Neutrophil Superoxide Release Is Required for Spontaneous and FMLP-Mediated but Not for TNFα-Mediated Apoptosis

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Abstract. Polymorphonuclear leukocyte (PMN) lifespan is characterized by both rapid production and apoptotic cell death. The mechanisms triggering apoptosis in PMN are not completely understood. In this study, the relationship of neutrophil activation and apoptosis as related to released superoxide was investigated. PMN apoptosis was detected by DNA fragmentation, and ultraviolet and light microscopy, and was quantified by flow cytometry; superoxide release was measured by superoxide dismutase-inhibitable reduction of ferricytochrome C. Incubation of PMN with 20 ng/ml tumor necrosis factor (TNF)α induced superoxide release (8.8 ± 7.5 nmol O₂⁻/30 min, n = 7) in normal PMN and also resulted in apoptosis within 2 h, whereas a subactivating dose of 2 ng/ml TNFα, which did not trigger superoxide release (3.1 ± 1.7 nmol O₂⁻, n = 10), did facilitate apoptosis, although to a lesser degree. PMN cultured under nonstimulating conditions underwent apoptotic cell death after 8 h. Exogenous superoxide dismutase did not inhibit apoptosis induced by 20 ng/ml TNFα. No upregulation of endogenous manganese superoxide dismutase mRNA expression was observed in response to TNFα as measured by reverse transcription PCR. Formyl-methionyl-leucyl-phenylalanine (FMLP) stimulation (10⁻⁷ M) resulting in superoxide release of 31.7 ± 6.1 nmol O₂⁻/30 min (n = 10) also significantly increased the percentage of apoptosis, but at 24 h (P < 0.05). Exogenous superoxide dismutase did inhibit FMLP-induced apoptosis, as well as apoptosis due to aging in culture. In conclusion, aging and FMLP-stimulated PMN undergo apoptosis by a superoxide release-dependent pathway, whereas TNFα-facilitated apoptosis appears to be unrelated to respiratory burst oxidase activity. (J Am Soc Nephrol 8: 1091–1100, 1997)

Neutrophils play a pivotal role in host protection from infectious organisms. Stimulated neutrophils respond with an NADPH-oxidase–dependent respiratory burst, degranulation of numerous antibacterial enzymes, and phagocytosis of particles. Although these mechanisms efficiently kill micro-organisms, they can have the unintended consequence of injuring host structures as well. Under basal conditions, approximately 10¹¹ human neutrophils are produced by the bone marrow each day (1), and because they have a short lifespan of 1 to 2 d, an immense number of neutrophils must be removed under normal conditions, with even more neutrophils being produced and removed under conditions of stress. Apoptosis regulates cell turnover, providing a physiological mechanism of eliminating single cells without causing structural or functional disintegration of surrounding tissue (2,3). Savill et al. first demonstrated that neutrophils aged in vitro undergo apoptosis and are recognized and removed by macrophages (4). Thus, neutrophils that undergo apoptosis and are subsequently engulfed by macrophages bypass further release of inflammatory agents and/or tissue-destructive granular contents. The factors controlling neutrophil apoptosis in aged neutrophils are not yet known, nor is it known what role neutrophil activation plays in the decision pathway leading to neutrophil apoptosis.

During activation, neutrophils generate and release large amounts of reactive oxygen species (ROS) via the NADPH oxidase system, allowing these cells to respond to various stimuli with a respiratory burst (5). An electron transfer from NADPH to molecular oxygen (O₂) generates superoxide anions (O₂⁻). Superoxide is then dismutated to form hydrogen peroxide, which reacts with halogens such as chloride to generate hypochloric acid. The latter reaction is promoted by myeloperoxidase, an enzyme of the primary neutrophil granules released during activation. In addition, a metal ion-independent and a metal ion-dependent pathway, known as the Haber-Weiss reaction, is responsible for the generation of hydroxyl radicals (6,7). Thus, we examined the following two questions: Does neutrophil activation result in apoptotic cell death?, and Is oxygen superoxide release a mediator of this process?

