 4 C. Supernatants were extracted with phenol and phenol/chloroform and precipitated with 0.5 volumes of 7.5 mol/L ammonium acetate and 2 volumes of ethanol for 2 hours at −80 C.

DNA was resuspended in TE (10 mmol/L Tris-Cl, pH 7.4, and 1 mmd/L EDTA, pH 8) and 1 µg was treated with 5 U of Klenow polymerase (Boehringer Mannheim Indianapolis, IN) and 5 µCi of [^{32}P]dCTP for 10 minutes in the presence of 10 mmol/L Tris, pH 7.5, and 1 mmol $MgCl_2$ as described.[21] Unincorporated nucleotides were removed by three consecutive precipitations. One-half of the labeled DNA was separated on 2% agarose gels, dried, and exposed to X-ray film.

Histochemical Detection of DNA Fragmentation

Cells were harvested, centrifuged onto glass slides, fixed with 0.25% glutaraldehyde for 1 minute, washed with water, and stained with 1 µg/ml 4′,6-dianidino-2-phenylindole dihydrochloride (DAPI) for 10 minutes at 37 C.[22] Slides were then washed with water, air dried, and covered with Vectashield (Vector Laboratories, Burlingame, CA) for analysis by fluorescence microscopy with a triple band filter set (61000; Chroma Technology, Brattleboro, VT) on an Olympus microscope.

DNA Labeling Technique for Flow Cytometric Analysis

Cells were fixed in 70% ethanol at 4 C for 60 minutes, washed, and incubated with RNAse (500 µg/ml) and propidium iodide (50 µg/ml) for 15 minutes at room temperature in the dark and kept at 4 C. Propidium iodide fluorescence of nuclei was measured by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA) with a 560-nm dichromatic mirror and a 600-nm band pass filter. Data are expressed as percent apoptotic (hypodiploid) nuclei.

Electron Microscopy

For electron microscopy, chondrocytes were grown as monolayers in flasks. After 2 days in culture, 2 mmol/L sodium nitroprusside (SNP) or *s*-nitro-N-acetyl-D-L-penicillamine (SNAP) was added for the times indicated and then cells were scraped, pelleted, rinsed with phosphate-buffered saline, fixed for 1 hour at room temperature in 2.5% glutaraldehyde buffered with 0.1 mol/L cacodylate (pH 7.2), rinsed in cacodylate buffer, postfixed for 1 hour in 2% OsO_4 buffered with cacodylate, dehydrated in a graded ethanol series, and embedded in Polybed 812 (Polysciences, Warrington, PA). Thin sections of cell pellets were stained with uranyl acetate and lead citrate.

Quantification of Nitrites

Chondrocytes were plated at 50,000 cells per well in 96-well plates and cultured for 48 hours at which time culture supernatants were collected for nitrite measurements.

NO formation was detected by NO_2^- accumulation in the culture supernatants by the Griess reaction with sodium nitrite as standard. Briefly, 50 µl of culture supernatant were incubated with 50 µl of 1% sulfanilamide, 0.1% N-1-naphthylethylenediamide dihydrochloride in 25% H_3PO_4 at room temperature for 5 minutes. Optical density was measured at 570 nm.

Reagents

Human recombinant IL-1β, transforming growth factor-β1, TNF-α, and IFN-γ were purchased from

R&D Systems (Minneapolis, MN) Tissue culture media were obtained from MA Bioproducts. Sodium nitroprusside (SNP), superoxide dismutase (SOD), N-methyl arginine (NMA), N-acetyl cysteine (NAC), lipopolysaccharide (LPS; from *Salmonella minnesota*), catalase, dimethylsulfoxide (DMSO), and 5,5′-dimethylpyrroline 1-oxide (DMPO) were purchased from Sigma. SNAP and 3-morpholinosynonimide-hydrochloride were from Alexis Corporation (San Diego, CA), and hypoxanthine and xanthine oxidase were from Calbiochem (La Jolla, CA).

Statistical Analysis

Results are expressed as mean ± SEM. For all results shown, at least two separate experiments with cells from different donors were performed. Within experiments each individual measurement was either performed in duplicate or triplicate. Statistical analysis was performed with the unpaired two-tailed *t*-test.

Results

NO Donors, but Not Oxygen Radicals, Induce Chondrocyte Apoptosis

NO, when applied through SNP, induced cell death in primary or subcultured human articular chondrocytes. When analyzed by light microscopy, the SNP-treated cultures contained cells with small bodies that were surrounding the chondrocytes or were budding off the cell membranes (Figure 1C, D). Chondrocytes appeared smaller in cell volume, and cells with increased volume characteristic of necrosis were not observed unless very high concentrations of SNP (>4 mmol/L) were used. Staining of the cell cultures with DAPI showed that the NO donors SNP and SNAP induced DNA condensation and margination (Figure 2). This was first detectable after 6 hours in a small fraction of the cells (<10%) and reached a maximum (83% of the cells) after 24 hours. For quantification of these changes the cells were stained with propidium iodide and analyzed by flow cytometry (Figure 3). This

