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IL-1 β Protects Human Chondrocytes from CD95-Induced Apoptosis¹

Klaus Kühn, Sanshiro Hashimoto, and Martin Lotz²

This study addresses the effects of IL-1 β on apoptosis induced by agonistic anti-CD95 (Fas) Ab. IL-1 β inhibited anti-CD95 Ab-induced apoptosis in all preparations of normal human articular chondrocytes tested. Inhibitors of nitric oxide synthase or cyclooxygenase did not influence the protective effect of IL-1 β , indicating that nitric oxide and PGs were not involved in the modulation of CD95-induced apoptosis. However, when the IL-1 β -dependent induction of NF- κ B was inhibited, the antiapoptotic effect of IL-1 β was partially reversed, suggesting that NF- κ B-mediated gene activation is part of the protective mechanism. In addition, IL-1 β significantly increased the expression of Bcl-2. The protein tyrosine kinase inhibitor herbimycin A completely eliminated the protective effect of IL-1 β on CD95-induced apoptosis. These findings suggest that IL-1 β modulates the CD95 death cascade in chondrocytes by mechanisms that involve tyrosine phosphorylation events and NF- κ B-dependent gene activation. *The Journal of Immunology*, 2000, 164: 2233–2239.

Cartilage degradation occurs in inflammatory arthropathies, such as rheumatoid arthritis and osteoarthritis (OA),³ the major age-associated joint disease. Chondrocyte death and the reduction of tissue cellularity may represent an important component in the pathogenesis of cartilage destruction (1). Apoptotic death of chondrocytes has been described in endochondral ossification (2, 3) and in human (4, 5) as well as experimentally induced OA in rabbits (6). In both types of OA, increased chondrocyte apoptosis was correlated with the severity of cartilage damage. However, the physiologic mechanisms that regulate chondrocyte apoptosis have been poorly characterized. We have recently shown that human chondrocytes express the death receptor CD95 (Fas/APO-1) on the cell surface and that primary cultures of chondrocytes can be induced to undergo apoptosis by agonistic anti-CD95 Ab (7). In addition, we have demonstrated that the expression and activity of the CD95/CD95L death receptor system in cultured chondrocytes are profoundly influenced by cell density (8). Chondrocyte apoptosis can also be induced by NO donors (9). However, endogenous NO, which is produced as a consequence of IL-1 β stimulation, does not induce apoptosis in chondrocytes. IL-1 β does induce apoptosis in these cells when used in combination with oxygen radical scavengers (9). Other cytokines, such as TNF- α or TGF- β , which have proapoptotic effects in other cell systems (10–13), do not induce apoptosis in chondrocytes (14). It is possible that the initial events that predispose articular chondrocytes to apoptosis as well as the activity of

intracellular apoptotic signaling cascades are regulated by cytokines.

This study addresses the effects of cytokines, in particular IL-1 β , on CD95-dependent chondrocyte apoptosis. We show that chondrocyte apoptosis induced by agonistic Ab to CD95 is inhibited by IL-1 β . We also demonstrate that the effects of IL- β are not mediated by nitric oxide or PGs. Our results suggest that tyrosine kinase-controlled signal transduction pathways as well as NF- κ B activation are major mediators of the antiapoptotic actions of IL-1 β in chondrocytes.

Materials and Methods

Reagents

DMEM, penicillin/streptomycin, L-glutamine, PBS, and FBS were purchased from Life Technologies (Gaithersburg, MD). All other chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise. Agonistic Ab directed against CD95 (clone CH-11) was purchased from Kamiya Biomedical (Seattle, WA). Abs to inducible NO synthase (iNOS) and caspase-3 were obtained from Transduction Laboratories (Lexington, KY). Monoclonal anti-Bcl-2 Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human IL-1 β and recombinant human TNF- α were purchased from InterGen (Purchase, NY) and PeproTech (Rocky Hill, NY), respectively. Recombinant human TGF- β was obtained from Austral Biologicals (San Ramon, CA). Indomethacin and PGE₂ were obtained from Cayman Chemical (Ann Arbor, MI). Pyrrolidine dithiocarbamate (PDTC), N-monomethyl-L-arginine (L-NMA), and 6-aminoguanidine were purchased from Alexis (San Diego, CA). Herbimycin A and staurosporine were obtained from Calbiochem (San Diego, CA).

Cell culture

Human chondrocytes were obtained from macroscopically normal knee articular cartilage of donors without a recorded history of joint disease and were cultured as described previously (9). The chondrocytes used in the experiments reported here were treated as follows. After initial isolation the cells were kept in DMEM (high glucose) supplemented with 10% FBS, L-glutamine, and antibiotics and allowed to attach to the surface of the culture flasks. After the cells had grown to confluence they were split once (passage 1) and grown to confluence again. For use in the experiments the cells were then trypsinized, washed once, and plated at confluence at 3×10^6 cells/100-mm plate, $1.5\text{--}2 \times 10^6$ cells/60-mm dish (Becton Dickinson, Franklin Lakes, NJ), $6\text{--}8 \times 10^5$ cells/six-well plate (Costar, Cambridge, MA), or at 2×10^5 cells/12-well plate (Costar).

Quantification of nitrites

Chondrocytes were plated at 40,000 cells/well in 96-well plates in the presence of 10% FBS. After 48 h the medium was changed, and the cells

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³ Abbreviations used in this paper: OA, osteoarthritis; iNOS, inducible NO synthase; PDTC, pyrrolidine dithiocarbamate; L-NMA, N-monomethyl-L-arginine.

were stimulated as described in *Results*. NO production was detected as NO₂ accumulation in the culture supernatants by the Griess reaction as described previously (14).

