

# Tumor Necrosis Factor- $\alpha$ Protects Synovial Cells from Nitric Oxide Induced Apoptosis Through Phosphoinositide 3-Kinase Akt Signal Transduction

QINGQUAN CHEN, BRUNO CASALI, LAURA PATTACINI, LUIGI BOIARDI, and CARLO SALVARANI

**ABSTRACT.** *Objective.* To investigate the anti-apoptotic role of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and its signaling pathways in cultured human fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis. *Methods.* FLS were cultured in Dulbecco's modified Eagle's medium. Apoptotic cells were identified by TUNEL assay and Hoechst staining. Cell viability was determined by the MTT method. Expression of phospho-Akt and phospho-BAD was measured by Western blotting. *Results.* A 24-h TNF- $\alpha$  treatment prevented FLS apoptosis induced by nitric oxide (NO) donor sodium nitroprusside dihydrate (SNP), achieving 70% protection. At 1–10 ng·ml<sup>-1</sup> concentrations, TNF- $\alpha$  induced phosphorylation of Akt and BAD in a time and concentration-dependent manner. This effect was blocked by treatment with both LY294002 and nuclear factor- $\kappa$ B inhibitor pyrrolidine-dithiocarbamate. *Conclusion.* TNF- $\alpha$  has an anti-apoptotic effect in human FLS. Activation of Akt and BAD may have an important role in this process. (J Rheumatol 2006;33:1061–8)

## Key Indexing Terms:

TUMOR NECROSIS FACTOR- $\alpha$   
APOPTOSIS

SIGNAL TRANSDUCTION

NITRIC OXIDE  
SYNOVIAL CELLS

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a major mediator of inflammation and apoptotic processes<sup>1</sup>. It can protect cells derived from neck squamous cell carcinoma and smooth muscle cells<sup>2,3</sup> from apoptosis. TNF- $\alpha$  is also a critical cytokine in the immunopathogenesis of rheumatoid arthritis (RA). Indeed it can recruit other proinflammatory factors, increase enzymes that cause cartilage erosion, and drive pathologic reactions such as overproliferation or resistance to apoptosis that lead to synovial hyperplasia<sup>4-6</sup>.

The pleiotropic effect of TNF- $\alpha$  is due to its ability to induce a complicated signal transduction network<sup>7</sup>. TNF- $\alpha$  is able to activate the phosphoinositide 3-kinase (PI3K) Akt signal pathway<sup>8-10</sup>, which is involved in survival of a broad range of fibroblastic, epithelial, and neuronal cells. Akt is a serine/threonine protein kinase that can be activated by PI3K. BAD is a pro-apoptotic Bcl-2 family member localized in the cytosol of living cells; in the presence of a pro-apoptotic stimulus it migrates to mitochondria, where it interacts and inactivates the anti-apoptotic members Bcl-2 and Bcl-X<sub>L</sub>. BAD phosphorylation by Akt inhibits its translocation, blocking its pro-apoptotic activity<sup>11</sup>.

Synovial hyperplasia is a major hallmark of RA.

Fibroblast-like synoviocytes (FLS) are relatively resistant to apoptosis in RA, and such resistance may be relevant in the formation of synovial hyperplasia<sup>12-14</sup>.

We investigated the possible relationship between TNF- $\alpha$  and apoptosis in FLS and the signal transduction pathway in this process.

## MATERIALS AND METHODS

*Patient selection.* Synovial membranes were obtained from patients with RA during total knee replacement and patients with osteoarthritis (OA) undergoing hip prosthesis surgery. All patients met the American College of Rheumatology (formerly, American Rheumatism Association) 1987 revised criteria for RA<sup>15</sup> and OA<sup>16</sup>. None were receiving corticosteroids at the time of surgery.

*Materials and chemicals.* All media and fetal calf serum (FCS), bisbenzimidazole H 33258, dimethylthiazol-diphenyltetrazolium bromide (MTT), pyrrolidine-dithiocarbamate (PDTC), wortmannin, z-VAD-fmk, Ac-ESMD-CHO, and sodium nitroprusside dihydrate (SNP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TNF- $\alpha$  and LY294002 were purchased from Calbiochem Inc. (San Diego, CA, USA) and methotrexate was from Wyeth Inc. Anti-phospho-Akt and phospho-BAD antibodies and *in situ* cell death detection kits were produced by Roche Molecular Biochemicals (Milan, Italy).

*Human synovial fibroblast culture.* Human synovial fibroblasts were prepared as described<sup>17</sup>. Synovial tissue was cut into small pieces (~1 mm<sup>3</sup>), then incubated 2 h at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 1 mg/ml type I collagenase. After dissociation of the fibroblasts, harvested cells were centrifuged at 500 g and seeded into 75 cm<sup>2</sup> flasks in DMEM with 10% FCS. Cells were cultured at 37°C in 5% CO<sub>2</sub>. Confluent cells were subcultured by 0.1% trypsin treatment. All experiments were carried out using primary synovial cell cultures between passage numbers 4 and 6.

*Cell viability and proliferation.* Cell viability was measured by MTT assay. Vital cells can convert MTT salt into soluble formazan dye through mitochondrial NADH/NADPH-dependent dehydrogenase activity<sup>18</sup>. Formation of MTT-formazan is positively correlated with the viability of cells. Cells were seeded into 24-well plates and treated according to the experiment design. At

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the end of treatment, MTT, dissolved in phenol-red free M1640 to a concentration of 5 g/l, was added to cell cultures with an amount of 10% of the cultured medium volume. After incubation for 1 h at 37°C, formazan dye was dissolved with solubilization solution containing 10% (v/v) Triton X-100 and HCl 0.1 mol/l in isopropanol. Metabolic activity was quantified by measuring light absorbance of the solution at 550 nm.

