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The loss of susceptibility to apoptosis in exudated tissue neutrophils is associated with their nuclear factor-κB activation

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Abstract

Tissue neutrophils, human salivary neutrophils donated from healthy subjects and synovial fluid neutrophils collected from patients with rheumatoid arthritis were compared with circulating blood neutrophils. Concomitant treatment of circulating blood neutrophils with tumor necrosis factor-α (TNF-α) and cycloheximide induced neutrophil apoptosis, whereas the same treatment failed to induce significant apoptosis in salivary and synovial fluid neutrophils. Caspase-3 activation by TNF-α was observed in these tissue neutrophils, although its activity was significantly weaker than that in circulating blood neutrophils. In circulating blood neutrophils, TNF-α induced activation of nuclear factor-κB (NF-κB), whereas, in tissue neutrophils, NF-κB had been already activated without any stimulation, and no further activation was induced by the treatment with TNF-α. Furthermore, while pretreatment of neutrophils with an NF-κB inhibitor produced typical apoptotic changes in circulating blood neutrophils, this inhibitor did not produce any morphological apoptotic changes induced by TNF-α in tissue neutrophils. These results indicate that neutrophils undergo marked functional changes such as altered sensitivity to apoptosis-inducing stimuli in association with their exudation from blood into tissue, and that NF-κB activation is involved in the acquisition of resistance to TNF-α-induced apoptosis.

Keywords: Neutrophil, circulating blood; Neutrophil, tissue; Apoptosis; Transcription factor

1. Introduction

Tissue neutrophils, which have migrated from circulating blood, usually play crucial roles in host defense mechanisms, such as phagocytosis of bacteria and/or production of superoxide and various cytokines. It has been reported that salivary neutrophils, which are tissue neutrophils with a higher viability, exhibit a variety of functional responses when stimulated, such as production of active oxygen species for a longer period of time than circulating blood neutrophils (Yamamoto et al., 1991). We previously reported that the reactivity of salivary neutrophils to cyclic AMP and cyclic AMP-elevating agents is decreased in the presence of a chemotactic factor, resulting in a relatively higher production of superoxide (Al-Essa et al., 1995; Kanamori et al., 1997). We made similar observations in the rabbit when comparing peritoneal neutrophils and circulating blood neutrophils (Al-Essa et al., 1995; Kanamori et al., 1997). Thus, tissue neutrophils, such as salivary neutrophils, are likely to play important roles in various pathological states. If these cells are eliminated from sites of injury/infection too early by apoptotic processes, this could attenuate self-defense mechanisms and could lead to pathological sequelae. Recently, we and others have reported that

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tissue neutrophils are resistant to tumor necrosis factor-α (TNF-α)-induced apoptosis in both rabbits (Tsuchida et al., 1995) and humans (Niwa et al., 1997). A hypothesis is that these apoptosis-resistant tissue neutrophils are beneficial for the host defense. However, it is not known which mechanisms underlie this attenuation of susceptibility to apoptotic stimuli in neutrophils.

Several endogenous proteins are involved in apoptosis regulation, for example, the nuclear factor-κB (NF-κB) pathway produces antiapoptotic proteins. Recently, it has been reported that TNF-α-induced neutrophil apoptosis may be modulated by the activation of the NF-κB pathway via the production of antiapoptotic proteins (Ward et al., 1999; Niwa et al., 2000). In this report, we demonstrate that not only salivary neutrophils from healthy donors but also synovial fluid neutrophils from rheumatoid arthritis patients show resistance to TNF-α-induced apoptosis. Furthermore, we examined the role of activation of the NF-κB pathway in both tissue and circulating blood neutrophils from humans as a potential mechanism, which may underlie the relative susceptibility to TNF-α-induced apoptosis.

2. Materials and methods

2.1. Materials

Cycloheximide, actinomycin D, phorbol 12-myristate 13-acetate (PMA) and histopaque were purchased from Sigma (MO, USA). *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Calbiochem (CA, USA). Hoechst 33258 and pyrrolidine derivative of dithiocarbamate (PDTC) were obtained from Amersham Japan and Wako (Osaka, Japan), respectively. SN50 and SN50-M were purchased from BioMol (USA). HEPES was purchased from Dojin (Kumamoto, Japan). 2-Methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo [1,2-α]pyrazin-3-one (MCLA) was purchased from Tokyo Kasei (Tokyo, Japan). Recombinant human TNF-α was a gift from Dainippon Pharmaceutical (Osaka, Japan).

2.2. Preparation of neutrophils

Human circulating blood neutrophils from healthy donors were isolated by a direct purification method by using Mono–Poly resolving medium (Dainippon Pharmaceutical) (Boyum, 1968; Ting and Morris, 1971). Purified circulating blood neutrophils were resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 300 mg/ml L-glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin (RPMI 1640 medium).

Human salivary neutrophils were prepared by nylon mesh filtration followed by Histopaque centrifugation (Al-Essa et al., 1994). Briefly, intensive mouth washings with saline were collected from each healthy donor, filtered through a nylon mesh into 50-ml centrifuge tubes, and centrifuged at $250 \times g$ for 10 min. The pellets obtained were suspended in Hank's Balanced Salt Solution containing 10 mM HEPES, pH 7.4 (HBSS). The suspension was cushioned carefully on Histopaque ($d = 1.083$) and centrifuged at $420 \times g$ for 30 min at 20 °C. Pure salivary neutrophils were collected from the interface between Histopaque and HBSS, washed and resuspended in RPMI 1640 medium.

Human synovial fluid neutrophils were isolated from synovial fluid freshly aspirated from the knee joints of five rheumatoid arthritis patients aged 52–72 (mean: 61) years. All patients met the American Rheumatology Association 1987 revised criteria for the classification of rheumatoid arthritis (Arnett et al., 1988). All were being treated with various doses of non-steroidal anti-inflammatory drugs. Three patients also received disease-modifying anti-rheumatic drugs and one received low-dose oral steroids. All human experiments were performed in accordance with protocols approved by the Human Subjects Research Committee at our Institution, and informed consent was obtained from all patients and volunteers.

To avoid neutrophil clumping, synovial fluid was aspirated by using a plastic container containing EGTA (1 mM as final concentration), to which the same volume of ice-cold saline was added. After centrifugation of synovial fluid at $250 \times g$ for 10 min, synovial cells were washed in HBSS, and neutrophils were isolated using Histopaque ($d = 1.077$). Purified synovial fluid neutrophils were collected, washed and resuspended in RPMI 1640 medium.

The purity of each neutrophil type was greater than 95%. Cell number was counted by using a Coulter counter model Z1 (Coulter, England). Cells were diluted in RPMI 1640 medium to the final required concentrations and kept on ice until examined. The viability of the neutrophils used was more than 95% evaluated by Trypan blue exclusion test.