More recently, it has been suggested that intracellular accumulation of ROS promotes apoptosis (8–11). Here, we hypothesize that the release of generated ROS by human neutrophils not only provides a defense mechanism against target cells and structures, but also affects neutrophil survival. The effect of activating agents on apoptosis of normal polymorphonuclear leukocyte (PMN) was explored in this study. Neutrophils that
were challenged with tumor necrosis factor (TNF) α rapidly underwent apoptotic cell death that was not dependent on superoxide release. In contrast, a delayed form of apoptotic cell death was observed in neutrophils aged in vitro and in FMLP-stimulated neutrophils that was mediated by superoxide release. We suggest that inflammation results in feedback mechanisms that affect neutrophil survival by modifying programmed cell death, and that ROS, like oxygen superoxide and inflammatory cytokines such as TNFα, are mediators of this process.

Materials and Methods

Buffers and Reagents

Phosphate-buffered saline (PBS) and Hank’s balanced salt solution with calcium and magnesium (HBSS++) or without (HBSS) were obtained from the University of North Carolina Lineberger Cancer Center Tissue Culture Facility (Chapel Hill, NC). The reagents formyl-methionyl-leucyl-phenylalanine (FMLP); bovine erythrocyte superoxide dismutase (SOD) (2500 to 7000 U/mg protein); ferricytochrome C; cytochalasin B; propidium iodide (PI); Ficoll-Hypaque; modified Wright Giemsa; 4′,6-diamidino-2-phenylindole (DAPI); and sulforhodamin 101 (SR 101) were purchased from Sigma (St. Louis, MO). Tumor necrosis factor α (rTNFα) was purchased from Genzyme Corp. (Boston, MA), and sodium dithionite was from Fisher (Fair Lawn, NJ).

Isolation of Human Neutrophils and Determination of Cell Counts and Viability

Neutrophils were prepared from heparinized whole blood of healthy volunteers. The isolation of neutrophils included sedimentation of red blood cells by Plasmagel (Cellular Products Inc., Buffalo, NY) followed by Ficoll-Hypaque density gradient centrifugation, and hypotonic red cell lysis. Cells were resuspended at 10^7/ml in HBSS when used for superoxide assay or in RPMI 1640 supplemented with 2 mM glutamine and penicillin/streptomycin when cultured. Trypan blue exclusion was used to determine cell viability. Ten microliters of neutrophils in suspension was incubated with 40 µl trypan blue (Gibco, Grand Island, NY) for 5 min at room temperature. Cells were counted in duplicate using a hemocytometer and considered as viable if trypan blue could be excluded.

Assay to Detect Oxygen Superoxide Release by Human Neutrophils

Superoxide was measured using the standard assay of SOD-inhibitable reduction of ferricytochrome C (12,13). PMN were pretreated with 5 µg/ml cytochalasin B for 15 min at 4°C. Cells (2.5 × 10^6) were incubated with 2 or 20 ng/ml TNFα, 10^{-7} M FMLP, or an equal amount of HBSS++ and 50 µM ferricytochrome C. The mixtures were incubated for 30 min at 37°C, and the reactions were stopped by placing the mixtures on ice for 3 min before centrifugation at 4°C. Supernatant absorbances at 550 nm were measured with and without 125 µg/ml SOD, using a Microplate Autoreader (BioTek Instruments). Sodium dithionite was added to the tubes containing no SOD to fully reduce all residual ferricytochrome C, and at 550 nm the absorptions were remeasured. The amount of generated superoxide was calculated from the amount of reduced ferricytochrome C, and the results are expressed in nanomoles of O_2^-/2.5 × 10^6 cells/30 min (14). All experiments were performed in duplicate.

Culture Conditions of Human Neutrophils

Five hundred microliters of neutrophils at 10^7/ml RPMI without fetal calf serum (FCS) were pipetted into 12 × 75-mm polystyrene culture tubes (Fisher, Fair Lawn, NJ), and 2 or 20 ng/ml TNFα, 10^{-7} M FMLP, or an equal volume of PBS was added, and the cells were incubated at 37°C for 10 min. An equal volume of supplemented RPMI 1640 containing 20% heat-inactivated FCS was added, giving a final concentration of 5 × 10^6 cells/ml in 10% FCS. SOD was used at concentrations from 1.25 to 250 µg/ml, with 125 µg/ml SOD being the dose used for blocking apoptosis in stimulated cells. Cell suspensions were incubated at 37°C in 5% CO₂ for up to 48 h. Where indicated, neutrophils were pretreated with 5 µg of cytochalasin B per 10^7 cells. It has been reported that endotoxic lipopolysaccharide inhibits neutrophil apoptosis (15,16). Therefore, all solutions and reagents used in cell culture were checked for the presence of endotoxin and were found to contain <0.05 ng/ml endotoxin as determined by the Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD).

