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TNF-α Protects Human Primary Articular Chondrocytes from Nitric Oxide-Induced Apoptosis Via Nuclear Factor-κB

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SUMMARY: TNF-α plays a key role in rheumatoid arthritis, but its effect on chondrocyte survival is still conflicting. In the present study, we tested how TNF-α influences chondrocyte survival in response to nitric oxide (NO)-related apoptotic signals, which are abundant during rheumatoid arthritis. Human primary articular chondrocytes or cartilage explants were pretreated with TNF-α for 24 hours and then treated with the proapoptotic NO donor sodium-nitro-prusside (SNP) for an additional 24 hours. TNF-α pretreatment markedly protected chondrocytes from SNP-induced cell death. Preincubation of chondrocytes with TNF-α inhibited both SNP-induced high-molecular weight DNA fragmentation and annexin V-FITC binding. Of interest, TNF-α induced persistent nuclear factor-κB (NF-κB)-DNA binding activity even in the presence of SNP, mirroring apoptosis protection effects. Both the TNF-α antiapoptotic effect and NF-κB-DNA binding activity were significantly inhibited by NF-κB inhibitors, Bay 11-7085, MG-132, and adenovirus-expressing mutated IκB-α. Phosphatidylinositol-3 kinase inhibitor LY 294002 also markedly inhibited the antiapoptotic effect of TNF-α. In primary chondrocytes, TNF-α induced expression of the antiapoptotic protein Cox-2, which persisted in the presence of SNP, and a specific Cox-2 inhibitor significantly blocked the TNF-α protective effect. We therefore conclude that TNF-α–mediated protection of chondrocytes from NO-induced apoptosis acts through NF-κB and requires Cox-2 activity. (*Lab Invest 2002, 82:1661–1672*).

Cartilage loss associated with rheumatoid arthritis (RA) leads to irreversible joint dysfunction. Cartilage destruction results from metalloproteinase-dependent matrix degradation and nitric oxide (NO)-induced chondrocyte apoptosis (Amin et al, 1999; Blanco et al, 1995; Lotz et al, 1999; Sakurai et al, 1995). Extracellular matrix modifications may also lead to chondrocyte apoptosis, as was shown in collagen II–deficient mice (Yang et al, 1997). Because chondrocytes are the only cells in the cartilage and the only matrix producers, their survival is the focus of many recent investigations (Blanco et al, 1998; Colnot et al, 2001; Hashimoto et al, 1998; Kim and Song, 1999).

The proinflammatory cytokines TNF-α and IL-1β are indirectly responsible for cartilage degradation and chondrocyte death because they stimulate the synthesis and release of metalloproteinases and NO from monocytes-macrophages, synoviocytes, and chondrocytes. In vitro, however, conflicting results have been reported about the effect of proinflammatory cytokines on chondrocyte apoptosis (Aizawa et al, 2001; Blanco et al, 1995; Fischer et al, 2000; Kuhn et al, 2000; Lotz et al, 1999).

Many TNF-α biologic activities are mediated by nuclear factor-κB (NF-κB). NF-κB is maintained in the cytoplasm of most cell types under an inactive form associated with an inhibitor from the IκB family, such as the ubiquitous IκB-α protein. After cellular stimulation with TNF-α, NF-κB nuclear activity is rapidly induced as a consequence of IKK kinase activation and IκB-α phosphorylation, ubiquitination, and degradation (Karin, 1999). NF-κB transcription factor controls the expression of a number of proinflammatory molecules, including cytokines (TNF-α, IL-1β, IL-6), chemokines (IL-8, macrophage inflammatory protein-1α), enzymes (COX-2, inducible nitric oxide synthase, cPLA2, metalloproteinases), and adhesion molecules (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) (Pahl, 1999). Interestingly, NF-κB also controls the expression of its inhibitor, IκB-α (Brown et al, 1993). However, in chronic inflammatory diseases, this negative regulating loop is overwhelmed by a positive one involving NF-κB activation by TNF-α and IL-1β and NF-κB–dependent expression of these two major proinflammatory cytokines.

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Indeed, chronic inflammatory diseases such as RA, asthma, or inflammatory bowel diseases are associated with persistent in situ NF-κB activity (Bureau et al, 2000; Handel et al, 1995; Weber et al, 2000). Moreover, animal models in which the genes coding for the IκB-α or A20 NF-κB inhibitors have been inactivated present important and even fatal inflammatory reactions (Beg et al, 1995; Lee et al, 2000). The constitutive NF-κB activity is required for cell survival (Bargou et al, 1997; Sovak et al, 1997; Wu et al, 1996), and NF-κB activity protects many cell types from apoptotic death. In the context of the chronic inflammatory diseases, this activity is likely to maintain immune and inflammatory cell viability and to participate in the persistence of inflammation (Bureau et al, 2000, 2002).

In this work we show that TNF-α protected chondrocytes from NO-mediated apoptosis and this protection required NF-κB, and its target antiapoptotic gene Cox-2, as well as phosphatidylinositol-3 kinase (PI-3K) activity.

Results

TNF-α Protects Primary Chondrocytes from Sodium-Nitro-Prusside (SNP)–Induced Apoptosis

Human primary chondrocytes were stimulated with TNF-α (10–30 ng/ml) and with IL-1β (5 ng/ml) for 24 hours and then treated with the apoptosis-inducing agent SNP (0.5–2 mM) (Blanco et al, 1995) for an additional 24 hours. Viability test [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS] showed that treatment with SNP induced primary chondrocyte cell death in a dose-dependent manner (Fig. 1, A and C), a significant decrease in cell viability being already observed at 0.5 mM SNP. TNF-α (Fig. 1, A and B) and IL-1β (Fig. 1C) markedly protected chondrocytes from SNP-induced cell death. Although TNF-α induces apoptosis in several cell types (Grell et al, 1994), all the tested concentrations (10–30 ng/ml) had a similar protective effect on chondrocyte mortality (Fig. 1A). TNF-α pretreatment for various times ranging from 30 minutes to 18 hours before

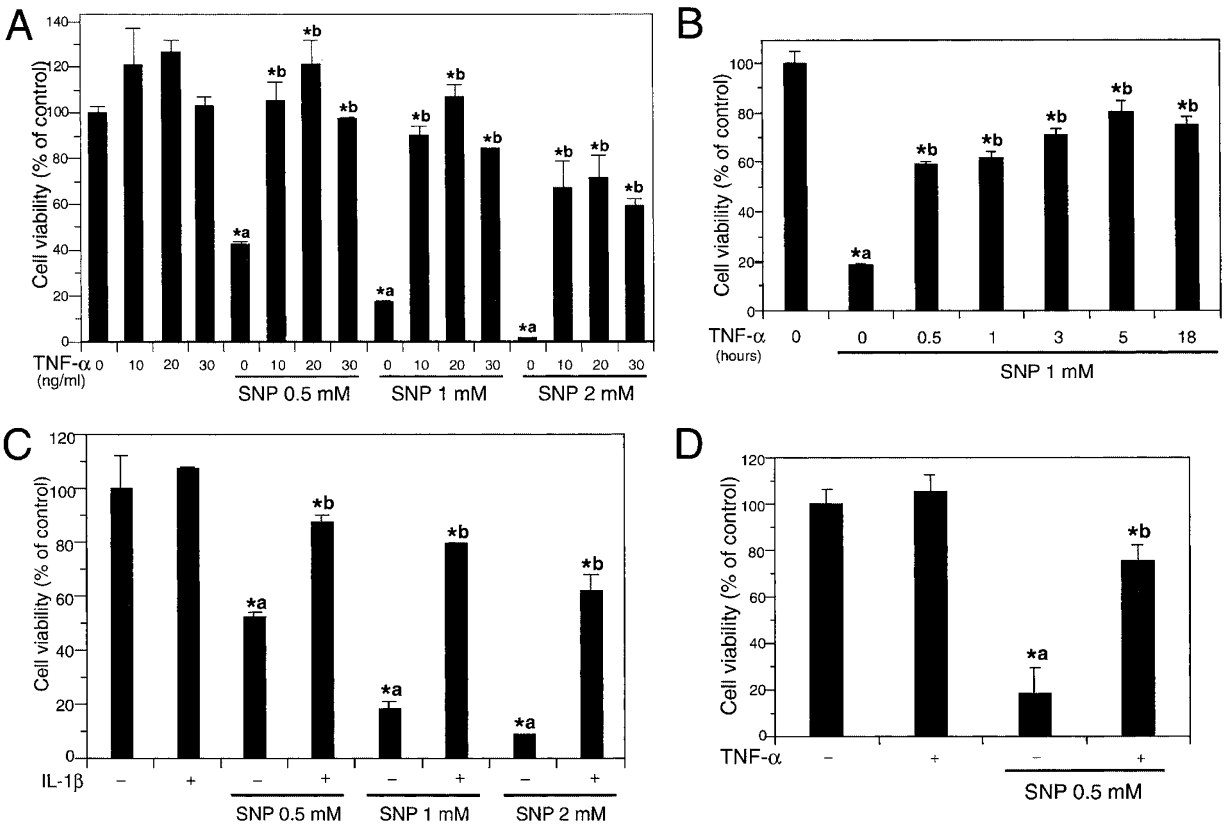

Figure 1.