Measurement of cell death

Fragmented nucleosomal DNA was measured using the Cell Death ELISA Plus kit from Roche (Indianapolis, IN) according to the manufacturer's protocol. Chondrocytes were plated confluent in 12-well plates in the presence of 5% FBS. After 48 h the medium was changed, and the cells were stimulated with CH-11 (0.75–1.0 μ g/ml) or mouse IgM (Harlan, Indianapolis, IN) at 0.75–1 μ g/ml as an isotype control, staurosporine (1 μ M), herbimycin A (1 μ M), IL-1 β (5 ng/ml), TGF- β (10 ng/ml), or TNF- α (25 ng/ml). The iNOS inhibitors L-NMA and 6-aminoguanidine were used at 5 mM. Indomethacin, a specific inhibitor of the cyclooxygenase activity of PGH synthase-1 and -2, was used at 15 μ M ($2 \times IC_{50}$), and PGE₂ was used at 20 ng/ml. In experiments involving the inhibition of NF- κ B activity chondrocytes were incubated for 3 h with 0.1 mM PDTC before the addition of other stimulating agents. All compounds were used at concentrations previously reported by us and others to be effective in chondrocytes. After the indicated periods of time in all cases the medium that contained floating cells was harvested, and the cells on the plate were trypsinized briefly. Floating and trypsinized cells were combined and spun down. The pellet, representing the total cell population in the well, was resuspended in 2 ml of DMEM supplemented with glutamine and antibiotics. The cells were counted, and viability was assessed by trypan blue dye exclusion. For each sample 5×10^4 cells were spun down in an Eppendorf tube, lysed in 0.2 ml of lysis buffer, and transferred to a microtiter plate. The nuclei were spun down at $300 \times g$ for 10 min, and 20 μ l of the supernatants were used in the cell death ELISA.

TUNEL assay

Chondrocytes were plated subconfluent and confluent in chamber slides (Nunc, Naperville, IL) in the presence of 5% FBS. After 48 h the medium was changed, and the cells were stimulated with mouse IgM or CH-11 at 1 μ g/ml for 14 h. The cells were then fixed with fresh 4% paraformaldehyde for 15 min at 4°C and permeabilized by treatment with 0.5% Tween 20 in PBS for 15 min at room temperature. Following permeabilization, the cells were incubated in 100 μ l of TdT labeling solution (MEBSTAIN Apoptosis Kit; Medical & Biological Laboratories, Watertown, MA) and incubated for 1 h at 37°C. After washing the cells were counterstained with 100 μ l of a 0.5 μ g/ml propidium iodide solution for 15 min at room temperature and examined by fluorescence microscopy.

Western blot analysis

Whole cell extracts were prepared from 3×10^6 chondrocytes stimulated as described in *Results* by lysing the cells on the plate with ice-cold lysis buffer (10 mM Tris (pH 7.6), 158 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, leupeptin, aprotinin (1 μ g/ml each), and 1 μ M PMSF, which was added immediately before use). The lysates were transferred to Eppendorf tubes and centrifuged at 14,000 rpm for 30 min at 4°C. The supernatants were transferred into fresh tubes, and the protein concentration was determined by Bradford assay. Similar amounts of protein were separated by 10 or 14% SDS-PAGE and transferred to nitrocellulose filters (Schleicher & Schuell, Keene, NH) by electroblotting. The filters were blocked overnight in 2% FBS/Tris-buffered saline solution supplemented with 0.1% Tween 20 (TBS-T). The membranes were then rinsed once with TBS-T and incubated with Ab against iNOS, caspase-3, or Bcl-2 for 2 h. The membranes were washed three times with TBS-T and then further incubated with HRP-conjugated secondary Ab for 1 h. Afterward the membranes were washed three times with TBS-T and developed using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

Northern blot analysis

Total RNA was isolated from 2×10^6 chondrocytes/sample using the STAT-60 reagent (Tel-Test, Friendswood, TX). The RNA for each sample was quantified photometrically, and 5 μ g were separated on 1.2% agarose/6% formaldehyde gels. After electrophoresis the gels were photographed, and the RNA was transferred onto Hybond-N nylon membranes (Amersham) by capillary blotting. The membranes were air-dried, and baked for 2 h at 80°C. Prehybridization was performed for 2 h at 60°C in $5 \times$ SSC, 1 mM EDTA, 0.2% SDS, and $5 \times$ Denhardt's solution. Radio-labeled probe was added, and hybridization was conducted overnight at 60°C. After hybridization, the filters were rinsed twice in $2 \times$ SSC/0.1% SDS and washed once in $2 \times$ SSC/0.1% SDS at 60°C and once in $0.2 \times$ SSC/0.1% SDS at 60°C. The membranes were covered with Saran wrap and exposed with intensifying screen for 12 h at -70°C . The probe used

for the hybridization was prepared as follows: a 236-bp fragment from the coding region of iNOS was obtained by RT-PCR using RNA from IL-1 β -stimulated chondrocytes. The primer sequences were: forward primer, 5'-TTCAATGGCTGGTACATGGGCAC-3'; and reverse primer, 5'-GGAGG GACCAGCCAAATCCAGTC-3'. The PCR fragment was gel purified and labeled using the random hexamer priming kit from Roche. Unincorporated nucleotides were removed using the NucTrap system (Stratagene, San Diego, CA), and the sp. act. of the probe was determined by scintillation counting. Before addition to the hybridization solution the probe was boiled for 5 min.