Measurement of Apoptosis Using PI-Stained Neutrophils by Flow Cytometry

Flow cytometry was performed following the procedure of Darzynkiewicz et al. (17) for measuring DNA content in RNase-treated cells that have been stained with PI. Degraded low molecular weight DNA fragments leak out of apoptotic cells during the washing and staining process, whereas DNA in nonapoptotic cells remains unchanged; DNA content of cells is then correlated with the light scatter properties reflecting cell size and granularity. The protocol was modified for use with human neutrophils because the multiple centrifugation steps caused extensive neutrophil disruption. Briefly, freshly isolated or cultured cells were spun at 200 × g for 5 min at 4°C and carefully resuspended in PBS containing 0.5 mM ethylenediaminetetra-acetic acid (EDTA). Chilled, 95% ethanol was added to a final concentration of 70%, and the cell mixtures were stored at −20°C for 1 to 3 d. Neutrophils were pelleted once (200 × g, 5 min, 4°C) and resuspended in 250 µl of PBS/0.5 mM EDTA/1% bovine serum albumin. A total of 250 µl of PBS containing 200 µg of DNase-free RNase and 500 µl of PBS containing 50 µg of PI were added. Cells were kept for 15 min in the dark at room temperature and then stored at 4°C. Separation between the apoptotic sub-G₀/G₁-population and the G₀/G₁ population was enhanced in our method by storing the neutrophils for 6 to 8 h in the staining mixture, allowing low molecular DNA fragments to leave permeabilized cells. Neutrophils were analyzed using a FACSscan (fluorescence-activated cell sorter; Becton Dickinson, San Jose, CA), and 25,000 events per sample were collected in list mode using Cytocaps software (Fort Collins, CO) for data acquisition and analysis.

Cell Staining with Wright Giemsa or DAPI and SR 101

Cytocentrifuge preparations of cells were either stained using modified Wright Giemsa stain or were fixed in ethanol and stained with a solution containing 1 µg/ml DAPI and 20 µg/ml SR 101. Morphologic features of apoptosis as described by Kerr et al. (2), including pyknotic nuclei, nuclear and cytoplasmic condensation, and the formation of apoptotic bodies, were considered as evidence for apoptosis.

DNA Fragmentation Assay

DNA fragmentation was studied using isolated, low molecular weight DNA. Neutrophils (5 × 10^7) were resuspended in lysis buffer (0.5 mM Tris-HCl, pH 7.5, 20 mM EDTA, pH 8.0, and 0.5% Triton X-100). Supernatants absorbances at 550 nm were measured with and without 5 µg/ml cytochalasin B, 10^{-4} M FMLP, or an equal volume of PBS was added, and the cells were incubated at 37°C for 10 min. An equal volume of supplemented RPMI 1640 containing 20% heat-inactivated FCS was added, giving a final concentration of 5 × 10^6 cells/ml in 10% FCS. SOD was used at concentrations from 1.25 to 250 µg/ml, with 125 µg/ml SOD being the dose used for blocking apoptosis in stimulated cells. Cell suspensions were incubated at 37°C in 5% CO₂ for up to 48 h. Where indicated, neutrophils were pretreated with 5 µg of cytochalasin B per 10^7 cells. It has been reported that endotoxic lipopolysaccharide inhibits neutrophil apoptosis (15,16). Therefore, all solutions and reagents used in cell culture were checked for the presence of endotoxin and were found to contain <0.05 ng/ml endotoxin as determined by the Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD).
X-100), and lysate was centrifuged at 20,000 \( \times g \) for 20 min at 4\(^{\circ}\)C to select for low molecular weight DNA. Supernatants were extracted twice with an equal volume of PCIA (Tris-saturated phenol:chloroform:isoamyl alcohol at 25:24:1) and twice with CIA (chloroform:isoamyl alcohol at 24:1). DNA was precipitated at -20\(^{\circ}\)C overnight by adding 2.2 vol of absolute ethanol in the presence of 0.3 M sodium acetate, pH 5.2. DNA was centrifuged at 13,500 \( \times g \) for 20 min at 4\(^{\circ}\)C, air-dried, and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) containing RNase Plus (5'-3'). After 30 min of incubation at 37\(^{\circ}\)C, DNA was quantified and electrophoresed in a 1.5% agarose gel containing 0.5 \( \mu g/ml \) ethidium bromide, visualized under ultraviolet light, and photographed.

