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Bid Truncation, Bid/Bax Targeting to the Mitochondria, and Caspase Activation Associated with Neutrophil Apoptosis Are Inhibited by Granulocyte Colony-Stimulating Factor

Nikolai A. Maianski,[1]*† Dirk Roos,† and Taco W. Kuijpers*†

Neutrophil apoptosis constitutes a way of managing neutrophil-mediated reactions. It allows coping with infections, but avoiding overt bystander tissue damage. Using digitonin-based subcellular fractionation and Western blotting, we found that spontaneous apoptosis of human neutrophils (after ~20 h of culture) was associated with translocation of two proapoptotic Bcl-2 homologues, Bid and Bax, to the mitochondria and truncation of Bid, with subsequent release of Omi/HtrA2 and Smac/DIABLO into the cytosol. These events were accompanied by processing and increased enzymatic activity of caspase-8, -9, and -3. A G-CSF-mediated reduction in apoptosis coincided with inhibition of all these reactions. The G-CSF-induced effects were differentially dependent on newly synthesized mediators. Whereas inhibition of Bax targeting to the mitochondria and inhibition of caspase activation by G-CSF were dependent on protein synthesis, Bid truncation and redistribution were prevented by G-CSF regardless of the presence of the protein synthesis inhibitor cycloheximide. Apparently, the observed Bid changes were dispensable for neutrophil apoptosis. Although the regulators of the inhibitor of apoptosis proteins (IAPs), Omi/HtrA2 and Smac/DIABLO, were released into the cytosol during apoptosis, we did not observe cleavage of X-linked IAP, which suggests that another mechanism of IAP deactivation is involved. Together our results support an integrative role of the mitochondria in induction and/or amplification of caspase activity and show that G-CSF may act by blocking Bid/Bax redistribution and inhibiting caspase activation. ***The Journal of Immunology*****, 2004, 172: 7024–7030.**

Regulation of neutrophil survival and turnover by apoptosis provides a fine balance between their function as effector cells of innate host defense and a safe removal of these potentially harmful cells. Neutrophils have a constitutively short life span, which may be further reduced or prolonged by various mediators. Among these agents, G-CSF is one of the most clinically relevant and widely used in the treatment of various conditions associated with neutropenia (1, 2). Besides stimulation of neutrophil myeloid precursors in bone marrow, this growth factor has a clear antiapoptotic effect on mature neutrophils. Mechanisms of that action as well as the regulation of neutrophil apoptosis in general have been the focus of a large number of studies (for recent reviews, see Refs. 3 and 4), but have not yet been fully elucidated.

As do other cell types, neutrophils express several members of the Bcl-2 family of proteins and capsases (3, 4), which are integrated in their function at the level of mitochondria. However, mitochondria in neutrophils have special features and are characterized by preserving death-mediating abilities while being inactive in many of the usual cellular metabolic activities. The neutrophil mitochondria have been shown to be targeted by the proapoptotic Bcl-2 homologue Bax and release factors such as cytochrome *c*, Smac/DIABLO, and Omi/HtrA2 into the cytosol upon apoptosis (5–9). These events together with cytochrome *c*-dependent oligomerization of Apaf-1 and formation of the apoptosome (10) constitute an internal (mitochondrial, stress-induced) route of apoptosis, which may be expected to be of importance in neutrophils as they are tuned for a rapid spontaneous cell death without the need to generate a positive death signal (3). If so, it would be logical to suggest that antiapoptotic signals generated by pro-survival factors would also influence this intrinsic mechanism. To check this assumption, in the present paper we have studied in more detail the mechanisms of G-CSF-mediated pro-survival effects with respect to the mitochondrial death pathway. Our data show that G-CSF-induced survival of neutrophils was associated with inhibition of cleavage of Bid, Bid/Bax translocation to the mitochondria, and prevention of subsequent release of the proapoptotic mitochondrial constituents, such as Omi/HtrA2 and Smac/DIABLO. Moreover, both processing of the initiator caspase-8 and caspase-9 and the executioner caspase-3 and their specific enzymatic activities in apoptotic neutrophils were pronouncedly blocked by G-CSF. Hence, we conclude that the antiapoptotic properties of G-CSF are mediated through inhibition of Bid/Bax-dependent mitochondrial dysfunction and caspase activation.

Materials and Methods

Neutrophil preparation and culture

Neutrophils were isolated from heparinized venous blood of healthy volunteers, after informed consent had been obtained, by density gradient centrifugation and subsequent isotonic erythrocyte lysis as previously described (6). After the last wash with PBS, the neutrophils (>97% pure) were resuspended in IMDM (BioWhittaker, Brussels, Belgium) supplemented with 10% heat-inactivated FCS (Life Technologies, Paisley, U.K.) and antibiotics at a final concentration of 5×10^6/ml. The cells were cultured for ~20 h in a 24-well plate (1 ml/well; Nunc, Roskilde, Denmark) without additions or with 100 ng/ml G-CSF (10^4 U/ml; Neupogen; Amgen, Breda, The Netherlands) alone or in combination with 5 μg/ml cycloheximide (CHX;[2] Calbiochem, Bad Soden, Germany) in a humidified CO_2 incubator at 37°C.