TNF-α and IL-1β protect chondrocytes from sodium-nitro-prusside (SNP)–induced cell death. A, Isolated chondrocytes were pretreated with different concentrations of TNF-α (10–30 ng/ml) for 24 hours. SNP at the indicated concentrations was then added for an additional 24 hours. Cell survival was estimated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) test, and results were expressed as percent of surviving cells compared with control nontreated cells (100%). *a, Statistically different from the nontreated control ($p < 0.05$); *b, statistically different from SNP-treated cells in the absence of TNF-α ($p < 0.05$). B, Chondrocyte survival after 30 minutes to 18 hours of TNF-α (10 ng/ml) pretreatment, followed by 24 hours of SNP treatment. *a, Statistically different from the nontreated control ($p < 0.05$); *b, statistically different from SNP-treated cells in the absence of TNF-α ($p < 0.05$). C, Experiment was performed as in A, except that cells were pretreated with IL-1β (5 ng/ml) instead of TNF-α. *a, Statistically different from the nontreated control ($p < 0.05$); *b, statistically different from SNP-treated cells in the absence of IL-β ($p < 0.05$). D, Cartilage explants were pretreated with TNF-α (10 ng/ml) for 24 hours. SNP (0.5 mM) was then added for an additional 24 hours. Cell survival was determined as in A. *a, Statistically different from the nontreated control ($p < 0.05$); *b, statistically different from SNP-treated cells in the absence of TNF-α ($p < 0.05$).

SNP treatment protected chondrocytes from cell death (Fig. 1B). A longer TNF-α pretreatment (48 hours) also efficiently protected chondrocytes from SNP-induced apoptosis (data not shown).

Similarly, preincubation (24 hours) of cartilage explants with TNF-α (Fig. 1D) or IL-1β (not shown) efficiently protected chondrocytes from SNP-induced cell death. These results show that both proinflammatory cytokines protected isolated and cartilage-associated chondrocytes from SNP-induced cell death.

SNP-induced chondrocyte apoptosis was analyzed by gel electrophoresis of ^{32}P end-labeled fragmented high-molecular weight (HMW) DNA (Relic et al, 2001; Susin et al, 2000) (Fig. 2). Radioactive labeling of total DNA from SNP-treated cells revealed an intense band, corresponding to the fragmented HMW DNA fraction (Fig. 2, *lane 2*). However, pretreatment with TNF-α prevented this DNA fragmentation (Fig. 2, *lane 3*).

To confirm SNP and TNF-α effects on apoptosis, chondrocytes were stained with annexin V-FITC. SNP treatment increased the percentage of annexin V–positive chondrocytes 5-fold, whereas TNF-α pretreatment reduced it back to basal level (Fig. 3A). Microscopic examination of cells confirmed that membranes of SNP-treated chondrocytes were strongly stained, whereas TNF-α pretreatment inhibited this effect (Fig. 3B). However, the chondrocyte death after 24 hours of SNP treatment was also characterized by propidium iodide uptake that stained condensed nuclei (not shown), as observed in late apoptosis (Foglieni et al, 2001) and secondary necrosis (Honda et al, 2000).

Kinetics of TNF-α–Induced NF-κB Activation

TNF-α is a known inducer of the NF-κB transcription factor that protects several cell types from apoptosis (Barkett and Gilmore, 1999; Bours et al, 2000). We analyzed the NF-κB activity in chondrocyte nuclear extracts. Constitutive NF-κB-DNA binding activity was observed in nuclear extracts from nontreated chondrocytes (Fig. 4A). TNF-α treatment further induced NF-κB-DNA binding activity, and this induction was inhibited by preincubation of chondrocytes with Bay 11-7085, a selective inhibitor of cytokine-induced IκB

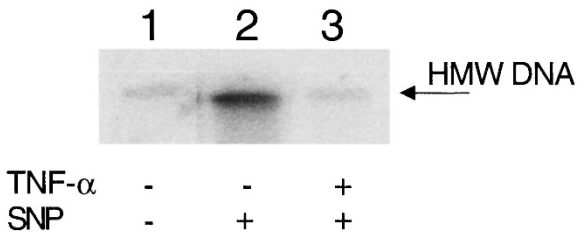

Figure 2.

TNF-α inhibits SNP-induced DNA fragmentation. Chondrocytes were pretreated or not with TNF-α (10 ng/ml), for 24 hours. SNP (1 mM) was then added for an additional 24 hours. Genomic DNA isolation and ^{32}P labeling were performed as in "Materials and Methods" to detect fragmented high-molecular weight (HMW) DNA (Relic et al, 2001). *Lane 1*, control cells; *lane 2*, SNP-treated cells; *lane 3*, TNF-α pretreated, SNP-treated cells.

phosphorylation (Pierce et al, 1997), whereas SNP did not modify NF-κB-DNA binding activity (Fig. 4A). NF-κB-DNA binding activity was strongly induced as early as 15 minutes after TNF-α stimulation and persisted for up to 72 hours (Fig. 4B). Of interest, the TNF-α–induced NF-κB binding activity persisted even in the presence of SNP (Fig. 4C). Supershift assays performed with antibodies to p50 and p65 showed that both components of NF-κB are present in nuclear extracts from unstimulated and TNF-α–treated chondrocytes (Fig. 4D, *lanes 3, 6, and 2, 5*, respectively). Application of c-Rel, Rel-B, and p52 antibodies did not cause a supershift, suggesting that these units are not present in nuclear extracts from TNF-α–stimulated chondrocytes (not shown).

Suppression of TNF-α Protective Effect by NF-κB Inhibitors

To test whether TNF-α–induced NF-κB activity is responsible for its antiapoptotic effect, cells were treated with the specific NF-κB inhibitor Bay 11-7085 (Pierce et al, 1997) before TNF-α stimulation and SNP treatment. Bay 11-7085 concentrations that inhibited TNF-α–induced NF-κB-DNA binding (Fig. 5A) suppressed the TNF-α cytoprotective effect on SNP-induced cell death as measured by the MTS test (Fig. 5B). Moreover, even in the absence of SNP, the same Bay 11-7085 concentrations reduced cell viability and caused chondrocyte death (Fig. 5B). ^{32}P end-labeling of total DNA from Bay 11-7085 (20 μM)–treated cells revealed a band corresponding to the fragmented HMW DNA fraction, suggesting apoptosis (Relic et al, 2001; Susin et al, 2000) (Fig. 5C). Bay 11-7085–induced chondrocyte death was also characterized by annexin-V-FITC–stained cell membranes (not shown).

To confirm these data, we tested another NF-κB inhibitor, an adenovirus vector expressing a mutated nondegradable IκB-α (Ad5-IκB) (DiDonato et al, 1996; Jobin et al, 1998). Chondrocytes were incubated with the adenovirus for 24 hours before TNF-α stimulation. Electrophoretic mobility shift assay (EMSA) showed that Ad5-IκB inhibited TNF-α–induced NF-κB-DNA binding activity (Fig. 6A), whereas the control adenovirus, Ad5-GFP, an adenoviral vector encoding the green fluorescent protein (GFP), did not have a significant effect. Chondrocytes pretreated with both Ad5-IκB and TNF-α before SNP treatment survived significantly less than chondrocytes pretreated with only TNF-α or with control AdGFP virus and TNF-α before SNP treatment (Fig. 6B). These results confirm that the inhibition of NF-κB activity decreased the TNF-α antiapoptotic effect.

