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Nuclear factor-κB activates dual inhibition sites in the regulation of tumor necrosis factor-α-induced neutrophil apoptosis

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Abstract

The objective of this study was to elucidate the role of the nuclear factor-κB (NF-κB) pathway in tumor necrosis factor-α (TNF-α)-induced neutrophil apoptosis. A single treatment with TNF-α produced significant caspase-3 activiation in a time- and concentration-dependent manner, while no significant morphological change in neutrophils was observed. After pretreatment of neutrophils with cycloheximide or actinomycin D, TNF-α produced morphologically typical apoptosis in a time- and concentration-dependent manner. Similarly, following pretreatment of neutrophils with the specific NF-κB inhibitors, pyrrolidine dithiocarbamate or SN50, TNF-α also produced neutrophil apoptosis (assessed morphologically). Caspase-3 activation by TNF-α was significantly enhanced by pretreatment with both cycloheximide and pyrrolidine dithiocarbamate. TNF-α-induced a rapid phosphorylation and degradation of IκB-α in neutrophils. Furthermore, TNF-α increased NF-κB DNA binding, which was abolished by pretreatment with pyrrolidine dithiocarbamate. These results indicate that the NF-κB pathway is crucial for neutrophil survival against TNF-α cell toxicity. Furthermore, it is proposed that NF-κB-induced proteins act on dual inhibitory sites, both upstream and downstream of caspase-3, to protect against apoptosis.

Keywords: Neutrophil; Caspase; Apoptosis inhibitory protein; Transcription factor; NF-κB (nuclear factor-κB); TNF-α (tumor necrosis factor-α)

1. Introduction

Neutrophils participate in host defense mechanisms against infection, and in inflammatory and allergic reactions, such as asthma. To fulfill this role, neutrophils migrate from blood to various tissues. The number of neutrophils in the circulating blood is maintained within a narrow range by the balance between the constant production of cells by bone marrow (Mauer et al., 1960) and their death following spontaneous apoptotic processes (Savill et al., 1989). Apoptotic senescent neutrophils in tissue are recognized and phagocytosed by macrophages. This apoptotic process has been suggested to represent an in vivo mechanism to limit the tissue injury by neutrophils in sites of inflammation in both rats (Tsuchida et al., 1995) and in humans (Niwa et al., 1997).

Tumor necrosis factor-α (TNF-α), a 17-kDa mammalian cell macrophage/monocyte-derived lymphokine, as originally defined for its anti-tumor activity, binds to specific receptors on most mammalian cells, having various effects on target cells (Tracey and Cerami, 1994). Recently, TNF-α has been shown to initiate apoptotic cell death and DNA fragmentation in several mammalian cell lines including human leukemia and murine fibrosarcoma cell lines (Obeid et al., 1993). While TNF-α is a potent neutrophil activator, stimulating neutrophil functions, such as adherence, phagocytosis, degranulation and oxidative metabolism (Klebanoff et al., 1986), it has also been reported that TNF-α induces apoptosis in neutrophils (Murray et al., 1997). This apoptosis is greatly enhanced in the presence of cycloheximide (Tsuchida et al., 1995; Niwa et al., 1997). Furthermore, it has also been reported that prolonged exposure to cycloheximide or actinomycin D accelerates spontaneous neutrophil apoptosis (Whyte et al., 1997). Although the mechanism of cycloheximide in this action has not yet been elucidated, endogenous sur-

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vival proteins may be involved in neutrophil apoptosis (Whyte et al., 1997).

Recently, it has been reported that in many cells in which TNF-α induces apoptosis, there is also activation of the transcription factor, nuclear factor-κB (NF-κB) (Baeuerle and Henkel, 1994). However, in neutrophils, the exact role of NF-κB in TNF-α-induced apoptosis has not yet been elucidated. Furthermore, NF-κB-independent cytoprotective pathways, that, is c-Jun N terminal kinase/stress-activated protein kinase (JNK/SAPK), also have been proposed, which originate at the TNF-α receptor in other types of cells (Natoli et al., 1998).

In this study, we first confirmed whether TNF-α stimulated NF-κB activation in neutrophils. Second, we elucidated the effect of protein or RNA synthesis inhibition and selective NF-κB inhibition on TNF-α-induced neutrophil apoptosis (evaluated both morphologically and by caspase-3 activation). Our results indicate that the NF-κB-dependent pathway makes a significant contribution to the TNF-α-induced cytoprotective response in neutrophils. Further, it is also suggested that the target site(s) of the NF-κB pathway in the TNF-α-induced apoptosis mechanism may be both upstream and down stream of caspase-3.