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[2] Abbreviations used in this paper: CHX, cycloheximide; AIF, apoptosis-inducing factor; AMC, 7-amino-4-methyl-coumarin; cIAP, cellular inhibitor of apoptosis protein; DFP, diisopropyl fluorophosphate; PIM, protease inhibitor mixture; XIAP, X-linked IAP.

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Apoptosis assay and fluorescent microscopy

Before or after a 20-h incubation, neutrophils were washed once in ice-cold PBS and split into three portions. One portion (1×10^5 cells) was stained using the annexin V-FITC apoptosis assay kit (Bender MedSystems, Vienna, Austria) and analyzed by FACScan (BD Biosciences, San Jose, CA) as described previously (6). Another two portions of neutrophils ($2–3 \times 10^5$ cells) were used for fluorescence microscopy (see detailed description in Ref. 6). Briefly, to estimate mitochondrial morphology, unfixed neutrophils were stained with 100 nM MitoTracker GreenFM (Molecular Probes, Eugene, OR) for 15–30 min at 37°C. Staining patterns of Bax were determined in 2% paraformaldehyde-fixed, saponin-permeabilized neutrophils labeled with an anti-Bax polyclonal Ab (final dilution, 1/250; BD PharMingen, San Diego, CA), followed by secondary staining with AlexaFluor-568-conjugated goat anti-rabbit IgG (Molecular Probes). After staining, the cells were analyzed by confocal laser scanning microscopy (LSM510; Carl Zeiss, Heidelberg, Germany). At least 300 neutrophils were counted in each sample, and the percentages of cells with clustered, rounded mitochondria or with aggregated Bax (as shown in Fig. 1, *b* and *c*, respectively) were determined.

Subcellular fractionation and Western blotting

Subcellular fractionation and Western blotting were performed essentially as previously described (6, 7). Whole-cell lysates were prepared as follows. The neutrophil pellets were resuspended in a protease inhibitor mixture (PIM; one tablet of Complete Mini protease inhibitor mixture (Roche, Mannheim, Germany) in 5 ml of PBS containing 5 mM EDTA) with 2 mM diisopropyl fluorophosphate (DFP; Acros Organics, Morris Plains, NJ) and incubated for 15 min on ice. After addition of an equal amount of 2× SDS sample buffer with 4% ME, the preparations were boiled for 15 min and kept at −20°C until use.

To obtain subcellular fractions, neutrophils treated under various conditions were washed in ice-cold PBS and resuspended in ice-cold cytosol extraction buffer (250 mM sucrose, 70 mM KCl, 250 μg/ml digitonin, PIM, and 2 mM DFP in PBS) at a final concentration of 100×10^6/ml. After a 10- to 15-min incubation on ice, when 80–90% cells had become trypan blue positive, the preparations were spun at $1,000 \times g$ for 5 min, and the supernatants were kept as cytosolic fractions. The pellets were resuspended in the same volume (as the cytosol extraction buffer) of ice-cold mitochondria lysis buffer (100 mM NaCl, 10 mM $MgCl_2$, 2 mM EGTA, 2 mM EDTA, 1% Nonidet P-40 (v/v), 10% glycerol (v/v), PIM, and 2 mM DFP in 50 mM Tris, pH 7.5) and incubated for 10 min on ice, followed by a 10-min centrifugation at $10,000 \times g$. The supernatants were taken as mitochondrial fractions. To prepare samples for Western blotting, 24 μl of either the cytosolic or the mitochondrial fraction was mixed with 8 μl of 4× SDS sample buffer, containing 8% ME, boiled for 5 min, and kept at −20°C until use.

Western blotting was performed as previously described (6). The blots were probed with mAbs against cytochrome *c* (7H8.2C12; BD PharMingen) and caspase-8 (1C12; Cell Signaling Technology, Beverly, MA) or polyclonal Abs against Smac/DIABLO (Ab-1; Oncogene, San Diego, CA), apoptosis-inducing factor (AIF; H-300), cellular inhibitor of apoptosis protein (cIAP-1; H-83), cIAP-2 (H-85; all three from Santa Cruz Biotechnology, Santa Cruz, CA), Bax (BD PharMingen), manganese-containing superoxide dismutase (Stressgen, Canada), Bid, X-linked IAP (XIAP), caspase-3, and caspase-9 (Cell Signaling Technology). All mAbs were used at a final concentration of 1 μg/ml, and polyclonal Abs were used at a 1/1000 dilution. Anti-Omi/HtrA2 polyclonal Ab (11) was provided by Dr. S. M. Srinivasula (Philadelphia, PA) and was used at a 1/5000 dilution.

Measurement of caspase-specific enzymatic activity

Caspase activity was fluorometrically assessed using an assay modified from a previously described method (12). The neutrophils were lysed in the caspase assay lysis buffer (1% Triton X-100, 10 mM sodium pyrophosphate, protease inhibitor mixture (see above), and 2 mM DFP in 10 mM Tris, pH 7.5) at a concentration of 20×10^6 cells/ml for 30 min on ice. Thereafter, the lysates were kept at −70°C. Before use, the samples were thawed at 37°C, spun down at $22,000 \times g$ for 10 min, and the supernatants were collected. To assay enzymatic reactions, 50 μl of the neutrophil lysate (equivalent of $\sim 1 \times 10^6$ cells) was placed in a white 96-well plate (Costar, Cambridge, MA) and mixed with 100 μl of a fluorogenic substrate diluted in the reaction buffer (80% HEPES buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, and 1.2 mM K_2HPO_4, pH 7.4), 20% glycerol, and 5 mM DTT). The final concentration of each substrate was 50 μM. The substrates used were as follows (all from Alexis Biochemicals, San Diego, CA): Ac-DMQD-7-amino-4-methyl-coumarin (Ac-DMQD-AMC; caspase-3), Ac-IETD-AMC (caspase-8), and Ac-LEHD-AMC (capsase-9).