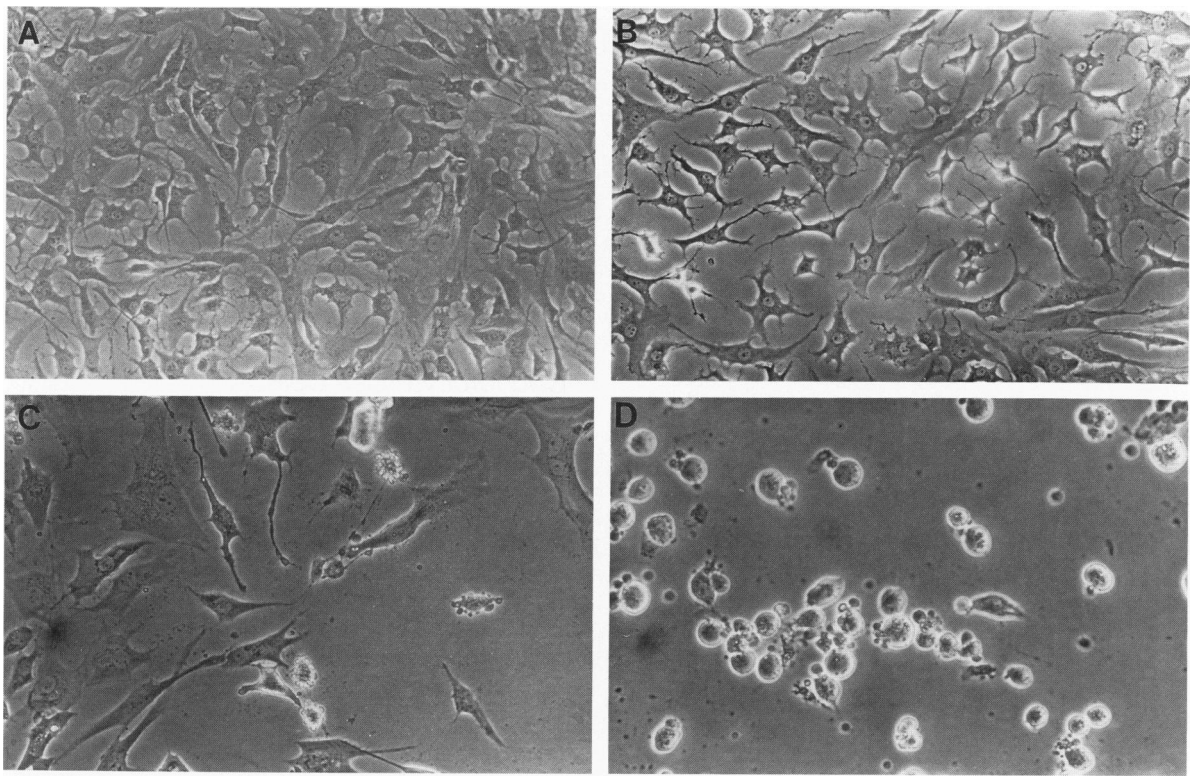

Figure 1. *The NO donor SNP induces apoptosis in chondrocytes. Chondrocytes (passage 1) were plated in tissue culture chamber slides and treated with SNP (2 mmol/L). Untreated cells (A) and SNP-treated cells were analyzed by light microscopy after 6 (B), 10 (C) and 18 (D) hours. After 6 hours cells start to change morphology. Cells reduce size and detach from flask (ring highlight). At 10 hours some cells show clear cytoplasmic and membranous apoptotic changes. By 18 hours cells are seen that have undergone complete fragmentation and essentially all cells at this time point show some degree of apoptosis.*