Although both Bay 11-7085 and Ad5-IκB inhibited the TNF-α antiapoptotic effect, Bay 11-7085 also caused cell death even in the absence of SNP. We thus tested another NF-κB inhibitor, the proteasome inhibitor MG-132. MG-132 also inhibited TNF-α–induced NF-κB-DNA binding (Fig. 7A) and the TNF-α antiapoptotic effect (Fig. 7B). However, in contrast to Bay 11-7085, in the absence of SNP, the effect of MG-132 on chondrocyte survival was moderate and

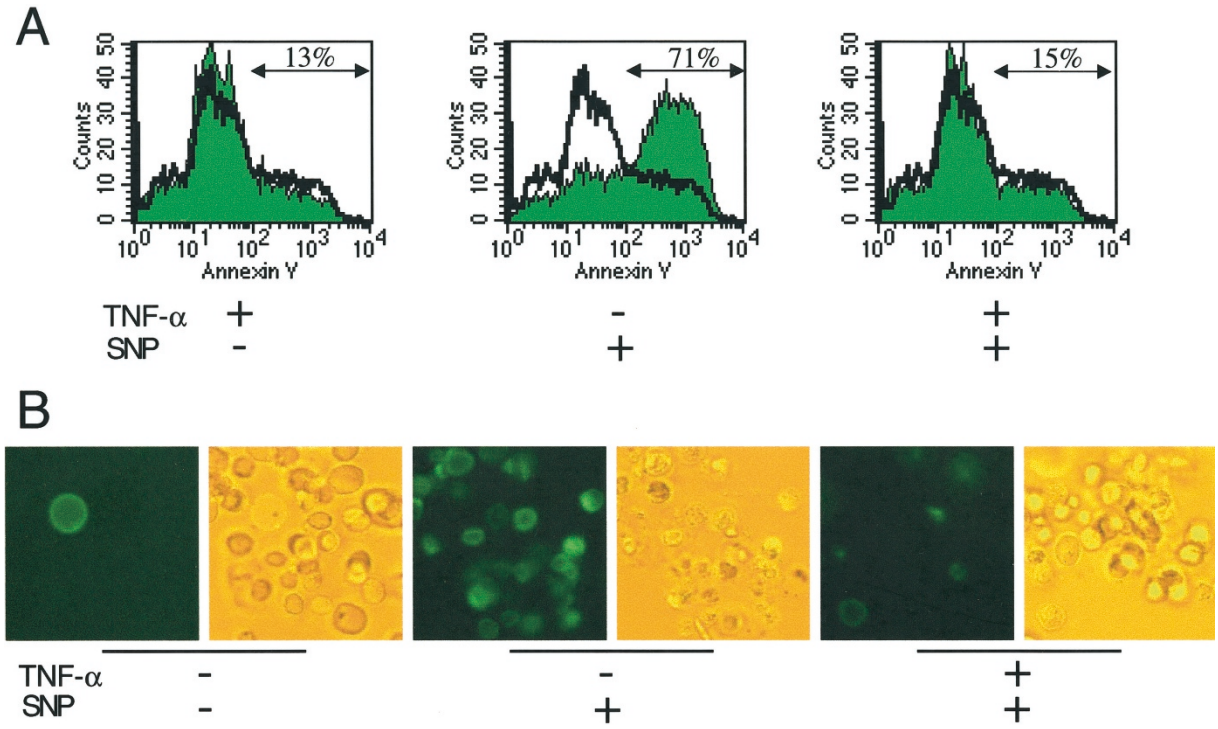

Figure 3.
TNF-α suppresses SNP-induced annexin V-FITC binding. Chondrocytes were pretreated or not with TNF-α (10 ng/ml) and then exposed to SNP for an additional 24 hours. Annexin V-FITC labeling was performed, and FITC was detected by FACScan in A or by fluorescence microscope in B. In A, black graphs represent chondrocytes in control conditions and green surfaces correspond to annexin V–positive cells (% indicated on the *arrow*). In B, each of three fluorescence figures is accompanied by a phase contrast image of the same microscopic field.

reached significance only at high concentrations (Fig. 7B).

PI-3K Inhibitor Suppresses TNF-α–Induced Antiapoptotic Activity

Because several recent studies showed that both PI-3K and its substrate Akt are involved in NF-κB activation (Ozes et al, 1999; Romashkova and Makarov, 1999; Sizemore et al, 1999; Sonoda et al, 2000), we tested the effect of the PI-3K–specific inhibitor LY 294002 on TNF-α antiapoptotic activity. Chondrocytes were pretreated with LY 294002 for 1 hour before TNF-α treatment for 24 hours, and SNP was then added for an additional 24 hours. LY 294002 at 10 μM (Fig. 8A) or 20 μM (not shown) markedly inhibited TNF-α–mediated protection from SNP-induced apoptosis. However, TNF-α–induced NF-κB-DNA binding activity was not modified by LY 294002 (Fig. 8B). LY 294002, at the concentration that inhibited the TNF-α antiapoptotic effect, was able to block Akt phosphorylation in stimulated chondrocytes (Fig. 9, *lanes 2 and 3*, respectively).

TNF-α–Induced Cox-2 Production Is SNP Resistant

The TNF-α–inducible NF-κB-dependent gene Cox-2 (Sakai et al, 2001) was induced upon TNF-α treatment (Fig. 10A, *lane 2*). Two protein forms were detected by anti-Cox-2 antibodies, as has been shown for human endothelial cells (Habib et al, 1993) and human neutrophils (McAdam et al, 2000).

Because Cox-2 has been shown to be involved in tumorigenesis through its antiapoptotic actions (Cao and Prescott, 2002; Shimada et al, 2000) and because TNF-α–induced Cox-2 expression (Fig. 10A, *lane 2*) persisted in the presence of SNP (Fig. 10A, *lane 4*), we studied its possible involvement in the TNF-α antiapoptotic effect. The specific Cox-2 inhibitor celecoxib significantly reduced the TNF-α cytoprotective effect, indicating that Cox-2 participates in the TNF-α inhibition of apoptosis, after the SNP treatment (Fig. 10B).

Discussion

Our present study shows that TNF-α protects chondrocytes from NO-induced cell death. Furthermore, we show that the TNF-α effect is NF-κB mediated because TNF-α induced lasting NF-$\kappa\beta$ activity that persisted even in the presence of SNP and because both NF-κB activity and the antiapoptotic effect were sensitive to the specific inhibitors Bay11-7085, MG-132, and Ad5-IκB, which prevent nuclear translocation of NF-κB. Thus our results are in agreement with reports showing that proinflammatory cytokines do not cause chondrocyte apoptosis (Blanco et al, 1995; Lotz et al, 1999) and even protect these cells from proapoptotic stimuli such as those triggered by anti-Fas antibodies (Kuhn et al, 2000). Our results concern-

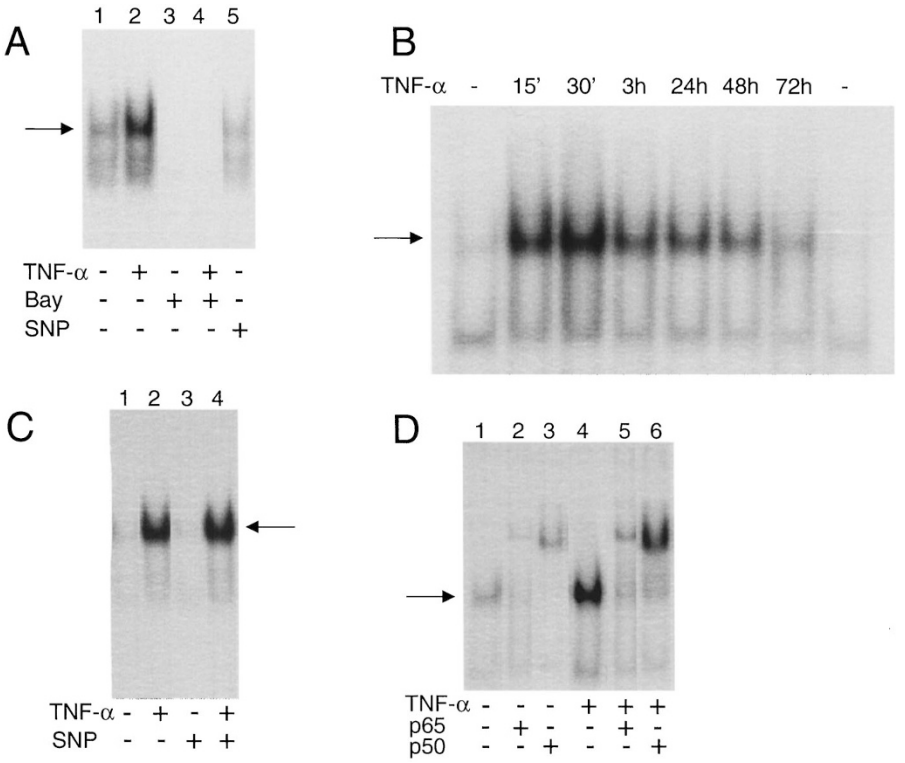

Figure 4.