RT-PCR analysis

Total RNA was isolated from IL-1 β -stimulated chondrocytes and reverse transcribed using the Superscript Preamplification System (Life Technologies, Gaithersburg, MD) with random hexamers. One microliter of the RT reactions was subjected to PCR analysis with primers specific for Bcl-2 and GAPDH. The sequences of the primers were chosen as follows: Bcl-2 forward primer, 5'-TGCCACCTGTGGTCCACCTGGCCCT-3'; Bcl-2 reverse primer, 5'-AACAGAGGCGCATGCTGGGGCCGT-3'; GAPDH forward primer, 5'-TGGTATCGTGAAGGACTCATG-3'; and GAPDH reverse primer, 5'-ATGCCAGTGAGCTTCCCGTTC-3'. The PCR conditions for Bcl-2 amplification were 2 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 62°C, and 40 s at 72°C. The conditions for GAPDH amplification were 2 min at 95°C, 25 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. The PCR products were separated on 1.5% agarose gels.

Statistical analysis

The results of the cell death ELISAs were statistically evaluated using StatView software (Abacus Concepts, Berkeley, CA). Individual experiments were repeated at least three times. Paired Student *t* test was employed to determine significance levels between datasets.

Results

Influence of IL-1 β on the induction of chondrocyte apoptosis

When IL-1 β was added to adherent first-passage chondrocytes, we observed morphological changes, including retraction of plasmopodia and rounding as well as shrinking of the cells (Fig. 1A), which were reminiscent of the changes described for proapoptotic stimuli such as the protein kinase inhibitor staurosporine (15) or the NO donor sodium nitroprusside. To test whether the morphological changes in these cells were associated with apoptotic death, we incubated confluent monolayer cultures for 14 h with 5 ng/ml IL-1 β and analyzed the cells by TUNEL. As shown in Fig. 1B the IL-1 β -stimulated chondrocytes showed the same low level of background apoptosis as the nonstimulated control cells. Neither the spread and adherent cells nor the cells that had adopted a rounded shape were apoptotic, indicating that the IL-1 β -induced morphologic changes were not associated with increased levels of apoptosis. To exclude the possibility of general effects on viability we measured mitochondrial activity in IL-1 β -stimulated cells by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay and nonapoptotic cell death by erythrosine B exclusion. The results did not indicate any differences in cell viability between normal controls and IL-1 β -stimulated cultures. In addition, intracellular ATP levels did not change in response to IL-1 β stimulation (data not shown). These results suggest that IL-1 β stimulation of chondrocytes did not lead to ATP depletion, changes in mitochondrial activity, and necrosis under our culture conditions.

Because IL-1 β did not induce apoptosis in monolayer cultures of chondrocytes, we asked whether apoptosis induced by known death stimuli was influenced by IL-1 β and other cytokines. We had previously shown that a subpopulation of chondrocytes expressed the death receptor CD95 and that agonistic anti-CD95 Ab (CH-11) induced apoptosis in these cells (7). It was therefore of interest to investigate the possible influence of cytokines on chondrocyte apoptosis induced by anti-CD95 Ab. Measurement of fragmented nucleosomal DNA by cell death ELISA showed that IL-1 β did not induce apoptosis after 14 h of stimulation (Fig. 2A), confirming the previous observation. In addition, no DNA fragmentation was