**RNA Extraction and Reverse Transcription PCR**

RNA was purified by resuspending 5 \( \times 10^6 \) to 1 \( \times 10^7 \) neutrophils in 1 ml of RNA-STAT 60 (Tel-Test "B", Inc., Friendswood, TX) following the Tel-Test "B" procedure, in which the RNA/RNA-STAT mixture is extracted with chloroform and the RNA is precipitated with isopropanol and dissolved in 1 mM EDTA, pH 8.0. Gene-specific RNA was analyzed by reverse transcription PCR using 1 \( \mu g \) of RNA per reaction. First-strand cDNA for two independent RNA samples and PCR (performed in triplicate) were prepared as described in the Clontech (Palo Alto, CA) 1st-strand cDNA synthesis and RT-PCR kits. A total of 5 \( \mu l \) of cDNA was used per 50-\( \mu l \) PCR reaction, and cycling conditions were 94\(^{\circ}\)C for 45 s, 58\(^{\circ}\)C for 1 mm, and 72\(^{\circ}\)C for 2 min for 35 cycles using a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers (Clontech) were used as an internal control for normalizing PCR product quantitation. Primers for manganese superoxide dismutase (Mn-SOD) PCR were selected using the MacVector (Kodak) primer pair program and included 5’-CGCCCCTGGAACCTCACATCAAC-3’ and 5’-CCCACACATCACCCACAGCAG-3’. The primers were synthesized using an Applied Biosystems model 394 DNA synthesizer (Foster City, CA) by the University of North Carolina Pathology Department Oligonucleotide Synthesis Facility. Confirmation of amplification of the correct gene was done by digesting the Mn-SOD-amplified product with BalI, which gave the expected bands of 336 and 112 bp (data not shown). PCR products were electrophoresed in 1.8% Metaphor (FMC BioProducts, Rockland, ME) agarose gels in TBE buffer (Tris, pH 8.0, 89 mM borate, and 2 mM EDTA), stained in 0.5 \( \mu g/ml \) ethidium bromide, and photographed. A control reaction was run on at least one sample from each RNA preparation with PCR of samples without reverse transcriptase to assess contamination by genomic DNA. No bands were seen in these lanes (data not shown). Densitometric analysis of scanned negatives was used to quantify differences in mRNA expression. At each time point, the ratio of G3PDH and Mn-SOD mRNA was determined and compared with the G3PDH/Mn-SOD mRNA ratio at time 0, which was set at 100%.

**Statistical Analyses**

The nonparametric Wilcoxon signed-rank test was used to compare results between paired groups to test whether the differences were unequal to 0, whereas the Wilcoxon rank-sum test was applied to test differences between nonpaired groups.

**Results**

**Superoxide Release by Neutrophils Stimulated with TNF\( \alpha \) or with FMLP**

We compared superoxide release of normal human neutrophils stimulated with either 2 or 20 ng/ml TNF\( \alpha \) or with 10\(^{-7}\) M FMLP (Figure 1). The dose of TNF\( \alpha \) determined whether a significant respiratory burst was observed; 20 ng/ml TNF\( \alpha \) triggered a significant release of \( O_2^- \), whereas PMN incubated with 2 ng/ml TNF\( \alpha \) did not result in more superoxide release than cells treated with buffer only. The highest amount of respiratory burst was observed in PMN that were challenged with FMLP. However, it must be understood that because of the techniques used to purify the neutrophils for these studies, the PMN are considered to be primed before being exposed to TNF\( \alpha \) or FMLP.

**Effect of TNF\( \alpha \) on Neutrophil Apoptosis**

Freshly isolated human neutrophils were cultured in the presence or absence of 20 ng/ml TNF\( \alpha \), and apoptotic cells were identified by flow cytometry. TNF\( \alpha \) stimulated apoptosis within 2 h in approximately 25% of cultured neutrophils. Apoptosis was accompanied by a loss in forward scatter, indicating cell shrinkage, and a slight increase in side scatter. As demonstrated by gating, the same population was also characterized by a decrease in DNA content as a result of the loss of low molecular DNA (Figure 2).