Kinetics of TNF-α–induced nuclear factor-κB (NF-κB)-DNA binding activity in chondrocyte nuclear extracts. A, NF-κB-DNA binding activity was determined in chondrocyte nuclear extracts by EMSA. *Lane 1*, control; *lane 2*, TNF-α (10 ng/ml, 1 hour); *lane 3*, Bay 11-7085 (20 μM, 1 hour); *lane 4*, Bay 11-7085 (1 hour) followed by TNF-α (1 hour); *lane 5*, SNP (1 mM, 2 hours). The *arrow* indicates the p65/50 complex. B, NF-κB activity from chondrocyte nuclear extracts after TNF-α (10 ng/ml) stimulation for the indicated times. The first and the last lanes show NF-κB-DNA binding activity in nuclear extracts from nontreated chondrocytes at the beginning and the end of the experiment, respectively. C, NF-κB activity in chondrocyte nuclear extracts pretreated with TNF-α (10 ng/ml) for 48 hours and then treated with SNP (1 mM) for an additional 10 hours. *Lane 1*, control; *lane 2*, TNF-α; *lane 3*, SNP (1 mM); *lane 4*, TNF-α followed by SNP. D, Supershift analysis. Nuclear extracts from unstimulated (*lanes 1–3*) and TNF-α–treated (*lanes 4–6*) chondrocytes were analyzed for DNA binding to a κB probe either in the absence of antibody (*lanes 1 and 4*) or in the presence of an anti-p65 (*lanes 2 and 5*) or an anti-p50 antibody (*lanes 3 and 6*).

ing the Bay 11-7085 apoptotic effect are also in agreement with the previous demonstration that vitamin C, another NF-κB inhibitor that blocks IL-1β and TNF-α–mediated IκB-α degradation (Bowie and O'Neill, 2000), caused apoptosis of human articular chondrocytes (Malicev et al, 2000). However, in contrast to Bay 11-7085, neither Ad5-IκB nor MG-132 caused chondrocyte apoptosis in the absence of SNP. It is possible that the inhibition of NF-κB activity by Ad5-IκB is less efficient than inhibition by Bay 11-7085 treatment because of the irreversible nature of this inhibitor (Pierce et al, 1997). Indeed, we have observed that when cells are transfected with an IκB-α super-repressor, a high level of IκB-α expression and complete NF-κB inactivation were required to observe a significant increase in TNF-α–induced cell death (Bours et al, 2000). Alternatively, Bay 11-7085 could also affect other signaling pathways than those involving inhibition of NF-κB (Hu et al, 2001; Pierce et al, 1997). These pathways probably do not involve p38 because Bay 11-7085 provoked the chondrocyte death, whether or not chondrocytes were pretreated with the p38-specific inhibitor SB203580 (not shown).

Opposite results have however been reported showing that TNF-α induces apoptosis of chicken chondrocytes (Aizawa et al, 2001) and of human chondrocytes in the absence of matrix but not in cartilage explants (Fischer et al, 2000). These results suggest that TNF-α proapoptotic or antiapoptotic effects may depend upon the experimental model. Nevertheless, in our model of SNP-induced apoptosis, TNF-α consistently protected human chondrocytes in cartilage explants, either as primary isolated cells or as dedifferentiated chondrocytes (our unpublished data).

TNF-α–induced NF-κB activation is achieved through IKK activity and IκB degradation and through the regulation of NF-κB transcriptional activity (Wang et al, 2000). These two distinct processes in NF-κB activation were also proposed as a mechanism for IL-1β action (Sizemore et al, 1999), illustrating that NF-κB DNA binding activity is necessary but not sufficient for NF-κB activity. Recently, PI-3K was recognized to be involved in NF-κB activation (Bertrand et al, 1998; Ozes et al, 1999; Reddy et al, 2000; Romashkova and Makarov, 1999; Sizemore et al, 1999). We show in this work that LY 294002 markedly inhibited the TNF-α antiapoptotic effect, but it did not influence TNF-α–induced NF-κB DNA binding activity. These results suggest that PI-3K could be involved either in stimulation of NF-κB transcriptional activity

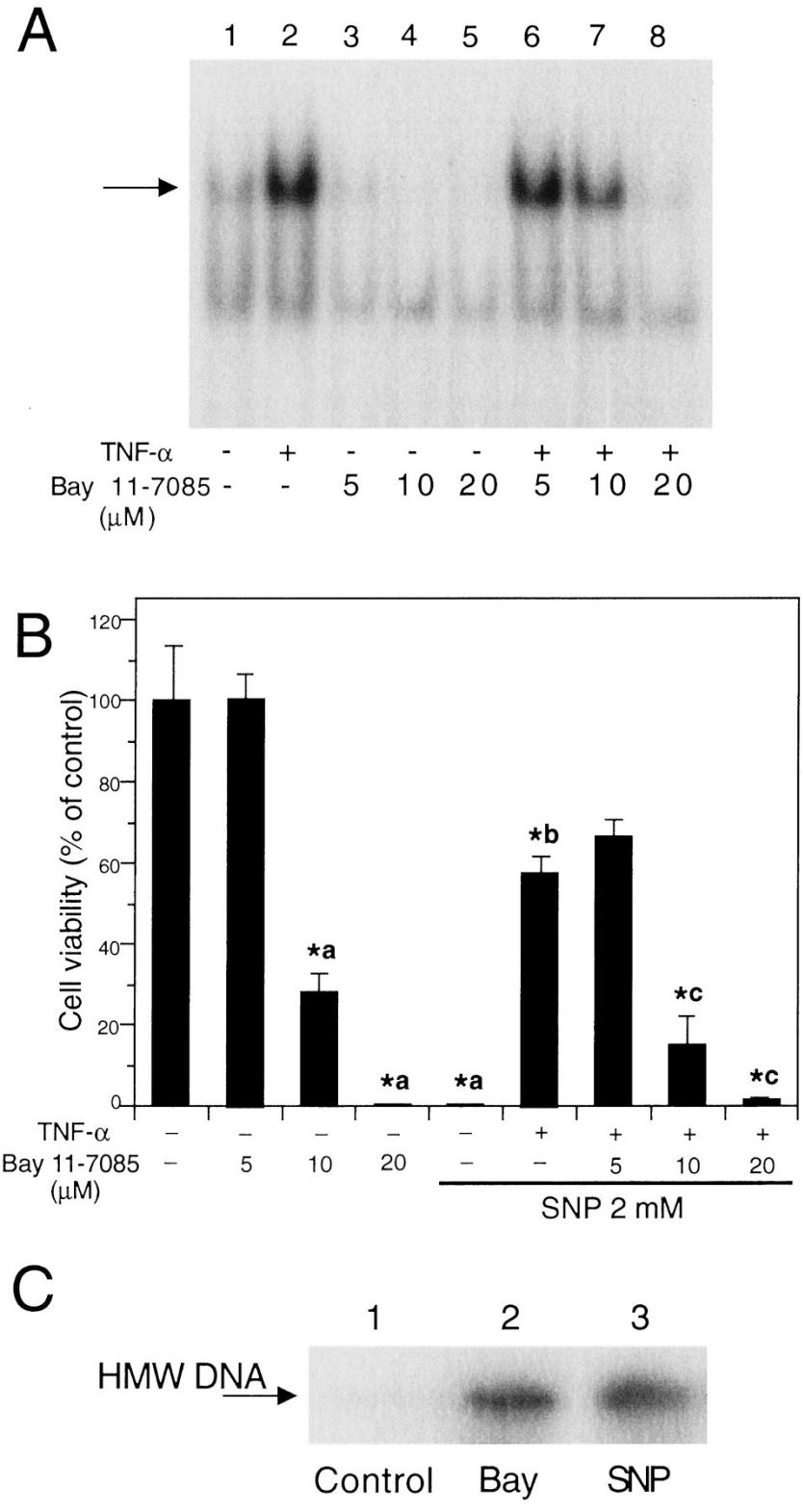

Figure 5.

NF-κB inhibitor Bay 11-7085 blocks TNF-α antiapoptotic effect and induces chondrocyte apoptosis. A, Effect of Bay 11-7085 on basal and TNF-α–induced NF-κB-DNA binding activity in chondrocyte nuclear extracts, determined by EMSA. Chondrocytes were pretreated with Bay 11-7085 for 1 hour, at the indicated concentrations, in the absence of any stimulus or before 24 hours of TNF-α (10 ng/ml) treatment. The *arrow* indicates the p65/50 complex. B, Effect of Bay 11-7085 on TNF-α protection and chondrocyte survival. Chondrocytes were pretreated with Bay 11-7085 at the indicated concentrations for 1 hour and then treated with TNF-α (10 ng/ml) for 24 hours. SNP was then added for an additional 24 hours. Cell survival was determined by MTS test as explained in Figure 1A. *a, Statistically different from the nontreated control ($p < 0.05$); *b, statistically different from SNP-treated cells in the absence of TNF-α ($p < 0.05$); *c, statistically different from SNP-treated cells in the presence of TNF-α ($p < 0.05$). C, HMW DNA fragmentation test. After treatment with Bay 11-7085 (20 μM) or SNP (2 mM) for 24 hours, genomic DNA was isolated and labeled as explained in "Materials and Methods" and in Figure 2. *Lane 1*, control untreated cells; *lane 2*, BAY 11-7085; *lane 3*, SNP.

