Chondrocyte Death in Experimental Osteoarthritis Is Mediated by MEK 1/2 and p38 Pathways: Role of Cyclooxygenase-2 and Inducible Nitric Oxide Synthase

JEAN-PIERRE PELLETIER, JULIO C. FERNANDES, DRAGAN V. JOVANOVIC, PASCAL REBOUL, and JOHANNE MARTEL-PELLETIER

ABSTRACT. ***Objective.*** To explore the mechanisms responsible for *in situ* induction of chondrocyte death in experimental dog osteoarthritic (OA) cartilage. The roles of 2 mitogen activated protein kinases (MAPK), MEK 1/2 and p38, nuclear factor-κB (NF-κB), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and the caspase cascade were investigated.

Methods. OA knee cartilage was obtained from dogs that had received sectioning of the anterior cruciate ligament and were sacrificed 12 weeks after surgery. Cartilage explants were cultured in different inhibitors: Z-DEVD-FMK (caspase 3 inhibitor), Z-LEHD-FMK (caspase 9 inhibitor), PD 98059 (MEK 1/2 inhibitor), SB 202190 (p38 inhibitor), SN-50 (NF-κB inhibitor), NS-398 (COX-2 inhibitor), N-iminoethyl-l-lysine (L-NIL) (iNOS inhibitor). Cartilage specimens were stained for TUNEL reaction and immunostained using specific antibodies for caspase 3, COX-2, iNOS, and nitrotyrosine. Morphometric analyses were performed.

Results. The significant level of chondrocyte death in OA cartilage was markedly decreased by caspase 3 and caspase 9 inhibitors. The two MAPK inhibitors, but not the NF-κB inhibitor, decreased chondrocyte death concomitant with the levels of caspase 3 and iNOS. COX-2 level was reduced by all 3 inhibitors. Specific inhibition of either COX-2 or iNOS reduced the level of chondrocyte death and caspase 3. There was evidence of crosstalk between these 2 latter systems; specific inhibition of COX-2 reduced the iNOS level, and selective inhibition of iNOS reduced COX-2 expression. COX-2 and iNOS seem to function in a positive autoregulatory manner that triggers transcription of their own biosynthetic machinery, since the specific inhibition of each system downregulates its expression.

Conclusion. This study shows that in the early lesions of experimental OA cartilage *in situ*, activation of the caspase cascade is responsible for induction of chondrocyte death. Marked inhibition of cell death by caspase inhibitors indicates a significant participation of apoptosis in the phenomenon. This phenomenon is linked to the activation of at least 2 major kinase pathways, MEK 1/2 and p38. These pathways are responsible for upregulating the expression of iNOS and COX-2, each of which seems essential for the induction of apoptosis. Data are provided about possible regulation and interregulation of the COX-2 and iNOS systems by prostaglandin E_2 and NO. (J Rheumatol 2001;28:2509–19)

Key Indexing Terms:
CHONDROCYTE DEATH OSTEOARTHRITIS APOPTOSIS
MITOGEN ACTIVATED PROTEIN KINASES

The cartilage lesions of osteoarthritis (OA) are characterized by a depletion of extracellular matrix macromolecules and a decreased number of chondrocytes — the only cell type existing in cartilage[1]. The gradual loss of chondrocytes in OA is likely related to both necrosis and apoptosis[2,3]. Studies have explored the mechanisms involved in induction of OA chondrocyte apoptosis. Evidence indicates that the level of chondrocyte apoptosis is related to the extent of matrix depletion, supporting the role of cell injury in this phenomenon[4].

Recent studies indicate a pivotal role of the excess production of nitric oxide (NO) in OA cartilage chondrocyte death by apoptosis[5-8]. This seems related, in part, to the upregulation of inducible NO synthase (iNOS) gene expression by inflammatory mediators such as proinflammatory cytokines[9,10]. Studies have also shown that most of the cells undergoing death are located in the superficial and middle zones of lesional areas of OA cartilage[5-7,11]. This is the area

From the Osteoarthritis Research Unit, Hôpital Notre-Dame, Centre hospitalier de l'Université de Montréal, Montréal, Québec, Canada.

Supported in part by a grant from Le Fonds de Recherche en Santé du Québec (FRSQ).

J-P. Pelletier, MD; J.C. Fernandes, MD, MSc; D.V. Jovanovic, MD, PhD; P. Reboul, PhD; J. Martel-Pelletier, PhD.

Address reprint requests to Dr. J-P. Pelletier, Unité de recherche en Arthrose, Hôpital Notre-Dame, Centre hospitalier de l'Université de Montréal, 1560 rue Sherbrooke est, Montréal, Québec, Canada H2L 4M1.

Submitted February 8, 2001; revision accepted May 15, 2001.

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2001. All rights reserved.

Downloaded on April 15, 2024 from www.jrheum.org

where the greatest loss in extracellular matrix including proteoglycan is found, as well as the highest level of iNOS and NO production[10,12,13].

Proof of the essential roles played by NO in OA chondrocyte death *in vivo* has recently been presented. Treatment with a selective and potent inhibitor of iNOS, N-iminoethyl-l-lysine (L-NIL), reduced the level of chondrocyte apoptosis *in vivo* in experimental OA[14]. This study[14] also revealed the role of the caspase cascade in this phenomenon[5].

Recent reports have outlined the role of cyclooxygenase-2 (COX-2) and prostaglandins in cell apoptosis, particularly in cancer cells[15-21]. Overexpression of the COX-2 gene protects cancer cells from apoptosis, and drugs that inhibit COX-2 were shown to induce programmed death in these cells[17]. Moreover, the use of a specific COX-2 inhibitor has been shown to reduce the size and number of neoplastic polyps in patients with familial polyposis[22]. The role of COX-2 and prostaglandins in chondrocyte death and apoptosis is still under investigation. We reported that, *in vitro* in monolayer cultures, NO mediated chondrocyte death by apoptosis is dependent on the induction of prostaglandin synthesis[23]. Under these experimental conditions, specific inhibition of COX-2 was found to block the induction of cell apoptosis.

We investigated, *in situ*, the major pathways that operate in OA cartilage chondrocyte death including apoptosis. Particular attention was given to the role of the mitogen activated protein kinase (MAPK) pathways as well as the iNOS and COX-2 systems and the interaction between these 2 systems.

MATERIALS AND METHODS

Experimental group. Nine adult crossbred dogs (2–3 years old), each weighing 20–25 kg, were used. Surgical sectioning of the anterior cruciate ligament (ACL) of the right knee through a stab wound was performed as described[24]. Before surgery, the animals were anesthetized intravenously with pentobarbital sodium (25 mg/kg) and intubated. After surgery, the dogs were sent to a housing farm where they were free to exercise in a large pen. All dogs were fed the same food. The dogs received no treatment and were sacrificed 12 weeks after surgery.