2. Materials and methods

2.1. Materials

Cycloheximide, histopaque and propidium iodide were purchased from Sigma. SN50 and SN50-M were purchased from BioMol. Triton X-100, pyrrolidine dithiocarbamate and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) were obtained from Kishida Kagaku (Japan), Amersham Japan and CalbioChem, respectively. Dextran (MW 208,000), HEPES and L-isoleucyl-L-glutamyl-L-threonyl-L-aspartic acid-fluoromethyl ketone (IETD-FMK) were purchased from Nacalai (Japan), DOJIN (Japan) and MBL (Japan), respectively. Acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid-α-(4-methyl-coumaryl-7-amide) (Ac-DEVD-MCA), acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid-1-aldehyde (Ac-DEVD-CHO) and acetyl-L-tyrosyl-L-valyl-L-alanyl-L-aspartic acid-1-aldehyde (Ac-YVAD-CHO) were purchased from Peptide Institute (Japan). Recombinant human TNF-α was a kind gift from Dainippon Pharmaceutical (Japan).

2.2. Preparation of neutrophils

Human neutrophils were isolated from blood by the Dextran–Histopaque method as previously described (Boyum, 1968) with minor modifications (Niwa et al., 1996). Purification of neutrophils was performed to minimize exposure of the cells to bacterial endotoxin. The purity of neutrophils was greater than 95%. Cell number was counted by a Coulter counter model ZM (Coulter, USA), and cells were diluted in RPMI 1640 medium to the final required concentrations and kept on ice until examined.

2.3. Evaluation of apoptosis

For morphological assessments, neutrophils were suspended at 2×10^6/ml in RPMI 1640 medium, and then incubated with TNF-α at 37°C for up to 3 h. Neutrophils incubated under specific conditions were spun down onto glass slides in a cytocentrifuge (CF-12SB, Sakurai-Seiki, Japan), dried in cool air, and stained with May-Giemsa solution (Merck, Germany) for light microscopic evaluation. The percentage of apoptotic cells was assessed by counting at least 500 cells/slide (Niwa et al., 1997). To confirm the appearance of nuclear chromatin condensation in apoptotic neutrophils, Hoechst 33258 staining was also performed.

2.4. Analysis of DNA content

The DNA content of neutrophils was analyzed by flow cytometry (Beckman–Coulter EPICS XL-MCL cytofluorometer) using propidium iodide. Briefly, neutrophils (10^6/500 μl) were washed with phosphate-buffered saline (PBS) supplemented with 0.5 mM ethylenediaminetetraacetate (EDTA) and resuspended 4% paraformaldehyde. Neutrophils were permeabilized for 20 min at 4°C, washed twice with PBS supplemented with 0.5% EDTA, and suspended in 500 μl PBS with 0.5 mM EDTA. After addition of 25 μg/ml RNAse, sample were incubated for 30 min at 37°C, then further incubated with 500 ng/ml propidium iodide for 15 min at room temperature and kept at 4°C until flow cytometric analysis.

2.5. Measurement of caspase-3 activity

Caspase-3 activity in neutrophils was measured as previously described (Niwa et al., 1999). Briefly, neutrophils were harvested after being exposed to TNF-α for the indicated periods and resuspended with hypotonic lysis buffer (25 mM HEPES, pH 7.5, containing 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A and 10 μg/ml leupeptin). Then cells were lysed by subjecting them to four cycles of freezing and thawing. After centrifugation ($15{,}000 \times g$, for 20 min at 4°C) of the cell lysates, supernatant was used to measure caspase activity. Caspase-3 activity of the cell extracts was determined by using Ac-DEVD-AMC, a specific caspase-3 substrate, as described previously (Nicholson et al., 1995). Caspase-3 activity is expressed as the amount of liberated AMC (7-amino-4-methylcoumarin) cleaved from Ac-DEVD-AMC, measured by using spectrofluorometer (Fluoroskan, Dainippon Pharmaceutical).

2.6. Western blotting

Whole cell extract was diluted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. After SDS-PAGE, the gels were transferred to 0.2 μm polyvinylidene difluoride (PVDF) membranes (BioRad) for 30 min. Blots were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 80 overnight at 4°C. The next day, blots were rinsed three times with Tris-buffered saline containing 0.1% Tween 80 for 10 min. Blots were then incubated with primary antibodies against IκBα and phospho-IκBα (New England BioRabs, MA). Antibodies were added at a dilution of 1:1000 for an overnight incubation at 4°C. Then blots were washed with Tris-buffered saline containing 0.1% Tween 80 and incubated for 1 h with the secondary antibody, goat anti-rabbit immunoglobulin G-peroxidase (Chemicon International, CA) at a dilution of 1:1000 in 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 80. Subsequently, blots were washed three times with Tris-buffered saline containing 0.1% Tween 80. Then blots were developed for 4 min with ECL reagents (Amersham Pharmacia Biotec, Tokyo, Japan).