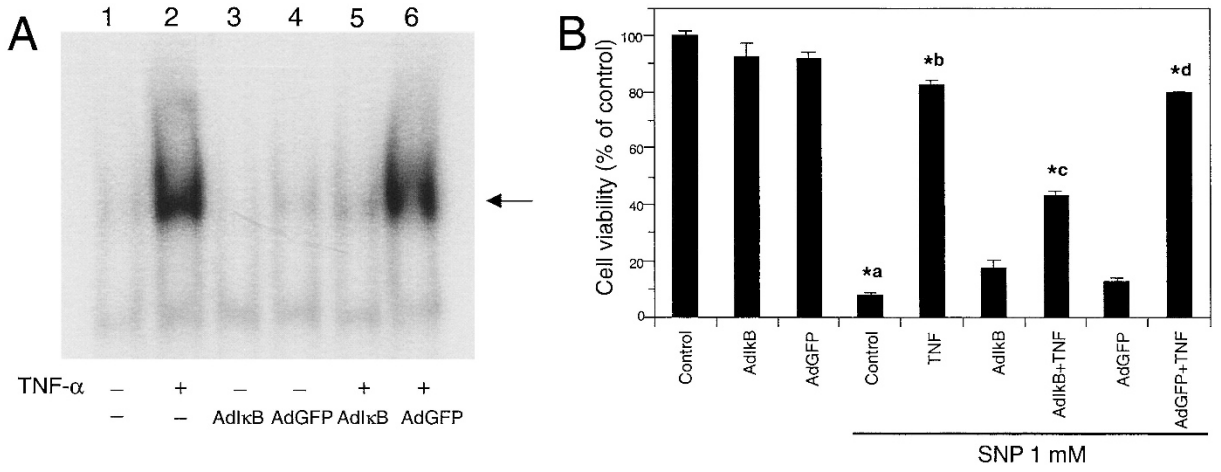

Figure 6.

Adenovirus-mediated IκB-α overexpression inhibits the TNF-α antiapoptotic effect. A, NF-κB-DNA binding activity in nuclear extracts from chondrocytes infected with adenovirus expressing mutated nondegradable IκB-α (Ad5-IκB) or control Ad5-GFP adenovirus for 24 hours, in the absence of any stimulus or before TNF-α (10 ng/ml) treatment for 24 hours. The *arrow* indicates the p65/50 complex. B, Chondrocytes were infected with Ad5-IκB for 24 hours, before 24 hours of TNF-α (10 ng/ml) treatment. SNP was then added for an additional 24 hours. Cell survival was estimated by MTS test, and results were expressed as percent of surviving cells compared with control untreated cells (100%). *a, Statistically different from the nontreated control ($p < 0.05$); *b, statistically different from SNP-treated cells in the absence of TNF-α ($p < 0.05$); *c, statistically different from SNP-treated cells in the presence of TNF-α ($p < 0.05$); *d, statistically different from SNP-treated cells in the presence of AdIkB and TNF-α ($p < 0.05$).

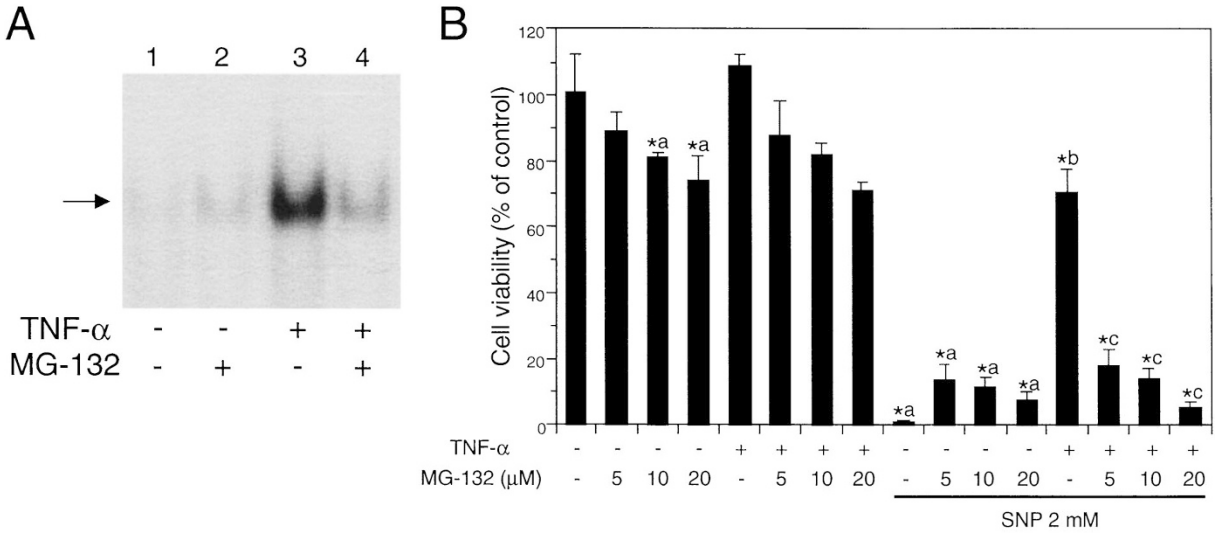

Figure 7.

The proteasome and NF-κB inhibitor MG-132 blocks the TNF-α antiapoptotic effect. A, Effect of MG-132 on basal and TNF-α–induced NF-κB-DNA binding activity in chondrocyte nuclear extracts, determined by EMSA. Chondrocytes were pretreated with MG-132 (20 mM) for 1 hour in the absence of any stimulus or before 24 hours of TNF-α (10 ng/ml) treatment. The *arrow* indicates the p65/50 complex. B, Effect of MG-132 on TNF-α protection and chondrocyte survival. Chondrocytes were pretreated with MG-132 at the indicated concentrations for 1 hour and then treated with TNF-α (10 ng/ml) for 24 hours. SNP (2 mM) was then added for an additional 24 hours. Cell survival was determined by MTS test as explained in Figure 1A. *a, Statistically different from the nontreated control ($p < 0.05$); *b, statistically different from SNP-treated cells in the absence of TNF-α ($p < 0.05$); *c, statistically different from SNP-treated cells in the presence of TNF-α ($p < 0.05$).

(Sizemore et al, 1999) or that PI-3K acts through another, NF-κB–independent, antiapoptotic pathway. The PI-3K pathway that can be stimulated with both insulin and IGF-1 is known to be important for chondrocyte survival, especially during osteoarthritis and aging (Loeser et al, 2000; Tardif et al, 1996).

Cox-2 prevents apoptosis in many cell types, including cancer cells (Cao and Prescott, 2002; Shimada et al, 2000). Moreover, Cox-2–specific inhibitors have already been applied in clinical practice for the treatment of inflammatory diseases. In primary chondrocytes Cox-2 expression was markedly induced by TNF-α, and the induction persisted in the presence of SNP. Pretreatment of chondrocytes with the selective Cox-2 inhibitor celecoxib markedly reversed the TNF-α protective effect against SNP, indicating that Cox-2 participates in this cytoprotection.

The NF-κB transcription factor is a major player of the inflammatory reaction, and in situ NF-κB activation is a hallmark of chronic inflammatory diseases including RA (Han et al, 1998; Handel et al, 1995; Miagkov et al, 1998). The role of NF-κB in the inflammatory

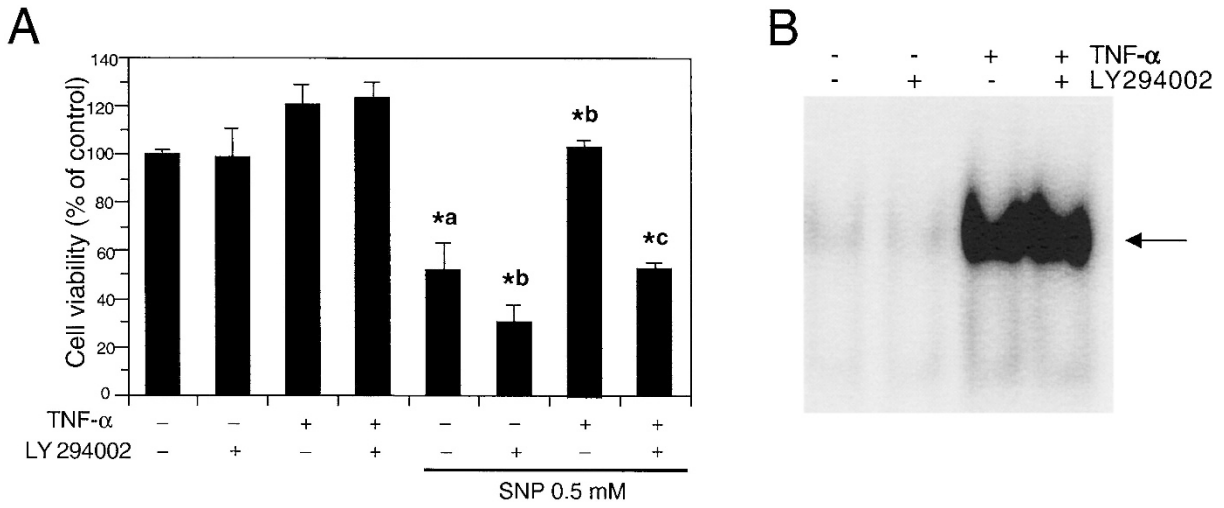

Figure 8.