Cartilage explant sampling and culture. Immediately after the dogs were killed, the right knees were dissected aseptically. Each knee was examined for gross morphologic changes. A total of 13 cartilage explants (8 from medial condyles, 5 from medial plateaus) were obtained by aseptically dissecting full thickness strips of cartilage across the lesioned areas of articular cartilage from femoral condyles and tibial plateaus, which were processed for culture. The cartilage was cut into duplicate pieces (roughly 150 mg each), rinsed several times in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL Life Technologies, Burlington, ON, Canada), and incubated (duplicate) 48 h at 37°C in a humidified atmosphere of 5% CO_2/95% air in a medium containing DMEM, 10% heat inactivated fetal calf serum (FCS; HyClone Laboratories, Logan, UT, USA), and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin) in the presence or absence of the following inhibitors: Z-DEVD-FMK (10 µM; caspase 3 inhibitor, Calbiochem-Novabiochem, San Diego, CA, USA), Z-LEHD-FMK (10 µM; caspase 9 inhibitor, Calbiochem-Novabiochem), PD 98059 (100 µM; MEK 1/2 inhibitor, Calbiochem-Novabiochem), SB 202190 (10 µM; p38 inhibitor, Calbiochem-Novabiochem), SN-50 [50 µM; nuclear factor κB (NF-κB) inhibitor, Biomol Research Laboratories, Plymouth Meeting, PA, USA], NS-398 (10 µM; COX-2 specific inhibitor, Cayman Chemicals, Ann Arbor, MI, USA), L-NIL (50 µM; iNOS specific inhibitor, Pharmacia Corp., St. Louis, MO, USA).

In situ detection of cell death (TUNEL). Cartilage explants were processed as above. Sections (5 µm) were floated onto Superfrost Plus slides (Fisher Scientific, Nepean, ON, Canada). *In situ* detection of cell death was performed using the Tacs 2TdT (TBL) kit (Trevigen, Gaithersburg, MD, USA) as described[5]. Briefly, sections were digested with chondroitinase ABC (0.25 units/ml; Sigma-Aldrich, Oakville, ON, Canada) in phosphate buffered saline (PBS; Sigma-Aldrich) for 45 min at 37°C and with proteinase K (20 µl; Sigma-Aldrich) for 15 min at room temperature. DNA was end labeled with biotinylated nucelotides using terminal transferase (TdT), followed by binding to horseradish peroxidase that was detected with TACS Blue Label (Trevigen). Sections were counterstained with Red Counterstain C (Trevigen).

Immunohistochemical studies. Cartilage explants were processed for immunohistochemical analysis as described[25]. Briefly, sections (5 µm) of paraffin embedded specimens were placed on Superfrost Plus slides, deparaffinized in toluene, hydrated in a series of graded dilutions of ethanol, and preincubated with chondroitinase ABC (0.25 units/ml) in PBS for 60 min at 37°C. The specimens were washed in PBS, then in 0.3% hydrogen peroxide/PBS for 20 min. Slides were further incubated with universal blocking solution (Dako Diagnostics, Mississauga, ON, Canada) for 15 min at room temperature, then blotted and overlaid for 18 h at 4°C in a humidified chamber with: (1) a rabbit polyclonal antibody (IgG) against iNOS (100 µg/ml, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA); (2) rabbit polyclonal (IgG) anti-nitrotyrosine antibody (dilution 1:1000, Dr. P. Manning, Pharmacia Corp.); (3) rabbit polyclonal antibody (IgG) against COX-2 (dilution 1:250, Oxford Biomedical Research, Oxford, MI, USA); and (4) rabbit polyclonal (IgG) anti-caspase 3 antibody (100 µg/ml, dilution 1:200, R&D Systems, Minneapolis, MN, USA), which recognized only the mature form (p20 subunit) of the enzyme.

Each slide was washed 3 times in PBS, pH 7.4, and stained using the avidin-biotin complex method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). This method entails incubation in the presence of the biotin conjugated secondary antibody for 30 min at room temperature, followed by the addition of the avidin-biotin-peroxidase complex for 30 min. All incubations were carried out in a humidified chamber, and color was developed with 3,3'-diaminobenzidine (Dako Diagnostics) containing hydroxide peroxide. The slides were counterstained with nuclear fast red stain (Digene Diagnostic, Silver Spring, MD, USA).

To determine the specificity of staining, 3 control procedures were used, according to the same experimental protocol: (1) use of adsorbed immune serum (1 h at 37°C) with a 20-fold molar excess of recombinant or purified antigen; (2) omission of the primary antibody; and (3) substitution of the primary antibody with an autologous preimmune serum. The purified antigens used in our study were recombinant human (rh) iNOS, rhCOX-2 (Pharmacia), nitrotyrosine (Sigma-Aldrich), and rhcaspase 3 (Upstate Biotechnology, Lake Placid, NY, USA). The primary antibodies used in this study have been shown to be specific in dogs[5,10].

Several sections were made from each block of cartilage, and slides from each specimen were processed for immunohistochemical analysis. Each section was examined under a light microscope (Leitz Orthoplan) and photographed with Kodak Ektachrome 64 ASA film.

Morphometric analysis. Three sections from each cartilage explant specimen were examined using a Leitz Diaplan microscope (×40), and each section was scored separately. These data were then integrated as a mean for each specimen. The number of chondrocytes staining positive in the superficial zone (superficial and upper intermediate layers) of cartilage for TUNEL reaction, COX-2, iNOS, nitrotyrosine, or caspase 3 was estimated as described[5,25]. Briefly, each section was divided into 3 different areas at the superficial zone of cartilage. For each specimen, it was ensured before the evaluation that an intact cartilage surface could be detected to be used as a marker to validate the morphometric analysis.

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2001. All rights reserved.

Downloaded on April 15, 2024 from www.jrheum.org

The total number of chondrocytes and the number of chondrocytes staining positive were quantitated separately. The final results were expressed as the percentage of positive chondrocytes (mean ± SEM). The maximum score for each cartilage specimen was 100%. Each slide was evaluated by 2 independent observers under blinded conditions (JCF and J-PP); variations between the observers' findings were < 5%.

Statistical analysis. All data were expressed as mean ± SEM and analyzed with the Mann-Whitney U test when appropriate. P values < 0.05 were considered significant.

RESULTS

Levels of chondrocyte death. The chondrocyte death level in OA cartilage explants was 28.6 ± 2.6% (Table 1, Figure 1). The levels were similar for condyles (30.3 ± 9.5%) and for plateaus (24.8 ± 9.2%). Similar results were also obtained in unincubated explants. The level of chondrocyte death in those was 24.6 ± 2.6%. These results are consistent with a report on the *in situ* level of chondrocyte death in experimental dog OA cartilage[5]. Moreover, these levels are much higher than that found in normal cartilage[5]. In this study, we found that the level of chondrocyte death was significantly reduced by the specific inhibition of either caspase 3 or caspase 9. The extent of inhibition was nearly similar for both inhibitors.

Additional experiments were performed to determine the role of the kinases, including the MAPK pathways, as well as the NF-κB system *in situ* in OA chondrocyte death. Data showed (Table 1, Figure 1) that MEK 1/2 as well as p38 inhibitors were almost equally effective at significantly reducing the level of cell death. Interestingly, inhibition of NF-κB had no effect on this process (Table 1).

The potential role of COX-2 and iNOS *in situ* in the death process was also explored[8,26]. As illustrated in Table 1 and Figure 1, specific inhibition of either COX-2 (by NS-398) or iNOS (by L-NIL) significantly reduced the level of chondrocyte death.