2.7. Electrophoretic mobility shift assay (EMSA)

EMSAs were carried out as described previously (Ward et al., 1999) using a kit (Promega, Southampton, UK). Nuclear extracts were prepared from 5×10^6 cells using a modification of the method of Dignam et al. (1983). Briefly, pelletted cells were resuspended in 200 μl of hypotonic buffer (buffer A: 10 mM Tris–HCl, pH 7.8, 1.5 mM EDTA, 10 mM KCl, 0.5 mM dithiothreitol, 1 μg/ml aprotinin, leupeptin, and pepstatin A, 1 μM 4-(2-aminoethyl) benzenesulfonyl fluoride, 1 mM sodium orthovanadate, 0.5 mM benzamidine, and 2 mM levamisol) and placed on ice for 10 min. Following the addition of 0.1 volume of 10% Nonidet P-40 (w/v), the cells were vortexed briefly and centrifuged at $12{,}000 \times g$ for 2 min at 4°C. The supernatant was discarded and the pellet was washed in 100 μl of buffer A minus Nonidet P-40 and recentrifuged. The pelleted nuclei were then resuspended in 50 μl of hypertonic buffer (buffer B: 20 mM Tris–HCl, pH 7.8, 150 mM NaCl, 50 mM KCl, 1.5 mM EDTA, 5 mM dithiothreitol, 1 μg/ml aprotinin, leupeptin, and pepstatin A, 1 μM 4-(2-aminoethyl) benzenesulfonyl fluoride, 1 mM sodium orthovanadate, 0.5 mM benzamidine, and 2 mM levamisol) and stored at −80°C until use. Two micrograms of nuclear extracts, as determined by bicinconinic acid (BCA) protein assay, were incubated in binding buffer (5% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris–HCl, pH 7.5, with poly(dI–dC)-poly(dI–dC)Pharmacia Bioteck, UK) with γ-^{32}P-labeled double-stranded oligonucleotide containing the decamericκB-binding site, by standard protocols using T4 kinase, at 4°C for 30 min. Samples were loaded onto 8% native acrylamide gel and run at 150 V for 2 h. The gel was then dried under vacuum and exposed to X-ray film.

3. Results

3.1. Effects of protein or RNA synthesis inhibitor on TNF-α-induced apoptosis and caspase-3 activation

We determined apoptosis in freshly isolated neutrophils from healthy donors by morphologic evaluation. After

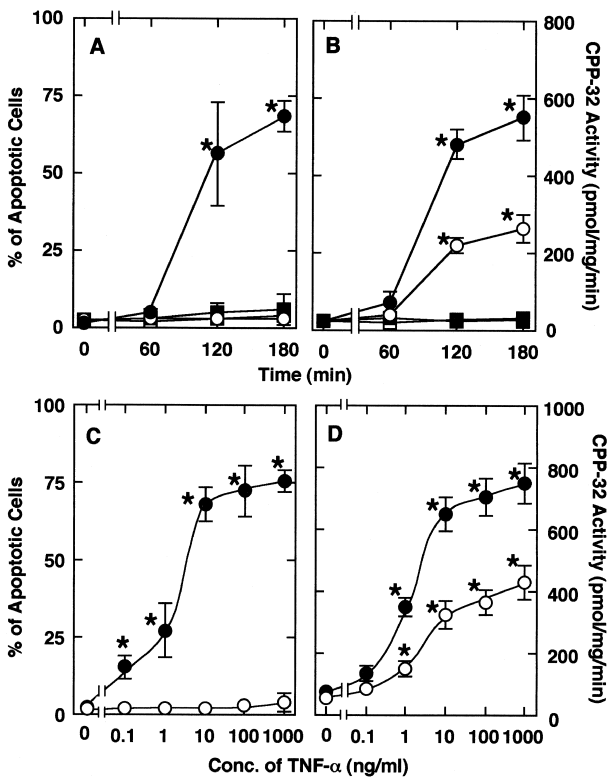

Fig. 1. Time course of morphological apoptotic changes (A) and caspase-3 activity (B), and effect of cycloheximide on TNF-α-induced neutrophil apoptosis (C) and caspase-3 activity (D) in human neutrophils. (A) Human neutrophils were incubated with vehicle (□), TNF-α (100 ng/ml, ○), cycloheximide (1 μg/ml, ■) or TNF-α + cycloheximide (●) at 37°C for the indicated periods of time (min). Then, May–Grünwald–Giemsa staining was performed and apoptotic cells were counted as described in Materials and methods. (B) Human neutrophils were treated in the same way as (A). Then, caspase-3 activity in neutrophils was determined as described in Materials and methods. (C and D) Human neutrophils were incubated with TNF-α at 37°C for the 3 h in the presence (●) or absence (○) of 1 μg/ml cycloheximide. Then, May–Grünwald–Giemsa staining (C) and caspase-3 activity (D) in neutrophils were determined as described in Materials and methods. Each value represents the mean ± S.D. of at least four separate experiments. Statistically significant differences ($P < 0.05$) in the percentage of morphologically apoptotic cells and in caspase-3 activity were determined by the Mann–Whitney U test and analysis of variance (ANOVA) with Fisher's Protected Least Significant Difference (Fisher's PLSD) test, respectively. * indicates a significant difference from the vehicle control.