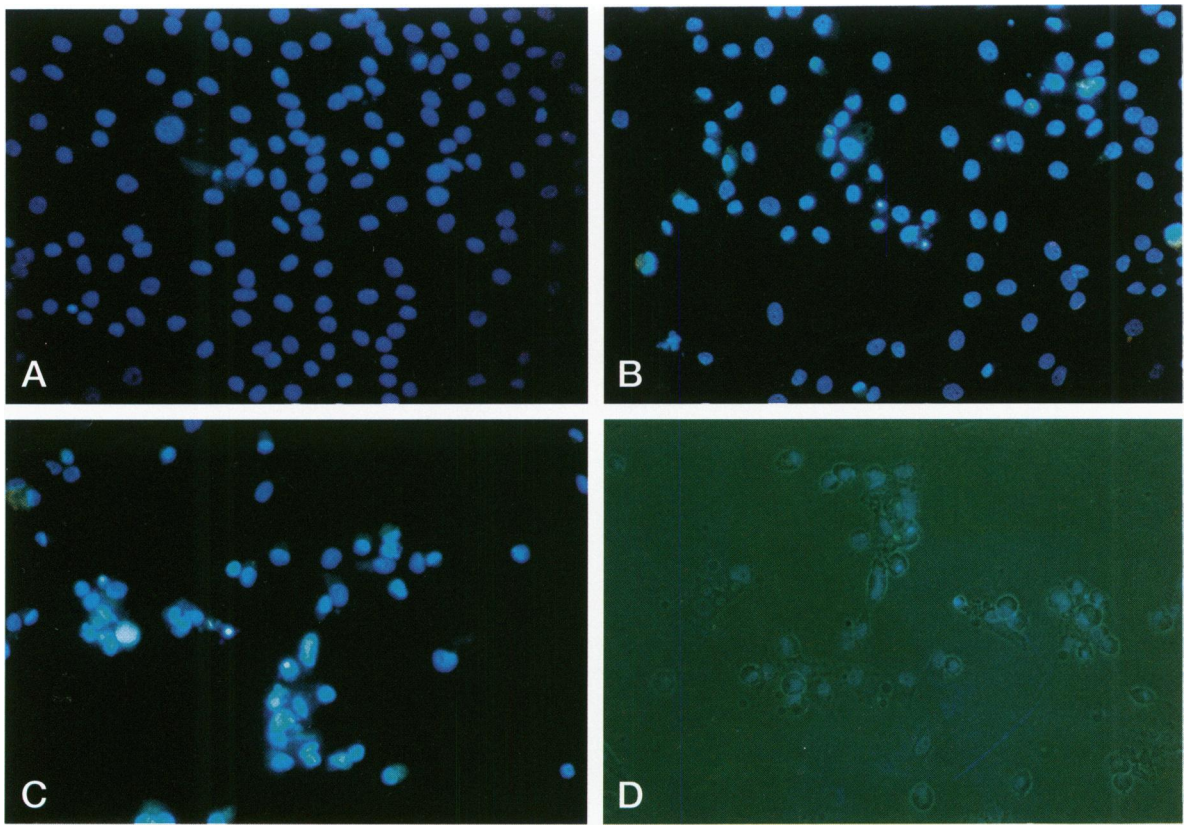

Figure 2. *DNA fragmentation induced by SNAP. DAPI staining analyzed by fluorescence microscopy* (**A–C**) *and by the combination of fluorescence and light microscopy* (**D**) *is shown.* **A:** *Untreated cells (control).* **B:** *Cells treated with SNAP (2 mmol/L) for 8 hours.* **C** *and* **D:** *Cells treated with SNAP (2 mmol/L) for 24 hours.*

showed that in response to treatment with SNP there was a progressive decrease in the diploid peak and an increase in the number of hypodiploid cells.

Electron microscopy confirmed the formation of membrane-limited bodies typical of apoptosis (Figure 4B, C). In addition, the earliest apoptotic changes were noted in the nucleolus where nucleoli were transformed from loosely organized irregularly shaped structures (Figure 4A) to smaller, compact, round nucleoli (Figure 4B). As a basis for comparison with apoptosis, a swollen necrotic cell is shown in Figure 4D. The overall effects of SNP and SNAP were similar, but the time course was different, with SNAP effects fully maximal at 8 hours compared with 16 hours for SNP.

To analyze whether chondrocytes show similar changes in response to other radicals we tested the effects of hypoxanthine and xanthine oxidase. However, different concentrations and incubation periods did not result in the induction of apoptotic changes, although there was a time- and dose-dependent reduction in cell viability (Figure 5).

Collectively, these results indicate that NO, when generated from exogenous donors, can induce morphological changes and DNA fragmentation characteristic of apoptosis. In contrast, oxygen radicals do not induce apoptotic changes in chondrocytes.

IL-1 and Other Inducers of Endogenous NO Synthesis Do Not Trigger Chondrocyte Cell Death

Previous studies have demonstrated that in human articular chondrocytes, stimulation with IL-1 results in the production of NO.[11–13] As the NO donor SNP induced chondrocyte apoptosis, we tested whether endogenously produced NO was capable of inducing similar effects. Chondrocytes were treated with IL-1 (0.1 to 10 ng/ml) for different time periods, but cell viability was not significantly reduced and there were no detectable apoptotic changes as analyzed by light microscopy and DAPI staining (Table 1). Furthermore, combinations of NO inducers such as TNF, IL-1, IFN-γ, and LPS did not induce apoptosis. Conditioned media were collected at 24 hours from replicate wells of the same cultures for the analysis of nitrite levels. This confirmed the expected induction of