Levels of active caspase 3. The level of caspase 3 found in the untreated OA explants was similar to that recorded *in situ* in experimental dog OA cartilage[5]. Moreover, the level of enzymes found in these explants before and after incubation was similar (31.8 ± 1.6 vs 28.7 ± 1.4). The specific inhibition of either MEK 1/2 or p38 very significantly reduced the level of active caspase 3 (Figures 2A, 2B). However, the inhibition of NF-κB had no effect on this caspase level (data not shown). Specific inhibition of either COX-2 or iNOS also very potently reduced the level of caspase 3, although slightly less inhibition was found with L-NIL (Figures 2A, 2B).

Levels of COX-2. Specific inhibition of MEK 1/2 or p38 significantly and markedly reduced the level of COX-2 (Figures 3A, 3B). Inhibition of NF-κB by SN-50 reduced the level of COX-2 (control: 27.5 ± 1.1; SN-50: 20.3 ± 4.1), but these differences were not significant. The specific inhibition of COX-2 by NS-398 reduced the COX-2 cell score almost to background levels. Specific inhibition of iNOS by L-NIL decreased the level of COX-2 by about half of the OA control, a much lesser extent than that found with NS-398 or the 2 MAP kinase inhibitors PD 98059 and SB 202190.

Levels of iNOS and nitrotyrosine. The inhibition of MEK 1/2 or p38 each markedly and significantly reduced the level of iNOS (Figures 4A, 4B). Similar results were obtained for nitrotyrosine (data not shown). The inhibition of iNOS by L-NIL reduced the level of iNOS. Interestingly, the COX-2 specific inhibitor, NS-398, also reduced the level of iNOS, and the results with nitrotyrosine were essentially identical to those of iNOS (data not shown). The inhibition of NF-κB by SN-50 had no effect on the levels of iNOS or nitrotyrosine.

DISCUSSION

There is increasing evidence that predisposition to cartilage degeneration and OA development may be related, at least in part, to gradual reduction in the number of cartilage cells. Chondrocyte death may represent one of the contributing factors in cartilage degradation, particularly in the superficial layers of this tissue where most of the chondrocyte apoptosis takes place. This mechanism of cell death, among other factors, may play a role in the pathogenesis of OA. Hence, we explored the intracellular signaling pathways involved in such a phenomenon *in situ* in OA cartilage. We used cartilage from the ACL dog model representing early OA lesions. Our data from cartilage explants are consistent with prior reports[5] in regard to chondrocyte death level *in*

Table 1. TUNEL cell scores.

	OA Control	Caspase 3 Inhibitor	Caspase 9 Inhibitor	MEK 1/2 Inhibitor	p38 Inhibitor	NF-κB Inhibitor	COX-2 Inhibitor	iNOS Inhibitor
Percentage of positive chondrocytes	28.6 ± 2.6	9.8 ± 1.9	8.8 ± 2.2	7.9 ± 1.0	5.5 ± 1.1	29.6 ± 3.9	7.5 ± 1.9	8.8 ± 1.1
p		0.01	0.002	0.001	0.0001	NS	0.002	0.001

Explants (n = 13 specimens) were incubated in the absence (OA control) or presence of different inhibitors: caspase 3 inhibitor (Z-DEVD--FMK, 10 µM); caspase 9 inhibitor (Z-LEHD-FMK, 10 µM); MEK 1/2 inhibitor (PD 98059, 100 µM); p38 inhibitor (SB 202190, 10 µM); NF-κB inhibitor (SN-50, 50 µM); COX-2 inhibitor (NS-398, 10 µM); iNOS inhibitor (L-NIL, 50 µM). Level of cell death was determined by TUNEL reaction, as described in Materials and Methods. Values are the mean ± SEM percentage of TUNEL positive chondrocytes. p Values indicate difference versus OA control. Statistical analysis by Mann-Whitney U test. NS: nonsignificant.

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2001. All rights reserved.

Downloaded on April 15, 2024 from www.jrheum.org

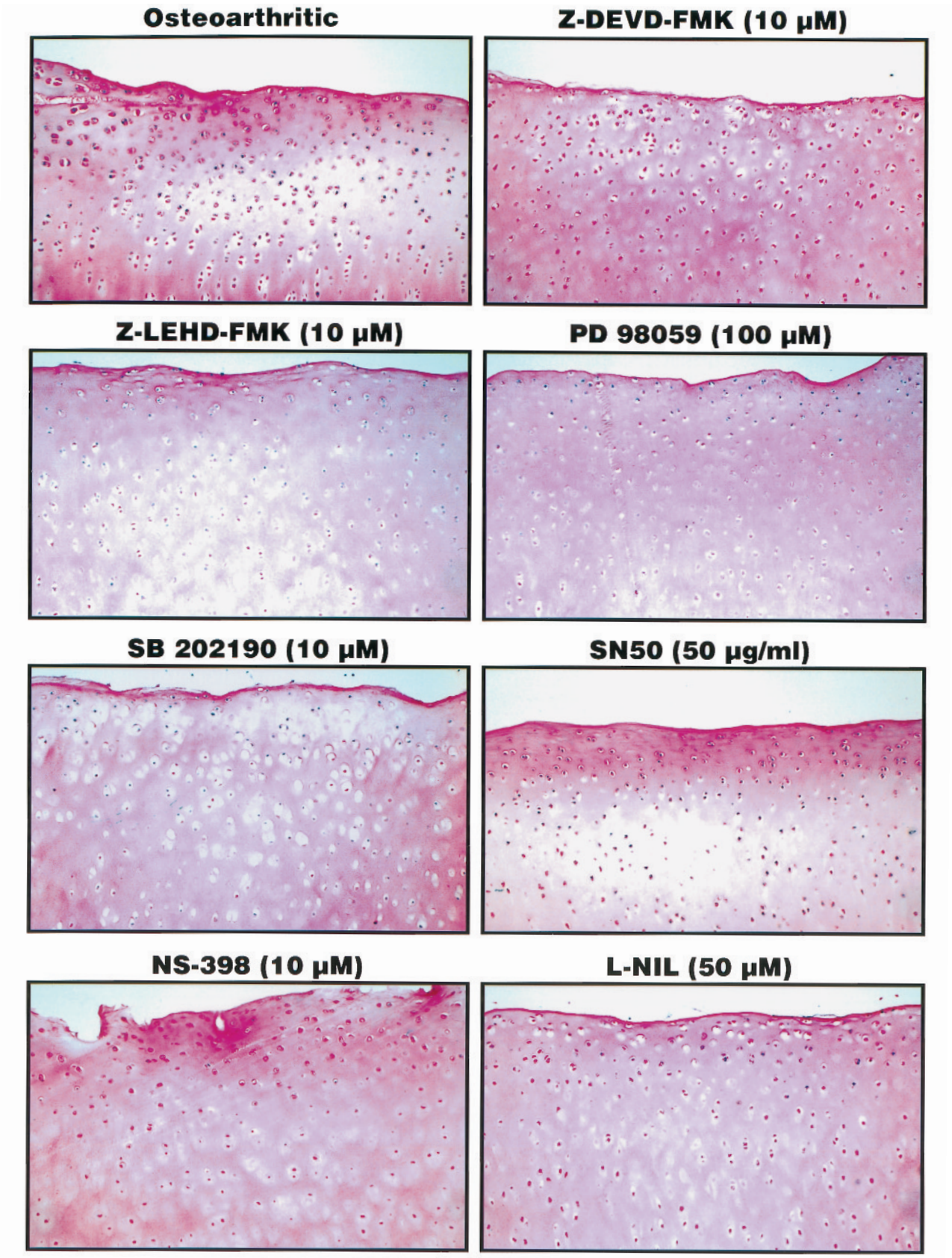

Figure 1. Representative sections of OA cartilage showing *in situ* detection of chondrocyte death by TUNEL reaction. Explants were incubated in the absence (osteoarthritic) or presence of the various inhibitors at the indicated concentrations (original magnification ×100).