2.3. Evaluation of apoptosis

For morphological assessment, neutrophils were suspended at 2×10^6/ml in RPMI 1640 medium, and then incubated with TNF-α at 37 °C for up to 4 h. Neutrophils incubated under specific conditions were spun down onto glass slides in a cytocentrifuzer (CF-12SB, Sakurai-Seiki, Japan), dried in cool air, and stained with May-Grünwald-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.

DNA fragmentation of neutrophils was analyzed by using agarose gel electrophoresis. Neutrophils (2×10^6) were harvested and incubated in 100 μl of 10 mM Tris–

HCl, pH 7.4, containing 10 mM EDTA and 0.5% Triton X-100 for 10 min at 4 °C, and then centrifuged at 22,000 × *g* for 20 min. The supernatant was collected and incubated with 2 μl of 20 mg/ml ribonuclease-A at 37 °C for 1 h. Two microliters of 20 mg/ml proteinase-K was then added and the incubation was continued for an additional 1 h. After incubation, the mixture was kept at −20 °C overnight with 120 μl of isopropyl alcohol and 20 μl of 5 M NaCl. Then the mixture was centrifuged at 22,000 × *g* for 15 min, the supernatant was discarded and the pellet was dissolved in 15 μl of 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 0.25% bromophenol blue and 40% sucrose. Samples were loaded into each well of the 2% agarose gels, and electrophoresis was carried out at 100 V for 1 h. The DNA in gels was visualized under ultraviolet light after staining with ethidium bromide (Niwa et al., 1999).

2.4. Measurement of caspase-3 activity

Neutrophils were harvested after being exposed to TNF-α plus cycloheximide for up to 4 h and were resuspended in 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 × *g* for 20 min at 4 °C) of the cell lysates, the supernatant was used to measure caspase activity. The 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 7-amino-4-methylcoumarin (AMC) cleaved from Ac-DEVD-AMC, measured by using spectrofluorometer (Fluoroskan, Dainippon Pharmaceutical).

2.5. Superoxide measurement

The superoxide-releasing activity of neutrophils was evaluated by a chemiluminescence development technique using a specific superoxide probe, MCLA (Niwa et al., 1996; Nakano et al., 1986; Suzuki et al., 1991). Aliquots of neutrophil preparation (2×10^5 cells) and MCLA (2 μM) were incubated at 37 °C for 10 min before the addition of fMLP or PMA. The development of chemiluminescence was continuously monitored with a six-channel photon counter (Biolumat LB 9505, Berthold).

2.6. Western blotting

Whole cell extract was diluted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. After SDS-PAGE at 20 mV for 90 min, the gels were transferred to 0.2-μm polyvinylidene difluoride (PVDF) membranes (BioRad, USA) at 10 V for 30 min. Blots were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) overnight at 4 °C. The next day, blots were rinsed three times with TBST for 10 min. Blots were then incubated with primary antibodies against IκB-α and phospho-IκB-α (Cell Signaling Technology, MA, USA). Antibodies were added at a dilution of 1:1000 in TBST containing 5% bovine serum albumin (fraction V) for an overnight incubation at 4 °C. The blots were then washed with TBST and incubated for 1 h with the secondary antibody, goat anti-rabbit immunoglobulin G-peroxidase (Chemicon International, CA, USA) at a dilution of 1:1000 in 5% skimmed milk in TBST. Subsequently, blots were washed three times with TBST. Then blots were developed for 4 min with ECL reagents (Amersham Pharmacia Biotec, Tokyo, Japan). The density of each band was quantified with an image analyzer using NIH image 1.60 software on a Macintosh computer system.

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 5×0^6 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 levamisole) 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 × *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 pelletted nuclei were then resuspended in 50 μl of hypotonic 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 levamisole) and stored at −80 °C until used. Two micrograms of nuclear extracts, as determined by bicinconinic acid (BCA) protein assay, was 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), Amersham Pharmacia Biotec) with γ-^{32}P-labeled double-stranded oligonucleotide containing the decameric κB-binding site, using standard protocols for T4 kinase, at 4 °C for 30 min. Samples were loaded onto an 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. Superoxide generation in circulating blood and tissue neutrophils

To evaluate the activity of the neutrophils used, fMLP- and PMA-stimulated superoxide generation was measured. FMLP-stimulated superoxide generation in tissue neutrophils, i.e. human salivary neutrophils from healthy donors and synovial fluid neutrophils from rheumatoid arthritis patients, was significantly higher than that in circulating blood neutrophils from healthy donors and rheumatoid arthritis patients (Fig. 1A). The magnitude of superoxide generation produced by PMA stimulation did not differ between the four types of neutrophils evaluated (Fig. 1B).

3.2. TNF-α-induced apoptosis and caspase-3 activation in circulating blood and tissue neutrophils

We determined apoptosis by morphologic evaluation of freshly isolated circulating blood neutrophils and tissue neutrophils (from saliva and synovial fluid) from healthy donors and from rheumatoid arthritis patients, respectively. Treatment of human circulating blood neutrophils and salivary neutrophils with 100 ng/ml TNF-α resulted in less than 5% neutrophils showing typical apoptotic phenomena up to 4 h (data not shown). However, when circulating blood neutrophils were pretreated with 1 μg/ml cycloheximide, TNF-α treatment produced typical apoptotic cells, which showed a diminution in cell volume and nuclear pyknosis in a time- and concentration-dependent manner, reaching a maximum of 75% of cells exhibiting apoptosis (Figs. 2A and 3A). In contrast, the same cycloheximide/TNF-α concomitant treatment did not produce any morphological changes in salivary neutrophils (Figs. 2A and 3A).

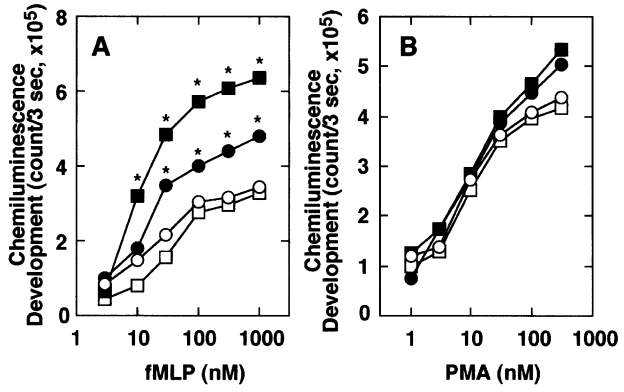

Fig. 1. Superoxide generation in circulating blood neutrophils (○) and salivary neutrophils (●) from healthy donors, and circulating blood neutrophils (□) and synovial fluid neutrophils (■) from rheumatoid arthritis patients. Neutrophils were stimulated with fMLP (A) or PMA (B), and production of superoxide was determined as described in Materials and methods. The results are expressed as the means of three to four independent experiments in duplicate. * indicates significant difference from control.