The percentage of apoptosis for a time course of samples cultured for 1, 2, 4, 8, and 24 h is shown in Figure 3. The results indicate that TNF\( \alpha \) at a dose of 20 ng/ml facilitates apoptosis when compared with untreated controls (data based on six separate donor neutrophil preparations). Apoptotic death in TNF\( \alpha \)-treated cultures occurred rapidly between the first and the second hour. A significantly higher percentage of apoptotic neutrophils was found as early as 2 h after cells were challenged with TNF\( \alpha \) compared with controls \( (P < 0.05) \). This difference between cells treated with 20 ng/ml TNF\( \alpha \) and untreated cells remained statistically significant at 4 and 8 h in culture \( (P < 0.05) \), whereas similar percentages of apoptotic cells were observed after 24 h. The viability of neutrophils was higher than 93% in both groups, as estimated by trypan blue exclusion, and no significant decrease in cell number was observed.

A lower dose of TNF\( \alpha \) (2 ng/ml) that did not trigger significant generation of superoxide did accelerate apoptosis (Figure 3). Both 2 and 20 ng/ml TNF\( \alpha \) triggered apoptotic cell death starting as early as 2 h after incubation; however, the 20 ng/ml TNF\( \alpha \) caused approximately twice as much apoptosis as 2 ng/ml TNF\( \alpha \) at the early time points, although the difference in percentage of apoptotic cells between 2 and 20 ng/ml up to 24 h did not reach statistical significance \( (P > 0.05, n = 3) \).

To confirm the results obtained by flow cytometry, we looked for morphological signs of apoptosis and for the presence of internucleosomal cleaved DNA, a hallmark of apoptosis. Human neutrophils cultured in the presence or absence of 20 ng/ml TNF\( \alpha \) were harvested after 30 min and 2, 4, 8, 12, and 24 h. Low molecular weight DNA was isolated from 5 \( \times 10^6 \) PMN and electrophoresed on agarose gels. Similar to the FACs analyses of TNF\( \alpha \)-treated neutrophils, apoptosis as indicated by DNA "ladders" was observed after 2 h, whereas untreated samples showed no fragmented low molecular DNA for up to 12 h in culture (data not shown). In addition, TNF\( \alpha \) treatment (20 ng/ml) resulted in morphological signs of apo-
Figure 1. Oxygen superoxide release by human neutrophils from healthy donors was detected using superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C. Data are expressed as means ± SD. Neutrophil response to each stimulus is compared with the response obtained when cells of the same preparation were exposed to buffer control only (Control). Neutrophils incubated with 20 ng/ml tumor necrosis factor (TNF) α generated 8.8 ± 7.5 nmol O$_2^-$/2.5×10$^6$ polymorphonuclear leukocyte (PMN)/30 min (controls, 2.8 ± 1.7 nmol O$_2^-$; n = 7, P < 0.01); 2 ng/ml TNFα did not result in significant respiratory burst (3.1 ± 1.7 nmol O$_2^-$/2.5×10$^6$ PMN/30 min compared with 2.4 ± 0.9 nmol O$_2^-$ in untreated cells; n = 10, P > 0.05). Superoxide release for 10$^{-7}$ M FMLP was 31.7 ± 6.1 nmol O$_2^-$/2.5×10$^6$ PMN/30 min versus 2.3 ± 1.4 nmol O$_2^-$ in untreated cells (n = 10, P < 0.01).

ptosis, including pyknotic nuclei, nuclear condensation, and formation of apoptotic bodies, as identified by staining with modified Wright Giemsa (data not shown) or with DAPI (Figure 4). Morphological features of apoptosis were not observed in untreated controls (Figure 4). In two experiments, the DNA fragmentation assay, flow cytometry, and cell staining were carried out simultaneously. We observed a good correlation between FACS and microscopy at each time point; however, cell staining resulted in approximately 10% fewer cells being identified as apoptotic (data not shown). Thus, analysis of DNA fragmentation is probably more sensitive than detection of morphological changes by microscopy.

Influence of Exogenous SOD on Neutrophil Apoptosis
To test whether released superoxide is responsible for facilitating apoptotic cell death in aged or in stimulated neutrophils, we added exogenous SOD to the cultures. SOD promotes the formation of hydrogen peroxide from oxygen superoxide, leading to a reduction of superoxide levels. Exogenous SOD did suppress apoptosis in untreated, aged neutrophils in a dose-dependent manner (Figure 5). In three separate experiments, SOD concentrations from 1.25 µg/ml up to 250 µg/ml were incubated with otherwise untreated neutrophils (Figure 5). Cells were cultured for 24 h, and apoptosis was assessed by flow cytometry. The results demonstrate an inverse dose-response relationship, because the percentage of apoptotic cells decreased as the SOD dose increased. Trypan blue exclusion results showed that cell counts did not change significantly and that viability was higher than 95% in all samples for up to 24 h (data not shown).