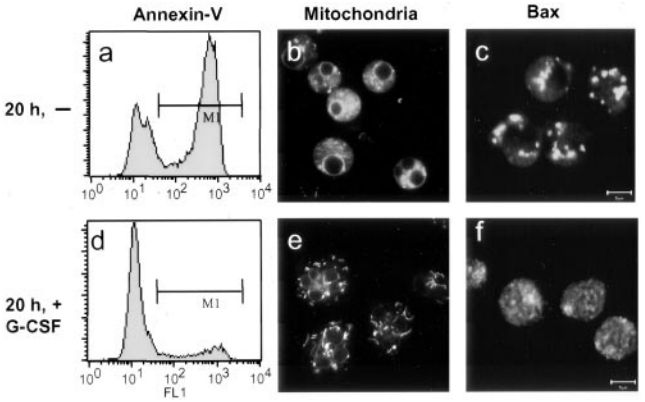

FIGURE 1. Events associated with neutrophil apoptosis and their prevention by G-CSF. After a 20-h culture without (*a–c*) or with 100 ng/ml G-CSF (*d–f*), neutrophils were labeled with annexin V-FITC (*a* and *d*), MitoTracker GreenFM (*b* and *e*), or anti-Bax polyclonal Ab (*c* and *f*), and analyzed by flow cytometry (*a* and *d*) or fluorescence microscopy (*b*, *c*, *e*, and *f*). M1 (*a* and *d*) marks apoptotic cells, which bind annexin V. Apoptotic cells showed clustered, degraded mitochondria (*b*) and aggregated Bax (*c*), whereas G-CSF-treated cells kept an accurate tubular shape of the mitochondria (*e*) and a punctate, cytoplasmically dispersed localization of Bax (*f*), which is typical for fresh intact cells (6). Bar = 5 μm. Representative images for four or five experiments are shown.

For a negative control (blank), substrates were mixed with 50 μl of caspase assay lysis buffer (without cells). Reactions were monitored by means of a HTS7000+ plate reader (PerkinElmer, Norwalk, CT) at 37°C, and the enzymatic activity was assessed after 2 h as maximum fluorescence (in relative fluorescence units) generated by the release of AMC (excitation, 405 nm; emission, 465 nm). The results were expressed as the percentage of enzymatic activity compared with that in untreated cultured neutrophils (see also Fig. 5).

Results

Novel markers to quantify neutrophil apoptosis

Previously, we have shown that in apoptotic human neutrophils, the mitochondria form clusters (Fig. 1*b*) to which the proapoptotic protein Bax is relocated (Fig. 1*c*) (6), whereas in fresh neutrophils the mitochondria have a tubular shape, and Bax displays a dispersed punctuate localization (not shown) (6). Moreover, the hemopoietic cytokine G-CSF can prevent these changes by keeping the original shape of the mitochondria intact (Fig. 1*e*) and preserving the punctate distribution of Bax (Fig. 1*f*). Thus, the mitochondrial and Bax staining patterns appear to be very distinct between apoptotic and intact cells. This clear difference stimulated us to quantify the proportion of cells with each phenotype and to correlate that to the well-known sign of apoptosis: annexin V binding. Using a fluorescence microscope, we counted the percentages of cells with clustered mitochondria (the phenotype shown in Fig. 1*b*) and aggregated Bax (the phenotype shown in Fig. 1*c*). In parallel, a flow cytometric analysis of the same cell suspensions was performed, and the fraction of annexin V^+ neutrophils was determined (Fig. 1, *a* and *d*, M1). The quantitative data are summarized in Fig. 2. Notably, the percentage of cells with either clumped mitochondria or clustered Bax closely correlated with the proportion of annexin V^+ neutrophils ($r > 0.9$ for both). This was true for neutrophils undergoing spontaneous apoptosis (a 20-h culture without additions), as well as for the G-CSF-induced survival (Fig. 2). All three parameters tested were hardly detectable in neutrophils before culturing (Fig. 2, 0 h). Routine morphological analysis of the May-Grünwald-Giemsa-stained cytospins confirmed these correlations (Fig. 2; apoptotic morphology was defined as rounding and separation of nuclei in apoptotic bodies, pronounced chromatin condensation, and cell shrinkage). Hence, the assessment of