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2001. All rights reserved.

Downloaded on April 15, 2024 from www.jrheum.org

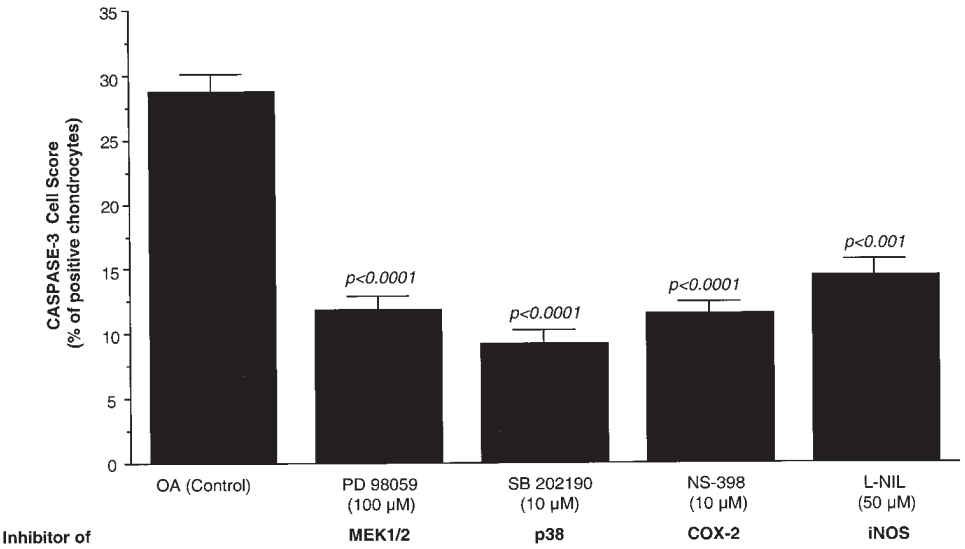

Figure 2A. Effect of inhibitors on the level of active caspase 3 in OA dog cartilage explants. Explants were incubated in the absence (osteoarthritic) or presence of the inhibitors at the indicated concentrations. (A) The level of caspase 3 was determined as described in Materials and Methods. Values are mean ± SEM percentage of caspase 3 positive chondrocytes. P values indicate difference versus OA control (Mann-Whitney U test).

vivo in experimental OA. They also conform with the established predominance of this phenomenon in chondrocytes from the superficial zone of the cartilage[5,6,11].

We demonstrated that the increased level of chondrocyte death in experimental OA cartilage was essentially associated with the activation of the caspase cascade. This brings into perspective the likelihood that a significant part of this phenomenon is related to apoptosis. This finding is also in accord with our previous study in this OA model[5]. This phenomenon was in turn related to the activation of at least 2 important kinase pathways, MEK 1/2 and p38. The increased production of both prostaglandins and NO, as reported[5,6,8,10,26,27], was also shown to have an essential role in the induction of OA chondrocyte death. There is evidence from this study that collaboration or crosstalk between the iNOS and COX-2 systems may exist and that it may play a role in the chondrocyte death phenomenon.

Apoptosis is a highly orchestrated and controlled form of cell death distinct from the pathologic process of necrosis that occurs as a result of cellular damage. Apoptosis involves specific initiating stimuli and intracellular signals, and requires expression of a well defined set of genes that accomplish the cellular program. In general, apoptosis involves sequential activation of a proteolytic cascade of enzymes named caspases[28]. In this study, chondrocyte death was clearly linked to the activation of the caspase cascade, as inhibitors of either caspase 3 or caspase 9 very effectively reduced the level of chondrocyte apoptosis. This finding provides important information, particularly on the relevance of the mitochondrial/cytochrome c pathway in this phenomenon. Indeed, the current understanding of caspase activation is that different initiator caspases are activated by apoptotic stimuli, and those primarily acting on mitochondria preferentially activate caspase 9[28,29]. Nitric oxide has been shown to be an inducer of cytochrome c release[30], and this mechanism may explain, in part, how L-NIL, a specific inhibitor of iNOS, can reduce OA chondrocyte apoptosis[5].

The level of chondrocyte death was very potently reduced by specific inhibition of COX-2 by NS-398, the extent of which was similar to that observed with the specific inhibition of iNOS by L-NIL. It must also be noted that there was a very close connection in the superficial layers of OA cartilage between cells that were undergoing death and those expressing COX-2 and iNOS. This finding is most interesting and indicates the important role played by COX-2 and prostaglandins (PG) in OA chondrocyte apoptosis. The level of COX-2 expression has been found by us and others to be upregulated in human and experimental OA cartilage[10,26]. Here we show that PGE_2 production is required for caspase dependent chondrocyte death, as shown by the concomitant reduction in the level of cell death and caspase 3 by NS-398. However, this contrasts with data from other cell types such as cancer cells and macrophages, in which COX-2 expression was shown to prevent apoptosis[19-21]. The reason for this might be that the role of COX-2 in apoptosis is tissue and/or cell-specific, which likely reflects differences in cellular responses to PGE_2.

We also observed that caspase related chondrocyte death was dependent on the activation of the MAPK pathways such as MEK 1/2 and p38, but not of NF-κB. The absence of effect of NF-κB on chondrocyte caspase dependent death

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2001. All rights reserved.