Table 1
Effect of actinomycin D and cycloheximide on TNF-α-induced apoptosis and caspase-3 activation in human neutrophils

Reagent (μg/ml)	TNF-α (100 ng/ml)	Apoptotic cells (%)	Caspase-3 activity (pmol/mg/min)
−	−	2.3 ± 1.5	54.5 ± 2.6
−	+	4.5 ± 2.5	315.0 ± 9.3[a]
Actinomycin D 0.1	+	18.7 ± 5.6[a,b]	324.6 ± 15.9[a]
1	+	43.5 ± 13.1[a,b]	588.3 ± 27.9[a,b]
10	+	80.6 ± 22.3[a,b]	807.2 ± 35.8[a,b]
Cycloheximide 1	+	75.7 ± 17.9[a,b]	698.7 ± 18.8[a,b]

The values represent the means ± S.D. of four separate experiments. Statistically significant differences ($P < 0.05$) in the proportion of morphologically apoptotic cells and in caspase-3 activity were determined by the Mann–Whitney *U* test and ANOVA with Fisher's PLSD test, respectively.

[a]Indicates significant difference from intact neutrophils (reagent: − and TNF-α: −).

[b]Indicates significant difference from TNF-α-treated control neutrophils (reagent: − and TNF-α: +).

treatment of neutrophils with 100 ng/ml TNF-α, fewer than 5% neutrophils showed typical apoptotic phenomena up to 3 h. However, when neutrophils were pretreated with 1 μg/ml cycloheximide, typical apoptotic cells, displaying diminution in cell volume and nuclear pyknosis, were readily observed in a time- and TNF-α concentration-dependent manner. Finally, apoptosis affected 75% of the population (Fig. 1A and C). Cycloheximide (1 μg/ml) alone elicited only weak apoptotic morphological changes in neutrophils (Fig. 1A).

Since many previous reports indicated that caspase-3 activation was involved in neutrophil apoptosis as a final step of the caspase cascade, we determined caspase-3 activity after treating neutrophils with TNF-α in the presence or absence of cycloheximide. TNF-α alone showed a significant activation of caspase-3 in a time- and TNF-α-concentration-dependent manner. The activation of caspase-3 by TNF-α was significantly enhanced in the presence of 1 μg/ml cycloheximide (Fig. 1B and D).

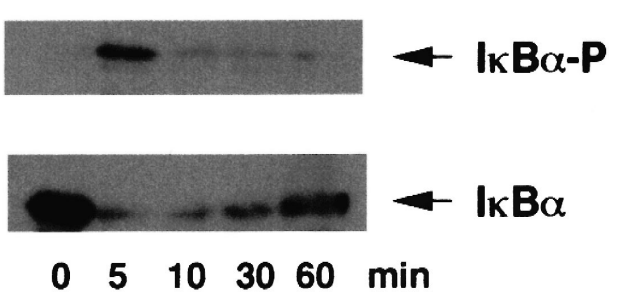

Fig. 2. Time course of TNF-α-induced phosphorylation and degradation of IκB-α in human neutrophils. Neutrophils were treated with TNF-α (100 ng/ml) and harvested at the end of the indicated time periods, then equal amounts of cytoplasmic extracts were separated on 10% SDS-PAGE. After transfer to PVDF membranes, the blots were probed with phosphorylated IκB-α and non-phosphorylated IκB-α specific antisera.

Similarly to cycloheximide, the RNA synthesis inhibitor actinomycin D also exhibited an enhancing effect on TNF-α-induced neutrophil apoptosis and caspase-3 activation (Table 1).

3.2. TNF-α-induced NF-κB activation in neutrophils

The results described above strongly suggest that TNF-α not only activates caspase cascades to induce apoptosis of neutrophils, but also enhances protein synthesis. These proteins may act to inhibit TNF-α-triggered neutrophil apoptosis. It has been reported that inflammatory stimuli, such as TNF-α or fMLP, activate the NF-κB pathway (McDonald et al., 1997), and that NF-κB transcription products subsequently act as inhibitors of apoptosis. Thus, it was evaluated whether NF-κB activation occurred in the TNF-α-stimulated neutrophils. Fig. 2 shows that in the whole extracts of cells stimulated with 100 ng/ml TNF-α, IκB-α levels were substantially decreased by 5 min. A trace of residual IκB-α protein was detectable following stimulation, and by 60 min levels had partly recovered to prestimulation values. Interestingly, phosphorylated IκB-α was not detectable prior to TNF-α stimulation but was detectable 5 min after TNF-α. Residual amounts of phosphorylated IκB-α protein were detected at 10, 30 and 60 min after TNF-α stimulation. Thus, the transient appearance of phosphorylated IκB-α in association with the reciprocal disappearance of IκB-α indicates that NF-κB was induced in neutrophils by TNF-α stimulation.

To confirm the NF-κB activation induced by TNF-α, we also performed an EMSA assay to detect NF-κB DNA binding activity. As shown in Fig. 3, after a 60-min incubation with TNF-α, NF-κB was activated in neutrophils. Furthermore, after pretreatment of neutrophils with pyrrolidine dithiocarbamate, an inhibitor of NF-κB activation (Schreck et al., 1992), for 2 h, TNF-α (100ng/ml)-induced NF-κB DNA binding was inhibited.