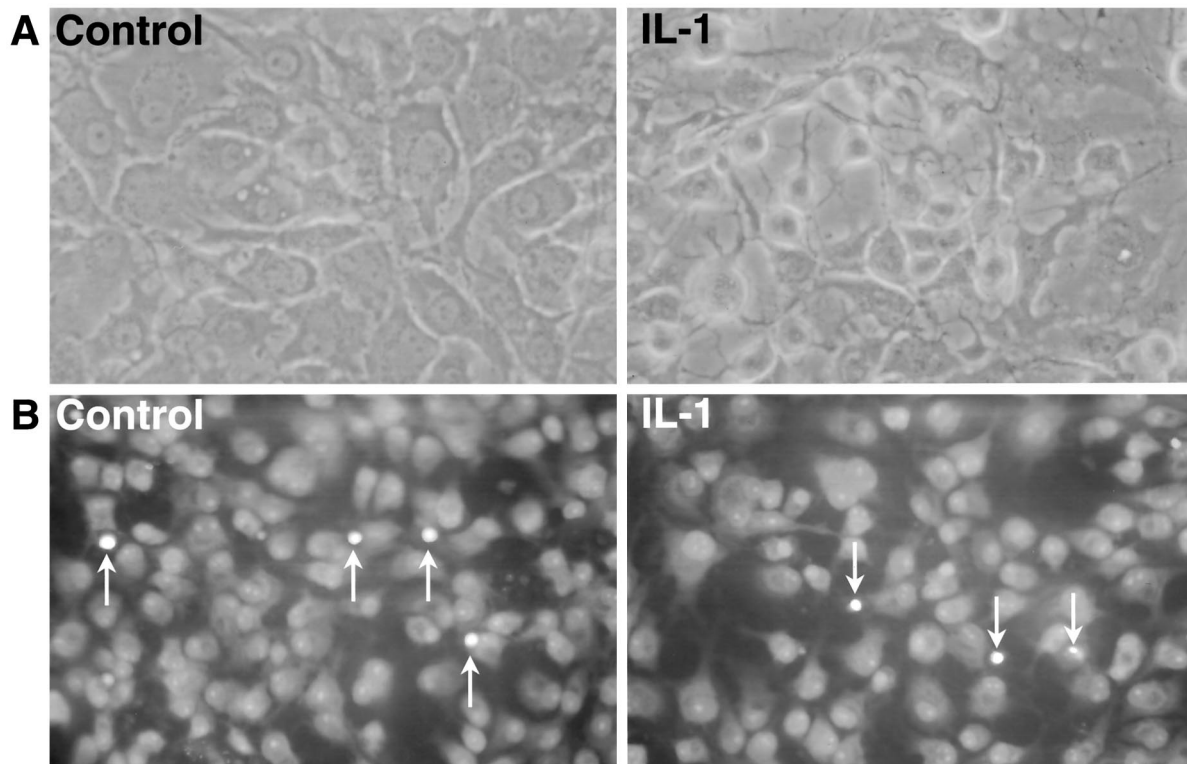


FIGURE 1. A, Unstimulated and IL-1 β -stimulated (14 h) confluent chondrocytes. Cells with rounded shape and smaller size are present only in the IL-1 β -stimulated culture. B, Chondrocytes were analyzed for DNA strandbreaks by TUNEL and were counterstained with propidium iodide. Similarly, low numbers of apoptotic cells (indicated by arrows) were found in both control and IL-1 β -stimulated cultures.

detected after prolonged periods (>24 h) of incubation (not shown). IL-1 β strongly inhibited (average inhibition, >80%) apoptosis induced by agonistic anti-CD95 Ab (Fig. 2A) in all the chondrocyte preparations from nine different cartilage donors. The extent of this inhibition was similar to that observed with the caspase inhibitor z-VAD.fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) (data not shown).

Many of the IL-1 β effects, such as the inhibition of proliferation in early passage chondrocytes (14) or IL-1 β -dependent cartilage destruction, are antagonized by TGF- β . In confluent monolayer chondrocytes TGF- β did not induce any morphological changes (not shown), but it protected chondrocytes from anti-CD95-induced apoptosis, although this effect was less pronounced than the protection induced by IL- β . In contrast, the effects of TNF- α , which induces similar morphological changes in first passage chondrocytes as IL-1 β , varied considerably and appeared to be donor dependent. TNF- α on its own did not increase apoptosis in any of seven independent experiments, but some donors (two of five) showed inhibition of CD95-dependent apoptosis, which was of similar magnitude as that produced IL-1 β . The remaining donors showed weak or no TNF- α inhibition of CH-11-induced apoptosis. Overall, the effects of TNF- α on CD95-dependent cell death were not statistically significant.

The inhibitory effects of IL-1 β on CD95-dependent cell death may either be stimulus specific or indicate a general protective function of this cytokine in chondrocytes regardless of the apoptotic stimulus. The protein kinase inhibitor staurosporine causes apoptosis in a wide variety of cell systems, including chondrocytes (15). Although the mechanisms by which staurosporine induces cell death are not known in detail, it is likely to activate different intracellular signal transduction pathways than CD95. Staurosporine (1 μ M) induced DNA fragmentation after 14 h of stimulation (Fig. 2B). Surprisingly, IL-1 β costimulation increased apoptosis

~3-fold, indicating that in the absence of protein kinase-dependent phosphorylation events the effects of exogenous IL-1 β were pro-rather than antiapoptotic.

Role of NO in IL-1 β -induced antiapoptosis

RT-PCR as well as FACS analyses showed that IL-1 β did not influence the expression of CD95 in our cell system (data not shown). This excluded the possibility that CD95 modulation was responsible for the antiapoptotic effect. Many IL-1 β effects on chondrocyte functions are mediated by NO (16, 17). Depending on the cell type, pro- as well as antiapoptotic actions have been described for NO (18, 19). We investigated whether inhibition of CD95-dependent apoptosis by IL-1 β was mediated by NO. First-passage chondrocytes were stimulated with IL-1 β or/and CH-11 in the presence or the absence of 5 mM L-NMA for 14 h. Western blot analysis showed that IL-1 β stimulated the expression of iNOS in chondrocytes, while CH-11 or L-NMA alone did not (Fig. 3A). Costimulation of chondrocytes with IL-1 β and CH-11 did not significantly change iNOS expression, nor did costimulation with IL-1 β , CH-11, and L-NMA. NO synthesis was induced by IL-1 β , but not by CH-11. Induction of NO by IL-1 β was completely abrogated when chondrocytes were costimulated with L-NMA (Fig. 3B). These stimulus combinations were then used to investigate the effects of iNOS inhibition on CD95-dependent apoptosis. As shown previously, IL-1 β did not induce apoptosis, nor did L-NMA (Fig. 3C). CH-11-induced chondrocyte death was not affected when NO synthesis was inhibited by L-NMA. A slight, but non-significant ($p > 0.3$), reduction of the antiapoptotic effect of IL-1 β on CD95-dependent apoptosis was observed when the cells were costimulated with CH-11, IL-1 β , and L-NMA. Similar results were observed with another inhibitor of iNOS, 6-aminoguanidine (not