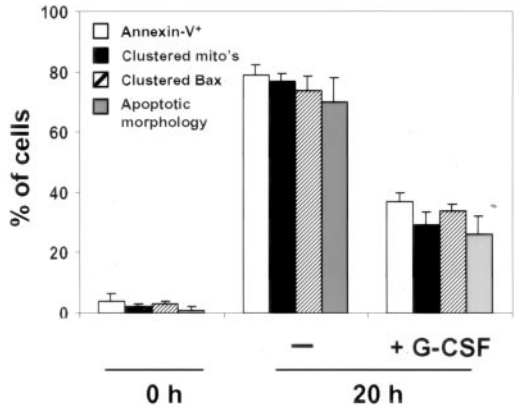

FIGURE 2. Events associated with neutrophil apoptosis and their prevention by G-CSF (quantitative data). Neutrophils before (0 h) or after a 20-h incubation without or with 100 ng/ml G-CSF were labeled and analyzed as described in Fig. 1. The bars represent the percentages of cells with the following features: □, annexin V^+ cells from M1 in Fig. 1, *a* and *d*; ■, cells with clustered mitochondria as in Fig. 1*b*; ▨, cells with clustered Bax as in Fig. 1*c*. ▨, The percentage of cells with typical apoptotic morphology (rounded nuclei and nuclei separated in apoptotic bodies, pronounced chromatin condensation, and cell shrinkage) counted in the May-Grünwald-Giemsa-stained cytospins by light microscopy. Shown are results (mean ± SEM) from four or five separate experiments.

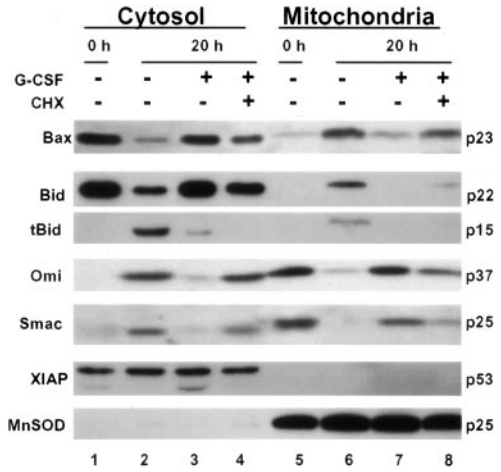

FIGURE 3. Western blot analysis of subcellular fractions of neutrophils cultured under various conditions. Using a digitonin-based subcellular fractionation, neutrophils were separated into cytosol-enriched (*lanes 1–4*) and mitochondria-enriched (*lanes 5–8*) fractions before culture (0 h; *lanes 1* and *5*) or after a 20-h culture without additions (*lanes 2* and *6*) or with 100 ng/ml G-CSF alone (*lanes 3* and *7*) or in combination with 5 μg/ml CHX (*lanes 4* and *8*). The fractions were subjected to SDS-PAGE. Western blot was performed with specific Abs for the indicated proteins. Each neutrophil fraction represents $\sim 2 \times 10^6$ cells. The exposition time in the tBid panel was extended compared with that in the Bid panel, because of reduced immunoreactivity of the cleaved form of Bid. The probe with anti-XIAP and anti-manganese-containing superoxide dismutase (MnSOD) Ab served as a reference for cytosol and mitochondria, respectively. The numbers on the *right* indicate the molecular weights of the corresponding proteins. Results represent three independent experiments.

mitochondrial morphology and localization patterns of Bax may serve as sensitive and reliable tools to measure apoptosis in human neutrophils.

G-CSF prevents truncation of Bid, Bid/Bax translocation to mitochondria, and subsequent mitochondrial leakage associated with neutrophil apoptosis

A growing amount of experimental data suggests that the mitochondria are critically involved in the apoptotic program of neutrophils (5–9, 13–15). In our recent report it was shown that, as in other cell types, the neutrophil mitochondria release a number of proapoptotic proteins into the cytosol upon TNF-α-induced apoptosis (7). This event is believed to induce and/or amplify the caspase cascade activation, eventually leading to apoptosis (10, 16, 17). The pro-survival effect of G-CSF has been circumstantially related to inhibition of mitochondria-dependent caspase-3 activation (6). The results presented above indicate that G-CSF blocks Bax relocalization to the mitochondria (Fig. 1, *c* and *f*, and Fig. 2), an event associated with permeabilization of the mitochondrial outer membrane and release of proapoptotic proteins (16, 17). Indeed, Western blot analysis of subcellular neutrophil fractions demonstrated that before culture Bax was predominantly present in the cytosol (Fig. 3, *lanes 1* and *5*), whereas in cultured neutrophils that have undergone spontaneous apoptosis, this protein was found in the mitochondria-enriched fraction (Fig. 3, *lanes 2* and *6*). G-CSF prevented this redistribution, trapping Bax in the cytoplasm (Fig. 3, *lanes 3* and *7*). These qualitative data support our quantitative estimation of Bax changes (Fig. 2).

Bax relocalization to mitochondria may be mediated by another proapoptotic Bcl-2 homologue, the Bcl-2 homology domain 3-only protein Bid (18, 19). Bid is activated by caspase-mediated proteolytic cleavage (truncation) of the original 22-kDa protein into a 15-kDa fragment (tBid), which is known to possess an increased pro-death activity (20–22). In intact neutrophils, only total Bid as a cytosolic protein was detectable (Fig. 3, Bid, *lane 1*). Spontaneous apoptosis in cultured cells led to appearance of tBid in the cytosol, which was absent in fresh cells (Fig. 3, tBid, *lanes 1* and *2*). Similar to Bax, the full-length Bid was detected in the mitochondrial fraction of apoptotic, but not fresh, cells (Fig. 3, Bid, *lanes 5* and *6*). Addition of G-CSF pronouncedly abrogated truncation of Bid, because it stayed largely uncleaved, with only a minute amount of detectable tBid. G-CSF also prevented the mitochondrial redistribution of total Bid, which remained in the cytosol (Fig. 3, Bid and tBid, *lanes 3* and *7*).