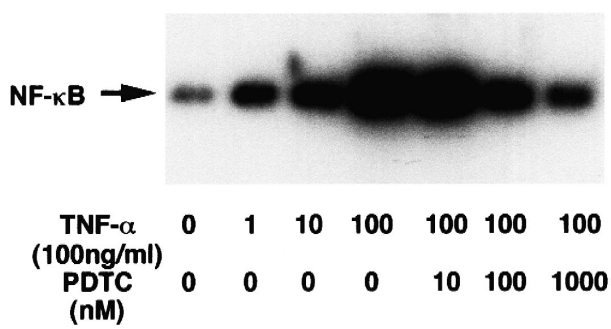

Fig. 3. TNF-α induced activation of NF-κB DNA binding, and their inhibition by pyrrolidine dithiocarbamate in human neutrophils. Neutrophils were preincubated for 2 h with the indicated concentrations of pyrrolidine dithiocarbamate followed by stimulation with TNF-α for 60 min. Nuclear extracts were prepared and equal amounts of protein were analyzed for κB-specific DNA binding in EMSA using a ^{32}P-labeled DNA probe (see Materials and methods).

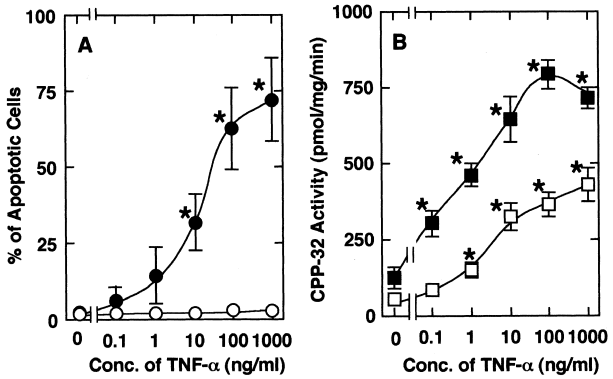

Fig. 4. Effect of a NF-κB inhibitor, pyrrolidine dithiocarbamate, on TNF-α-induced morphological apoptotic changes (A) and caspase-3 activity (B) in human neutrophils. Human neutrophils were incubated with (●) or without (○) pyrrolidine dithiocarbamate (100 nM) at 37°C for 2 h and, then TNF-α (100 ng/ml)-induced apoptosis was evaluated by May–Grünwald–Giemsa staining and caspase-3 activation as described in Materials and methods. Each value represents the mean ± S.D. of four separate experiments. Statistically significant differences ($P < 0.05$) in the percentage of morphologically apoptotic cells and in caspase-3 activity were determined by the Mann–Whitney *U* test and ANOVA with Fisher's PLSD test, respectively. * indicate significant difference from vehicle control.

3.3. Effects of NF-κB inhibitors on TNF-α-induced apoptosis and caspase-3 activation

Next, to determine whether NF-κB activation is the pathway responsible for the synthesis of the apoptosis inhibitory protein after TNF-α stimulation, we evaluated the effects of a specific inhibitor of NF-κB activation on TNF-α-stimulated apoptosis. After pretreatment of neutrophils with pyrrolidine dithiocarbamate (100 nM) for 2 h, TNF-α caused neutrophil apoptosis, as assessed morphologically, in a concentration-dependent manner (Fig. 4A). TNF-α-stimulated caspase-3 activation was also enhanced by the pretreatment with pyrrolidine dithiocarbamate (Fig. 4B), in similar manner to that produced by cycloheximide.

These results were also confirmed by flow cytometric observation of propidium iodide-stained and permeabilized neutrophils; apoptosis was detected by the loss of cellular DNA content. Fig. 5 shows original recordings of the fluorescence histograms obtained from neutrophils treated with vehicle (A), TNF-α (100 ng/ml: B), pyrrolidine dithiocarbamate (100 nM: C) and TNF-α + pyrrolidine dithiocarbamate (D). Unstimulated neutrophils showed only one prominent fluorescence peak, due to a uniform DNA content. Neutrophils stimulated by TNF-α or pyrrolidine dithiocarbamate showed negligibly weak apoptosis. Co-stimulation by pyrrolidine dithiocarbamate and TNF-α markedly augmented neutrophil apoptosis (loss of DNA content). Pretreatment of neutrophils with cycloheximide produced similar results (data not shown).

To confirm the effect of pyrrolidine dithiocarbamate, we also used the other inhibitor of NF-κB, SN50 (Maggirwar et al., 1998). SN50 is a synthetic oligopeptide that contains a hydrophobic cell-permeable motif, together with nuclear localization sequences from the p50 subunit of NF-κB (Lin et al., 1995). Following pretreatment of neutrophils with SN50 (100 μg/ml) for 15 min, TNF-α induced apoptosis, while the inactive control peptide of SN50, SN50-M (100 μg/ml), which is mutated within the nuclear localization sequence motif, did not show any effect on TNF-α-induced apoptosis in human neutrophils (Data not shown).