To test TNF- $\alpha$ 's effects on synovial cell proliferation, a cell count was performed using a hemacytometer. Briefly, fibroblasts were seeded in a 24-well plate in the presence or absence of TNF- $\alpha$ . Twenty-four hours later, cells were trypsinized, suspended in phosphate buffered saline (PBS)-trypan blue solution, and counted. The results were analyzed by Mann-Whitney test.

**Apoptosis detection.** Apoptosis of fibroblasts was identified by the terminal dUTP nick-end labeling (TUNEL) method using the *in situ* cell death detection kit<sup>19</sup>. Synovial fibroblasts were cultured on chamber slides to 80% confluence, then treated for 24 h. Cells were then fixed in 4% paraformaldehyde in PBS, pH 7.4, for 1 h, and washed with PBS. The cells were then permeabilized by incubation for 2 min with a solution of 0.1% Triton X-100 in 0.1% sodium citrate. Fixed and permeabilized cells were then labeled with TUNEL reaction mixture for 1 h at 37°C in the dark, according to the manufacturer's protocol. DNA fragmentation was identified by fluorescence microscopy.

**Microscopic quantification of apoptosis.** Apoptotic cells undergo distinctive morphologic changes such as shrinkage and nuclear DNA condensation and fragmentation. After staining with Hoechst 33258, apoptotic cells can easily be distinguished from normal cells because of morphological changes detectable by fluorescence microscope<sup>20</sup>. To calculate the apoptotic rate directly, FLS from different treatments were grown on glass slides and fixed with 3:1 methanol/acetic acid solution. After washing with PBS and drying, the fixed cells were stained with Hoechst 33258 (5 mg/ml) for 5 min and then rinsed and dried. Samples were observed under fluorescence microscope to discriminate apoptotic cells with condensed or fragmented nuclei and intact cells. The apoptotic rate was determined by counting the percentage of apoptotic cells in total cells in 6 randomly chosen fields of view.

**Subcellular fraction preparation.** At the end of the treatment, cells were lysed with 60  $\mu$ l of ice-cold lysis buffer (NaCl 50 mM, Na<sub>3</sub>VO<sub>4</sub> 2 mM, phenylmethylsulfonyl fluoride 0.5 mM, HEPES 10 mM, pH 7.4, 0.01% Triton X-100, and leupeptin 10 mg). Lysates were obtained by centrifugation at 16,000 g at 4°C for 15 min. Total cell protein was determined by Bradford analysis<sup>21</sup>.

**Western blotting.** Sample buffer [Tris-HCl 0.33 mol·l<sup>-1</sup>, SDS 10% (w/v), glycerol 40% (v/v), bromophenol blue 0.4%] was added to cell lysates. After boiling for 5 min, samples were separated by electrophoresis on SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The blot was blocked for 1 h at 25°C with 5% bovine serum albumin in TBST (Tris-HCl 50 mmol  $\times$  l<sup>-1</sup>, pH 7.4, NaCl 150 mmol·l<sup>-1</sup> containing 0.1% Tween-20), then incubated with the primary antibodies against phospho-Akt or phospho-BAD (1:1000 dilution) overnight at 4°C and with the secondary antibodies (horseradish peroxidase-conjugated) at room temperature for 1 h (1:2000 dilution). Immunoreactive signals were visualized by the ECL system according to the company protocol.

**Semiquantitative analysis.** For semiquantitative studies, the intensity of bands detected by Western blotting was measured by densitometry using x-ray films exposed to ECL reagents, using Scion Images software.

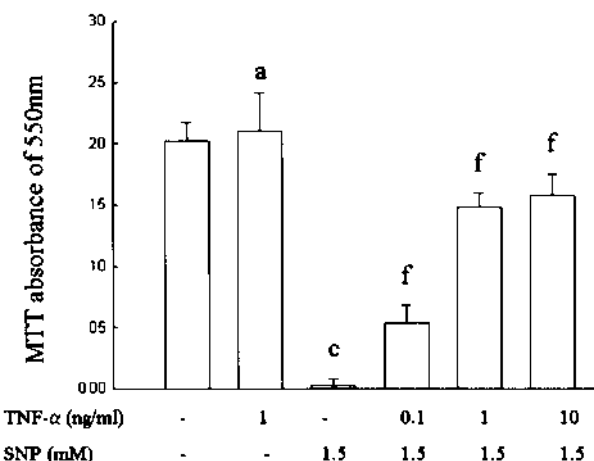
## RESULTS

**TNF- $\alpha$  preserves cell viability in the presence of SNP.** We studied the role played by TNF- $\alpha$  in the lack of apoptotic response to NO of FLS obtained from tissues of patients with OA or RA. To quantify the protective effect of TNF- $\alpha$  on synovial fibroblasts, cell viability was measured in the presence or absence of a NO donor, SNP. Our data show that TNF- $\alpha$  concentrations ranging from 0.1 to 10 ng/ml increase synovial cell viability in a concentration-dependent manner in the presence of SNP (Figure 1).

Since TNF- $\alpha$  has been shown to induce proliferation of FLS<sup>4,22</sup>, a proliferation assay was performed to demonstrate that the TNF- $\alpha$ -induced increase in cell viability was not due to augmented proliferation but to reduced cell death. Three separate experiments on different tissue samples were carried out. Table 1 shows the results as the mean of 2 counts. A Mann-Whitney test showed no significant difference in the proliferation rate between cells incubated with water alone and those incubated in the presence of TNF- $\alpha$ . This is probably because cell proliferation is already maximal in the presence of 10% FBS.