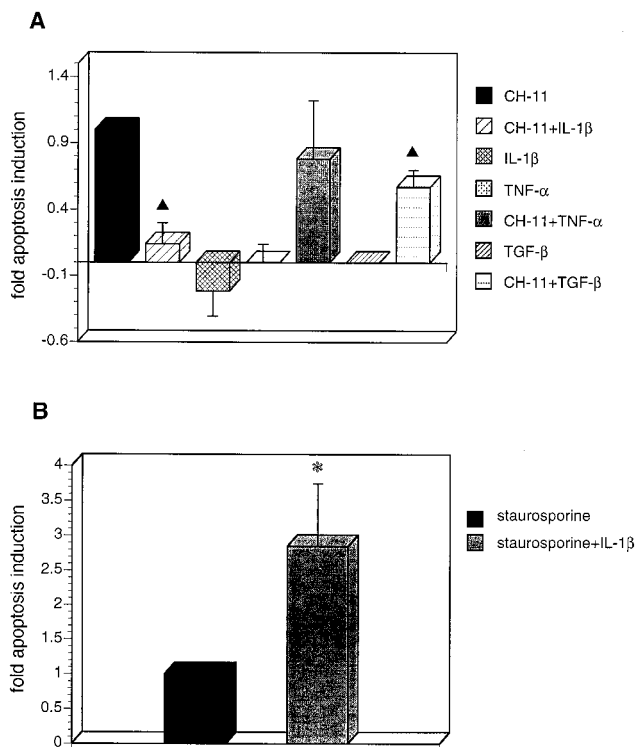


FIGURE 2. Differential interaction of cytokines and death stimuli. *A*, Effects of various cytokines on CD95-dependent apoptosis. *B*, Effect of IL-1 β on staurosporine-induced apoptosis. Chondrocytes were stimulated as indicated. After 14 h fragmented DNA was measured by cell death ELISA. Background apoptosis was determined by stimulating the cultures with IgM and was subtracted from apoptosis obtained with the other stimuli. Apoptosis induction was calculated as follows {[absorbance₄₀₅ (stimulus) - absorbance₄₀₅ (background)]/absorbance₄₀₅ (background)}. Apoptosis induction for CH-11 and staurosporine was set at 1. ▲, $p < 0.01$ vs CH-11; *, $p < 0.05$ vs CH-11.

shown). These data suggest that, unlike other functions of chondrocytes, modulation of the CD95 death pathway by IL-1 β is not influenced by the production of endogenous NO.

PGs are also induced by IL-1 β in chondrocytes and mediate a variety of cellular responses. IL-1 β stimulation inhibits chondrocyte proliferation via activation of a NO-dependent PGE₂ synthesis pathway (16). To examine the role of PGs in the antiapoptotic effects of IL-1 β , chondrocytes were stimulated with IL-1 β , CH-11, and indomethacin, a specific inhibitor of cyclooxygenase-1 and -2. Stimulation of the cells with CH-11 and indomethacin or with CH-11 and PGE₂ did not significantly affect apoptosis induction by CH-11 (Fig. 4). When chondrocytes were costimulated with CH-11, IL-1 β , and indomethacin or PGE₂, no influence on the antiapoptotic effect of IL-1 β was observed. These data indicate that down-regulation of CD95-dependent apoptosis by IL-1 β was not mediated by PGs.

Induction of Bcl-2 by IL-1 β

Expression of the apoptosis inhibitor Bcl-2 can block CD95-dependent cell death in various cell types. We examined whether IL-1 β stimulation affected the synthesis of endogenous Bcl-2. Chondrocytes were treated with IL-1 β for the indicated periods of time, and Bcl-2 mRNA as well as protein expression were measured. IL-1 β increased the expression of Bcl-2 mRNA (Fig. 5A). Induction was first seen after 2 h of incubation, and expression peaked after 14 h. Increased Bcl-2 protein expression correlated with the increase in mRNA expression after 14 h (Fig. 5B). Den-

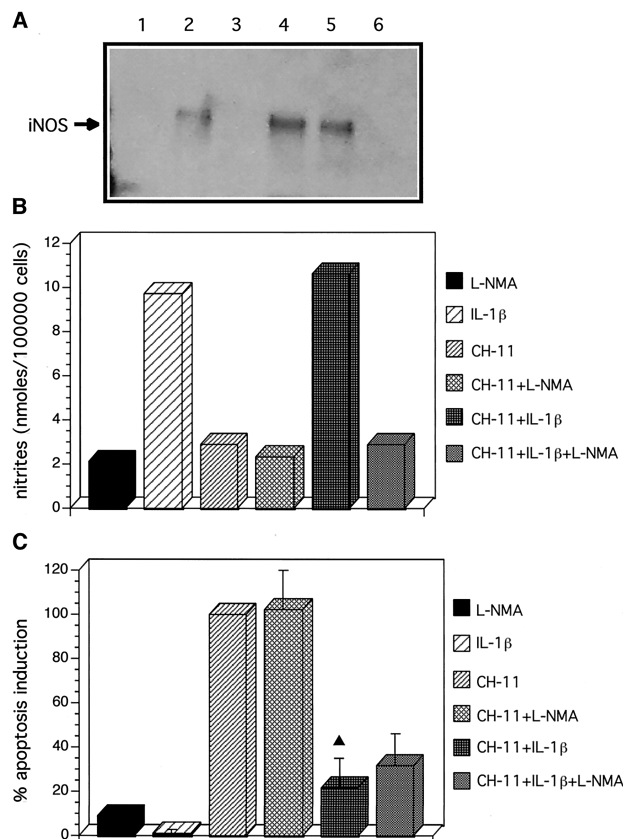


FIGURE 3. NO does not mediate the antiapoptotic IL-1 β effects. *A*, Chondrocytes were stimulated for 14 h, and iNOS expression was determined by Western blot analysis. Lane 1, L-NMA (5 mM); lane 2, IL-1 β (5 ng/ml); lane 3, CH-11 (0.75 μ g/ml); lane 4, CH-11 and IL-1 β ; lane 5, CH-11, IL-1 β , and L-NMA; lane 6, CH-11 and L-NMA. *B*, Chondrocytes were stimulated as indicated for 24 h, and NO production was measured. The results represent the average of two independent experiments. *C*, Cell death was measured by ELISA in chondrocytes stimulated as indicated for 14 h. The differences in the data between CH-11 plus IL-1 β and CH-11, IL-1 β , plus L-NMA were not significant ($p > 0.3$). ▲, $p < 0.02$ vs CH-11.

sitometry of the autoradiograph indicated an ~4-fold induction compared with that in the unstimulated control. The same samples were used to test the expression of caspase-3. No changes in caspase-3 protein expression as a result of IL-1 β stimulation were observed (Fig. 5B).