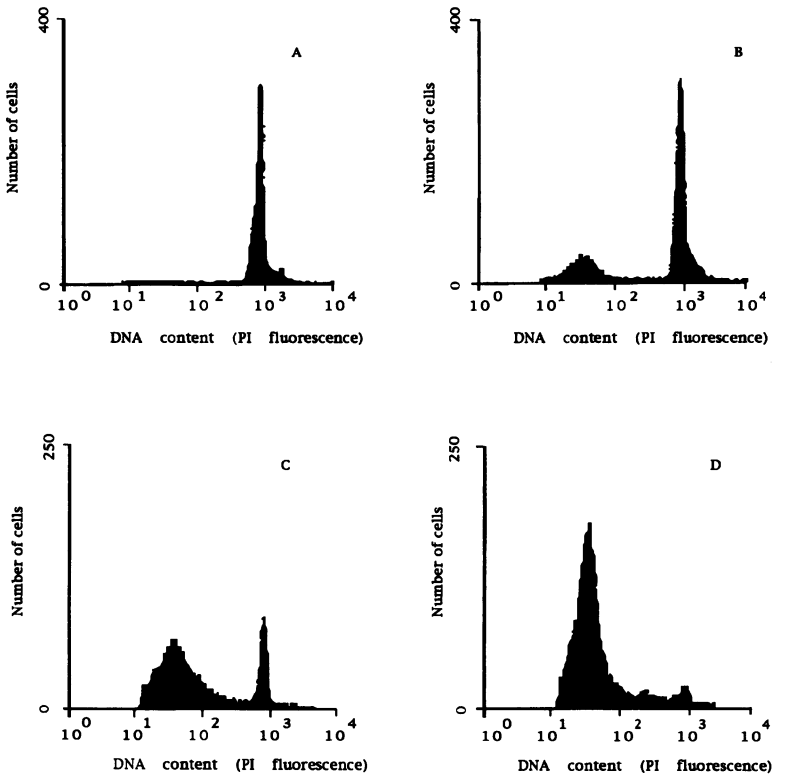

Figure 3. *Kinetics of SNP effects on chondrocytes analyzed by flow cytometry. Chondrocytes cultured in media alone or treated with SNP (2 mmol/L) for 12, 16, and 24 hours were stained with propidium iodide for analysis by flow cytometry.* A: *Typical DNA peak of untreated diploid chondrocytes.* B–D: *Progressive decrease in the diploid peak and increase in the number of hypodiploid cells.*

high levels of endogenous NO synthesis by IL-1 and the other stimuli (Table 1).

Interactions of NO and Oxygen Radicals in the Induction of Chondrocyte Apoptosis

IL-1-stimulated chondrocytes also produce oxygen radicals that can react with NO to form peroxynitrite, which reduces the levels of biologically active NO and induces necrosis in other cell systems. We thus tested combinations of IL-1 and oxygen radical scavengers. The addition of DMSO, DMPO, or NAC to IL-1-stimulated cultures did not reduce cell viability and, in the absence of IL-1, these reagents did not induce apoptotic changes. However, the combination of the radical scavengers and IL-1 caused a time-dependent increase in the number of apoptotic cells that reached maximal levels after 20 hours (Figure 6A). The three radical scavengers did not differ in the kinetics or magnitude of their effects at the concentrations tested. Catalase, but not SOD, used as physiologically relevant scavengers, caused similar induction of apoptosis in the presence of IL-1 (Figure 6B). DNA analysis showed the ladder pattern due to internucleosomal cleavage characteristic of apoptosis in cultures that were treated with IL-1 and DMSO but not in response to IL-1 alone (Figure 7).

IL-1-Induced Apoptosis in the Presence of Oxygen Radical Scavengers is Dependent on Endogenous NO Synthesis

To examine whether endogenous NO is involved with chondrocyte apoptosis induced by IL-1 in the presence of oxygen radical scavengers, we added the NO synthase inhibitor NMA. NMA dose-dependently reduced IL-1-induced apoptosis (Figure 8) and, with an optimal dose of NMA (1 mmol/L), there was a significant decrease (48 to 66%) in the number of apoptotic cells. These results indicate that endogenously produced NO is required for the IL-1 induction of apoptosis.

Oxygen Radicals Reduce NO-Mediated Apoptosis by Lowering the Levels of Biologically Active NO and NO Protects Chondrocytes from Oxygen Radical-Induced Necrosis

The following experiments addressed mechanisms by which oxygen radicals can reduce NO-induced