3.4. Effects of JNKs / SAPKs inhibitors on TNF-α-induced apoptosis and caspase-3 activation

To determine whether the TNF-α stimulated apoptosis inhibitory protein utilizes the JNK/SAPK pathway, we

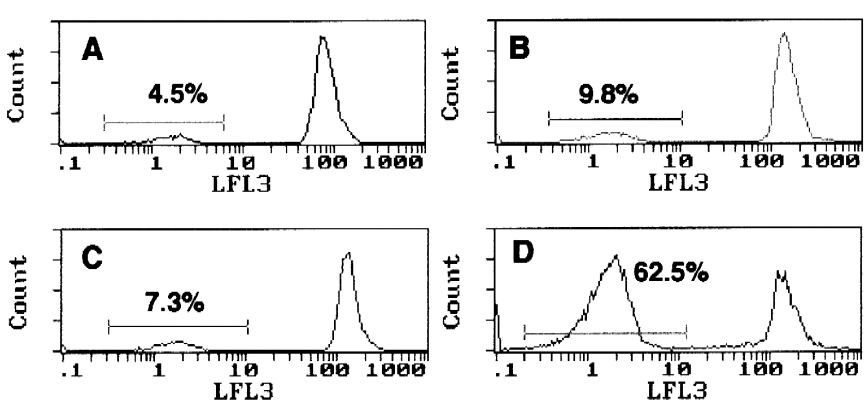

Fig. 5. Fluorescence histograms of propidium iodide-stained neutrophils evaluated by a fluorocytometry. Original recording of fluorescence histograms of neutrophils (10^6/samples) stimulated for 3 h with vehicle (A), TNF-α (100 ng/ml: B), pyrrolidine dithiocarbamate (100 nM: C) and TNF-α + pyrrolidine dithiocarbamate (D). Unstimulated neutrophils showed only a single prominent fluorescence peak, due to their uniform DNA content. Neutrophils stimulated by TNF-α or pyrrolidine dithiocarbamate also showed a single peak, indicating negligibly weak apoptosis. After pretreatment of neutrophils with pyrrolidine dithiocarbamate, TNF-α-induced apoptosis, as indicated by loss of DNA content, was markedly augmented.

evaluated the effects of an inhibitor of JNK/SAPK (NPPB (Chan and Riches, 1998)) activation on the apoptosis produced by TNF-α-stimulation. Pretreatment of neutrophils with NPPB (50–200 μM) for 30 min did not enhance the TNF-α-stimulated neutrophil apoptosis. TNF-α-stimulated caspase-3 activation was not affected by NPPB either (data not shown).

3.5. Desensitization of TNF-α + cycloheximide-induced apoptosis by TNF-α pretreatment

The results so far obtained suggest that, when neutrophils are stimulated by TNF-α, apoptosis-inhibitory proteins are synthesized via the NF-κB pathway. To evaluate the protective effect of these TNF-α-inducible proteins against apoptosis, we determined the effect of TNF-α-pretreatment on TNF-α + cycloheximide-induced neutrophil apoptosis. Pretreatment of neutrophils with TNF-α for more than 30 min, but not less than 30 min, significantly reduced the magnitude of apoptosis induced by TNF-α + cycloheximide (Fig. 6). The pretreatment of neutrophils with pyrrolidine dithiocarbamate (Fig. 6) abolished this protective effect.

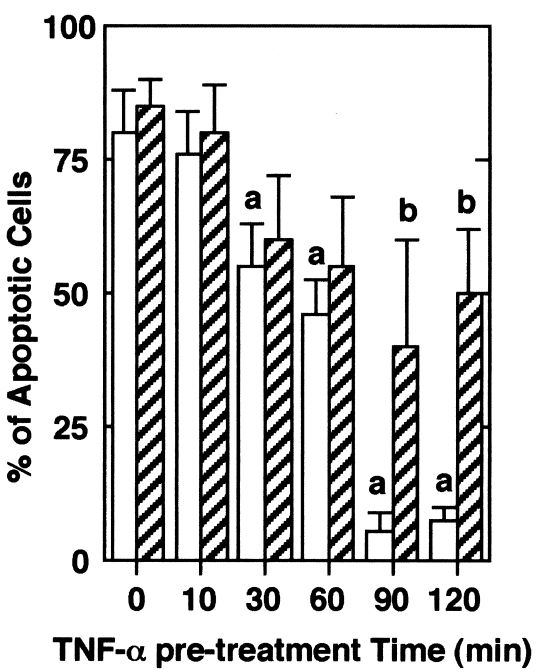

Fig. 6. Effect of TNF-α pretreatment on TNF-α + cycloheximide-induced neutrophil apoptosis. Neutrophils were pretreated with TNF-α (100 ng/ml) at 37°C for indicated periods, washed and then incubated with TNF-α (100 ng/ml) and cycloheximide (1 μg/ml) for 3 h (open column). Prior to the pretreatment with TNF-α (100 ng/ml), pyrrolidine dithiocarbamate (100 nM) was also incubated with neutrophils (shaded column). Then, May–Grünwald–Giemsa staining was evaluated as described in Materials and methods. Each value represents the mean ± S.D. of at least four separate experiments. Statistical significance was determined by the Mann–Whitney *U* test. [a] indicates significant difference from control (without TNF-α pretreatment) and [b] indicates significant difference from the case without pyrrolidine dithiocarbamate at $P < 0.05$.