In contrast, exogenous SOD did not inhibit TNFα-mediated apoptosis (Figures 6 and 9). Apoptosis in TNFα-treated (20 ng/ml) neutrophils with and without SOD pretreatment (125 µg/ml) was compared by measuring the percentage of apoptotic cells immediately after cell isolation and after culturing for 1, 2, 4, 8, and 24 h. The results indicate no significant influence of exogenous SOD on apoptosis in TNFα-treated cells (Figure 6; P > 0.05, n = 3).

Effect of TNFα on Expression of Mn-SOD mRNA
The expression of Mn-SOD mRNA in TNFα-treated neutrophils was evaluated by preparing total RNA from freshly isolated neutrophils and from cells cultured for 1, 2, and 4 h in the presence or absence of 20 ng/ml TNFα. Two independent studies were carried out with PCR performed in triplicate on each sample and quantified by optical densitometry. A representative PCR experiment is shown in Figure 7 and demonstrated no significant change in Mn-SOD RNA expression.

Effect of FMLP Treatment on Neutrophil Apoptosis
Compared with TNFα-treated neutrophils, no significant effect on apoptosis was observed over the first 8 h when cells were stimulated with FMLP, as determined by flow cytometry. In contrast, incubation of PMN with FMLP (10$^{-7}$ M) resulted
Figure 2. Characteristics of neutrophil apoptosis in flow cytometry studies. Loss of DNA content, decrease in forward scatter, and slightly increased side scatter are features of ethanol-permeabilized apoptotic neutrophils stained with propidium iodide. Cells cultured for 2 h were analyzed in the absence (controls) or presence of 20 ng/ml TNFα (TNF).

in a statistically significant increase in the percentage of apoptotic neutrophils at 24 h (Figure 8). Because of the superoxide release-enhancing effect, neutrophils were pretreated in some experiments (Figure 8) with cytochalasin B (18). In all cases, cell viability was higher than 95%.

**Discussion**

We have characterized TNFα-induced apoptosis, and we conclude that it is not mediated by the respiratory burst oxidase based on the following lines of evidence. First, TNFα rapidly mediated apoptosis not only at the activating concentration of 20 ng/ml, but also at the subactivating dose of 2 ng/ml. Second, exogenous SOD that markedly decreased released superoxide levels in an in vitro assay was sufficient to inhibit apoptosis associated with FMLP, but failed to inhibit TNFα-mediated apoptosis. Third, FMLP stimulated a considerably greater amount of respiratory burst superoxide release than TNFα but did not cause significant early apoptosis. Even the low-dose TNFα treatment facilitated apoptosis in approximately 25% of cultured cells at 8 h, whereas FMLP did not cause significant apoptosis at this time point. Fourth, TNFα-induced apoptosis was not associated with a change in endogenous Mn-SOD RNA expression up to 4 h in neutrophils, although upregula-
tion of Mn-SOD RNA in response to TNF has been demonstrated in a murine adipogenic cell line and in a pulmonary adenocarcinoma cell line. Interestingly, only a subpopulation of PMN appears to be susceptible to TNFα-mediated apoptosis. This is an interesting fact that requires further investigation. Better understanding of this finding may help elucidate underlying mechanisms of TNFα action.

In contrast, apoptosis in aged human neutrophils and in FMLP-treated neutrophils did appear to be facilitated by the release of superoxide. Incubation of neutrophils with FMLP triggered the strongest respiratory burst and significantly accelerated apoptotic cell death at 24 h. Additionally, exogenous SOD did suppress apoptosis in a dose-dependent manner in nonstimulated and in FMLP-stimulated neutrophils at 24 h. This result provides evidence for a causal effect of respiratory burst superoxide release on neutrophil apoptosis in cultured neutrophils.