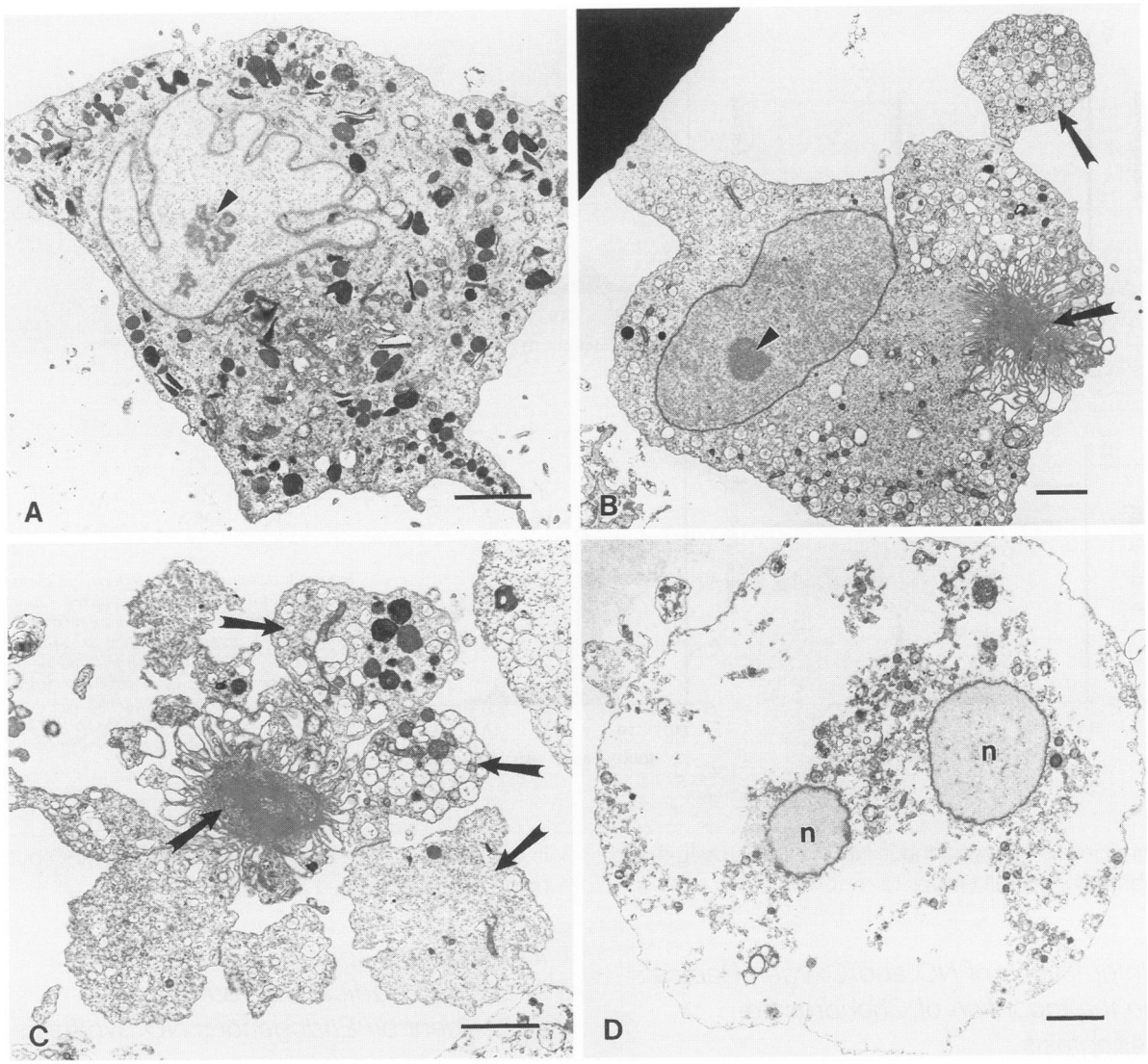

Figure 4. *Electron microscopy of cultured chondrocytes.* **A:** *Control; untreated chondrocytes were characterized by an elaborate cytoplasm containing many organelles and a nucleus containing a loosely organized irregularly shaped nucleolus (arrowhead).* **B:** *After treatment with SNAP for 8 hours, the nucleolus (arrowhead) was round and compact, and presumptive apoptotic bodies (arrows) were observed pinching off from the cytoplasm. Note the paucity of cytoplasmic organelles.* **C:** *After treatment with 2 mmol/L SNP for 16 hours, many cells were detached from the substrata and various kinds of membrane-limited apoptotic bodies (arrows) were observed, some of which resembled those noted in* **B**. **D:** *After treatment with 2 mmol/L SNP for 16 hours, an occasional necrotic cell was observed that contained swollen nuclei (n) and a small amount of cytoplasm with few identifiable organelles. Bar equals 2 μ in* **A–D**.

apoptosis. As IL-1-stimulated chondrocytes produce both NO and oxygen radicals, it was possible that reactions between the different radicals reduced the levels of biologically active NO. This notion was confirmed in experiments in which the addition of DMSO, DMPO, or NAC increased nitrite levels in IL-1-stimulated chondrocyte cultures (Figure 9). These results also revealed a correlation between high NO levels and the induction of apoptosis in chondrocytes.

Oxygen radicals, when generated by hypoxanthine/xanthine oxidase, did not induce apoptotic changes but caused necrosis in a dose-dependent manner. It was therefore unexpected that induction of endogenous oxygen radical synthesis by IL-1 and other proinflammatory stimuli was not associated with chondrocyte necrosis. The interaction of different radicals in the regulation of chondrocyte apoptosis suggested that similar mechanisms may govern the induction of necrosis. To examine this hypothesis we stimulated chondrocytes with the combination of IL-1, TNF, and LPS that did not induce apoptosis or necrosis. However, when the production of high levels of NO in these cultures was inhibited by NMA, there was induction of necrosis (Figure 10).

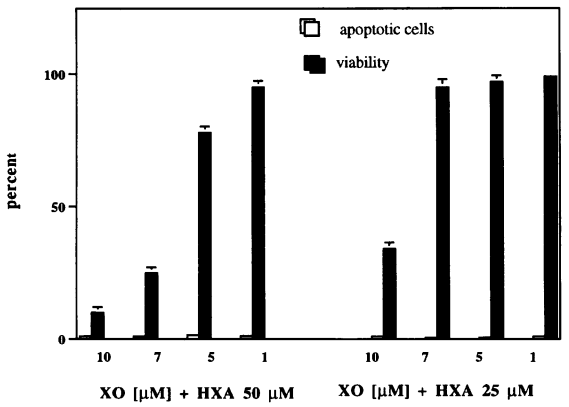

Figure 5. *Effects of oxygen radicals on chondrocyte viability and apoptosis. Chondrocytes were incubated in the presence of the indicated concentrations of hypoxanthine and xanthine oxidase[41,42] for 24 hours. The cells were stained with erythrocin red for the analysis of viability and DAPI for the detection of apoptotic changes. Results are shown as mean percentage ± SEM.*

Table 1. *Endogenous NO Production Does Not Correlate with Cell Death*

Treatment	Nitrites (μmol)	% Apoptotic cells	% Viability
Media	0.8	<1	96
IL-1 (5 ng/ml)	26.2	<1	93
TNF (10 ng/ml)	18.4	<1	91
LPS (1 μg/ml)	21.1	<1	92
IL-1 + TNF + LPS	29.8	<1	91
SNP (2 mmol/L)	42	80	90

Chondrocytes were cultured in the presence of stimuli as indicated. Conditioned media were collected after 48 hours for the analysis of nitrites. Viability was determined by erythrosin staining.[43]

Discussion

Articular cartilage shows a profound decrease in cellularity as a function of aging[2–6] and this may provide one important component in development of osteoarthritis, the most common age-related disease in humans.[1] The basis for this age-related cellular change is unknown. During development, chondrocytes in the growth plate undergo a sequence of events that includes proliferation, hypertrophy, and cell death.[23] Mechanisms of cell death in cartilage development are essentially uncharacterized, and information is limited to ultrastructural evidence for apoptosis in terminal hypertrophic chondrocytes.[24] The present study is the first analysis of mechanisms and patterns of cell death that can be induced in human articular chondrocytes.