Downloaded on April 15, 2024 from www.jrheum.org

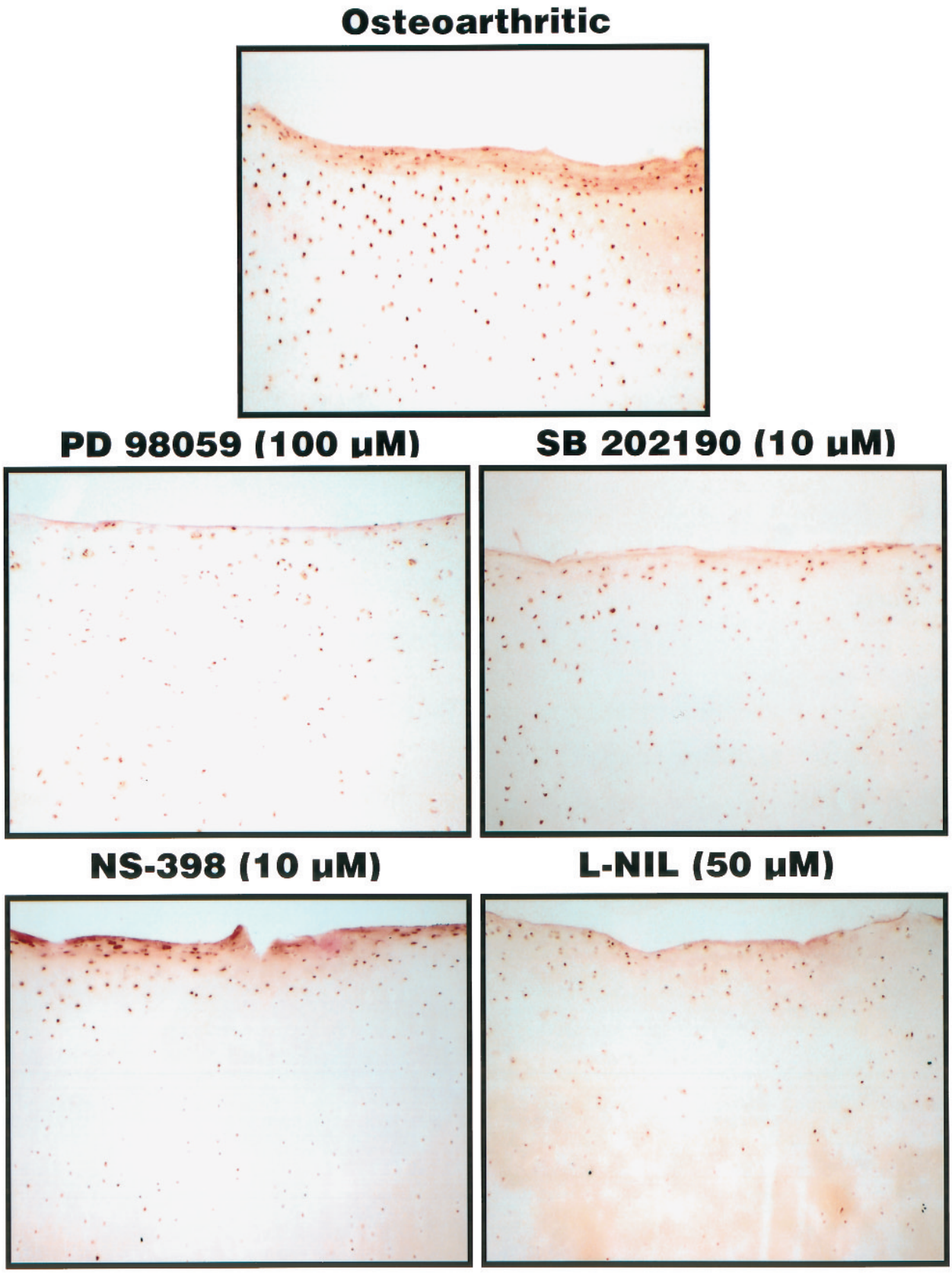

Figure 2B. Representative sections of OA cartilage showing *in situ* detection of caspase 3. Controls showed only background staining (not illustrated) (original magnification ×100).

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2001. All rights reserved.

Downloaded on April 15, 2024 from www.jrheum.org

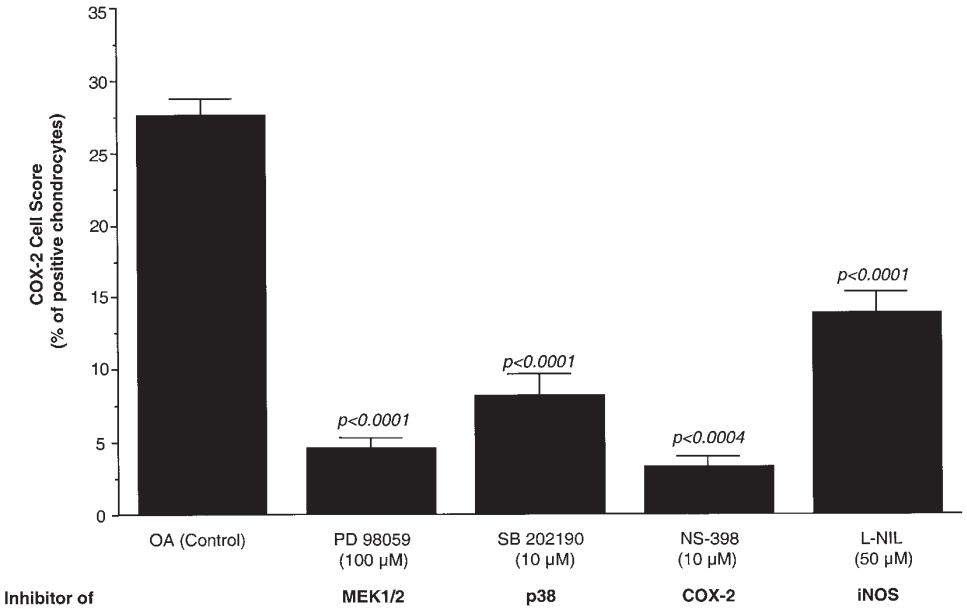

Figure 3A. Effect of inhibitors on the level of chondrocyte COX-2 in OA dog cartilage explants. Explants were incubated in the absence (osteoarthritic) or presence of the inhibitors at the indicated concentrations. (A) The level of COX-2 was determined as described in Materials and Methods. Bars show the mean ± SEM percentage of COX-2 positive chondrocytes. P values indicate the difference versus OA control (Mann-Whitney U test).

contrasts with the data reported on T lymphocytes[31], but is supported by the fact that in our system, inhibition of NF-κB also had no net effect on the level of caspase 3 positive chondrocytes. The activation of these MAPK pathways in OA chondrocytes is likely related to activation of proinflammatory cytokines, such as interleukin 1ß (IL-1ß) and tumor necrosis factor-α[1,32]. Our findings concerning the role of p38 in chondrocyte apoptosis are interesting, since the activation of this kinase has been associated with apoptosis in other cell types including the mesangial cells[33-35], and was associated with an increase in the level of iNOS[36,37].

The anti-apoptotic effect of NF-κB could not be detected in OA cartilage, as the level of chondrocyte death remained quite high, even with the inhibition of NF-κB. The inhibitor used in our system, SN-50, is a cell permeable peptide that inhibits the translocation of NF-κB from the cytoplasm to the nuclei. Its efficacy in *in vitro* experiments using OA chondrocytes has been demonstrated[23]. In our system, NF-κB activation was proven by the reduction in COX-2 production, which is a gene known to be regulated by this pathway. COX-2 inhibition induced by SN-50 was less marked than that found with the 2 MAPK inhibitors and also had no effect on iNOS levels. This implicates other pathways that are operative in OA cartilage regulating the expression of these genes. It can be hypothesized that the absence of a change in chondrocyte death level seen in this study upon inhibition of NF-κB could relate to the differential effect of this system. On one hand, the inhibition of NF-κB could have reduced the synthesis of anti-apoptotic factors; on the other hand, the inhibition of NF-κB reduced the level of COX-2, which has pro-apoptotic activity through the excess synthesis of PGE_2. The net effect of the 2 opposing influences may be one explanation for the absence of a net change in the level of cell death. Moreover, since NO has been shown to inhibit the DNA-binding activity of the NF-κB family proteins[38], this may be another explanation why SN-50 had no effect in our system.