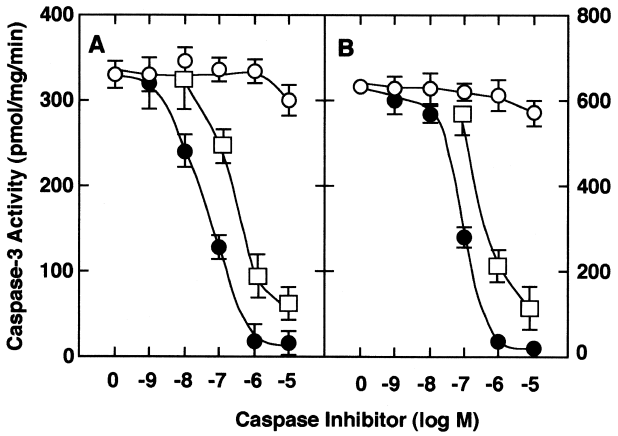

Fig. 7. Effects of caspase inhibitors on TNF-α (A) or TNF-α + cycloheximide (B) induced caspase-3 activation in human neutrophils. Neutrophils were preincubated with Ac-YVAD-CHO (○), Ac-DEVD-CHO (●) or IETD-FMK (□) at 37°C for 10 min, and further incubated with TNF-α (100 ng/ml) or TNF-α (100 ng/ml) + cycloheximide (1 μg/ml) for 3 h. Then, neutrophils were harvested and caspase-3 activity was determined as described in Materials and methods.

3.6. Effect of caspase inhibitors on TNF-α-induced caspase-3 activation

To determine whether activation of caspases contributes to the expression of apoptosis-inhibitory protein(s), the effect of selective inhibitors of caspase-1, -3 and -8 on the caspase-3 activation was evaluated. Ac-YVAD-CHO, a selective caspase-1 inhibitor, did not produce any effect on either TNF-α-induced or TNF-α + cycloheximide-induced caspase-3 activation (Fig. 7). Furthermore, neither TNF-α alone nor TNF-α + cycloheximide elicited caspase-1 activity in neutrophils (data not shown). Both the selective inhibitors against caspase-3 and -8, Ac-DEVD-CHO and IETD-FMK, respectively, inhibited the activation of caspase-3 induced by TNF-α alone and TNF-α + cycloheximide, in a dose-dependent manner (Fig. 7). Morphological evaluation indicated that Ac-YVAD-CHO did not alter the appearance of apoptotic neutrophils triggered by TNF-α + cycloheximide, whereas both Ac-DEVD-CHO and IETD-FMK strongly inhibited apoptosis.

4. Discussion

Most cells, whether normal or neoplastic, are generally resistant to TNF-α cytotoxicity unless co-treated with a protein or RNA synthesis inhibitor such as cycloheximide or actinomycin D (Baeuerle and Henkel, 1994). This phenomenon indicates the existence of some inhibitory protein(s), which act to save cells from TNF-α-induced apoptosis. To elucidate the existence of apoptosis-inhibitory proteins and their contribution to TNF-α-induced neutrophil apoptosis, we studied the effects on TNF-α-induced apoptosis of protein or RNA synthesis inhibitors, and an

inhibitor of NF-κB activation. Neutrophils were resistant to apoptosis after treatment with TNF-α alone, as determined by morphological assessment, although caspase-3 was activated at the same time. This resistance was highly diminished by the co-treatment of neutrophils with a protein or RNA synthesis inhibitor. Co-treatment with inhibitors of NF-κB also revealed apoptosis of neutrophils in response to TNF-α. These inhibitors also enhanced TNF-α-induced caspase-3 activation. In addition, TNF-α induced a rapid phosphorylation and degradation of IκB-α in neutrophils, and also increased NF-κB DNA binding activity, while preconditioning stimulation of neutrophils with TNF-α strongly suppressed the apoptotic changes induced by TNF-α + cycloheximide. This suppression was abolished by the further pretreatment with pyrrolidine dithiocarbamate. These results suggest that TNF-α synthesizes some protein(s) that may act as inhibitors of TNF-α-induced apoptosis through NF-κB activation. Furthermore, the sites of action of these apoptosis-protective protein(s) are both upstream and downstream of caspase-3.