**SNP-induced apoptosis is prevented by TNF- $\alpha$ .** DNA fragmentation is a characteristic feature of apoptotic cell death that can be detected by TUNEL assay. To determine the effects of NO on synovial fibroblasts, cells were treated for 24 h with SNP in the presence or absence of TNF- $\alpha$ , and a TUNEL assay was performed. SNP incubation strongly induced apoptosis of FLS; this effect was completely reversed by TNF- $\alpha$  treatment (Table 2, Figure 2). No cells with morphological features of necrosis were detected. No difference in apoptotic behavior was observed between synovial tissues from patients with the 2 studied pathologies, RA and OA (Table 2, Figure 2).

When viability was assayed on cells exposed to another



**Figure 1.** TNF- $\alpha$  pre-treatment preserves cell viability in presence of SNP. Cells were pretreated with TNF- $\alpha$  at the indicated concentrations for 24 hours; SNP (1.5mM) was then added for 24 hours. Cell viability was measured by MTT assay. Values correspond to the absorbance of 550 nm, and error bars to the SD, n = 4 experiments. Average of duplicate constitutes one determination. <sup>a</sup>p > 0.05, <sup>c</sup>p < 0.01 vs control. <sup>f</sup>p < 0.01 vs SNP 1.5 mM. Data are mean  $\pm$  SD

**Table 1.** Effect of TNF- $\alpha$  on synovial cell proliferation. Three different samples were plated in 6-well plates and incubated with water or with TNF- $\alpha$ ; after 24 h, cells were trypsinized and counted. Trypan blue dye was used to exclude dead cells. Each experiment was carried out in duplicate.

| - TNF           | + TNF           |
|-----------------|-----------------|
| 60.25 $\pm$ 6.1 | 36.00 $\pm$ 9.2 |
| 68.25 $\pm$ 6.7 | 52.75 $\pm$ 1.8 |
| 82.25 $\pm$ 5.3 | 83.00 $\pm$ 4.9 |

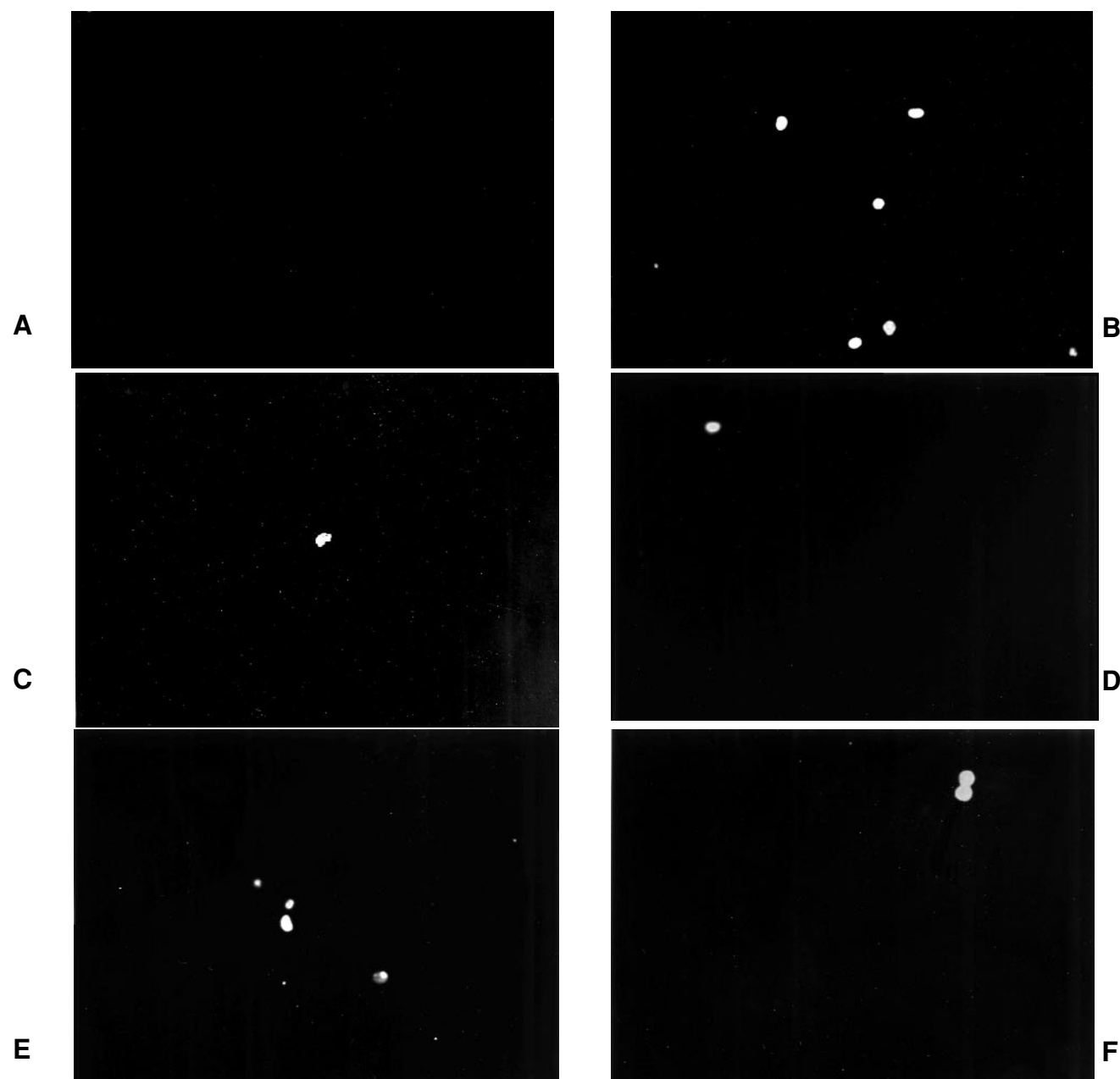
damaging agent, methotrexate, TNF- $\alpha$  failed to show the same protective effect (Table 3).