The levels of both COX-2 and iNOS were shown to be effectively regulated by MEK 1/2 and p38 kinases. The MAP kinases, and in particular p38, have been implicated in the upregulation of iNOS gene expression and NO production by IL-1ß stimulated in OA chondrocytes[39]. Moreover, IL-1ß induced COX-2 has recently been shown[40] to be mediated by the inhibition of protein phosphatases that results in a shutdown of the MEK 1/2 cascade, probably due to an increase in cAMP dependent protein kinase activity. Recent data also suggest that the regulation of COX-2 level by MAPK could have different mechanisms. The stimulation of the p38 MAPK signaling pathway increases mRNA stability, while stimulation of extracellular signal regulated kinase pathways regulates COX-2 at the transcription level[41]. Data from this study strongly indicate that the reduction in caspase dependent cell death by MAPK inhibitors was likely related to the COX-2/iNOS combined effect — we observed that PGE_2 alone did not affect the caspase 3 level in chondrocytes[23]. However, the reduced level of caspase 3 found in specimens treated with a specific COX-2 inhibitor alone is quite interesting, and brings into perspective the complexity of the interaction between the COX-2 and iNOS systems.

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2001. All rights reserved.

Downloaded on April 15, 2024 from www.jrheum.org

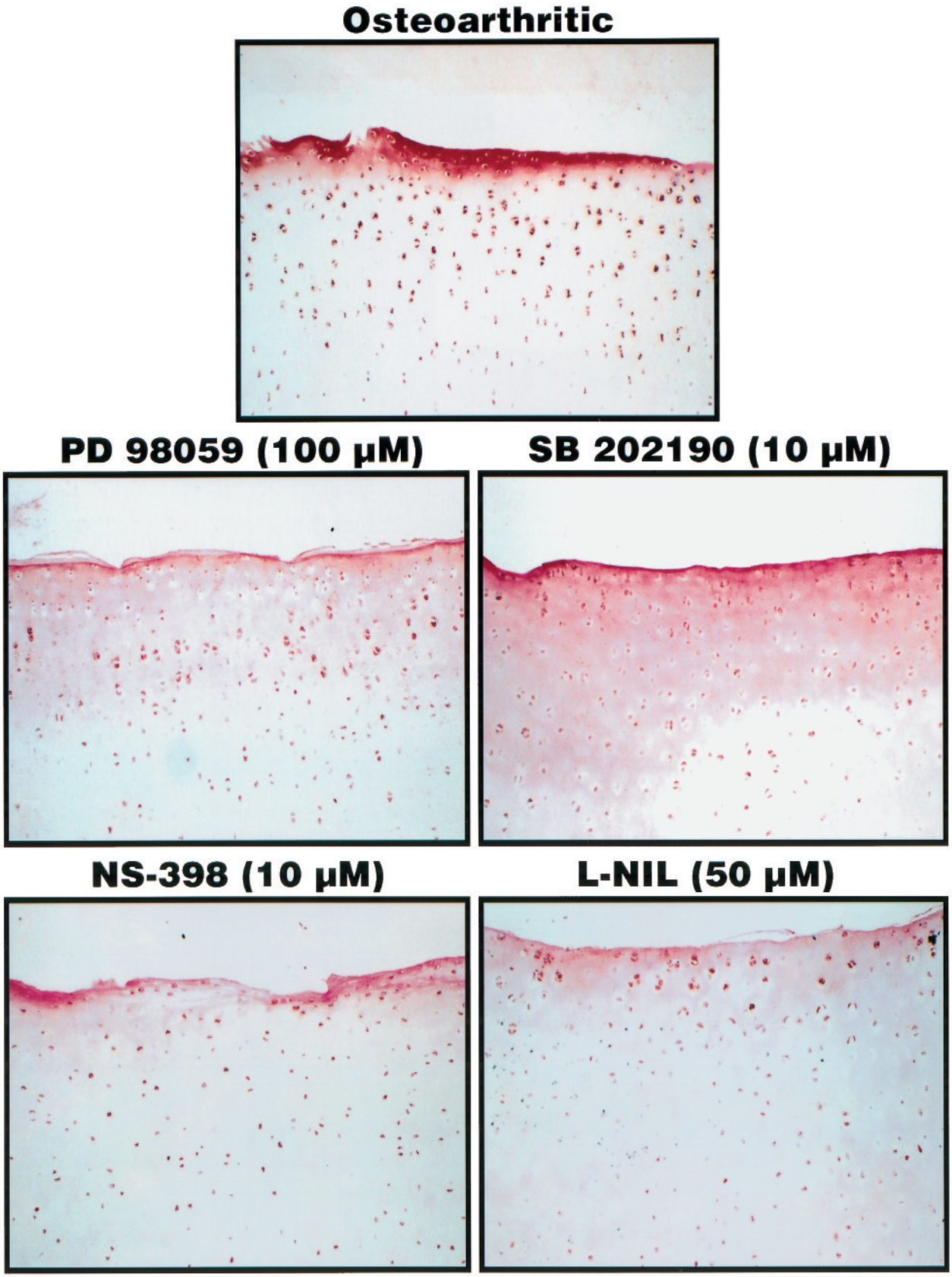

Figure 3B. Representative sections of OA cartilage showing *in situ* detection of COX-2. Controls showed only background staining (not illustrated) (original magnification ×100).

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2001. All rights reserved.