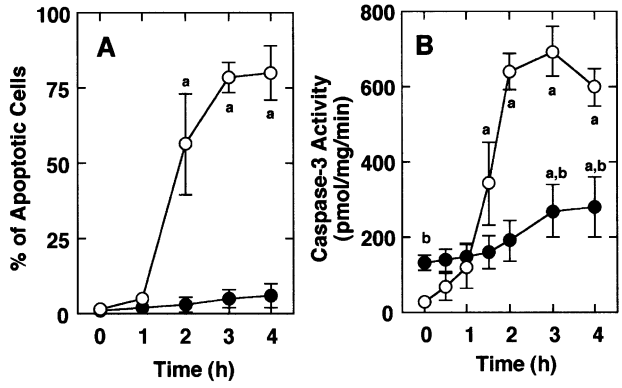

Fig. 2. Time course of apoptotic morphological changes (A) and caspase-3 activity (B) in neutrophils, induced by concomitant treatment with TNF-α and cycloheximide. (A) Circulating blood neutrophils (○) and salivary neutrophils (●) from healthy donors were incubated with TNF-α (100 ng/ml) and cycloheximide (1 μg/ml) at 37 °C for the indicated times (h). 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. The results are expressed as the means ± S.D. of three to four independent experiments in duplicate. Statistical significance ($P < 0.05$) of differences in the number of apoptotic cells and in caspase-3 activity was determined by the Mann–Whitney *U*-test and analysis of variance (ANOVA) with Fisher's Protected Least-Significant Difference (Fisher's PLSD) test, respectively. [a] Indicates significant difference from control (time zero). [b] Indicates significant difference from values for circulating blood neutrophils.

TNF-α plus cycloheximide treatment produced similar morphological changes in circulating blood neutrophils from rheumatoid arthritis patients: apoptotic neutrophils were observed in a concentration-dependent manner, while synovial fluid neutrophils exhibited only minor morphological changes as compared with those of circulating blood neutrophils from rheumatoid arthritis patients (Fig. 4A). Cycloheximide (1 μg/ml) alone elicited almost no apoptotic morphological changes in these neutrophils, circulating blood neutrophils, salivary neutrophils or synovial fluid neutrophils (data not shown).

Since several previous reports indicated that caspase-3 activation was involved in neutrophil apoptosis as a final step of the caspase cascade, we compared caspase-3 activity in circulating blood neutrophils and salivary neutrophils from healthy donors after TNF-α plus cycloheximide treatment. In circulating blood neutrophils, TNF-α, in the presence of 1 μg/ml cycloheximide, significantly activated caspase-3 in a time- and TNF-α concentration-dependent manner (Figs. 2B and 3B). In salivary neutrophils, TNF-α

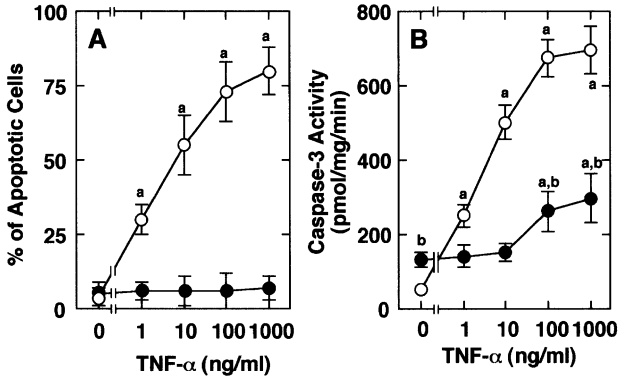

Fig. 3. Concentration dependency of TNF-α-induced apoptosis (A) and caspase-3 activation (B) in human neutrophils. (A) Circulating blood neutrophils (○) and salivary neutrophils (●) from healthy donors were incubated with the various concentration of TNF-α indicated at 37 °C for 3 h in the presence of cycloheximide (1 μg/ml). 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. The results are expressed as the means ± S.D. of three to four independent experiments in duplicate. Statistical significance ($P<0.05$) of differences in the number of apoptotic cells and in caspase-3 activity was determined by the Mann–Whitney *U*-test and ANOVA with Fisher's PLSD test, respectively. [a] Indicates a significant difference from control (time zero). [b] Indicates significant difference from values for circulating blood neutrophils at the same concentration of TNF-α.

also induced caspase-3 activation in the presence of cycloheximide but the magnitude of the activation was significantly less than that in circulating blood neutrophils (Figs. 2B and 3B).

Similar results were obtained with neutrophils from rheumatoid arthritis patients: in circulating blood neutrophils, caspase-3 was activated in a TNF-α concentration-dependent manner, while in synovial fluid neutrophils, TNF-α plus cycloheximide produced a significantly smaller caspase-3 activation compared to that in circulating blood neutrophils (Fig. 4B).

Similar to the effects of cycloheximide, the RNA synthesis inhibitor, actinomycin D, also enhanced TNF-α-induced apoptosis in circulating blood neutrophils, but not in salivary neutrophils (Table 1).

To confirm the appearance of nuclear chromatin condensation in apoptotic neutrophils, Hoechst 33258 staining was performed. In intact circulating blood neutrophils, almost no nuclear condensation was observed (Fig. 5A), but after TNF-α plus cycloheximide treatment, nuclear chromatin condensation was observed (Fig. 5B). In contrast, few apoptotic cells were observed in salivary neutrophils after TNF-α plus cycloheximide treatment (Fig. 5C).

The viability of both control neutrophils and neutrophils after treatment with TNF-α and/or cycloheximide was >95%. Almost no necrotic cells were observed in neutrophils after TNF-α plus cycloheximide treatment.

3.3. TNF-α-triggered DNA fragmentation in circulating blood and tissue neutrophils

To determine whether the above-mentioned morphological changes in neutrophils were accompanied by DNA fragmentation, which is regarded as another criterion of apoptosis, DNA was extracted from both types of neutrophils, circulating blood neutrophils and synovial fluid neutrophils, from the same rheumatoid arthritis patient 2 h after the incubation of the cells with reagents and was electrophoresed on agarose gels (Fig. 6). In synovial fluid neutrophils, the electrophoretic pattern of DNA after either cycloheximide and/or TNF-α stimulation showed little fragmentation (Fig. 6, lane 1–5). In contrast, in circulating blood neutrophils, marked DNA fragmentation was observed after concomitant treatment with both reagents (Fig. 6, lane 8 and 9).