Antiapoptotic effects of IL-1 β depend on NF- κ B activation and tyrosine phosphorylation

IL-1 β causes activation of the transcription factor NF- κ B in chondrocytes (our unpublished observation). Many of the effects of IL-1, including antiapoptotic effects in other cell types, are mediated through the activation of this transcription factor (20, 21). We determined whether NF- κ B activation was required for the antiapoptotic effect of IL-1 β seen in our cell system. In human monocytes as well as lung and liver cell lines NF- κ B-dependent gene expression can be effectively inhibited by PDTC (22, 23). To test the effectiveness of PDTC in chondrocytes, we measured its influence on NF- κ B-dependent induction of iNOS mRNA expression. When used at 0.1 mM, PDTC effectively inhibited IL-1 β -induced iNOS gene activation (Fig. 6A). The iNOS mRNA was virtually undetectable after 14 h of costimulation with IL-1 β and PDTC. CH-11 stimulation did not influence the IL-1 β -dependent induction of iNOS mRNA, and in the presence of CH-11 the induction of iNOS mRNA by IL-1 β was also blocked by PDTC. We

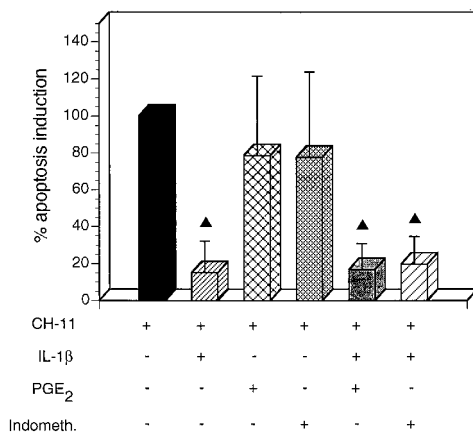


FIGURE 4. The antiapoptotic effect of IL-1 β is not mediated by PGE₂. Chondrocytes were stimulated as described for 14 h before they were harvested for DNA fragmentation assay. Apoptosis induction with CH-11 was set at 100%. Differences in the values for CH-11, IL-1 β , plus PGE₂ and CH-11, IL-1 β , plus indomethacin vs CH-11 plus IL-1 β were not significant. ▲, $p < 0.01$ vs CH-11.

then tested the role of NF- κ B activation in IL-1 β -dependent modulation of CD95-induced cell death. IL-1 β and PDTC alone or the combination of IL-1 β and PDTC did not induce apoptosis. However, when chondrocytes were costimulated with CH-11, IL-1 β , and PDTC, a significant inhibition of the antiapoptotic effect of IL-1 β was observed (Fig. 6B). Coincubation with CH-11 and PDTC did not lead to increased apoptosis compared with incubation with CH-11 alone (not shown). This indicates that NF- κ B activation is required at least in part for the antiapoptotic effect of IL-1 β .

In chondrocytes and other cell types tyrosine phosphorylation events are triggered in response to IL-1 β stimulation (24–26). We tested whether herbimycin A, a broad specificity inhibitor of ty-

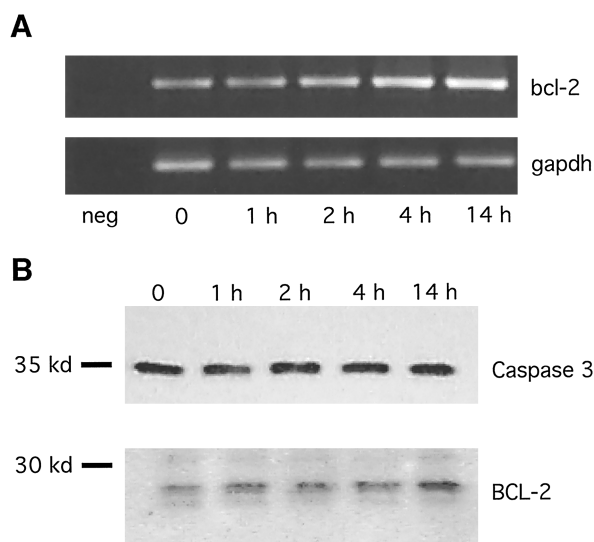


FIGURE 5. Induction of Bcl-2. Chondrocytes were stimulated for the indicated periods of time, after which total RNA or protein was isolated as described in *Materials and Methods*. *A*, RT-PCR analysis with Bcl-2-specific primers. PCR for GAPDH was performed to normalize the samples. neg, control reaction without cDNA. *B*, Western blot analysis of IL-1 β -stimulated chondrocytes. Two gels were run under identical conditions and probed separately for Bcl-2 and caspase 3 expressions. The data shown represent one of two similar experiments with two different donors.