Inhibition of the TNF-α antiapoptotic effect by phosphatidylinositol-3 kinase (PI-3K) inhibition. A, LY 294002 effect on TNF-α cytoprotective effect. Chondrocytes were cultured in DMEM supplemented with 2% FCS and pretreated or not with LY 294002 (10 μM) for 1 hour before TNF-α (10 ng/ml) treatment for 24 hours. SNP (0.5 mM) was then added for an additional 24 hours. Cell survival was estimated by MTS test, and results were expressed as percent of surviving cells compared with control untreated cells (100%). *a, Statistically different from the nontreated control ($p < 0.05$); *b, statistically different from SNP-treated cells in the absence of TNF-α ($p < 0.05$); *c, statistically different from SNP-treated cells in the presence of TNF-α ($p < 0.05$). B, EMSA showing NF-κB activity in nuclear extracts from chondrocytes pretreated or not with LY 294002 (20 μM) for 1 hour followed by 1 hour of TNF-α (10 ng/ml) treatment. The *arrow* indicates the p65/50 complex.

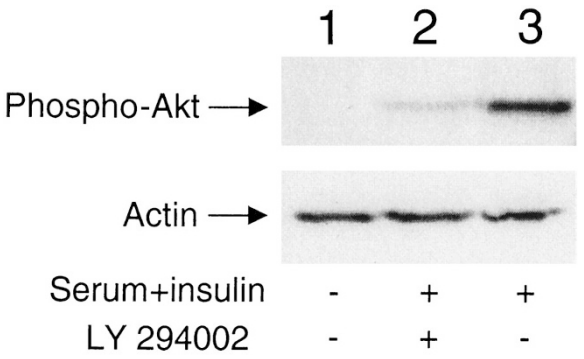

Figure 9.

LY 294002 effect on Akt phosphorylation in primary chondrocytes. Chondrocytes were starved for 24 hours in serum-free DMEM medium, pretreated (*lane 2*) or not (*lanes 1 and 3*) with LY 294002 (20 μM) for 1 hour, and then stimulated for 15 minutes with DMEM medium containing 10% FCS and 1 μM insulin (*lanes 2 and 3*). Phosphorylated Akt was detected by antibodies specific for Ser473 (New England Biolabs).

process is mediated through the expression of a large number of proinflammatory molecules (Pahl, 1999), and there is evidence of NF-κB–dependent mechanisms of cartilage destruction in RA (Bondenson et al, 2000; Miagkov et al, 1998). Indeed, inhibition of NF-κB activity by Bay 11-7085 application (Pierce et al, 1997) and intra-articular adenoviral gene transfer of super-repressor IκB and NF-κB decoys (Miagkov et al, 1998) were efficient in an experimentally induced animal model of arthritis. Furthermore, TNF-α inhibition is an established therapeutic strategy in RA (Feldmann and Maini, 2001). Thus, it seems likely that in vivo the destructive effect of prolonged TNF-α production on the cartilage matrix overwhelms its protective effect on chondrocyte survival. The long-term consequences of prolonged TNF-α blockade and whether NF-κB inhibition could became a future therapeutic target in RA remain important issues. Our results indicate that the inhibition of NF-κB activity during therapies would have to be carefully balanced without interfering with chondrocyte basal NF-κB activity, as discussed recently (Makarov, 2001).

The signaling pathways regulating proinflammatory and antiapoptotic genes in response to TNF-α–induced NF-κB activity are partially distinct, because they also involve other transcription factors and mediators. The identification of these specific pathways and of NF-κB target genes (Aupperle et al, 2001) will characterize molecules acting downstream of NF-κB, some of which could also constitute specific therapeutic targets. The present study points out a possible drawback to the use of nonspecific NF-κB inhibitors for the treatment of inflammatory diseases.

Materials and Methods

Chondrocyte Isolation and Culture

Human cartilage was obtained postmortem from 22- to 77-year-old patients who were not admitted to the hospital for joint diseases. Cartilage was cut in 1- to 2-mm^3 explants and digested at 37° C with gentle agitation successively by hyaluronidase (Sigma-Aldrich, St. Louis, Missouri) (0.5 mg/ml) for 30 minutes, pronase (Merck KGaA, Darmstadt, Germany) (1 mg/ml) for 1 hour, and collagenase (Sigma-Aldrich) (0.8 mg/ml) for 16 to 20 hours. After digestion, cells were filtered through 70 μM Nylon membrane (Falcon, Becton Dickinson, Franklin Lakes, New Jersey), washed four times, and seeded in DMEM containing or lacking phenol red (BioWhittaker, Walkersville, Maryland) and supplemented with 10% FCS. Only primary chondrocytes were used, ie, all experiments began immedi-

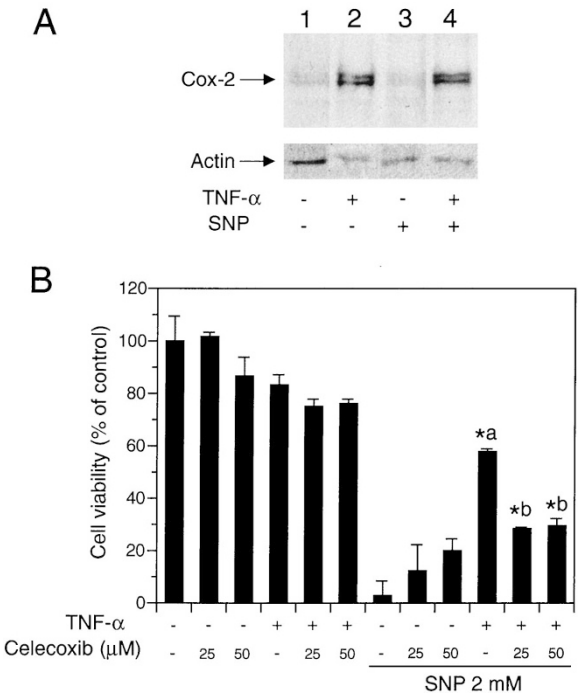

Figure 10.

The TNF-α–induced antiapoptotic effect is mediated by Cox-2. A, TNF-α effect on Cox-2 production as determined by Western blot. Chondrocytes were pretreated with TNF-α (10 ng/ml) for 24 hours and then with SNP (2 mM) for an additional 24 hours. B, Effect of Cox-2 inhibitor celecoxib on TNF-α antiapoptotic activity. Chondrocytes were pretreated with celecoxib at the indicated concentrations for 1 hour and then treated with TNF-α (10 ng/ml) for 24 hours. SNP (2 mM) was then added for an additional 24 hours. Cell survival was estimated by MTS test, and results were expressed as percent of surviving cells compared with control nontreated cells (100%). *a, Statistically different from SNP-treated cells in the absence of TNF-α ($p < 0.05$); *b, statistically different from SNP-treated cells in the presence of TNF-α ($p < 0.05$).

ately on the day of chondrocyte isolation and lasted for up to 82 hours maximum.

Cell Treatment

Isolated chondrocytes were cultured in 96-well plates at a density of 5×10^4 cells/100 μl of DMEM supplemented with 10% FCS, except in experiments concerning LY 294002, in which 2% FCS was used. Cells were pretreated with TNF-α or IL-1β (R&D Systems, Minneapolis, Minnesota) for 30 minutes to 48 hours. The NO donor SNP (Sigma-Aldrich) was then added to the cells for an additional 10 to 24 hours. In some experiments, Bay 11-7085, LY 294002 (Alexis Corporation, San Diego, California), MG-132 (Sigma-Aldrich), or Ad5-IκB (DiDonato et al, 1996; Jobin et al, 1998) were added before cytokine treatment. Experiments with cartilage explants were done in the same way except that 24-well plates, each containing 500 μl of medium and five explants (1–2 mm^3), were used.

Survival Assay

Cell survival was measured as mitochondrial NADH/NADPH–dependent dehydrogenase activity, resulting in the cellular conversion of methyltetrazolium salt MTS (Promega, Madison, Wisconsin) into a soluble formazan dye (Buttke et al, 1993). An electron coupling agent, phenazine methosulfate, was obtained from Sigma-Aldrich. Colorimetric measurement of formazan dye was performed at 490 nm.