3.4. NF-κB activity in circulating blood and tissue neutrophils

The results described above strongly suggest that, in circulating blood neutrophils, TNF-α not only activates the caspase cascade to induce apoptosis of neutrophils, but also enhances protein synthesis. They also indicate that these proteins act to inhibit TNF-α-triggered neutrophil

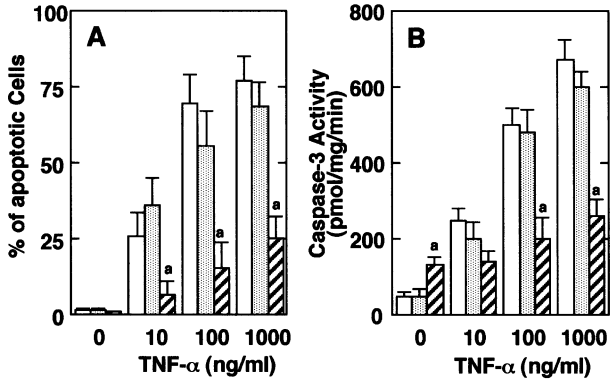

Fig. 4. Concentration dependency of TNF-α-induced morphological apoptosis (A) and caspase-3 activation (B) in human neutrophils. (A) Circulating blood neutrophils from healthy donors (open column) and rheumatoid arthritis patients (dotted column), and synovial fluid neutrophils from rheumatoid arthritis patients (hatched column) were incubated with the various concentrations of TNF-α indicated at 37 °C for 3 h in the presence of cycloheximide (1 μg/ml). 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. The results are expressed as the means ± S.D. of four to five independent experiments in duplicate. Statistical significance ($P<0.05$) of differences in the number of apoptotic cells and in caspase-3 activity was determined by the Mann–Whitney *U*-test and ANOVA with Fisher's PLSD test, respectively. [a] Indicates significant difference from values for circulating blood neutrophils at the same concentration of TNF-α.

Table 1
Effect of actinomycin D and cycloheximide on TNF-α-induced apoptosis in circulating blood neutrophils and salivary neutrophils

Reagent (μg/ml)		TNF-α (100 ng/ml)	Apoptotic cells (%)	
			Circulating blood	Salivary
–		–	2.3 ± 1.5	3.5 ± 2.6
–		+	4.5 ± 2.5	4.1 ± 2.5
Actinomycin D	1	+	39.3 ± 9.1[a]	4.3 ± 3.1
	10	+	81.3 ± 12.4[a]	4.5 ± 3.8
Cycloheximide	1	+	77.5 ± 13.8[a]	5.2 ± 3.2

The values represent the means ± S.D. of four separate experiments. Statistical significance ($P < 0.05$) of differences in apoptosis (based on cell morphology) was determined by the Mann–Whitney *U*-test. Reagent: – and TNF-α: +.

[a] Indicates a significant difference from TNF-α-treated control neutrophils.

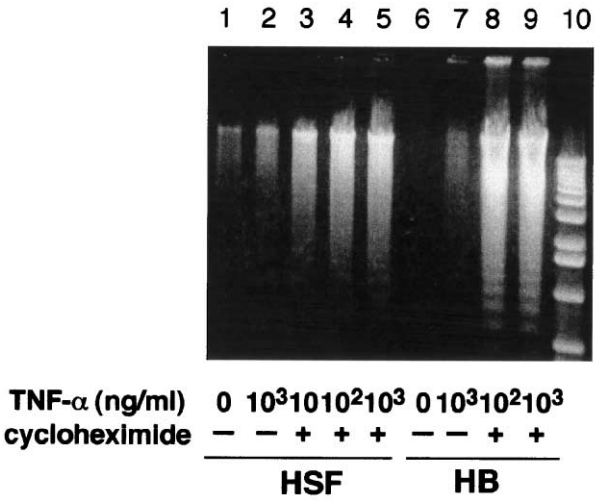

Fig. 6. DNA fragmentation induced by the treatment with TNF-α plus cycloheximide of circulating blood neutrophils (HB) and synovial fluid neutrophils (HSF) from rheumatoid arthritis patients. Neutrophils were incubated with cycloheximide (1 μg/ml) and/or TNF-α as indicated for 2 h. DNA extracted from synovial fluid neutrophils (lane 1–5) and circulating blood neutrophils (lane 6–9) from the same rheumatoid arthritis patient were electrophoresed on 2% agarose gels. Lane 10: DNA marker.

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. Our recent report indicates that the possible targets of these proteins are upstream and also downstream of caspase-3 to inhibit apoptosis in circulating blood neutrophils (Niwa et al., 2000). It is likely that the NF-κB pathway is activated in tissue neutrophils, which are resistant to TNF-α-induced apoptosis. Therefore, we compared NF-κB activity in circulating blood and tissue neutrophils. Fig. 7C and F shows that in the whole extracts of circulating blood neutrophils stimulated with 100 ng/ml TNF-α, IκB-α levels were substantially and significantly decreased after 5 min. A trace of residual IκB-α protein was detected following stimulation, and by 60-min levels partly recovered to prestimulation values. Phosphorylated IκB-α was not detected prior to TNF-α stimulation but was detected 5 min after TNF-α stimulation. A residual amount of phosphorylated IκB-α protein was detected at 10, 30 and 60 min after TNF-α stimulation (Fig. 7C and F). Thus, the transient appearance of phosphorylated IκB-α in association with the reciprocal disappearance of IκB-α indicates that NF-κB activation was induced in circulating blood neutrophils by TNF-α stimulation. Additionally, cycloheximide by itself did not affect the amount of either IκB-α or phosphorylated IκB-α (data not shown).

In contrast, phosphorylated IκB-α was already detected without TNF-α stimulation in human salivary neutrophils. Furthermore, TNF-α stimulation failed to change the amount of phosphorylated IκB-α protein (Fig. 7A and D). Also, the amount of IκB-α protein was not affected by TNF-α stimulation in salivary neutrophils (Fig. 7A and D). Similar results were observed in synovial fluid neutrophils

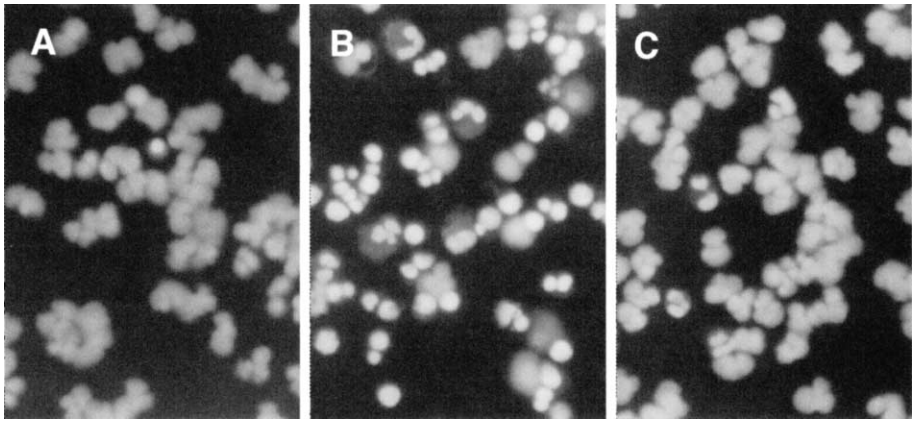

Fig. 5. Photomicrographs of human neutrophils, demonstrating the nuclear condensation of apoptosis. Circulating blood neutrophils (A and B) and salivary neutrophils (C) were incubated with (B and C) or without (A) TNF-α (100 ng/ml) and cycloheximide (1 μg/ml) for 3 h, and then cytospin preparation of neutrophils were stained with Hoechst 33258.