Hoechst 33258 staining allowed us to calculate the apoptotic rate, because chromatin appears condensed in apoptotic nuclei and distinguishes them from normal cells. The assay showed that TNF- $\alpha$  can reduce apoptosis induced by SNP from about 99% to 22% (Table 4). This experiment confirmed the data obtained with TUNEL assay.

*PI3K and nuclear factor- $\kappa$ B (NF- $\kappa$ B) mediate TNF- $\alpha$ 's anti-apoptotic role.* Since TNF- $\alpha$  has been shown to exhibit anti-

**Table 2.** Effect of TNF- $\alpha$  on SNP-induced synovial cell apoptosis. Cells from patients with RA or OA were incubated with or without TNF- $\alpha$ , and then SNP 1.5 mM was added into the culture as indicated for another 24 h. Apoptotic cell number was determined by TUNEL assay. Data are mean value  $\pm$  SD obtained from 5 different view fields.

| Treatment                                | Apoptotic Cells/Field |
|--|-----------------------|
| Control (RA)                             | 0 $\pm$ 0.0           |
| SNP 1.5 mM (RA)                          | 41.6 $\pm$ 9.3        |
| TNF- $\alpha$ 10 ng/ml + SNP 1.5 mM (RA) | 0.2 $\pm$ 0.4         |
| Control (OA)                             | 0.6 $\pm$ 0.5         |
| SNP 1.5 mM (OA)                          | 41.8 $\pm$ 2.9        |
| TNF- $\alpha$ 10 ng/ml + SNP 1.5 mM (OA) | 2.0 $\pm$ 1.58        |



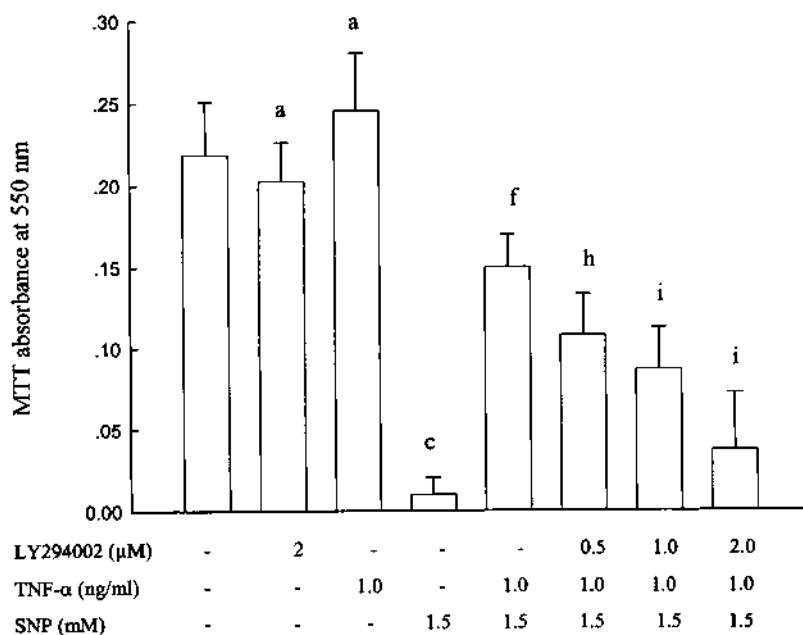
**Figure 2.** Apoptosis induced by SNP and in response to TNF- $\alpha$  treatment in rheumatoid arthritis synovial fibroblasts. Apoptosis was determined by TUNEL method showing DNA fragmentation (Staining: FITC. Original magnification: 40 $\times$ ). A. Control RA cells. B. RA cells treated with SNP (1.5 mM) for 24 hours. C. RA cells were pre-treated with TNF- $\alpha$  (1 ng/ml) for 24 hours and consecutively with SNP. D. Control OA cells. E. OA cells treated with SNP (1.5 mM) for 24 hours. F. OA cells were pre-treated with TNF- $\alpha$  (1 ng/ml) for 24 hours and consecutively with SNP.

**Table 3.** Effect of TNF- $\alpha$  on methotrexate-induced synovial cell apoptosis. Cells were incubated with or without 1 ng/ml TNF- $\alpha$  for 24 h, and then 3 different concentrations of methotrexate (MTX; 20, 50, 100  $\mu$ g/ml) were added and maintained for 24 h. Cell viability was measured by MTT assay. Values correspond to the average, expressed as a percentage, of 3 different experiments.

| Treatment                                  | Living Cells, %  |
|--|------------------|
| Control                                    | 100 $\pm$ 0.0    |
| TNF- $\alpha$ 10 ng/ml                     | 96.8 $\pm$ 3.31  |
| MTX 20 $\mu$ g/ml                          | 91.67 $\pm$ 3.01 |
| MTX 20 $\mu$ g/ml + TNF- $\alpha$ 1 ng/ml  | 93.15 $\pm$ 1.63 |
| MTX 50 $\mu$ g/ml                          | 78.44 $\pm$ 4.42 |
| MTX 50 $\mu$ g/ml + TNF- $\alpha$ 1 ng/ml  | 84.24 $\pm$ 1.16 |
| MTX 100 $\mu$ g/ml                         | 81.91 $\pm$ 7.46 |
| MTX 100 $\mu$ g/ml + TNF- $\alpha$ 1 ng/ml | 83.83 $\pm$ 1.29 |