As mentioned above, Bid-induced activation of Bax and targeting of the mitochondria by Bid and Bax are important steps in mitochondrial permeabilization. To check whether the observed Bid and Bax apoptotic changes were accompanied by the release of proapoptotic factors from the mitochondria, we monitored the subcellular expression of Omi and Smac proteins, which have been shown to relocate from the mitochondria into the cytoplasm upon TNF-α-mediated neutrophil apoptosis (7). In fresh neutrophils, Omi and Smac were only present in the mitochondria (Fig. 3, *lane 5*), and the cytoplasm was free of those proteins (Fig. 3, *lane 1*). In contrast, apoptosis was associated with the liberation of Omi and Smac into the cytosol and a concomitant decrease in their signal in the mitochondrial fraction (Fig. 3, *lanes 2* and *6*). G-CSF suppressed the cytosolic release of both proteins, preserving their mitochondrial localization (Fig. 3, *lanes 3* and *7*). The role of cytochrome *c* in neutrophil apoptosis is questionable because of its scarcity (5, 7, 14), although a minor amount of cytochrome *c* was detectable in the cytosolic fraction of apoptotic cells by Western blot after overexposure of the film (7). Also, the proapoptotic mitochondrial protein AIF, which in other cell types has been shown to relocate to the nucleus upon apoptosis (17), was found in neutrophils to remain in the mitochondria regardless of apoptosis (7). This could be explained by the recent finding that AIF may be associated with the mitochondrial inner membrane (23). Taken together, these data suggest that in neutrophils the truncation of Bid

and Bid/Bax translocation to the mitochondria may contribute to the mitochondrial permeabilization and leakage of the apoptosis-related proteins, and that G-CSF is able to block these reactions.

G-CSF mediates inhibition of caspase processing and enzymatic activity

Activation of the cascade of caspase proteases is the major aim of the apoptotic machinery, because caspase enzymes are responsible for cleavage and degradation of important intracellular targets, inducing disassembly of a dying cell. The described events (Bid activation, Bid/Bax translocation to the mitochondria, and subsequent release of proapoptotic proteins) are the intermediate steps, which serve to induce and/or amplify caspase activation (10, 16, 17). The upstream caspase-8 and caspase-9 initiate extrinsic and intrinsic pathways of apoptosis, respectively, which converge at the level of the executioner caspase-3 activation. In intact neutrophils, all tested caspases were present as inactive full-length proenzymes (Fig. 4, *lane 1*). Apoptotic cells displayed cleavage (activation) of the caspases (Fig. 4, *lane 2*). In case of caspase-9, activation was detected by the decrease in total 47-kDa protein. The cleavage products of caspase-8 (p43/41 and p18) and caspase-3 (p17) processing were readily found in the lysates of apoptotic neutrophils together with a reduction in signal from the full-length proteins (Fig. 4, *lane 2*). The processing of the caspases was blocked by G-CSF (Fig. 4, *lane 3*). This agent almost completely prevented cleavage of caspase-9 and caspase-3 and, to a lesser extent, processing of caspase-8 (Fig. 4, *lane 3*). Interestingly, the expression of Bax, used as a control for protein loading, remained stable under all conditions tested (Fig. 4, Bax), although in apoptotic cells this protein underwent major changes in subcellular localization, being redistributed from the cytosol to the mitochondria (Fig. 1, *c* and *f*, and Fig. 3, Bax). Also the expressions of Omi (Fig. 4) and Smac (not shown) remained stable. These findings underscore the importance of post-transcriptional qualitative regulation of the apoptotic mediators in neutrophils, and experiments with the inhibitor of protein synthesis cycloheximide support that idea (see below).

To show that the cleavage of caspases really resulted in their activation, we measured specific caspase enzymatic activity in the neutrophil cell lysates. To this end, assays based on preferential substrate specificity of different caspases were used (24). The results are summarized in Fig. 5. As expected, the highest caspase activity was determined in apoptotic neutrophil preparations after a 20-h culture, which was set at 100% (for absolute values, see Fig. 5). In fresh cells (0 h) the enzymatic activity of the tested caspases was negligible. In neutrophils from the G-CSF-treated cultures the caspase enzymatic activity was reduced by 60–70% (Fig. 5), which is in agreement with the Western blot data showing diminished caspase cleavage under G-CSF treatment (Fig. 4, *lane 3*).