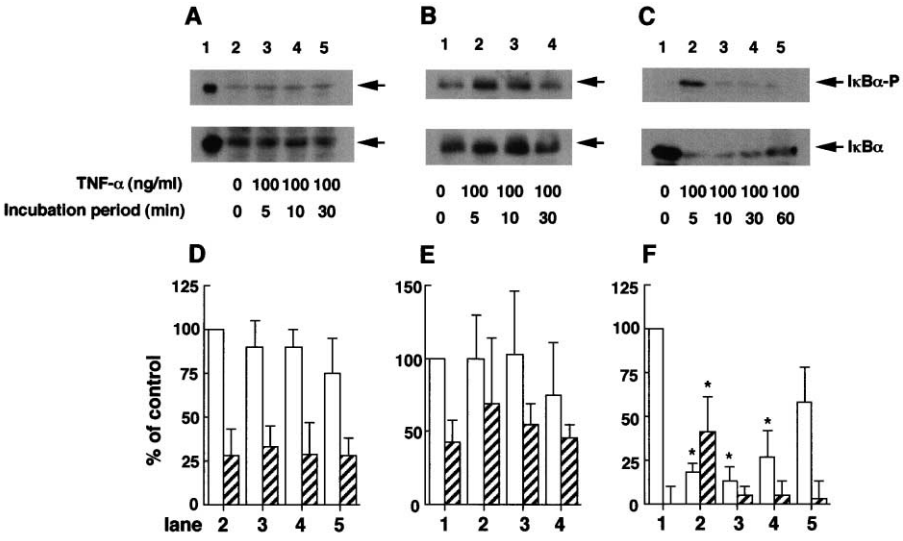

Fig. 7. Time course of TNF-α-induced phosphorylation and degradation of IκB-α in human neutrophils. Salivary neutrophils, synovial fluid neutrophils and circulating blood neutrophils were treated with TNF-α (100 ng/ml), harvested at the times indicated, and then equal amounts of extracts were separated by 10% SDS-PAGE. After transfer to PVDF membranes, the blots were probed with phosphorylated IκB-α-and IκB-α-specific antisera, as described in Materials and methods. Similar experiments were repeated three times and representative results are shown ((A) salivary neutrophils, (B) synovial fluid neutrophils and (C) circulating blood neutrophils). The relative intensity of each band was quantitated and standardized with that of the control value of IκB-α for neutrophils ((D), (E) and (F) in salivary, synovial fluid and circulating blood neutrophils, respectively), using an image analyzer. The results in (D), (E) and (F) are expressed as means ± S.D. of three independent experiments. Statistical significance ($P < 0.05$) of differences was determined by the ANOVA with Fisher's PLSD test. * indicates significant difference from each control (time zero). IκB-α-p: phosphorylated IκB-α. In (A), lane 1: positive control for phosphorylated IκB-α and IκB-α. Open column and hatched column in (D), (E) and (F) indicates relative amounts of IκB-α and phosphorylated IκB-α, respectively.

from rheumatoid arthritis patients (Fig. 7B and E). Specifically, phosphorylated IκB-α was detected without TNF-α stimulation in human synovial fluid neutrophils, and TNF-α did not significantly increase phosphorylated IκB-α levels, although there was a trend for an increased level of phosphorylated IκB-α, with no decrease in the level of IκB-α protein (Fig. 7E). This trend of an elevation of phosphorylated IκB-α in synovial fluid neutrophils might explain our observations of weak morphological signs of apoptosis after TNF-α and cycloheximide stimulation of human salivary neutrophils.

To confirm the NF-κB activity in circulating blood and tissue neutrophils induced by TNF-α, we also performed EMSAs for the detection of NF-κB DNA binding activity. As shown in Fig. 8A, TNF-α (100 ng/ml) induced NF-κB activation in circulating blood neutrophils. Furthermore,

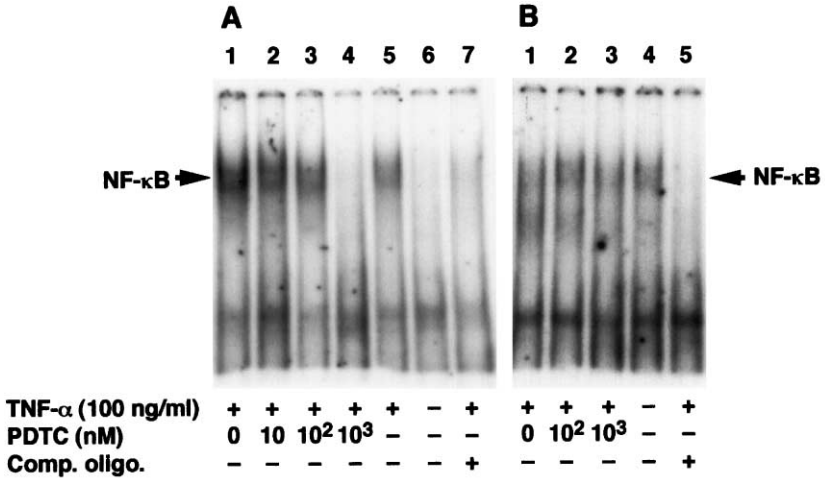

Fig. 8. TNF-α-induced activation of NF-κB DNA binding in circulating blood neutrophils (A) and salivary neutrophils (B). Neutrophils were stimulated with TNF-α for 60 min. Nuclear extracts were prepared and equal amounts of protein were analyzed for κB-specific DNA binding in EMSA using a ^{32}P-labeled DNA probe (see Materials and methods). Comp. Oligo.: competitive cold oligopeptide.