Downloaded on April 15, 2024 from www.jrheum.org

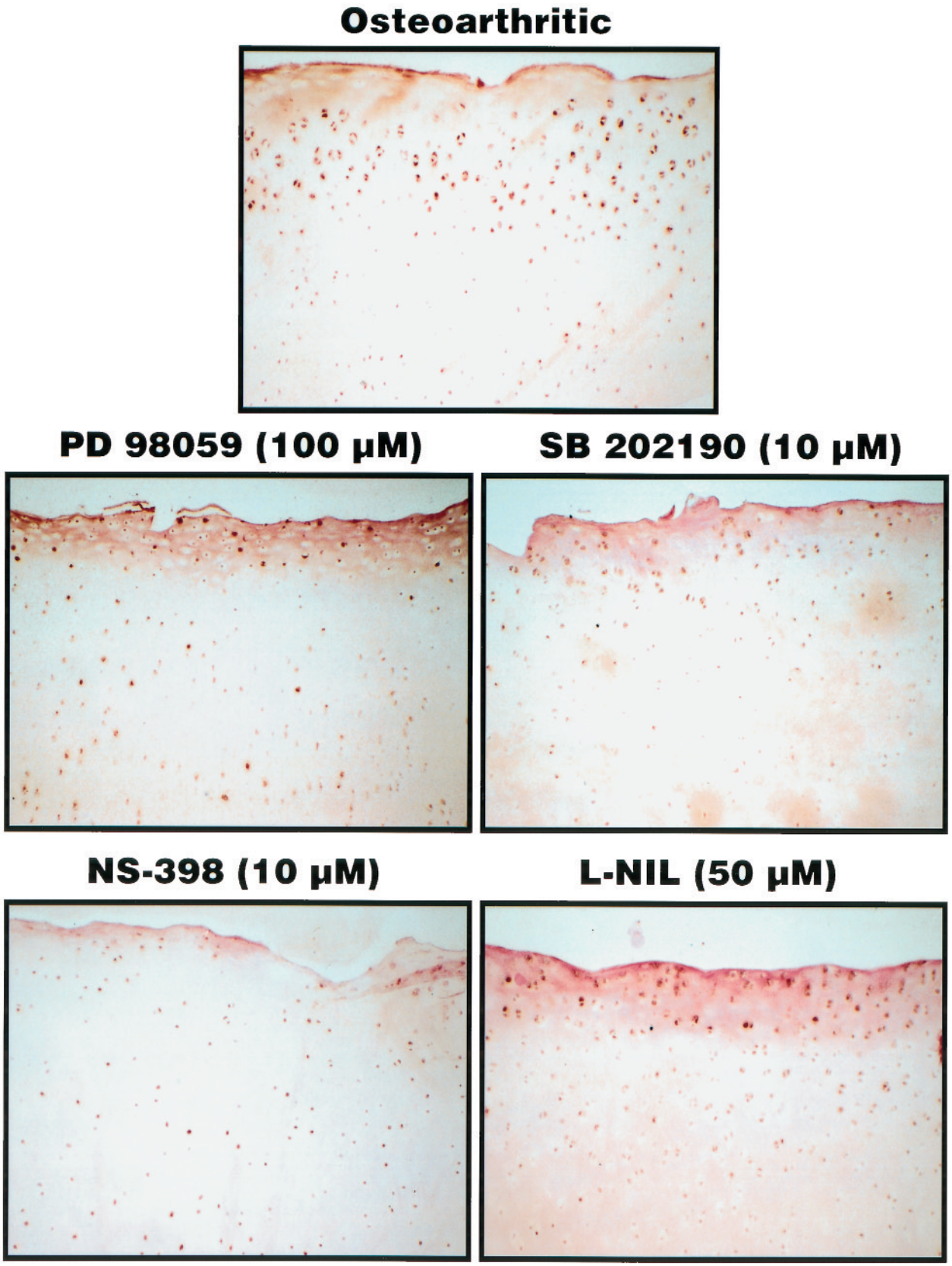

Figure 4A. Representative sections of OA cartilage showing *in situ* detection of iNOS. Controls showed only background staining (not illustrated) (original magnification ×100).

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2001. All rights reserved.

Downloaded on April 15, 2024 from www.jrheum.org

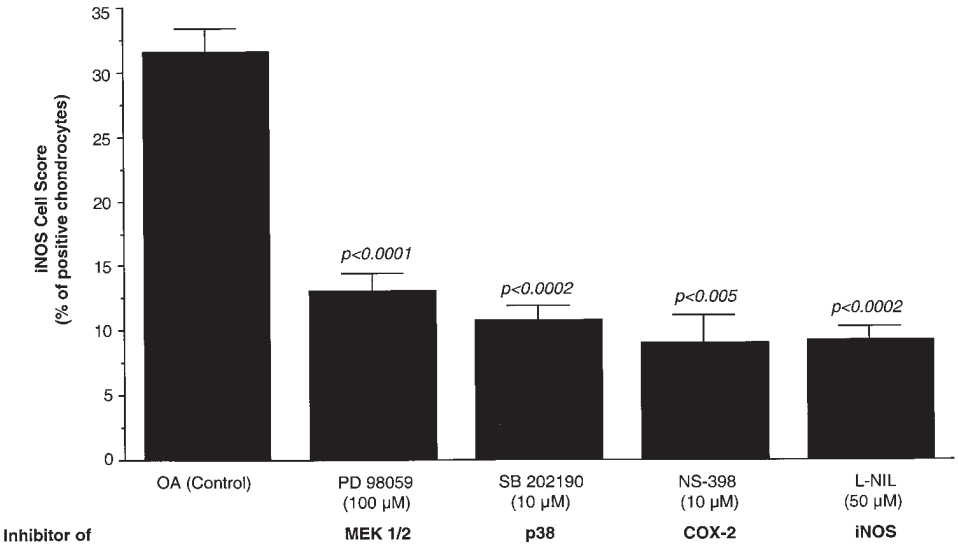

Figure 4B. Effect of inhibitors on the level of chondrocyte iNOS in OA dog cartilage explants. Explants were incubated in the absence (osteoarthritic) or presence of the inhibitors at the indicated concentrations. (B) The level of iNOS apoptosis was determined as described in Materials and Methods. Values show the mean ± SEM percentage of iNOS positive chondrocytes. P values indicate the difference versus OA control (Mann-Whitney U test).

The exact role of NO in the regulation of COX-2 expression/activity is still not completely clear. This may be related to the different experimental systems used. In one recent study, NO was found to upregulate PGE_2 production in OA cartilage, while COX-2 inhibitor did not affect the production of NO[26]. That the specific inhibition of iNOS by L-NIL *in situ* significantly reduces COX-2 levels gives credence to the link between these 2 systems. In addition, our findings indicate that specific inhibition of COX-2 by NS-398 can downregulate iNOS expression, suggesting the strong possibility of crosstalk between these 2 systems in OA chondrocytes. However, one must be careful about the exact meaning of results from these experiments. It is also possible that these findings may be related to inhibition of the synthesis of cytokines, such as IL-1ß, by these 2 inhibitors. This hypothesis is interesting, since we have shown that in dog OA cartilage, the chondrocytes that expressed IL-1 are preferentially located in the superficial layers[1].

Based on these results, it is likely that the inhibition of chondrocyte death by NS-398 or L-NIL may also be related to the combined inhibition of PGE_2 and NO production. Most interesting was the reduction in the level of COX-2 positive cells induced by specific COX-2 inhibition. Similar findings were observed with specific inhibition of iNOS, which induced a reduction in iNOS levels. These results are in agreement with our findings in human OA synoviocytes[42]. These findings suggest that in OA chondrocytes, the expression of COX-2 is autoregulated by PGE_2, and expression of iNOS is regulated by NO. This last finding is in agreement with a report in mesangial cells[43]. This suggests that in OA cartilage pathways other than, for example, proinflammatory cytokines are operating in the regulation of iNOS and COX-2 gene expression.

This study found that the *in situ* increase in chondrocyte death/apoptosis in experimental OA is mainly caspase dependent and is influenced by upregulation of the levels of both COX-2 and iNOS. In turn, enhancement of the level of these 2 enzymes seems to be induced, at least in part, by a crosstalk phenomenon between them. These data also indicate that both MAPK pathways, MEK 1/2 and p38, contribute to the increased level of both enzymes as well as to induction of chondrocyte death. Since PGE_2 alone does not induce OA chondrocyte death or DNA fragmentation[23], then the action of both COX-2 and iNOS *in situ* seems necessary for induction of these phenomena. Further, this process could be amplified by the positive regulation that exists between the COX-2 and iNOS systems. Although the excess production of both prostaglandins and NO appears responsible for the induction of chondrocyte death, the exact mechanisms by which these 2 factors collaborate in this phenomenon remain to be elucidated.

ACKNOWLEDGMENT

The authors express their appreciation to Viorica Lascau for her technical expertise and kind assistance in the preparation of this manuscript. Special thanks are due to Santa Fiori and Heather Yampolsky for help in writing this manuscript.

REFERENCES

1. Pelletier JP, Martel-Pelletier J, Howell DS. Etiopathogenesis of osteoarthritis. In: Koopman WJ, editor. Arthritis and allied conditions. A textbook of rheumatology. 14th ed. Baltimore: Lippincott Williams & Wilkins; 2000:2195-245.

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2001. All rights reserved.