Differential effects of cycloheximide on G-CSF-mediated pro-survival reactions

It is known that the G-CSF antiapoptotic signaling is mediated by de novo-synthesized effectors, although they remain obscure (3, 6). In attempt to elucidate whether protein synthesis is required for the G-CSF-induced prevention of the described apoptotic reactions, we applied inhibition of protein synthesis by CHX during culture of the cells with G-CSF. In G-CSF-stimulated neutrophils treated with CHX, Bax translocation to the mitochondria and the release of Omi and Smac from the mitochondria occurred (Fig. 3, *lanes 4* and *8*), as it did in "normal" apoptotic cells (without G-CSF or CHX; Fig. 3, *lanes 2* and *6*). Moreover, in the presence of CHX, the processing of caspases, which was inhibited by G-CSF, was restored (Fig. 4, *lane 4*), and specific caspase enzymatic activity was increased and reached 70–90% of that measured in apoptotic untreated cells (Fig. 5). This was accompanied by reduced survival (~25% annexin V^+ cells in G-CSF alone vs ~60% annexin V^+ cells in G-CSF plus CHX; see Fig. 5). Despite the effect on survival, CHX did not block the G-CSF-induced prevention of Bid changes. In the presence of G-CSF, tBid was hardly detectable, in

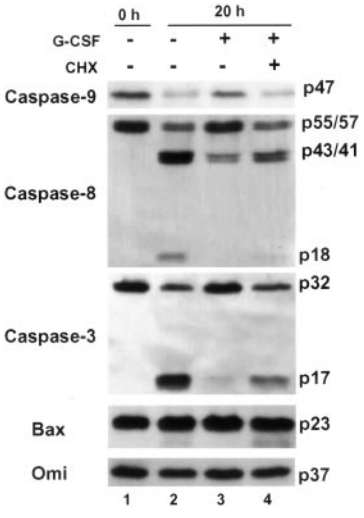

FIGURE 4. Processing of caspases in neutrophils. Total cell lysates were prepared from fresh neutrophils (0 h; *lane 1*) and from neutrophils cultured for 20 h without additions (*lane 2*) or with 100 ng/ml G-CSF alone (*lane 3*) or in combination with 5 μg/ml CHX (*lane 4*). Lysates of ~1 × 10^6 cells were subjected to SDS-PAGE, and Western blot was performed with specific Abs for the indicated proteins. The expression of Bax, which has been shown to be stable in neutrophils during culturing (6), was used as a control for protein loading. Results are representative of three independent experiments.

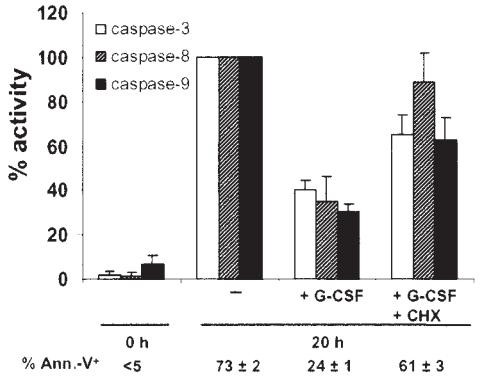

FIGURE 5. Specific enzymatic activities of caspase-3, -8, and -9 in neutrophils. Triton X-100 cell lysates obtained from neutrophils cultured as described in Fig. 4 were incubated with specific fluorogenic substrates for caspase-3 (Ac-DMQD-AMC; □), caspase-8 (Ac-IETD-AMC; ▨), and caspase-9 (Ac-LEHD-AMC; ■). The reactions were performed in a plate reader to monitor an increase in fluorescence after release of AMC. The maximum fluorescence (in relative fluorescence units) after a 2 h-incubation was used as a measure of caspase activity. The results are expressed as a percentage of activity (mean ± SEM; $n = 4$) relative to that in the cultured untreated neutrophil lysates, which was set at 100%. Absolute values for the individual caspase activities in those preparations were as follows (maximum relative fluorescence units ± SEM): caspase-3, 172 ± 11; caspase-8, 90 ± 10; and caspase-9, 93 ± 4. For comparison, the proportion of annexin V^+ (Ann.-V^+; apoptotic) neutrophils in the same preparation is shown (mean ± SEM; $n = 4$).

contrast to the untreated cultured neutrophils, and full-length Bid mainly resided in the cytosol regardless of CHX (Fig. 3, Bid and tBid). Obviously, G-CSF inhibited Bid activation by means of a pre-existing molecule(s). This differential dependence of the pro-survival events mediated by G-CSF on protein synthesis may explain the incomplete inhibition of G-CSF-induced survival by CHX. Bid truncation was also prevented in the presence of the general caspase inhibitor zVAD-fmk (not shown), whereas Bax translocation to the mitochondria and mitochondrial leakage were still observed under those circumstances despite inhibition of apoptosis (6).

Discussion

In the present study several important issues concerning the apoptotic process in primary human neutrophils have been addressed. We have identified apoptotic markers that could be used for quantitative analysis of neutrophil apoptosis. The unique tubular shape of the mitochondria in intact neutrophils and its dramatic change during apoptosis (formation of punctate clusters; see Fig. 1, *b* and *e*) provided an easy discrimination between live and apoptotic cells. This feature seems to be specific for neutrophils, because in other tested cell types, including eosinophils, lymphocytes, monocytes, and HL-60 cells, the mitochondria had a punctate appearance regardless of apoptosis (see, for instance, the HL-60 cell mitochondria in Ref. 7). Also, the subcellular redistribution and aggregation of Bax during neutrophil apoptosis proved to be a quantitative marker of neutrophil cell death (see Fig. 1, *c* and *f*). Both apoptotic features (mitochondrial changes and Bax translocation) were in close correlation with the traditional and well-known methods of apoptosis registration, such as annexin V staining and morphology (see Fig. 1, *a* and *d*, and Fig. 2). Importantly, mitochondrial clustering and Bax aggregation also occurred during TNF-α-induced neutrophil apoptosis (15), which means that these phenomena are universal and do not depend on the mechanism (spontaneous or induced) of apoptosis. Despite the availability of a broad spectrum of tools to study and register apoptosis, the new markers do not seem to be superfluous. In some instances a single parameter-based assessment of apoptosis can be unreliable (15, 25), and the described markers could be helpful in this respect.