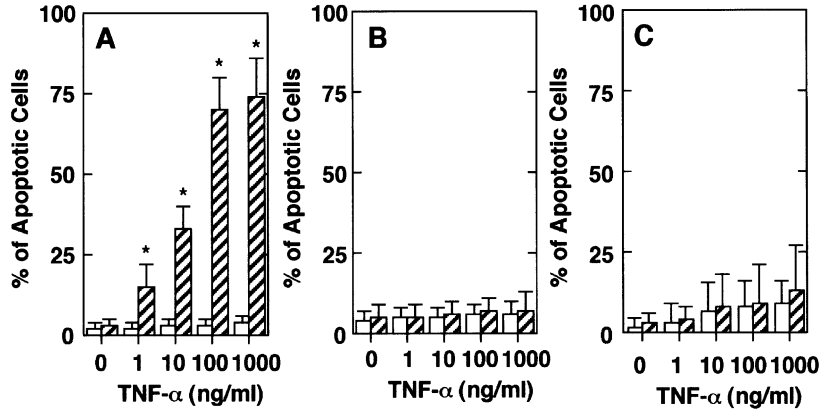

Fig. 9. Effect of an NF-κB inhibitor on TNF-α-induced apoptotic morphological changes in circulating blood neutrophils (A), salivary neutrophils (B) and synovial fluid neutrophils (C). Neutrophils were incubated with (hatched column) or without (open column) PDTC (100 nM) at 37 °C for 2 h and then TNF-α (100 ng/ml)-induced apoptosis was evaluated with May-Grünwald-Giemsa staining as described in Materials and methods. Each value represents the mean ± S.D. of three to four separate experiments. * indicates significant difference from control at $P<0.05$ by the Mann–Whitney *U*-test.

following pretreatment of neutrophils with pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB activation, for 2 h, TNF-α (100 ng/ml)-induced NF-κB DNA binding was inhibited. In salivary neutrophils (tissue neutrophils), NF-κB DNA binding activity was detected before TNF-α stimulation (Fig. 8B). Furthermore, neither treatment with TNF-α nor concomitant treatment with TNF-α plus PDTC affected NF-κB DNA binding activity (Fig. 8B). The specificity of NF-κB DNA binding activity was confirmed by the addition of excess cold oligopeptide.

3.5. Effect of NF-κB inhibitor on TNF-α-induced apoptosis in circulating blood and tissue neutrophils

The results described above indicated that NF-κB system was already activated in tissue neutrophils during their exudation from circulating blood. Next, we determined whether inhibition of NF-κB affected the neutrophil apoptosis induced by TNF-α stimulation. After pretreatment of circulating blood neutrophils with 100 nM PDTC for 2 h, TNF-α induced significant apoptosis (Fig. 9A). In contrast, in tissue neutrophils, salivary neutrophils and synovial fluid neutrophils, PDTC pretreatment did not significantly enhance TNF-α-induced apoptosis (Fig. 9B and C). To confirm the effect of PDTC on circulating blood neutrophils, we also used another 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 p50 subunit of NF-κB (Lin et al., 1995). After pretreatment of circulating blood neutrophils with SN50 (100 μg/ml) for 15 min, TNF-α induced significant apoptosis in human circulating blood neutrophils, while an inactive control peptide of SN50, SN50-M (100 μg/ml), which is mutated within the nuclear localization sequence motif, did not affect TNF-α-induced apoptosis (Fig. 10). In contrast, in tissue neutrophils, salivary neutrophils and synovial fluid neutrophils, SN50 pretreatment did not significantly enhance TNF-α-induced apoptosis (data not shown).

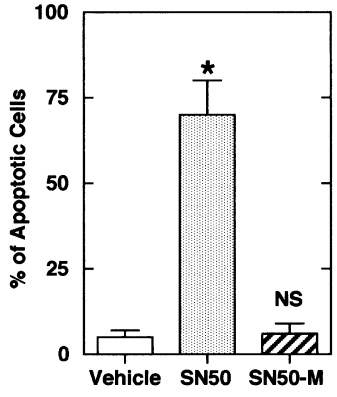

Fig. 10. Effect of an NF-κB inhibitor, SN50, on TNF-α-induced apoptotic morphological changes in circulating blood neutrophils and salivary neutrophils. Neutrophils were incubated with vehicle (open column), 100 μg/ml SN50 (dotted column) or 100 μg/ml SN50-M (hatched column) at 37 °C for 15 min and then TNF-α (100 ng/ml)-induced apoptosis was evaluated with May-Grünwald-Giemsa staining as described in Materials and methods. Each value represents the mean ± S.D. of three separate experiments. * indicates significant difference from control at $P<0.05$ by the Mann–Whitney *U*-test.

4. Discussion

In this study, we demonstrated that human tissue neutrophils, salivary neutrophils and synovial fluid neutrophils, exudated from circulating blood, acquired resistance to TNF-α-induced apoptosis, in contrast to the marked apoptosis seen in human circulating blood neutrophils induced by TNF-α. These findings strongly suggest that circulating blood neutrophils are transformed to become more resistant to apoptotic stimuli when exudated from blood into inflammatory sites. Furthermore, our data in this report also indicated that the activated NF-κB pathway is involved in the mechanism of this resistance. Similar results, namely the

lack of susceptibility to neutrophil apoptosis, were reported by Tsuchida et al. (1995), who compared TNF-α-induced apoptosis in rat peritoneal neutrophils (tissue neutrophils) and circulating blood neutrophils. The study did not evaluate which mechanisms mediate these effects. To our knowledge, our report is the first to attempt to clarify the mechanisms underlying the resistance of tissue neutrophils to TNF-α stimuli, and to describe that the heterogeneity of susceptibility to apoptotic induction in neutrophils depends on their location and may be generalized irrespective of species.

Tissue neutrophils, which have migrated from circulating blood, usually play a crucial role in host defense mechanisms, such as phagocytosis of bacteria and/or production of superoxide and various cytokines. It has been reported that salivary neutrophils, which have a higher viability, exhibit a variety of functional responses when stimulated, such as the production of active oxygen species for a longer period than circulating blood neutrophils (Yamamoto et al., 1991). Furthermore, we reported (Al-Essa et al., 1995; Kanamori et al., 1997) that the reactivity of salivary neutrophils to cyclic AMP and cyclic AMP-elevating agents is decreased, resulting in a relatively higher production of superoxide in the presence of, for example, a chemotactic factor. In this report, we found that not only salivary neutrophils from healthy donors but also synovial fluid neutrophils from rheumatoid arthritis patients showed a greater ability to produce superoxide than did circulating blood neutrophils (from normal and rheumatoid arthritis subjects) stimulated with a chemotactic factor, but not with a protein kinase C activator. Interestingly, fMLP-induced superoxide generation in circulating blood neutrophils from rheumatoid arthritis patients was the same as that seen in neutrophils from healthy donors. Thus, tissue-derived neutrophils, salivary neutrophils and synovial fluid neutrophils are likely to play important roles in protecting oral mucosa and joint cavities in pathological states. Without this mechanism, these cells would be eliminated from the reactive site too early by apoptotic processes, which would attenuate the self-defense mechanisms, leading to unfavorable results. Therefore, this attenuated susceptibility to apoptotic stimuli of tissue neutrophils, salivary neutrophils and synovial fluid neutrophils could be beneficial for the host defense. However, a contrasting hypothesis is that these activated neutrophils are responsible for tissue damage.