It is shown that chondrocytes can undergo cell death with morphological and ultrastructural changes and DNA fragmentation characteristic of apoptosis. Analysis of extracellular stimuli showed that none of a broad spectrum of chondrocyte regulatory factors induced apoptosis. IL-1, TNF, LPS, IFN-γ, and combinations of these cytokines that stimulate the expression of proinflammatory mediators and matrix metalloproteinases did not induce apoptosis or necrosis. NO, a recently identified product of cytokine-activated chondrocytes, was the major trigger for apoptosis. In contrast, oxygen radicals not only failed to induce apoptosis but prevented endogenously produced NO from inducing apoptosis.

NO has been shown to induce cell death and several mechanisms have been suggested. In murine macrophages that produce NO, the activity of NO synthase correlates inversely with their life span, and apoptosis induced in these cells by LPS and IFN-γ is dependent on NO.[16,17] As one possible mechanism for the NO induction of cell death in these and other cell types, inhibition of aconitase in the tricarboxylic acid cycle has been proposed. This interferes with oxidative metabolism and respiration and leads to a dependence on glycolysis. This is consistent with observations that NO induces apoptosis under conditions of glucose deprivation, which can be found in the intra-articular milieu during joint inflammation. Synovial fluid, in particular from patients with rheumatoid arthritis, has reduced O_2 partial pressure (pO_2), decreased pH, and increased pCO_2, suggesting a change of local metabolism from aerobic to anaerobic glycolytic metabolism. Low pH and high lactate and pCO_2 values correlate with low pO_2 values,[25–27] and synovial fluid acidosis correlates with joint destruction.[28]

In rat insulinoma cells, the combination of IL-1, TNF and IFN-γ induces high levels of NO, mitochondrial damage, DNA fragmentation, and cell death, changes that are inhibited by the NO synthase inhibitor L-NG-monomethyl arginine. Although IL-1 alone also induced high levels of NO, it did not cause apoptosis. Nicotinamide, which inhibits ADP ribosylation and scavenges oxygen radicals, prevented cell death without affecting NO levels. NO may thus be necessary, but is apparently not sufficient, for the induction of apoptosis in this cell system.[29]

Glutamate-induced neurotoxicity has been suggested to involve both superoxide[30,31] and NO.[15] The NO-mediated neurotoxicity correlated with activation of poly(ADP) ribose synthetase, which can lead to cell death through depletion of β-nicotinamide adenine dinucleotide and ATP.[32] Benzamidine, an inhibitor of poly(ADP) ribose synthetase, blocked NO-mediated neurotoxicity.[32] NO can also deaminate purine and pyrimidine bases and result in mutations and strand

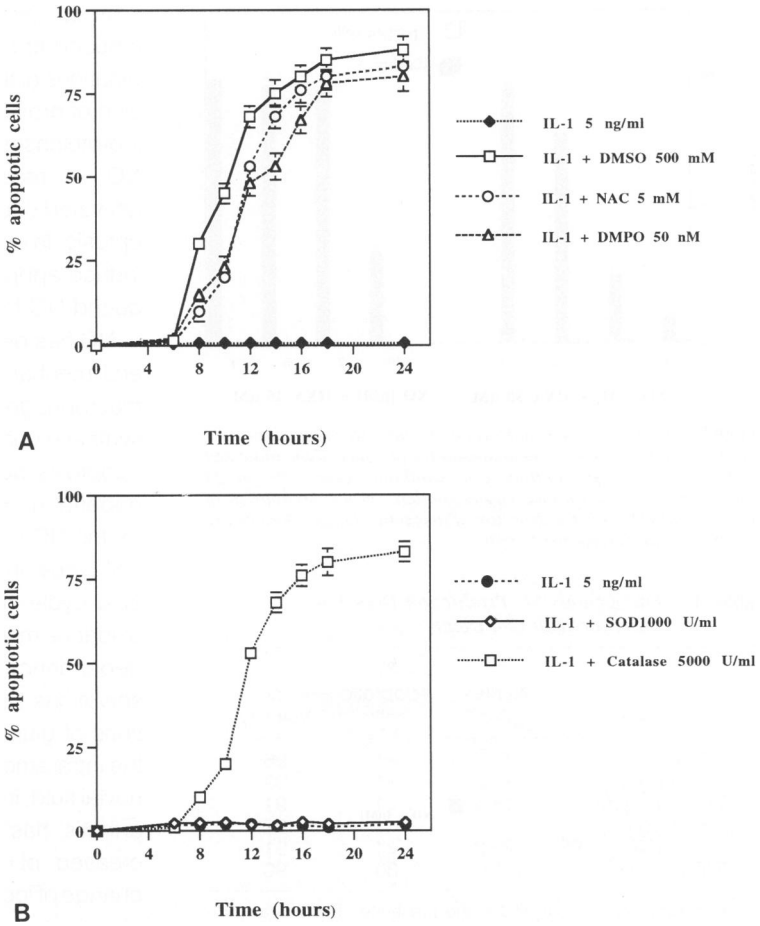

Figure 6. *IL-1 induces chondrocyte apoptosis in the presence of oxygen radical scavengers* (A) *and catalase* (B). *A: Chondrocytes were incubated with IL-1 plus DMSO, NAC, or DMPO and analyzed by flow cytometry for the percentage of apoptotic cells.* B: *The effects of SOD and catalase. Comparison of values obtained with IL-1 alone* versus *the presence of radical scavengers at 6 and 8 hours,* $P \leq 0.01$; *at all other time points,* $P \leq 0.001$.