Our present data not only provide useful tools, but also shed light on the mechanism of neutrophil apoptosis and the G-CSF-mediated pro-survival signaling, pointing to the mitochondria as integrative regulators (10, 16, 17). In support of fluorescence microscopy data, Western blot analysis has objectively shown that Bax translocates upon apoptosis from the cytosol to the mitochondria (see Fig. 3, Bax, *lanes 1, 2, 5,* and *6*). In intact cells Bax is present as a cytosolic protein, which targets the mitochondria once apoptosis is induced. There it can be integrated into the outer mitochondrial membrane and, forming pores, facilitates the release of proapoptotic mitochondrial constituents (19, 21, 26–28). As is generally assumed, this happens after another proapoptotic Bcl-2 homologue, the Bcl-2 homology domain 3-only protein Bid, interacts with Bax to trigger conformational changes in Bax, leading to its oligomerization and anchoring into the outer mitochondrial membrane (18, 19). Alternatively, as our data show, the full-length Bid may directly translocate to the mitochondria (see Fig. 3, Bid, *lanes 1, 2, 5,* and *6*), where it may cause effects similar to those of Bax (18). The Bid/Bax interaction appears to be critical for mitochondrial permeabilization, because in the mitochondria from Bax-deficient tumor cell lines, Bid-induced release of cytochrome *c* was minimal when Bid was added alone, but was dramatically increased when Bid and Bax were present together (18). In addition, a cell-free system has demonstrated that Bid, Bax, and mitochondrial lipids cooperate to form supramolecular openings in the outer mitochondrial membrane (29). Our present data suggest that this mechanism is also operative in primary human neutrophils, resulting in the release of proapoptotic mitochondrial factors, such as Smac/Diablo and Omi/HtrA2, into the cytosol. Moreover, inhibition of neutrophil apoptosis by G-CSF was associated with the prevention of both Bax and Bid redistribution to the mitochondria and release of the proapoptotic factors, underlining the importance of the mitochondrial death pathway in the control of neutrophil survival.

A number of studies in cell lines have established that the cleavage (truncation) of Bid enhances its pro-death properties; tBid targets the mitochondria and is capable of directly inducing cytochrome *c* release from purified mitochondria or, after overexpression, causing mitochondrial damage, cell shrinkage, and nuclear condensation (20–22). In this study we also observed Bid cleavage in neutrophils, which correlated with spontaneous apoptosis. However, tBid remained in the cytosol of apoptotic cells and hardly bound to the mitochondria (see Fig. 3, tBid), in contrast to the full-length protein (see Fig. 3, Bid). G-CSF prevented both Bid translocation to the mitochondria and Bid cleavage regardless of CHX, indicating that this effect was independent of protein synthesis. The latter finding was confirmed by the detection of Bid cleavage in apoptotic neutrophil-derived cytoplasts, which lack nuclei (6) (not shown).

As in other cell types, Bid cleavage in neutrophils was mediated by caspase activity and was prevented by zVAD-fmk. Besides a traditional view on Bid as a link between death receptors and the mitochondria, it has been shown that caspase-3-catalyzed cleavage of Bid distal to cytochrome *c* release may represent a feedback loop for the amplification of mitochondrial dysfunction during cytotoxic drug- and UV-induced apoptosis (30). Perhaps such a mechanism is operative in neutrophils. Yet caspase activity in itself was not sufficient for Bid truncation, as was evident during G-CSF plus CHX treatment, when Bid remained unprocessed even though caspases were active. Apparently, G-CSF produced a signal (not dependent on protein synthesis) that kept Bid intact. As G-CSF is known to influence multiple protein kinases, it is worthwhile to speculate that this effect of G-CSF could be mediated through regulation of the phosphorylation state of Bid, because phosphorylated Bid is resistant to caspase-8 cleavage in vitro (31, 32). This implies a differential dependence on protein synthesis of the G-CSF-induced pro-survival effects. However, despite the inhibition of Bid changes, the G-CSF- plus CHX-treated neutrophils still underwent apoptosis, displaying Bax redistribution to the mitochondria, release of Omi/HtrA2 and Smac/DIABLO, caspase activation, and annexin V binding, which were all similar to the changes seen in untreated cells undergoing spontaneous apoptosis. We may conclude that both the truncation and the relocation of Bid to the mitochondria are not strictly required for neutrophil apoptosis. In contrast, the interaction of Bid with Bax and the activation of Bax may represent a crucial element in the role of Bid in neutrophil cell death. Furthermore, a recent publication has demonstrated that Bid-deficient mice spontaneously develop myeloid hyperplasia over time that progresses to fatal chronic myelomonocytic leukemia, suggesting an important role for Bid in myeloid homeostasis (33).