Recently, TNF-α has been shown to induce neutrophil apoptosis (Takeda et al., 1993), which is accelerated by concomitant treatment with cycloheximide. Our previous report (Niwa et al., 2000) also indicated that neutrophils were resistant to TNF-α-induced apoptosis, although caspase-3 was activated. This resistance was markedly diminished by co-treatment of neutrophils with a protein or RNA synthesis inhibitor. Additionally, the presence of inhibitors of NF-κB accelerated the apoptosis of neutrophils in response to TNF-α (Niwa et al., 2000). These results, taken together, strongly suggest that TNF-α-induced apoptosis of neutrophils may be inhibited by the de novo synthesis of protein(s), and that the NF-κB system plays a key role in modulating apoptotic signals in neutrophils (Niwa et al., 2000; McDonald et al., 1997). In this study, we found that the NF-κB signaling system was constitutively active in tissue neutrophils (salivary neutrophils and synovial fluid neutrophils), while NF-κB activation was evoked in circulating blood neutrophils following TNF-α stimulation. Thus, we speculate that apoptosis-inhibiting protein(s) are synthesized through an activated NF-κB signaling system in exudated neutrophils, salivary neutrophils and synovial fluid neutrophils, thereby conferring resistance to TNF-α-induced apoptosis. Under these conditions, treatment with inhibitors of protein synthesis or of the NF-κB pathway can not enhance TNF-α-induced apoptosis in these neutrophils. Although the exact mechanism of the activation of the NF-κB pathway in tissue neutrophils is unknown at present, it might occur during their exudation from circulating blood. Because significant changes in the cell signaling system of tissue neutrophils, such as caspase-3 activity (Nakahara et al., 1998), prostanoid response (Kanamori et al., 1997), response to cyclic AMP and related agents (Al-Essa et al., 1995), Bcl-2 concentration (Nakahara et al., 1998), cytokine receptor affinity (Niwa et al., 1996) and active oxygen production (Yamamoto et al., 1991; Nakahara et al., 1998; Ueta et al., 1993) in salivary neutrophils, and macrophage inflammatory protein 1 alpha in synovial fluid neutrophils (Hatano et al., 1999), have been reported, it is not unreasonable to hypothesize that the NF-κB system is also modified in tissue neutrophils, salivary neutrophils or synovial fluid neutrophils. Furthermore, it is also not clear why the NF-κB inhibitor failed to restore the cell sensitivity to TNF-α in tissue neutrophils in our experiment. We hypothesize that the NF-κB pathway is irreversibly activated during neutrophil exudation from circulating blood, and that apoptosis-inhibiting protein(s) are continuously produced even after treatment with an NF-κB inhibitor.

Nakahara et al. (1998) reported that spontaneous caspase-3, but not caspase-1, activity was higher in salivary neutrophils than in circulating blood neutrophils. In the present study, we also showed that spontaneous caspase-3 activity in salivary neutrophils was significantly higher than that in circulating blood neutrophils. Furthermore, concomitant treatment of tissue neutrophils with TNF-α and cycloheximide resulted in a significantly lower caspase-3 activity than that in circulating blood neutrophils.

Recently, we have reported that TNF-α stimulates the synthesis of protein(s) that may act as inhibitors of TNF-α-induced apoptosis by activating NF-κB. The site of action of these apoptosis-protective protein(s) appears to be both upstream and downstream of caspase-3 (Niwa et al., 2000). This evidence strongly suggests that the caspase cascade is activated by TNF-α, and that caspase-3 activation can block the morphological appearance of apoptosis in circulating blood neutrophils, since apoptosis-inhibiting protein(s) are produced by caspase-3. In tissue neutrophils,

caspase-3 is spontaneously activated, as mentioned above, resulting in reduced morphological evidence of apoptosis, due to the constitutive synthesis of apoptosis-inhibiting protein(s) produced through the NF-κB system.

Since the target of apoptosis-inhibiting proteins synthesized through NF-κB activation triggered by TNF-α may be upstream and downstream of caspase-3 (Niwa et al., 2000), it is possible that caspase-3 activity induced by TNF-α in the presence of cycloheximide is lower in tissue neutrophils than in circulating blood neutrophils. Accumulating evidence implicates the activation of the caspase cascade as an essential process in apoptosis-mediated cell death in several cell types, including neutrophils (Niwa et al., 1999; Yamashita et al., 1999). Within the caspase cascade, caspase-3 activation is the final enzymatic step which catalyzes apoptotic events, such as caspase-activated DNase activation for DNA fragmentation, acinus activation for nuclear chromatin condensation, etc., following cell death signaling (Enari et al., 1998; Sakahira et al., 1998; Sahara et al., 1999). Therefore, incomplete activation of caspase-3 in tissue neutrophils would prevent TNF-α-induced neutrophil apoptosis. This evidence strongly suggests that even when the caspase cascade, including caspase-3, is activated by TNF-α, apoptosis is abolished in circulating blood neutrophils since apoptosis-inhibiting protein(s) act downstream of caspase-3. In tissue neutrophils, caspase-3 is spontaneously activated, as mentioned above, resulting in reduced morphological evidence of apoptosis, due to the constitutive synthesis of apoptosis-inhibiting protein(s) produced through the NF-κB system.

It has been reported that the caspase cascade mediates apoptotic events and downregulates oxygen radical production in TNF-α-treated neutrophils. Moreover, inhibition of caspase activation simultaneously suppresses apoptosis and prolongs the TNF-α-induced augmentation of superoxide generation (Yamashita et al., 1999). We show here that tissue neutrophils, which are resistant to TNF-α-induced apoptosis, also have a greater ability to generate superoxide. These phenomena have important implications for understanding the balance between pro-inflammatory and anti-inflammatory effects of TNF-α. As we mentioned above, caspase-3 is upstream of the final enzymatic step, which catalyzes apoptotic events, caspase-activated DNase or acinus. The present results suggest that caspase-3-mediated proteolysis also plays a role in apoptosis-associated suppression of superoxide generation, although it may lead to neutrophil-mediated tissue damage in some cases.

We have reported that the affinity of TNF-α receptors on neutrophil membranes is decreased in tissue neutrophils, human salivary neutrophils and rabbit peritoneal neutrophils, but that receptor density is not changed (Niwa et al., 1996). The decreased receptor affinity also contributes to the mechanism of decreased susceptibility to apoptosis produced by TNF-α and cycloheximide in tissue neutrophils. Since we showed, in preliminary experiments, that there was also partial resistance to fas-mediated apoptosis in tissue neutrophils, both heterogeneity in tissue neutrophils, NF-κB activation and receptor affinity all contribute to the resistance to apoptosis of tissue neutrophils.

In conclusion, the exudation of neutrophils from blood into tissue is associated with marked changes in their functions, including alteration in their sensitivity to apoptosis-inducing stimuli, and the activation of NF-κB during exudation may be involved in this resistance to apoptosis. Furthermore, our results also indicate a somewhat intriguing role of NF-κB in neutrophil survival, a role 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-inhibiting proteins in human neutrophils.

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References

Al-Essa, L.Y., Niwa, M., Kohno, K., Tsurumi, K., 1994. A proposal for purification of salivary polymorphonuclear leukocytes by combination of nylon mesh filtration and density-gradient method: a validation by superoxide-and cyclic AMP-generating responses. Life Sci. 55, PL333–PL338.