DNA Fragmentation Test

Chondrocyte apoptosis was evaluated by a HMW DNA fragmentation test (Relic et al, 2001), with slight modifications. Briefly, at the first day of isolation, 5×10^6 chondrocytes were seeded into 10-cm Petri dishes containing 10 ml of medium (DMEM, 10% FCS). The same day, cells were pretreated or not with TNF-α and incubated for 24 hours before SNP stimulation for an additional 24 hours. Total DNA was isolated from nonadherent cells harvested by supernatant centrifugation (300 ×*g*, 5 minutes) and from adherent cells harvested by trypsinization. Harvested cells were incubated in 1 ml of lysis buffer (100 mM NaCl; 10 mM TRIS-Cl, pH 8; 25 mM EDTA, pH 8; 0.5% SDS) supplemented with 0.1 mg/ml of proteinase K (Roche, Mannheim, Germany) for 16 hours, at 42° C. After lysis, an equal volume of phenol was used for DNA extraction. After 2 minutes of centrifugation (3200 ×*g*), DNA was precipitated and recovered by centrifugation (2 minutes, 3500 ×*g*), and the DNA pellet was dissolved in distillated water. One microgram of total DNA was labeled with Klenow enzyme (Feinberg and Vogelstein, 1984), in 1× buffer H (Roche) and 10 μCi of both ^{32}PdCTP and ^{32}PdATP. DNA was electrophoresed on standard 1% agarose gel in 1× TAE buffer. Gel was fixed in 10% acetic acid for 1 hour at 4° C, vacuum dried, and exposed to x-ray film for 10 to 30 minutes.

FACS Analysis

At the day of isolation, chondrocytes were plated in 6-well plates at a density of 10^6 cells/2 ml of DMEM supplemented with 10% FCS. Cells were pretreated with TNF-α for 24 hours before exposure to SNP for an additional 24 hours. Cells were collected, washed in PBS, and labeled with annexin V-FITC and propidium iodide (Annexin V fluos staining kit; Roche). Cell images were captured under fluorescence microscope. Fluorescent cells were counted by FACScan.

Nuclear Protein Extraction and EMSA

Nuclear extracts and EMSA were performed as described (Dejardin et al, 1995) and adjusted for chondrocyte cultures with modification of centrifugation conditions at 300 ×*g* for collecting and washing steps. Each nuclear extract was made from 5×10^6 chondrocytes seeded in 10-cm Petri dishes containing 10 ml of culture medium. The palindromic κB probe oligo was 5'-TTGGCAACGGCAGGGGAATTCCCCTCTCCTTA-3'. Gels were migrated at 260V for 2 to 3 hours. Supershifts were performed with p50 and p65 antibodies (UBS, Lake Placid, New York).

Adenovirus Vectors

The inhibition of NF-κB-DNA binding activity was obtained by infection with 100 plaque forming unit per cell of Ad5-IκB (adenovirus type 5, replication incompetent, deleted for E1 and E3 sequences and expressing a mutated undegradable IκB-α [Ser 32–36] under the control of the cytomegalovirus promoter) (DiDonato et al, 1996; Jobin et al, 1998). As a control, an adenoviral vector encoding GFP was used (Ad5-GFP).

Western Blot

Chondrocytes (5×10^6 cells per treatment) were collected in ice-cold PBS and lysed for 30 minutes on ice in 100 μl of buffer (25 mM HEPES, 150 mM NaCl, 0.5% Triton, 10% glycerol, 1 mM dithiothreitol) containing phosphatase inhibitors (1 mM Na_3VO_4, 25 mM β-glycerophosphate, 1 mM NaF). Western blot analysis was performed on a 10% SDS-polyacrylamide gel. Total proteins (40 μg) were separated by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore Corporation, Bedford, Massachusetts). Cox-2 was detected with goat polyclonal IgG (200 ng/ml) (Santa Cruz Biotechnology, Santa Cruz, California), and the reaction was revealed with horseradish peroxidase-conjugated 1:5000 diluted anti-goat antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland) and ECL chemiluminescent reagents (Amersham Biosciences, Arlington Heights, Illinois). For detection of phosphorylated Akt, chondrocytes were starved for 24 hours and pretreated or not with LY 294002 (20 μM) for 1 hour before 15-minute stimulation with DMEM medium containing 10% FCS and 1 μM insulin. Phosphorylated Akt protein was detected with 1:1000 diluted Phospho-Akt–specific (Ser473) antibody (New England Biolabs, Beverly, Massachusetts) and 1:2000 diluted anti-rabbit secondary antibodies (DACO A/S, Glostrup, Denmark).

Statistics

p values were obtained using the Mann-Whitney test and considered significant when lower than 0.05.

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References

Aizawa T, Kon T, Einhorn TA, and Gerstenfeld LC (2001). Induction of apoptosis in chondrocytes by tumor necrosis factor-alpha. J Orthop Res 19:785–796.

Amin AR, Attur M, and Abramson SB (1999). Nitric oxide synthase and cyclooxygenases: Distribution, regulation, and intervention in arthritis. Curr Opin Rheumatol 11:202–209.

Aupperle K, Bennett B, Han Z, Boyle D, Manning A, and Firestein G (2001). NF-kappa B regulation by I kappa B kinase-2 in rheumatoid arthritis synoviocytes. J Immunol 166:2705–2711.

Bargou RC, Emmerich F, Krappmann D, Bommert K, Mapara MY, Arnold W, Royer HD, Grinstein E, Greiner A, Scheidereit C, and Dorken B (1997). Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. J Clin Invest 100:2961–2969.

Barkett M and Gilmore TD (1999). Control of apoptosis by Rel/NF-kappaB transcription factors. Oncogene 18:6910–6924.

Beg AA, Sha WC, Bronson RT, and Baltimore D (1995). Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. Genes Dev 9:2736–2746.

Bertrand F, Atfi A, Cadoret A, L'Allemain G, Robin H, Lascols O, Capeau J, and Cherqui G (1998). A role for nuclear factor kappaB in the antiapoptotic function of insulin. J Biol Chem 273:2931–2938.

Blanco FJ, Ochs RL, Schwarz H, and Lotz M (1995). Chondrocyte apoptosis induced by nitric oxide. Am J Pathol 146:75–85.

Blanco FJ, Guitian R, Vazquez-Martul E, de Toro FJ, and Galdo F (1998). Osteoarthritis chondrocytes die by apoptosis: A possible pathway for osteoarthritis pathology. Arthritis Rheum 41:284–289.

Bondenson J, Brennan F, Foxwell B, and Feldmann M (2000). Effective adenoviral transfer of IkBα into human fibroblasts and chondrosarcoma cells reveals that the induction of matrix metalloproteinases and proinflammatory cytokines is nuclear factor-κB dependent. J Rheumatol 27:2078–2089.

Bours V, Bentires-Alj M, Hellin AC, Viatour P, Robe P, Delhalle S, Benoit V, and Merville MP (2000). Nuclear factor-kappa B, cancer, and apoptosis. Biochem Pharmacol 60:1085–1089.

Bowie A and O'Neill LA (2000). Oxidative stress and nuclear factor-kappaB activation: A reassessment of the evidence in the light of recent discoveries. Biochem Pharmacol 59:13–23.

Brown K, Park S, Kanno T, Franzoso G, and Siebenlist U (1993). Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. Proc Natl Acad Sci USA 90:2532–2536.

Bureau F, Delhalle S, Bonizzi G, Fievez L, Dogne S, Kirschvink N, Vanderplasschen A, Merville MP, Bours V, and Lekeux P (2000). Mechanisms of persistent NF-kappa B activity in the bronchi of an animal model of asthma. J Immunol 165:5822–5830.

Bureau F, Vanderplasschen A, Jaspar F, Minner F, Pastoret PP, Merville MP, Vincent Bours, and Lekeux P (2002). Constitutive nuclear factor-κB activity preserves homeostasis of quiescent mature lymphocytes and granulocytes by controlling the expression of distinct Bcl-2 family proteins. Blood 99:3683–3691.

Buttke TM, McCubrey JA, and Owen TC (1993). Use of an aqueous soluble tetrazolium/formazan assay to measure viability and proliferation of lymphokine-dependent cell lines. J Immunol Methods 157:233–240.

Cao Y and Prescott SM (2002). Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. J Cell Physiol 190:279–286.

Colnot C, Sidhu SS, Balmain N, and Poirier F (2001). Uncoupling of chondrocyte death and vascular invasion in mouse galectin 3 null mutant bones. Dev Biol 229:203–214.