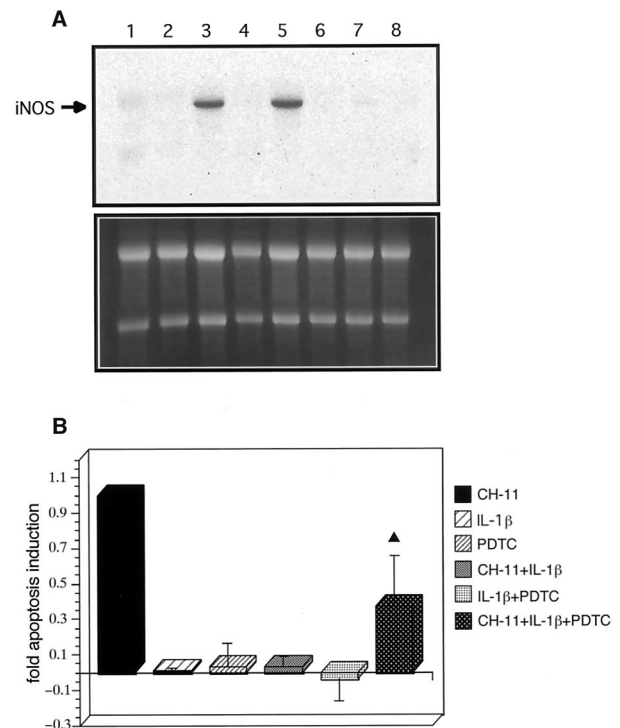


FIGURE 6. Influence of NF- κ B activation. *A*, Northern blot analysis of iNOS mRNA expression. For each sample 5 μ g of total RNA were loaded. The blot was probed with a radiolabeled RT-PCR fragment specific for iNOS (see *Materials and Methods*). Cells were stimulated for 14 h with: lane 1, IgM; lane 2, CH-11; lane 3, IL-1 β ; lane 4, PDTC; lane 5, CH-11 plus IL-1 β ; lane 6, CH-11 plus PDTC; lane 7, IL-1 β plus PDTC; lane 8, CH-11, IL-1 β , plus PDTC. The ethidium bromide-stained gel demonstrates equal loading of the samples. *B*, Chondrocytes were stimulated as indicated and harvested for cell death ELISA after 14 h. ▲, $p < 0.05$ vs CH-11 plus IL-1 β .

rosine kinases, influenced the effects of IL-1 β on cell death. At 1 μ M, herbimycin A in the absence of other stimuli did not induce chondrocyte apoptosis. When confluent chondrocytes were costimulated with IL-1 β and herbimycin A (1 μ M), apoptosis was also not detected (Fig. 7). Thus, in contrast to the effects of protein kinase inhibition by staurosporine, selective inhibition of tyrosine

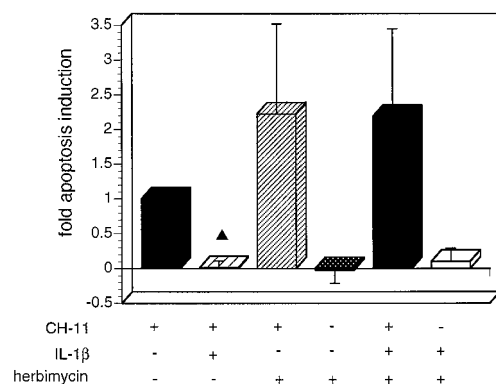


FIGURE 7. Tyrosine phosphorylation is required for the antiapoptotic effects of IL-1 β . Chondrocytes were stimulated as indicated and harvested for cell death ELISA after 14 h. Herbimycin A was used at 1 μ M. Differences between CH-11 plus herbimycin A and CH-11, IL-1 β , plus herbimycin A vs CH-11 alone were not significant, nor were differences between IL-1 β plus herbimycin A vs herbimycin A alone. ▲, $p < 0.01$ vs CH-11.

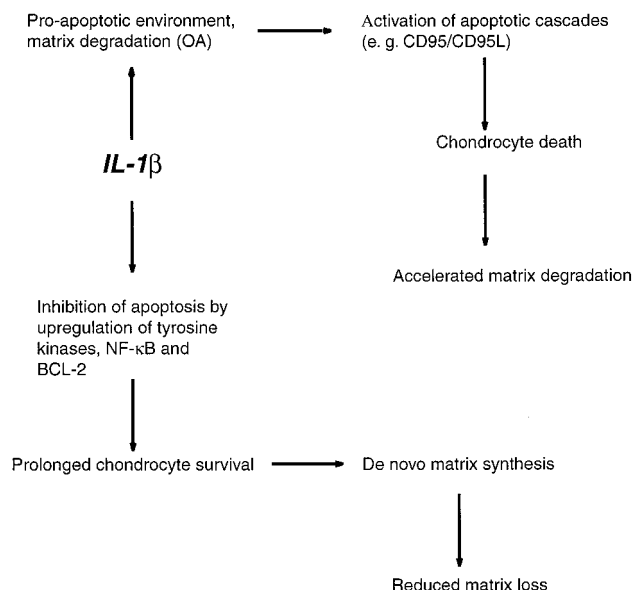


FIGURE 8. Possible role for IL-1 β in matrix loss and chondrocyte survival. IL-1 β may contribute to reduced matrix loss in pathogenic situations by promoting chondrocyte survival. Disease progression is determined by the balance between IL-1 β -dependent pro- and antiapoptotic events.

kinases in the absence or the presence of IL-1 β did not lead to apoptosis. However, herbimycin A enhanced (~2-fold) anti-CD95-induced apoptosis and, in addition, completely abrogated the antiapoptotic effect of IL-1 β . These data suggest that tyrosine kinase activity is essential for the IL-1 β inhibition of CD95-dependent cell death in chondrocytes and point to the existence of tyrosine phosphorylation-based antiapoptotic mechanisms that are activated by IL-1 β .