Al-Essa, L.Y., Niwa, M., Kohno, K., Nozaki, M., Tsurumi, K., 1995. Heterogeneity of circulating and exudated polymorphonuclear leukocytes in superoxide-generating response to cyclic AMP and cyclic AMP-elevating agents. Investigation of the underlying mechanism. Biochem. Pharmacol. 49, 315–322.

Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H., Luthra, H.S., Medsger Jr., T.A., Mitchell, D.M., Neustadt, D.H., Pinals, R.S., Schaller, J.G., Sharp, J.T., Wilder, R.L., Hunder, G.G. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 31, 315–324.

Boyum, A., 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. 97, 77–89.

Dignam, J.D., Lebovitz, R.M., Roeder, R.G., 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11, 1475–1489.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., Nagata, S., 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391, 43–50.

Hatano, Y., Kasama, T., Iwabuchi, H., Hanaoka, R., Takeuchi, H.T., Jing, L., Mori, Y., Kobayashi, K., Negishi, M., Ide, H., Adachi, M., 1999. Macrophage inflammatory protein 1 alpha expression by synovial fluid neutrophils in rheumatoid arthritis. Ann. Rheum. Dis. 58, 297–302.

Kanamori, Y., Niwa, M., Kohno, K., Al-Essa, L.Y., Matsuno, H., Kozawa, O., Uematsu, T., 1997. Migration of neutrophils from blood to tissue: alteration of modulatory effects of prostanoid on superoxide generation in rabbits and humans. Life Sci. 60, 1407–1417.

Lin, Y.Z., Yao, S.Y., Veach, R.A., Torgerson, T.R., Hawiger, J., 1995. Inhibition of nuclear translocation of transcription factor NF-kappa B

by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. J. Biol. Chem. 270, 14255–14258.

Maggirwar, S.B., Sarmiere, P.D., Dewhurst, S., Freeman, R.S., 1998. Nerve growth factor-dependent activation of NF-kappaB contributes to survival of sympathetic neurons. J. Neurosci. 18, 10356–10365.

McDonald, P.P., Bald, A., Cassatella, M.A., 1997. Activation of the NF-kappaB pathway by inflammatory stimuli in human neutrophils. Blood 89, 3421–3433.

Nakahara, H., Sato, E.F., Ishisaka, R., Kanno, T., Yoshioka, T., Yasuda, T., Inoue, M., Utsumi, K., 1998. Biochemical properties of human oral polymorphonuclear leukocytes. Free Radical Res. 28, 485–495.

Nakano, M., Sugioka, K., Ushijima, Y., Goto, T., 1986. Chemiluminescence probe with Cypridina luciferin analog, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one, for estimating the ability of human granulocytes to generate O_2^-. Anal. Biochem. 159, 363–369.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., Munday, N.A., Raju, S.M., Smulson, M.E., Yamin, T.T., Yu, V.L., Miller, D.K., 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376, 37–43.

Niwa, M., Al-Essa, L.Y., Kohno, K., Kanamori, Y., Matsuno, M., Abe, A., Uematsu, T., 1996. The loss of recombinant human granulocyte colony-stimulating factor and recombinant human TNF-alpha priming effects on the superoxide-generating response in exudated neutrophils is associated with a decrease in their receptor affinities. J. Immunol. 157, 4147–4153.

Niwa, M., Hara, A., Kanamori, Y., Yoshimi, N., Mori, H., Uematsu, T., 1997. Comparison of susceptibility to apoptosis induced by rhTNF-α and cycloheximide between human circulating and exudated neutrophils. Life Sci. 61, 205–215.

Niwa, M., Hara, A., Kanamori, Y., Matsuno, H., Kozawa, O., Yoshimi, N., Mori, H., Uematsu, T., 1999. Inhibition of tumor necrosis factor-alpha induced neutrophil apoptosis by cyclic AMP: involvement of caspase cascade. Eur. J. Pharmacol. 371, 59–67.

Niwa, M., Hara, A., Kanamori, Y., Hatakeyama, D., Saio, M., Takami, T., Matsuno, H., Kozawa, O., Uematsu, T., 2000. Nuclear factor-kappaB activates dual inhibition sites in the regulation of tumor necrosis factor-alpha-induced neutrophil apoptosis. Eur. J. Pharmacol. 407, 211–219.

Sahara, S., Aoto, M., Eguchi, Y., Imamoto, N., Yoneda, Y., Tsujimoto, Y., 1999. Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation. Nature 401, 168–173.

Sakahira, H., Enari, M., Nagata, S., 1998. Protein, Nucleotide, Structure Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391, 96–99.

Suzuki, N., Suetsuna, K., Mashiko, S., Yoda, B., Nomoto, T., Toya, Y., Inaba, H., Goto, T., 1991. Reaction rates for the chemiluminescence of Cypridina luciferin analogues with superoxide: a quenching experiment with superoxide dismutase. Agric. Biol. Chem. 55, 157–160.

Takeda, Y., Watanabe, H., Yonehara, S., Yamashita, T., Saito, S., Sendo, F., 1993. Rapid acceleration of neutrophil apoptosis by tumor necrosis factor-alpha. Int. Immunol. 5, 691–694.

Ting, A., Morris, P.J., 1971. A technique for lymphocyte preparation from stored heparinized blood. Vox Sang. 20, 561–563.

Tsuchida, H., Takeda, Y., Takei, H., Shinzawa, H., Takahashi, T., Sendo, F., 1995. In vivo regulation of rat neutrophil apoptosis occurring spontaneously or induced with TNF-alpha or cycloheximide. J. Immunol. 154, 2403–2412.

Ueta, E., Osaki, T., Yoneda, K., Yamamoto, T., 1993. Functions of salivary polymorphonuclear leukocytes (SPMNs) and peripheral blood polymorphonuclear leukocytes (PPMNs) from healthy individuals and oral cancer patients. Clin. Immunol. Immunopathol. 66, 272–278.

Ward, C., Chilvers, E.R., Lawson, M.F., Pryde, J.G., Fujihara, S., Farrow, S.N., Haslett, C., Rossi, A.G., 1999. NF-kappaB activation is a critical regulator of human granulocyte apoptosis in vitro. J. Biol. Chem. 274, 4309–4318.

Yamamoto, M., Saeki, K., Utsumi, K., 1991. Isolation of human salivary polymorphonuclear leukocytes and their stimulation-coupled responses. Arch. Biochem. Biophys. 289, 76–82.

Yamashita, K., Takahashi, A., Kobayashi, S., Hirata, H., Mesner, P.W., Kaufmann, S.H., Yonehara, S., Yamamoto, K., Uchiyama, T., Sasada, M., 1999. Caspases mediate tumor necrosis factor-alpha-induced neutrophil apoptosis and downregulation of reactive oxygen production. Blood 93, 674–685.