**Table 4.** Effect of TNF- $\alpha$  on SNP-induced synovial cell apoptosis. Cells were incubated with or without TNF- $\alpha$  at concentration ranging from 0.1 to 10 ng/ml for 24 h, and then SNP 1.5 mM was added into the culture as indicated for 24 h. Cells were stained with Hoechst 33258 and observed under a fluorescence microscope. Apoptotic cells with condensed or fragmented nuclei were identified. In 5 randomly chosen view fields, apoptotic rate was calculated as percentage of apoptotic cells to total cells (n = 4 experiments) <sup>a</sup> p > 0.05, <sup>c</sup> p 0.01 vs control; <sup>f</sup> p < 0.01 vs SNP (1.5 mM).

| Treatment                            | Apoptotic Rate, mean $\pm$ SD |
|--------------------------------------|-------------------------------|
| Control                              | 0 $\pm$ 0.005                 |
| TNF- $\alpha$ 10 ng/ml               | 0 $\pm$ 0.002 <sup>a</sup>    |
| SNP 1.5 mM                           | 0.99 $\pm$ 0.005 <sup>c</sup> |
| TNF- $\alpha$ 0.1 ng/ml + SNP 1.5 mM | 0.71 $\pm$ 0.064 <sup>f</sup> |
| TNF- $\alpha$ 1 ng/ml + SNP 1.5 mM   | 0.31 $\pm$ 0.037 <sup>f</sup> |
| TNF- $\alpha$ 10 ng/ml + SNP 1.5 mM  | 0.22 $\pm$ 0.036 <sup>f</sup> |



**Figure 3.** Effects of PI3K inhibitor LY294002 on the anti-apoptotic activity of TNF- $\alpha$ . LY294002 was added to the culture medium at the indicated concentrations 15 minutes before adding TNF- $\alpha$  1.0 ng/ml; after 24 hours of incubation, SNP 1.5 mM was added for additional 24 hours. Cell viability was measured by MTT assay. Average of duplicate constitutes one determination, n = 4 experiments. <sup>a</sup>p > 0.05, <sup>c</sup>p < 0.01 vs control, <sup>f</sup>p < 0.01 vs SNP 1.5 mM. <sup>h</sup>p < 0.05, <sup>i</sup>p < 0.01 vs TNF- $\alpha$  + SNP.

apoptotic effects, at least in part, through the PI3K-Akt signal transduction pathway<sup>2,23</sup>, the effect of the PI3K inhibitor LY294002 was tested. LY294002 markedly decreased the protective effect of TNF- $\alpha$  as shown by the MTT assay (Figure 3).

The PI3K-Akt signal transduction pathway is closely related to NF- $\kappa$ B<sup>24</sup>, and NF- $\kappa$ B plays a key role in TNF- $\alpha$ 's effects<sup>25</sup>. To determine the role of NF- $\kappa$ B in the anti-apoptotic effect of TNF- $\alpha$  on synovial cells, we tested the effect of the NF- $\kappa$ B inhibitor PDTC by adding it to the culture medium 1 hour before TNF- $\alpha$ . PDTC decreased TNF- $\alpha$  protection, significantly reducing cell viability (Figure 4).

When the same assay was repeated following a 30 min pre-treatment of synovial fibroblasts with the pan-caspase inhibitor z-VAD-fmk, the SNP-induced decrease in cell viability was reversed, and it could not be restored in the presence of either LY294002 or PDTC (Figure 5). Since it has been reported that apoptosis induction mediated by NO is strictly dependent on caspase-3 activation in OA synovial fibroblasts<sup>26</sup>, the specific caspase-3 inhibitor Ac-ESMD-CHO (50  $\mu$ M) was used to examine its role in RA FLS. One hour pretreatment with this molecule was followed by exposure to SNP. Cell death was then detected by TUNEL assay, which showed a complete absence of cell death induction by SNP (Table 5).

To determine whether PI3K and NF- $\kappa$ B inhibition is involved in apoptotic cell death, a TUNEL assay was performed. The PI3K inhibitors LY292004 and wortmannin alone had no effects on apoptosis induction, but when tested together with SNP and TNF- $\alpha$ , they significantly reduced the protective effect of TNF- $\alpha$  compared to the cells treated only with SNP and TNF- $\alpha$ . A more striking effect was observed using the NF- $\kappa$ B inhibitor PDTC (Figure 6).