Discussion

This study examined the effects of cytokines on apoptosis in human articular chondrocytes. In metabolically normal cells IL-1 β and TNF- α , which are major mediators of cartilage destruction, did not induce apoptosis. A significant antiapoptotic effect of exogenous IL-1 β on CD95-dependent programmed cell death was observed. In contrast to IL-1 β , TNF- α , which influences many chondrocyte functions in a similar way (27–29), affected anti-CD95-induced apoptosis donor-dependently, while TGF- β had a small, but reproducible, antiapoptotic effect. The variability of the TNF- α effects may be explained by donor-specific differences in surface expression of the TNF-receptor p55 (30).

A previous study, which used different experimental conditions, suggested that in primary human chondrocytes IL-1 β does not induce apoptosis despite high levels of NO production (9). The present study supports this finding and in addition demonstrates that endogenous NO induced by IL-1 β has no synergistic effect with respect to CD95-dependent apoptosis. On the contrary, the results of this study provide clear evidence for an antiapoptotic role of IL-1 β in chondrocytes. The antiapoptotic action of IL-1 β was not mediated by NO, because inhibition of NO production did not significantly influence this effect. These results are in contrast to those obtained in other cell systems (31) for which a protective role for NO was demonstrated. It is possible that in chondrocytes potential proapoptotic effects of IL-1 β -induced NO are counteracted by the simultaneous activation of protective pathways, which remain to be characterized in future investigations.

PGs are also produced by IL-1 β -activated chondrocytes and are known as modulators of apoptosis in other cell types (32, 33). The

role of PGs in chondrocyte apoptosis was addressed by adding exogenous PGs or by inhibiting endogenous PG synthesis. The results show that exogenous PGE₂ did not alter the levels of CD95-induced chondrocyte apoptosis, and inhibition of PG synthesis by indomethacin did not influence the antiapoptotic effects of IL-1 β .

The present study also analyzed the role of NF- κ B in the regulation of chondrocyte apoptosis. IL-1 β -induced NF- κ B activation can be effectively inhibited by PDTC (21, 23), and as shown here, PDTC completely blocks IL-1 β -induced iNOS mRNA expression in chondrocytes. Under conditions where NF- κ B activation is inhibited by PDTC, the protective effect of IL-1 β against CD95-mediated chondrocyte apoptosis is also reduced. This suggests that IL-1 β induces antiapoptotic effects against CD95-dependent apoptosis via NF- κ B-dependent gene expression. This finding is consistent with the observation that IL-1 β -induced NO can be proapoptotic in cultured human chondrocytes when antioxidants such as DMSO or *N*-acetyl cysteine are present (9). These antioxidants are, similar to PDTC, inhibitors of NF- κ B activation (34, 35).

The activation of protein kinases, specifically tyrosine kinases, is also required for the antiapoptotic effects of IL-1 β . Increased tyrosine phosphorylation in response to IL-1 β has been described in many cell systems (24, 36, 37). As shown in this study, inhibition of tyrosine phosphorylation by herbimycin in the absence or the presence of IL-1 β stimulation did not lead to chondrocyte apoptosis. This is in contrast to the observations with staurosporine, which showed a more potent proapoptotic effect in combination with IL-1 β . Interestingly, herbimycin A increased CD95-dependent apoptosis in chondrocytes and completely blocked the antiapoptotic capacity of IL-1 β . This suggests that constitutive as well as IL-1 β -induced tyrosine phosphorylation events are essential in the protection of chondrocytes from CD95-dependent apoptosis. In human melanoma cells activation of the transcription factor NF- κ B by IL-1 β can be blocked by herbimycin A (37), suggesting that tyrosine kinase activation may be part of the signaling pathway leading to the activation of this transcription factor in this cell type. In chondrocytes, herbimycin A does not effectively reduce the induction of NF- κ B activity by IL-1 β , but it does block the induction of iNOS and COX-2 gene expression,⁴ suggesting that tyrosine kinase-dependent signaling is not essential for NF- κ B activation and that NF- κ B activation by IL-1 β is not sufficient for the induction of these genes. In chondrocytes the antiapoptotic effects of IL-1 β stimulation were at least in part mediated through NF- κ B activation, because inhibition of NF- κ B-dependent gene expression by PDTC partially neutralized the protective effect of IL-1 β . Our data suggest that IL-1 β triggers protection against death receptor-induced apoptosis via the activation of tyrosine kinases and NF- κ B. Whether these events are linked or are two separate pathways mediating the antiapoptotic actions of IL-1 β in human chondrocytes remains to be determined.

IL-1 β also leads to the induction of Bcl-2 expression in chondrocytes. Elevated levels of Bcl-2 may contribute to the protective effects of IL-1 β . Although we did not determine whether Bcl-2 expression confers protection against CD95-induced apoptosis in chondrocytes, this antiapoptotic regulator is known to interfere with apoptotic cascades involving mitochondria.

The observation that IL-1 β can protect chondrocytes against apoptosis is relevant to the understanding of mechanisms that regulate cell survival in arthritic cartilage. Under conditions that are associated with matrix remodeling, the survival-promoting effect of extracellular matrix may be compromised (38). Furthermore,

⁴ Geng, Y., J. Valbracht, K. P. Zhixing, and M. Lotz. 2000. Submitted for publication.

these conditions may be associated with apoptosis-promoting circumstances such as those found in osteoarthritic cartilage. The present findings suggest a mechanism by which, in an otherwise proapoptotic environment, IL-1 β promotes chondrocyte survival through the induction of antiapoptotic pathways (Fig. 8). This may have long term beneficial effects, because the rate of matrix loss may be decreased.

In conclusion, the present study demonstrates that IL-1 β induces chondrocyte responses that are protective against CD95-dependent apoptosis. The effects are not dependent on NO or PGs, but require the activity of tyrosine kinases and are influenced by NF- κ B and possibly Bcl-2 induction.

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