Previous observations indicated that NF-κB activation requires the liberation of free NF-κB in the cytoplasm, after which NF-κB migrates into the nucleus and binds to the appropriate DNA region to promote transcription (Baeuerle and Henkel, 1994). One of the most important intracellular events for NF-κB activation is proteolytic cleavage of inhibitor proteins such as IκB-α, which allows the liberation of free NF-κB (Griscavage et al., 1996). Recent evidence indicates that phosphorylation of IκB-α at positions Ser^{32} and Ser^{36} triggers a rapid activation of NF-κB (Baldwin, 1996). Interestingly, IκB-α thus phosphorylated is reported to be rapidly degraded (Israel, 1995). Our results were consistent with this, indicating that the treatment of neutrophils with TNF-α led to the rapid disappearance of IκB-α within 10 min, followed by its reappearance after 30 min. Concomitantly, phosphorylated IκB-α was rapidly induced within 10 min, and thereafter rapidly degraded. Additionally, by using EMSA assay, we confirmed that TNF-α could stimulate NF-κB DNA binding activation and its inhibition by an NF-κB inhibitor, pyrrolidine dithiocarbamate. Recently, it has been reported that pyrrolidine dithiocarbamate is not a specific inhibitor of NF-κB, e.g., pyrrolidine dithiocarbamate can activate transcription factor AP-1 (Hartsfield et al., 1998). Moreover, in some systems, pyrrolidine dithiocarbamate does not inhibit NF-κB activation (Watanabe et al., 1999). However, our present results clearly indicate that pyrrolidine dithiocarbamate acts as an NF-κB inhibitor in neutrophils. Ward et al. (1999) also reported a similar result with pyrrolidine dithiocarbamate inhibiting NF-κB in neutrophils. Taken together, the data suggest that TNF-α induces NF-κB activation in human neutrophils.

Recently, it has been reported (William et al., 1998) that two different proinflammatory stimuli, lipopolysaccharide and granulocyte-macrophage-CSF (GM-CSF), upregulate the expression of both caspase-1 and interleukin-1-β in human neutrophils, and that the caspase-1-dependent cleavage of pro-interleukin-1-β results in a delayed expression of programmed cell death. Furthermore, this apoptotic delay by lipopolysaccharide and GM-CSF was blocked either by inhibiting NF-κB activation or by inhibiting protein synthesis (William et al., 1998). It indicates the possibility that interleukin-1-β is an inhibitory protein in programmed neutrophil death. In contrast to these findings, it is unlikely that interleukin-1-β is a candidate for the TNF-α-induced protein that inhibits TNF-α-dependent neutrophil apoptosis because the specific caspase-1 inhibitor, Ac-YVAD-CHO, did not inhibit caspase-3 activation induced by either TNF-α alone or combined with cycloheximide. The lack of caspase-1 activation by TNF-α or TNF-α + cycloheximide also supports the above speculation. Although it has been reported that sequential activation of caspase-1 and caspase-3 is essential for Fas-mediated apoptosis in mouse lymphoma cells (Enari et al., 1996), our results suggest that caspase-1 activation is independent of TNF-α-induced neutrophil apoptosis.

Recent observations strongly indicate that caspase-8 activation is involved in Fas or TNF-α-induced cell death in neutrophils (Niwa et al., 1999; Yamashita et al., 1999). TNF-α alone or TNF-α + cycloheximide-induced activation of caspase-3 as well as morphological apoptotic changes was inhibited by selective inhibitors of both caspase-3 and -8. These results indicate that the products of protein synthesis induced by TNF-α do not mediate caspase-3 and -8 activation.

It has been reported that TNF-α-induced apoptosis inhibitory proteins, such as TRAF-1, TRAF-2, c-IAP1 and c-IAP2, are synthesized through the activation of the NF-κB pathway, and that these newly synthesized proteins suppress caspase-8 activation (Wang et al., 1998). Although we have no direct evidence, these caspase-8 inhibitory proteins are candidates for the proteins induced by TNF-α receptor activation in neutrophils.

Intracellular signal transduction in neutrophils in response to a wide variety of stimuli appears to utilize the mitogen-activated protein (MAP) kinase cascade. Three distinct MAP kinases have been identified in mammalian cells: p42/p44 ERKs are activated by growth factors (Davis, 1994); JNK/SAPK is potently activated by irradiation and other environmental stress such as hypoosmolarity (Derijard et al., 1994); and p38 MAP kinase is activated by inflammatory cytokines, osmotic stress, and UV irradiation (Raingeaud et al., 1995). It is also suggested that the activation of MAP kinase(s) is involved in triggering apoptosis. Of note, Natoli et al. (1998) recently proposed that not only NF-κB, but also the JNK/SAPK system contributes to TNF-α-induced cytoprotection in other types of cells. However, our result indicates that the JNK/SAPK system is not involved in the inhibition of neutrophil apoptosis triggered by TNF-α. Mature neutrophils have the shortest life span of the various leukocytes and die

rapidly via apoptosis in vivo and in vitro. Recently, it has been reported that p38 MAP kinase is involved in spontaneous apoptosis in human neutrophils. From our present results, it is of interest to know whether NF-κB activation is involved in spontaneous apoptosis in human neutrophils. In our preliminary experiment, caspase-3 activity was increased after 24-h culture of neutrophils, and this caspase-3 activity was inhibited in the presence of pyrrolidine dithiocarbamate. These observation suggest that NF-κB activation is not involved in the prevention of spontaneous neutrophil apoptosis. Additional experiments to clarify the contribution of NF-κB to the spontaneous apoptosis in neutrophils are now in progress.

In conclusion, our results indicate somewhat intriguing roles of NF-κB in neutrophil survival that may form the basis for innovative therapeutic approaches against both inflammatory and proliferative diseases. Studies are in progress to further define the potential involvement of NF-κB activation and subsequent expression of apoptosis inhibitory proteins in human neutrophils.

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