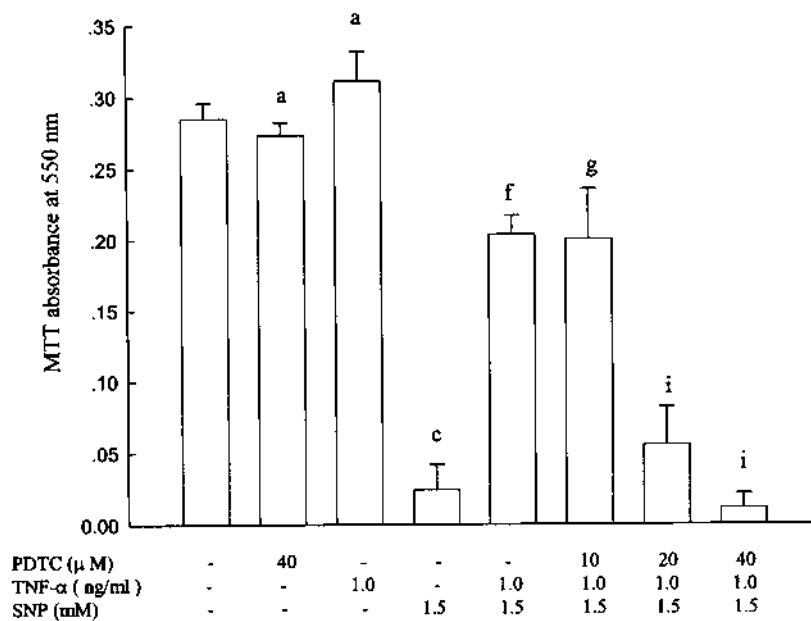


Figure 4. NF-κB inhibitor PDTC reduces in a concentration dependent manner the protective effect of TNF-α. PDTC was added 1 hour before TNF-α treatment. After 24 hours of incubation, cells were incubated with or without SNP for another 24 hours. Cell viability was measured by MTT assay. Average of duplicate constitutes one determination, n = 4 experiments. <sup>a</sup>p > 0.05, <sup>c</sup>p < 0.01 vs control. <sup>f</sup>p < 0.01 vs SNP 1.5 mM. <sup>g</sup>p > 0.05, <sup>i</sup>p < 0.01 vs TNF-α + SNP.

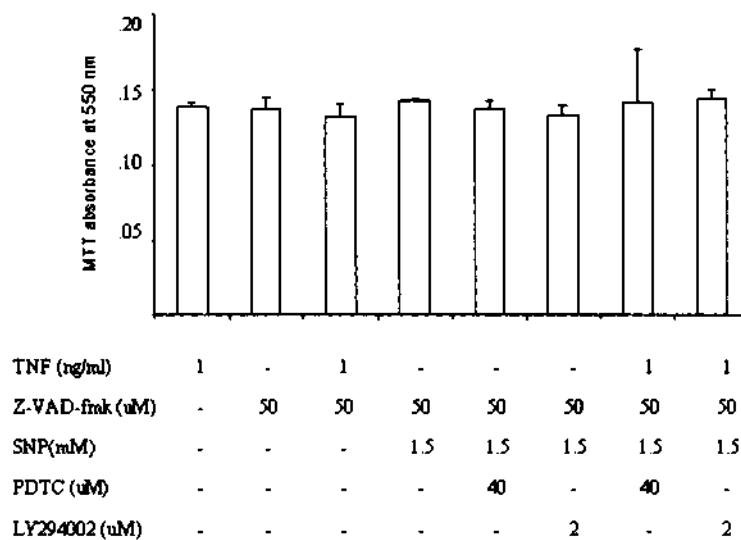


Figure 5. The TNF-α protective effect against SNP-induced apoptosis is lost in the presence of either PI3-K or NF-κB inhibitors. Synovial fibroblasts were grown on microscope slides and treated with different compounds at the concentrations mentioned above. The graph shows the mean values (± SD) of apoptotic cell numbers detected by TUNEL assay in 5 different view fields.

Together, these results indicate that both PI3K and NF-κB play a pivotal role in the anti-apoptotic effect of TNF-α in synovial cells.

*TNF-α induces high levels of Akt phosphorylation in synovial cells.* The anti-apoptotic effect of TNF-α suggests induction of survival signals. The viability results suggest the possible involvement of PI3K-Akt signal pathway activation. To deter-

mine whether PI3K-Akt is activated by TNF-α in FLS, Akt phosphorylation was measured by Western blotting and a semiquantitative analysis was performed. Synovial cells were treated with TNF-α for different times. The results showed that Akt phosphorylation reached a maximum 6 hours after TNF-α treatment, with a 3-fold increase compared to baseline level (Figure 7A). Data also showed that the activation of Akt



**Table 5.** SNP failed to induce cell death. Cells were incubated with or without TNF- $\alpha$  for 24 h. A 1-h pretreatment with Ac-ESMD-CHO (50  $\mu$ M) was followed by 24 h exposure to SNP 1.5 mM. Apoptotic cell number was determined by TUNEL assay. Data are mean values  $\pm$  SD obtained from 5 different view fields.

| Treatment  | Apoptotic Cells/Field,<br>mean $\pm$ SD |
|--|---|
| Ac-ESMD-CHO 50 $\mu$ M   | 0.8 $\pm$ 0.8                           |
| SNP 1.5 mM, Ac-ESMD-CHO 50 $\mu$ M                             | 2.0 $\pm$ 1.6                           |
| TNF- $\alpha$ 10 ng/ml + SNP 1.5 mM,<br>Ac-ESMD-CHO 50 $\mu$ M | 2.4 $\pm$ 1.1                           |

by TNF- $\alpha$  was concentration-dependent (Figure 7B), and both LY294002 and PDTC could decrease such enhancement, almost restoring control levels.

**TNF- $\alpha$  increases BAD phosphorylation in synovial cells.** The major anti-apoptotic mechanism of PI3K-Akt is BAD phosphorylation, which consequently is sequestered in the cytoplasm by 14-3-3, separated from Bcl-2 and Bcl-X<sub>L</sub><sup>11</sup>. To determine whether this mechanism is also present in synovial cells, the level of phospho-BAD was determined by Western blot analysis of lysates from cells with and without TNF- $\alpha$  treatment. Our results showed that TNF- $\alpha$  induced a 2-fold increase in BAD phosphorylation in synovial cells. This effect can be blocked by LY294002 and PDTC. Such enhancement lasts for 24 hours (Figure 8A, 8B).