breaks,[33] a stimulus for the activation of poly(ADP) ribose synthetase.

These studies not only suggest distinct and possibly tissue-specific mechanisms of NO-induced cell death but also emphasize the importance of NO interactions with other radicals. Cultured chondrocytes produce hydroxyl radical and hydrogen peroxide, superoxide, and singlet oxygen in response to activation by cytokines, phorbol esters, or anoxia-reoxygenation.[34–36] More recently, chondrocytes have been characterized as a major intra-articular cell source of NO.[11–13] Hydrogen peroxide[37] and NO[38] inhibit proteoglycan synthesis in chondrocytes[37] and other synthetic processes, including total protein and DNA synthesis. This correlated with reduced ATP levels[39] and oxidative inactivation of glyceraldehyde 3-phosphate dehydrogenase.[40] Intracellular oxygen radical damage may thus occur through inhibition of glyceraldehyde 3-phosphate dehydrogenase and subsequent depletion of intracellular ATP. This information led us to analyze interactions of NO and oxygen radicals in the induction of apoptosis in chondrocytes. Based on the effects of several exog-

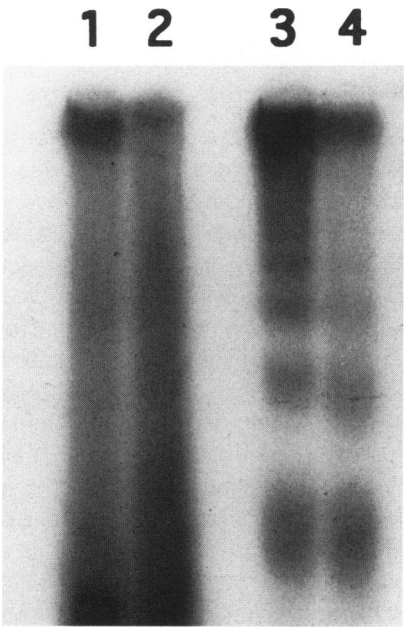

Figure 7. *IL-1-induced apoptosis is associated with internucleosomal DNA cleavage. Chondrocytes were cultured in media alone* (lane 1), *IL-1* (lane 2), *SNAP* (lane 3), *or the combination of IL-1 and DMSO* (lane 4). *Cells were collected after 12 hours for the extraction of DNA, which was end labeled and separated on agarose gels as described.*[20]

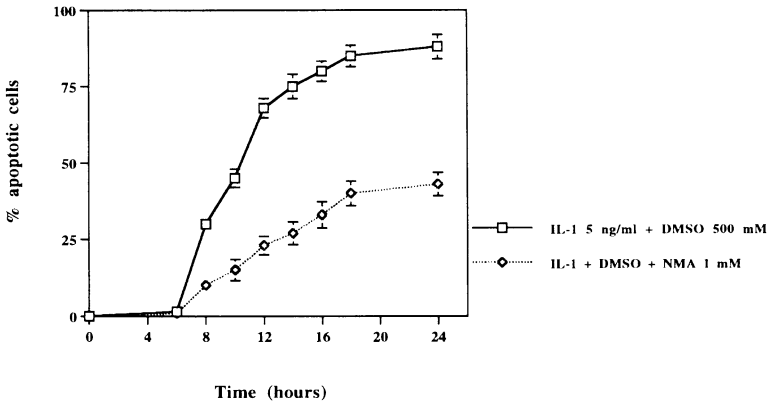

Figure 8. *IL-1-induced apoptosis in the presence of oxygen radical scavengers is dependent on endogenous NO synthesis. Chondrocytes were treated with IL-1 (5 ng/ml) or DMSO (500 mmol/L) in the absence or presence of the NO synthesis inhibitor NMA (at optimal concentration of 1 mmol/L). Cells were collected at the times indicated and analyzed by flow cytometry for the percentage of apoptotic cells. Comparison of values obtained with IL-1 + DMSO versus IL-1 + DMSO + NMA at 6 and 8 hours showed differences at* $P \leq 0.01$; *for all other time points,* $P \leq 0.001$.