Downloaded on April 15, 2024 from www.jrheum.org

2. Lotz M, Hashimoto S, Kuhn K. Mechanisms of chondrocyte apoptosis. Osteoarthritis Cartilage 1999;7:389-91.
3. 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.
4. Hashimoto S, Ochs RL, Komiya S, Lotz M. Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. Arthritis Rheum 1998;41:1632-8.
5. Pelletier J-P, Jovanovic DV, Lascau-Coman V, et al. Selective inhibition of inducible nitric oxide synthase reduces the progression of experimental osteoarthritis *in vivo*: possible link with the reduction in chondrocyte apoptosis and caspase-3 level. Arthritis Rheum 2000;43:1290-9.
6. 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.
7. Blanco FJ, Ochs RL, Schwarz H, Lotz M. Chondrocyte apoptosis induced by nitric oxide. Am J Pathol 1995;146:75-85.
8. Amin AR, Abramson SB. The role of nitric oxide in articular cartilage breakdown in osteoarthritis. Curr Opin Rheumatol 1998;10:263-8.
9. Clancy RM, Amin AR, Abramson SB. The role of nitric oxide in inflammation and immunity. Arthritis Rheum 1998;41:1141-51.
10. 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.
11. Kim HA, Lee YJ, Seong SC, Choe KW, Song YW. Apoptotic chondrocyte death in human osteoarthritis. J Rheumatol 2000;27:455-62.
12. Sakurai H, Kohsaka H, Liu M, et al. Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. J Clin Invest 1995;96:2357-63.
13. 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.
14. 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.
15. Ho L, Osaka H, Aisen PS, Pasinetti GM. Induction of cyclooxygenase (COX)-2 but not COX-1 gene expression in apoptotic cell death. J Neuroimmunol 1998;89:142-9.
16. Williams CS, Mann M, DuBois RN. The role of cyclooxygenases in inflammation, cancer, and development. Oncogene 1999; 18:7908-16.
17. Chan TA, Morin PJ, Vogelstein B, Kinzler KW. Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. Proc Natl Acad Sci USA 1998;95:681-6.
18. Miwa M, Saura R, Hirata S, Hayashi Y, Mizuno K, Itoh H. Induction of apoptosis in bovine articular chondrocyte by prostaglandin E_2 through cAMP-dependent pathway. Osteoarthritis Cartilage 2000;8:17-24.
19. von Knethen A, Brune B. Cyclooxygenase-2: an essential regulator of NO-mediated apoptosis. FASEB J 1997;11:887-95.
20. Janne PA, Mayer RJ. Chemoprevention of colorectal cancer. N Engl J Med 2000;342:1960-8.
21. Prescott SM. Is cyclooxygenase-2 the alpha and the omega in cancer? J Clin Invest 2000;105:1511-3.
22. Steinbach G, Lynch PM, Phillips RK, et al. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N Engl J Med 2000;342:1946-52.
23. Notoya K, Jovanovic D, Reboul P, Martel-Pelletier J, Mineau F, Pelletier JP. The induction of cell death in human osteoarthritis chondrocytes by nitric oxide is related to the production of prostaglandin E_2 via the induction of cyclooxygenase-2. J Immunol 2000;165:3402-10.
24. Fernandes JC, Martel-Pelletier J, Otterness IG, et al. Effects of tenidap on canine experimental osteoarthritis: I. Morphologic and metalloprotease analysis. Arthritis Rheum 1995;38:1290-303.
25. Moldovan F, Pelletier JP, Hambor J, Cloutier JM, Martel-Pelletier J. Collagenase-3 (matrix metalloprotease 13) is preferentially localized in the deep layer of human arthritic cartilage *in situ*: *In vitro* mimicking effect by transforming growth factor beta. Arthritis Rheum 1997;40:1653-61.
26. Amin AR, Attur MG, Patel RN, et al. Superinduction of cyclooxygenase-2 activity in human osteoarthritis-affected cartilage. Influence of nitric oxide. J Clin Invest 1997;99:1231-7.
27. Amin AR, Di Cesare PE, Vyas P, et al. The expression and regulation of nitric oxide synthase in human osteoarthritis affected chondrocytes: evidence for an inducible "neuronal-like" nitric oxide synthase. J Exp Med 1995;182:2097-102.
28. Thornberry N, Lazebnik YA. Caspases: enemies within. Science 1998;281:1312-6.
29. Nagata S. Apoptosis by death factor. Cell 1997;88:355-65.
30. Ushmorov A, Ratter F, Lehmann V, Droge W, Schirrmacher V, Umansky V. Nitric-oxide-induced apoptosis in human leukemic lines requires mitochondrial lipid degradation and cytochrome C release. Blood 1999;93:2342-52.
31. Kolenko V, Bloom T, Rayman P, Bukowski R, Hsi E, Finke J. Inhibition of NF-kappa B activity in human T lymphocytes induces caspase-dependent apoptosis without detectable activation of caspase-1 and -3. J Immunol 1999;163:590-8.
32. Pelletier J-P, Martel-Pelletier J, Abramson SB. Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. Arthritis Rheum 2001;44:1237-47.
33. Verheij M, Bose R, Lin XH, et al. Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 1996;380:75-9.
34. Kawakami Y, Miura T, Bissonnette R, et al. Bruton's tyrosine kinase regulates apoptosis and JNK/SAPK kinase activity. Proc Natl Acad Sci USA 1997;94:3938-42.
35. Kummer JL, Rao PK, Heidenreich KA. Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen activated protein kinase. J Biol Chem 1997;272:20490-4.
36. Guan Z, Buckman SY, Springer LD, Morrison AR. Both p38alpha (MAPK) and JNK/SAPK pathways are important for induction of nitric-oxide synthase by interleukin-1beta in rat glomerular mesangial cells. J Biol Chem 1999;274:36200-6.
37. Sandau K, Pfeilschifter J, Brune B. The balance between nitric oxide and superoxide determines apoptotic and necrotic death of rat mesangial cells. J Immunol 1997;158:4938-46.
38. Matthews JR, Botting CH, Panico M, Morris HR, Hay RT. Inhibition of NF-κB DNA binding by nitric oxide. Nucleic Acids Res 1996;24:2236-42.
39. Badger AM, Cook MN, Lark MW, et al. SB 203580 inhibits p38 mitogen-activated protein kinase, nitric oxide production, and inducible nitric oxide synthase in bovine cartilage-derived chondrocytes. J Immunol 1998;161:467-73.
40. Miller C, Zhang M, He Y, et al. Transcriptional induction of cyclooxygenase-2 gene by okadaic acid inhibition of phosphatase activity in human chondrocytes: Co-stimulation of AP-1 and CRE nuclear binding proteins. J Cell Biochem 1998;69:392-413.
41. Matsuura H, Sakaue M, Subbaramaiah K, et al. Regulation of cyclooxygenase-2 by interferon gamma and transforming growth factor alpha in normal human epidermal keratinocytes and squamous carcinoma cells. Role of mitogen-activated protein kinases. J Biol Chem 1999;274:29138-48.
42. Fahmi H, He Y, Zhang M, Martel-Pelletier J, Pelletier JP, Di Battista JA. Nimesulide reduces interleukin-1ß-induced cyclooxygenase-2 gene expression in human synovial fibroblasts. Osteoarthritis Cartilage 2001;9:332-40.
43. Muhl H, Pfeilschifter J. Amplification of nitric oxide synthase expression by nitric oxide in interleukin 1 beta-stimulated rat mesangial cells. J Clin Invest 1995;95:1941-6.

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2001. All rights reserved.

Downloaded on April 15, 2024 from www.jrheum.org