Colotta et al. studied the effect of several cytokines, including TNFα and FMLP, on neutrophil survival (19). The investigators reported an increase in survival by a delay in apoptosis when cells were incubated with TNFα for 48 to 96 h. FMLP treatment did not modify cell survival. The authors did not show cell numbers, but in our experience there is a progressive decrease in cell number and viability beyond 24 h when cultured cells are stimulated. Without knowledge of cell counts, the apoptotic cells that have been phagocytosed or disintegrated can be missed. However, our data confirmed the results of Takeda et al. (20), showing that the effect of facilitated apoptosis by TNFα happened within the first 2 h and that there was no difference in the amount of apoptosis at 24 h.

Recent studies provide evidence that TNFα induces apoptosis in several cell types. It was accepted for many years that TNFα cytotoxicity spares normal cells and affects only some transformed cells. Laster et al. demonstrated that TNFα can induce apoptosis and that the type of cell death, apoptotic or nonapoptotic, differed among various transformed fibroblast cell lines (21). However, it became apparent that TNFα also causes apoptosis in nontransformed cells, as demonstrated in endothelial cells (22,23), hepatocytes (24), murine T cells (25), and human neutrophils (20). Biological effects of TNFα are mediated by two different TNF receptors, p55 or TNF R1 and p75 or TNF R2. Engagement of these receptors may result in activation of different pathways including the generation of ROS (26,27). Further studies must be designed to investigate which receptor type mediates TNFα-facilitated apoptosis in human neutrophils.

It has been suggested that ROS mediate apoptosis. Studies in nerve cells (8,10) and lymphocytes (9,11) have demonstrated that intracellular accumulated superoxide plays an important role in apoptotic cell death. To date, the relationship between ROS and apoptotic cell death remains rather descriptive. Cell culture conditions that are known to increase oxidative stress also facilitate apoptosis. High oxygen tension (28), deprivation of nerve growth factor (8), diethyl maleate (10), and β-amyloid (29) are examples of those in vitro conditions and are associated with apoptosis in neuronal cells. Familial amyotrophic lateral sclerosis is a fatal disease with motor neuron death; patients are characterized by a mutation of copper/zinc SOD, suggesting a role of ROS in vivo (30,31). Further circumstantial evidence supporting a role of ROS in apoptosis derives from the fact that Bcl-2 can protect from apoptotic cell death in a variety of conditions, including suppression of cell death mediated by free radicals (reviewed in reference 32). The suggested mechanisms of the protective effect of Bcl-2 include...
a reduction of ROS generation and a suppression of biological effects of ROS (9,10). Bcl-2 is localized in mitochondria, endoplasmic reticulum, and nuclear membrane (33–35). All three loci contain e-transport chains necessary to generate superoxide. Human neutrophils do not express the Bcl-2 protein (36). Because the phagocyte-specific NADPH-oxidase system generates high amounts of superoxide in activated neutrophils, their lack of Bcl-2 might contribute to their susceptibility for undergoing apoptosis. The observation by Lagasse and Weissman that Bcl-2 expression in mice leads to a significant delay in apoptosis of neutrophils aged in vitro supports this suggestion (37).

No differences were observed in the expression of Mn-SOD mRNA associated with TNFα-facilitated neutrophil apoptosis.
A role for endogenous Mn-SOD in TNFα-mediated cytotoxicity has been demonstrated by Wong et al., studying a human embryonic kidney cell line (38). In this system, overexpression of Mn-SOD resulted in improved survival after TNFα treatment for 24 h. Thus, endogenous Mn-SOD may be necessary, but not sufficient, to provide protection against TNFα-mediated cytotoxicity. TNFα itself can upregulate Mn-SOD mRNA, as shown in a murine adipogenic cell line and in a pulmonary adenocarcinoma cell line (39). When these cells were pretreated with TNFα and consecutively exposed to paraquat, an intracellular superoxide generator, improved survival was observed (29), presumably due to increased expression of endogenous Mn-SOD. In our experiments on human neutrophils, however, TNFα does not appear to upregulate Mn-SOD message.

Taken together, our data suggest that apoptosis in human neutrophils in vitro can be modulated by superoxide release. Aging of neutrophils or treatment with FMLP results in a delayed superoxide-release-modulated apoptosis when compared with TNFα treatment that rapidly facilitates apoptosis by a pathway unassociated with released superoxide. We propose that both mechanisms have host-protective significance by facilitating apoptosis in aging, as well as in activated neutrophils. Apoptotic neutrophils can be removed by macrophages preventing neutrophil disintegration with the result of preventing acceleration of tissue inflammation.

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