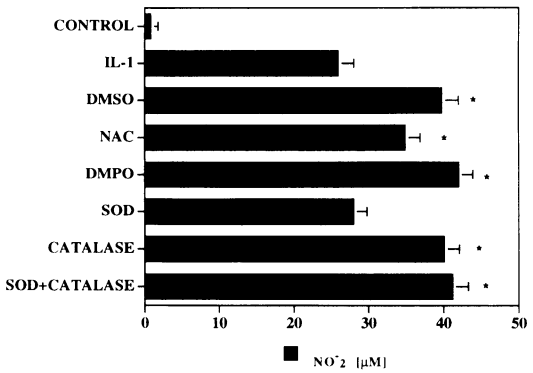

Figure 9. *Inhibition of oxygen radicals increases NO levels in IL-1-stimulated chondrocyte cultures. Chondrocytes were cultured in the presence of IL-1 (5 ng/ml) alone or IL-1 plus DMSO (200 mmol/L), NAC (1 mmol/L), DMPO (5 mmol/L), SOD (100 U/ml), catalase (1000 U/ml), or both SOD and catalase. Conditioned media were collected after 48 hours and nitrite levels determined in the Griess reaction.* $^{*}P \leq 0.001$ *in comparison with IL-1.*

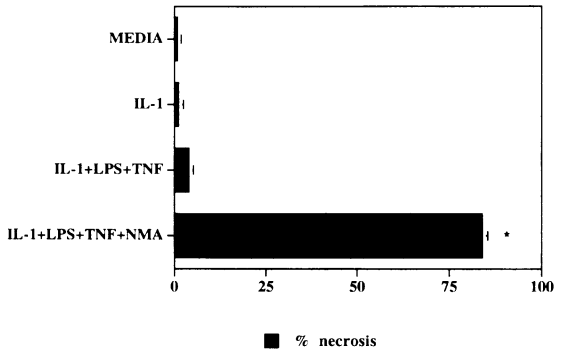

Figure 10. *Inhibition of NO synthesis in the presence of proinflammatory mediators results in chondrocyte necrosis. Chondrocytes were stimulated with IL-1, TNF-α, and LPS in the presence or absence of NMA. Cell viability was determined by staining with erythrosin red.* $^{*}P \leq 0.001$ *in comparison with media.*

enous NO donors including SNP, SNAP, and 3-morpholinosynonimide-hydrochloride the oxygen radical scavengers were dependent on NO as indicated by the protective effect of NMA. NO appeared sufficient to induce apoptosis in chondrocytes. The effects of the NO donors were typical for apoptotic cell death by morphological, ultrastructural, and biochemical criteria. The results obtained with the exogenous NO donors were in contrast to the findings with inducers of endogenous NO synthesis. IL-1, TNF, IFN-γ and LPS, as well as combinations of these stimuli that caused the expected increase in NO synthesis, did not reduce chondrocyte viability or induce apoptotic changes. Oxygen radicals generated by hypoxanthine/xanthine oxidase did not induce apoptosis but lowered levels of biologically active NO. Quenching oxygen radicals resulted in the induction of apoptosis by IL-1. This was seen with different compounds, including DMSO, DMPO, NAC, and catalase, and associated with increased levels of NO as compared with cultures treated with IL-1 alone. The apoptotic changes seen with IL-1 in the presence of the oxygen radical scavengers were dependent on NO as indicated by the protective effects of NMA.

The induction of apoptosis via endogenously produced NO is a characteristic that distinguishes chondrocytes from fibroblasts as this latter cell type does not produce detectable levels of NO or iNOS mRNA.[14] The expression of NO, its antiproliferative effects, and the ability to induce apoptosis may thus represent a basis for the profound age-related changes in chondrocytes and cartilage.

These findings on the interactions of different radicals suggest that NO is the major trigger for apoptosis in chondrocytes. The observations that high levels of NO derived from exogenous sources induce apoptosis but that endogenously produced NO only induces apoptosis in the presence of oxygen radical scavengers support the conclusion that the relative balance of different radicals will determine their influence on chondrocyte function and survival. Among other radicals, it appears that hydrogen peroxide may be more important than superoxide, as catalase, but not SOD, facilitated IL-1-induced, NO-dependent apoptosis. Oxygen radicals induced necrosis in chondrocytes when generated from hypoxanthine/

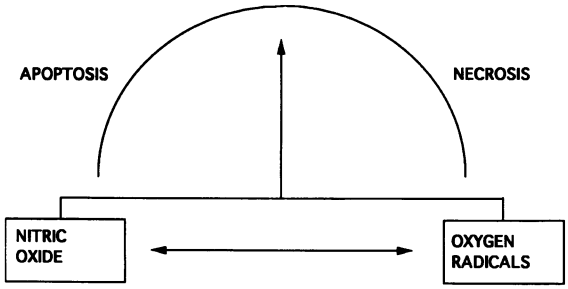

Figure 11. *The balance between NO and oxygen radicals and cell death in chondrocytes. Increased NO levels are associated with apoptosis, and increased levels of oxygen radicals cause necrosis. Not only the rate of production but also interactions between the different radicals determines effects on chondrocyte survival and the type of cell death.*

xanthine oxidase. Endogenous oxygen radicals induced by inflammatory mediators caused necrosis only under conditions which the simultaneous production of NO was reduced. Thus, not only chondrocyte survival but also the type of cell death is determined by the balance between the different radicals (Figure 11). The formation of the different types of radicals is influenced by tissue-specific factors that include oxygen tension, pH, and levels of radical-generating and -scavenging enzymes. The avascularity of cartilage and the relatively low oxygen tension of synovial fluid favor the production of NO by cytokine-activated chondrocytes, and these conditions support the concept that NO-mediated programmed cell death is a central mechanism in cartilage development and contributes to the unique age-related changes in this tissue.

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