Dejardin E, Bonizzi G, Bellahcene A, Castronovo V, Merville MP, and Bours V (1995). Highly-expressed p100/p52 (NFKB2) sequesters other NF-kappa B-related proteins in the cytoplasm of human breast cancer cells. Oncogene 11:1835–1841.

DiDonato J, Mercurio F, Rosette C, Wu-Li J, Suyang H, Ghosh S, and Karin M (1996). Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation. Mol Cell Biol 16:1295–1304.

Feinberg AP and Vogelstein B (1984). "A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity." Addendum. Anal Biochem 137:266–267.

Feldmann M and Maini RN (2001). Anti-TNF alpha therapy of rheumatoid arthritis: What have we learned? Annu Rev Immunol 19:163–196.

Fischer BA, Mundle S, and Cole AA (2000). Tumor necrosis factor-alpha induced DNA cleavage in human articular chondrocytes may involve multiple endonucleolytic activities during apoptosis. Microsc Res Tech 50:236–242.

Foglieni C, Meoni C, and Davalli AM (2001). Fluorescent dyes for cell viability: An application on prefixed conditions. Histochem Cell Biol 115:223–229.

Grell M, Zimmermann G, Hulser D, Pfizenmaier K, and Scheurich P (1994). TNF receptors TR60 and TR80 can mediate apoptosis via induction of distinct signal pathways. J Immunol 153:1963–1972.

Habib A, Creminon C, Frobert Y, Grassi J, Pradelles P, and Maclouf J (1993). Demonstration of an inducible cyclooxygenase in human endothelial cells using antibodies raised against the carboxyl-terminal region of the cyclooxygenase-2. J Biol Chem 268:23448–23454.

Han Z, Boyle DL, Manning AM, and Firestein GS (1998). AP-1 and NF-kappaB regulation in rheumatoid arthritis and murine collagen-induced arthritis. Autoimmunity 28:197–208.

Handel ML, McMorrow LB, and Gravallese EM (1995). Nuclear factor-kappa B in rheumatoid synovium: Localization of p50 and p65. Arthritis Rheum 38:1762–1770.

Hashimoto S, Ochs RL, Komiya S, and Lotz M (1998). Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. Arthritis Rheum 41:1632–1638.

Honda O, Kuroda M, Joja I, Asaumi J, Takeda Y, Akaki S, Togami I, Kanazawa S, Kawasaki S, and Hiraki Y (2000). Assessment of secondary necrosis of Jurkat cells using a new microscopic system and double staining method with annexin V and propidium iodide. Int J Oncol 16:283–288.

Hu X, Janssen WE, Moscinski LC, Bryington M, Dangsupa A, Rezai-Zadeh N, Babbin BA, and Zuckerman KS (2001). An IkappaBalpha inhibitor causes leukemia cell death through a p38 MAP kinase-dependent, NF-kappaB-independent mechanism. Cancer Res 61:6290–6296.

Jobin C, Panja A, Hellerbrand C, Iimuro Y, Didonato J, Brenner DA, and Sartor RB (1998). Inhibition of proinflammatory molecule production by adenovirus-mediated expression of a nuclear factor kappaB super-repressor in human intestinal epithelial cells. J Immunol 160:410–418.

Karin M (1999). How NF-kappaB is activated: The role of the IkappaB kinase (IKK) complex. Oncogene 18:6867–6874.

Kim HA and Song YW (1999). Apoptotic chondrocyte death in rheumatoid arthritis. Arthritis Rheum 42:1528–1537.

Kuhn K, Hashimoto S, and Lotz M (2000). IL-1 beta protects human chondrocytes from CD95-induced apoptosis. J Immunol 164:2233–2239.

Lee EG, Boone DL, Chai S, Libby SL, Chien M, Lodolce JP, and Ma A (2000). Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. Science 289:2350–2354.

Loeser RF, Shanker G, Carlson CS, Gardin JF, Shelton BJ, and Sonntag WE (2000). Reduction in the chondrocyte response to insulin-like growth factor 1 in aging and osteoarthritis: Studies in a non-human primate model of naturally occurring disease. Arthritis Rheum 43:2110–2120.

Lotz M, Hashimoto S, and Kuhn K (1999). Mechanisms of chondrocyte apoptosis. Osteoarthritis Cartilage 7:389–391.

Makarov SS (2001). NF-κB in rheumatoid arthritis: A pivotal regulator of inflammation, hyperplasia, and tissue destruction. Arthritis Res 3:200–206.

Malicev E, Woyniak G, Knezevic M, Radosavljevic D, and Jeras M (2000). Vitamin C induced apoptosis in human articular chondrocytes. Pflugers Arch 440:R46–R48.

McAdam BF, Mardini IA, Habib A, Burke A, Lawson JA, Kapoor S, and FitzGerald GA (2000). Effect of regulated expression of human cyclooxygenase isoforms on eicosanoid and isoeicosanoid production in inflammation. J Clin Invest 105:1473–1482.

Miagkov AV, Kovalenko DV, Brown CE, Didsbury JR, Cogswell JP, Stimpson SA, Baldwin AS, and Makarov SS (1998). NF-kappaB activation provides the potential link between inflammation and hyperplasia in the arthritic joint. Proc Natl Acad Sci USA 95:13859–13864.

Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, and Donner DB (1999). NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 401:82–85.

Pahl HL (1999). Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 18:6853–6866.

Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, and Gerritsen ME (1997). Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. J Biol Chem 272:21096–21103.

Reddy SA, Huang JH, and Liao WS (2000). Phosphatidylinositol 3-kinase as a mediator of TNF-induced NF-kappa B activation. J Immunol 164:1355–1363.

Relic B, Guicheux J, Mezin F, Lubberts E, Togninalli D, Garcia I, van den Berg WB, and Guerne PA (2001). IL-4 and IL-13, but not IL-10, protect human synoviocytes from apoptosis. J Immunol 166:2775–2782.

Romashkova JA and Makarov SS (1999). NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature 401:86–90.

Sakai T, Kambe F, Mitsuyama H, Ishiguro N, Takigawa M, Iwata H, and Seo H (2001). Tumor necrosis factor alpha induces expression of genes for matrix degradation in human chondrocyte-like HCS-2/8 cells through activation of NF-kappaB: Abrogation of tumor necrosis factor alpha effect by proteasome inhibitors. J Bone Miner Res 16:1272–1280.

Sakurai H, Kohsaka H, Liu MF, Higashiyama H, Hirata Y, Kanno K, Saito I, and Miyasaka N (1995). Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. J Clin Invest 96:2357–2363.

Shimada T, Hiraishi H, and Terano A (2000). Hepatocyte growth factor protects gastric epithelial cells against ceramide-induced apoptosis through induction of cyclooxygenase-2. Life Sci 68:539–546.

Sizemore N, Leung S, and Stark GR (1999). Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. Mol Cell Biol 19:4798–4805.

Sonoda Y, Matsumoto Y, Funakoshi M, Yamamoto D, Hanks SK, and Kasahara T (2000). Anti-apoptotic role of focal adhesion kinase (FAK): Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. J Biol Chem 275:16309–16315.

Sovak MA, Bellas RE, Kim DW, Zanieski GJ, Rogers AE, Traish AM, and Sonenshein GE (1997). Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer. J Clin Invest 100:2952–2960.

Susin SA, Daugas E, Ravagnan L, Samejima K, Zamzami N, Loeffler M, Costantini P, Ferri KF, Irinopoulou T, Prevost MC, Brothers G, Mak TW, Penninger J, Earnshaw WC, and Kroemer G (2000). Two distinct pathways leading to nuclear apoptosis. J Exp Med 192:571–580.

Tardif G, Reboul P, Pelletier JP, Geng C, Cloutier JM, and Martel-Pelletier J (1996). Normal expression of type 1 insulin-like growth factor receptor by human osteoarthritic chondrocytes with increased expression and synthesis of insulin-like growth factor binding proteins. Arthritis Rheum 39:968–978.

Wang D, Westerheide SD, Hanson JL, and Baldwin AS Jr (2000). Tumor necrosis factor alpha-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II. J Biol Chem 275:32592–32597.

Weber CK, Liptay S, Wirth T, Adler G, and Schmid RM (2000). Suppression of NF-kappaB activity by sulfasalazine is mediated by direct inhibition of IkappaB kinases alpha and beta. Gastroenterology 119:1209–1218.

Wu M, Lee H, Bellas RE, Schauer SL, Arsura M, Katz D, FitzGerald MJ, Rothstein TL, Sherr DH, and Sonenshein GE (1996). Inhibition of NF-kappaB/Rel induces apoptosis of murine B cells. EMBO J 15:4682–4690.

Yang C, Li SW, Helminen HJ, Khillan JS, Bao Y, and Prockop DJ (1997). Apoptosis of chondrocytes in transgenic mice lacking collagen II. Exp Cell Res 235:370–373.