Inhibition of Bax translocation to the mitochondria by G-CSF was dependent on de novo protein synthesis, but the critical molecules induced by G-CSF are as yet unknown. A good candidate might be an antiapoptotic Bcl-2 homologue, considering the possibility that it could prevent Bid-induced Bax activation or directly antagonize insertion of Bax, Bid, or their complexes into the mitochondrial membrane, as has been shown for Bcl-2 itself (not expressed in neutrophils (3, 4)) and Bcl-x_L (18–20, 29, 34). The latter might be of particular interest, because it is able to directly

interact with and inhibit both full-length and truncated Bid. However, the expression of Bcl-x_L in neutrophils is a matter of debate (3, 4). Another Bcl-2 homologue, Mcl-1, has been proposed to be involved in GM-CSF-induced neutrophil survival (3, 35, 36), because it is coimmunoprecipitated with Bax in surviving cells (36). However, our previous results did not reveal the changes in the expression of Mcl-1 (6). Neutrophils have also been shown to express mRNA for the Bcl-2-related protein A1 (Bfl-1), which is constitutively present in mature neutrophils and up-regulated by G-CSF (Ref. 3 and references therein). This may indicate that A1 participates in the regulation of neutrophil apoptosis, but the lack of data on the A1 protein level does not allow a more definitive statement at present.

Other factors that are involved in the regulation of apoptosis comprise a family of IAPs (37). In healthy cells, these cytosolic proteins may directly inhibit caspases, whereas in apoptotic cells IAPs undergo deactivation by Omi/HtrA2 and Smac/Diablo released from the mitochondria. Recent studies have shown that IAPs may be substrates for Omi/HtrA2 (38, 39). Neutrophils reportedly express XIAP, cIAP-1 and cIAP-2 (14, 40, 41). Kobayashi et al. (40) observed XIAP degradation during TNF-α/CHX-induced or spontaneous neutrophil apoptosis, which was mediated by calpain. In another study G-CSF-induced up-regulation of cIAP-2 coincided with increased neutrophil survival (41). In our experiments we did not find XIAP degradation in either the cytosolic fraction or total neutrophil lysates (see Fig. 3, XIAP). We also did not observe any change in cIAP-2 (and cIAP-1) expression on Western blot (not shown) regardless of the extent of apoptosis as measured after a 20-h culture. This could be explained by the differences in the experimental set-up, because, for instance, a modest up-regulation of cIAP-2 was reported in a short term culture with G-CSF (41), which could have disappeared after longer culturing such as that used in the present study. However, IAPs are not exclusively regulated by transcription, and they do not need to be degraded for inactivation. For instance, Smac/DIABLO binds XIAP (and probably other IAPs) in a manner that displaces caspases from XIAP (37). Our results favor this model of IAP activity control, because of the massive release of the IAP regulatory proteins, Omi/HtrA2 and Smac/DIABLO, from the mitochondria as well as activation of caspases upon neutrophil apoptosis in the absence of any cleavage of the tested IAPs.

Our present data suggest that the main result of G-CSF-mediated antiapoptotic reactions is keeping the mitochondria intact. Most likely, it is achieved by prevention of Bax translocation to the mitochondria through a newly synthesized protein(s). The mechanism of this translocation during the neutrophil senescence and spontaneous apoptosis as well as in other cell models of apoptosis induced by, for instance, UV irradiation, serum withdrawal, and some cytotoxic drugs remains unclear. A recent study has proposed caspase-2 to be an upstream initiator caspase required for Bax translocation to the mitochondria (42). However, neutrophils do not express caspase-2 (14) (N. A. Maianski, unpublished observations), and Bax translocation to the mitochondria is a caspase-independent event in these cells (6). Moreover, the mitochondrial dysfunction and cytochrome *c* release take place under caspase inhibition in purified mitochondria or living cells (43, 44), suggesting that other mechanisms are involved. Notably, neither Bax translocation nor mitochondrial leakage in itself is sufficient to support apoptosis under conditions of caspase inhibition. Apparently, a coordinate action of pre- and postmitochondrial effectors is required for the proper propagation of cell death. Perhaps, mitochondria themselves could generate signals that contribute not only to the propagation, but also to the initiation of cell death. For example, these organelles are capable of neoepitope expression, such as that recognized by Apo2.7 mAb, very early in the course of apoptosis with an as yet unknown biological meaning (45). The peculiarities of the neutrophil mitochondria, which have a defective respiration, but maintain transmembrane potential indicative of ongoing electron transport and a potential source of reactive oxygen metabolites (7), may add to the negative regulation of neutrophil survival. In agreement with this hypothesis is the finding that apoptosis of neutrophils (in contrast to other cell types) is dramatically reduced under hypoxic conditions (3, 46, 47), and caspase-independent neutrophil cell death is mediated by mitochondria-derived reactive oxygen species (15).

In conclusion, our present data bear on the mechanism of neutrophil apoptosis and its modulation by G-CSF. Our results underline an integrative role of the mitochondria in the regulation of this process. A better understanding of mechanisms underlying neutrophil cell death would help in the understanding of neutrophil physiology and contribute to the search for new approaches for handling of pathology related to disturbances in neutrophil apoptosis.

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