## DISCUSSION

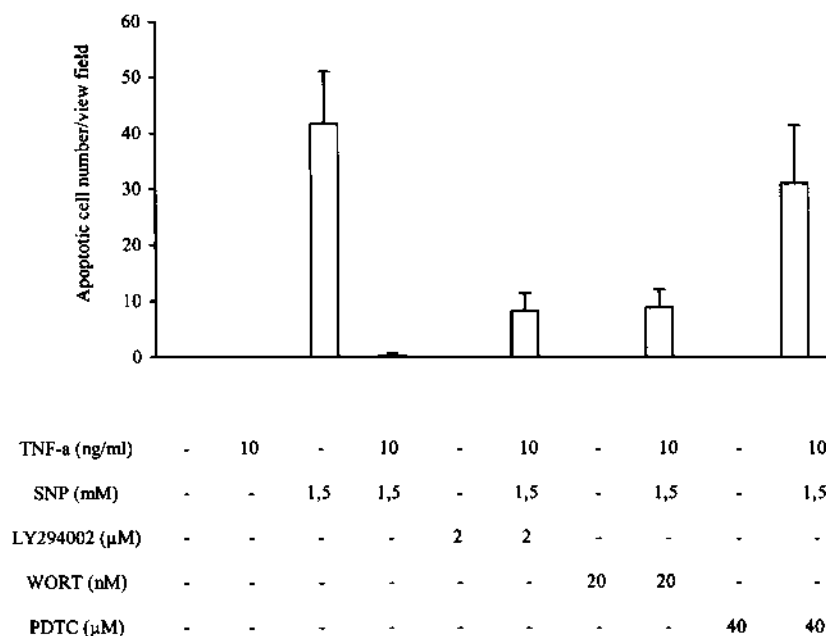
TNF- $\alpha$  is a critical cytokine in the immunopathogenesis of RA, since it can recruit other proinflammatory factors, increase enzymes involved in cartilage erosion, and promote

synovial proliferation<sup>4-6</sup>. Recent trials have established the efficacy of anti-TNF- $\alpha$  agents in relieving symptoms and signs of RA<sup>27</sup>. Further, anti-TNF- $\alpha$  therapy seems to prevent joint damage in RA.

Increased production of nitric oxide due to activation of inducible NO synthase (iNOS) is one of the features of RA. NO concentrations have been shown to be higher in synovial fibroblast supernatant and serum of patients with RA than in controls<sup>28</sup>; moreover, NO is a pro-apoptotic stimulus for FLS. Nevertheless, RA synovial cells can expand because of an abnormal proliferation as well as reduction in the response to apoptotic stimuli, forming a tumor-like structure known as pannus<sup>12-14</sup>.

We observed that TNF- $\alpha$  is able to protect synovial cells uniquely from NO-induced apoptosis. As well, the protective effect of TNF- $\alpha$  was not observed when fibroblasts were exposed to methotrexate. FLS obtained from patients with OA or RA showed the same behavior, proving that the uniqueness of the pathway is restricted to the apoptotic stimulus that is used, not to the pathology.

The pro-survival effect of TNF- $\alpha$  in synovial cells exposed to SNP was first detected and quantified measuring cell viability through MTT assays. Thus, the possibility that TNF- $\alpha$  could protect synovial cells from apoptotic stimuli, from NO in particular, was explored using 2 different assays, TUNEL and Hoechst staining; this allowed us to identify apoptotic cells through their specific characteristics, excluding the presence of necrosis, and estimate the apoptotic rate. However, both TUNEL assay and Hoechst staining can lead to underestimation of apoptosis because of the loss of cellular adherence characteristic of apoptotic cells. To obtain more accurate quantitative measurement of cell survival we turned to the



**Figure 6.** Caspase inhibition by z-VAD-fmk reversed proapoptotic effects of SNP and impaired the ability of Akt and NF- $\kappa$ B to restore SNP activity. Z-VAD-fmk (50  $\mu$ M) was added 30 minutes before SNP treatment. Cell viability was measured by MTT assay. WORT: wortmannin.

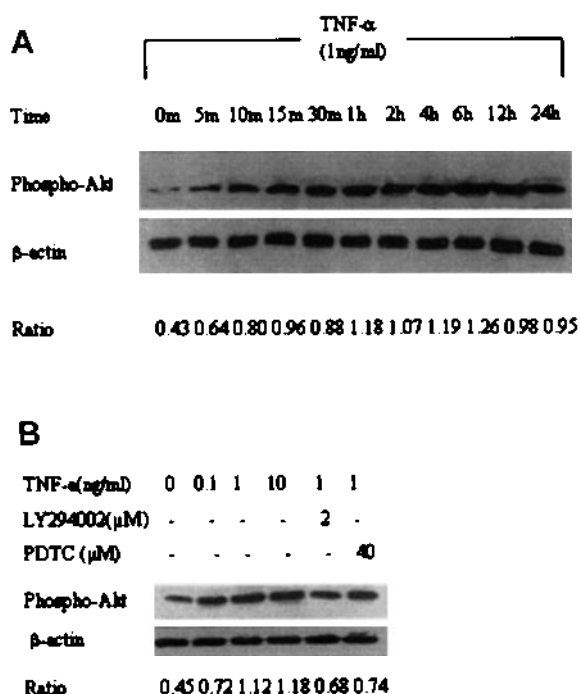


Figure 7. Effect of TNF- $\alpha$  on Akt phosphorylation in synovial cells. A. TNF- $\alpha$  (1 ng/ml) enhances phospho-Akt levels in a time-dependent manner for 24 hours; B. TNF- $\alpha$  increases Akt phosphorylation in a concentration dependent manner. Synovial cells were pretreated either with LY294002 (2 mM) for 15 minutes or with PDTC (40 mM) for 1 hour, and then TNF- $\alpha$  was added for 24 hours. Akt phosphorylation was detected by western blot.

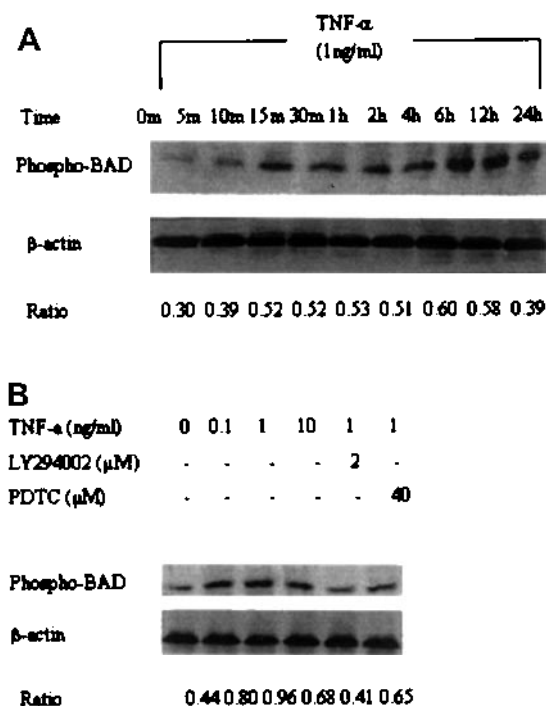


Figure 8. TNF- $\alpha$  affects BAD phosphorylation in synovial cells. A. Cells were incubated with TNF- $\alpha$  (1 ng/ml) for the indicated times. TNF- $\alpha$  treatment increases BAD phosphorylation with a time-dependent pattern. B. TNF- $\alpha$  increases BAD phosphorylation in a concentration-dependent manner. Pretreatment with LY294002 for 15 minutes or PDTC for 1 hour significantly decreases such enhancement.

viability assay. Since the most frequent mechanisms of cell death identified to date are apoptosis and necrosis, and since we excluded induction of necrosis in our model, we can state that apoptosis accounted for the decreased rate of cell viability that was observed.

With this approach, TNF- $\alpha$ 's anti-apoptosis protective effect induced by SNP in synovial cells was proved, and 2 molecular pathways involved in the effect were identified.

The PI3K-Akt pathway is a pro-survival signaling pathway activated by TNF- $\alpha$ <sup>29</sup>. Using 2 different PI3K inhibitors, LY294002 and wortmannin, at concentrations reported to adequately block this pathway<sup>30,31</sup>, we observed that these 2 molecules can prevent TNF- $\alpha$  protection, thus increasing cell death. The effect on apoptotic cell numbers estimated with the TUNEL assay was comparable for the 2 inhibitors.

First recognized as an anti-apoptotic factor in studies of the insulin-like growth factor signal pathway in cerebellar neurons, Akt belongs to an important survival signal pathway in many cell lines<sup>8,10,32</sup>, activated by the PI3K cascade. The relationship between Akt and NF- $\kappa$ B is still unclear. Some studies have shown that Akt is able to activate NF- $\kappa$ B by phosphorylation of I- $\kappa$ B, which promotes the nuclear translocation of NF- $\kappa$ B<sup>11,27,33</sup>. Activation of NF- $\kappa$ B by TNF- $\alpha$  contributes to the anti-apoptotic effect regulating gene expression of the anti-apoptotic proteins Bcl-1/A1 and Bcl-X<sub>L</sub><sup>34</sup>. Moreover, it has been demonstrated that NF- $\kappa$ B is required for TNF-mediated Akt activation as well<sup>35</sup>. TNF- $\alpha$  has been shown to increase Akt activation in synovial cells<sup>33</sup>, but the molecular pathway causing this activation remains to be elucidated.

We observed that the effect of PDTC is 3 times more powerful in restoring NO-induced apoptosis than PI3K inhibitors. This could be explained, at least in part, by the ability of NF- $\kappa$ B to act upstream as well as downstream of Akt.

Since Akt and NF- $\kappa$ B can activate the apoptotic pathway in a caspase-dependent or independent way, we examined this issue using the pan-caspase inhibitor z-VAD-fmk. We observed that a 30-minute pretreatment with this compound not only protected the cells from a decrease in cell survival mediated by SNP exposure, but also that Akt and NF- $\kappa$ B inhibition in either the presence or absence of TNF- $\alpha$  failed to restore SNP's pro-apoptotic effect, highlighting the importance of caspases in this molecular pathway. Since z-VAD-fmk can act on the 3 effector caspases -3, -6, and -7, we investigated the role of caspase-3 in the apoptotic process induced by SNP by using the specific caspase-3 inhibitor Ac-ESMD-CHO. We observed by TUNEL assay that in the absence of active caspase-3 the FLS failed to undergo apoptosis, thus highlighting the role of this caspase in SNP-induced apoptosis.

Moreover, our study shows that TNF- $\alpha$  increases phosphorylated Akt levels in synovial cells in a time and concentration-dependent manner, and that in the presence of PI3K and NF- $\kappa$ B inhibitors, Akt phosphorylation is similar to that detected in control cells.

Akt acts as a pro-survival protein by phosphorylating a serine residue of BAD<sup>36</sup>, which is then bound by 14-3-3 and seg-

regulated in the cytoplasm, where it cannot execute its pro-apoptotic function. Once phosphorylated, BAD is recognized by 14-3-3 and cannot migrate to mitochondria to exert anti-apoptotic effects by binding to Bcl-2 and Bcl-X<sub>L</sub>. In our study, Western blot results indicated that TNF- $\alpha$  enhances BAD phosphorylation, indicating that the activated Akt induces cell survival signals in synovial cells.

Our study shows that TNF- $\alpha$  protected synovial cells from SNP-induced apoptosis, which is relevant to the pathophysiology of RA. The anti-apoptotic effect of TNF- $\alpha$  is due, at least partially, to activation of the Akt signal transduction pathway, and such activation may involve the activation of NF- $\kappa$ B.

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