Temperature-dependent Arrest of Neutrophil Apoptosis

FAILURE OF Bax INSERTION INTO MITOCHONDRIA AT 15 °C PREVENTS THE RELEASE OF CYTOCHROME c

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Apoptosis is essential for the resolution of neutrophilic inflammation. To define the mechanisms triggering the execution phase of apoptosis we developed and utilized a model in which culture of human neutrophils at 15 °C for 20 h arrested apoptosis and subsequent warming to 37 °C triggered a synchronous burst of apoptosis. Treatment of 15 °C cultured neutrophils with the pan-caspase inhibitor zVAD-fmk just before warming to 37 °C inhibited the morphological changes associated with apoptosis, but did not prevent the insertion of the proapoptotic protein Bax into mitochondria nor the inhibition of secretion and the externalization of phosphatidyserine, indices of neutrophil apoptosis. In both intact neutrophils and a cell-free extract, cytochrome c released from mitochondria induced proteolytic cleavage of procaspase-3. At 15 °C the binding of Bax to mitochondria was uncoupled from Bax insertion into the mitochondrial membrane required for the release of cytochrome c. Apoptosis was also inhibited by low pH during warming to 37 °C, suggesting that changes to the conformation of Bax, necessary for membrane insertion, were being inhibited. Bax insertion was only sensitive to zVAD-fmk when added at the start of the 15 °C culture period, suggesting that a cytoplasmic substrate of the effector caspases may mediate in the mechanism of Bax insertion into mitochondria.

Successful resolution of the inflammatory response requires that granulocytes, neutrophils and eosinophils, trigger an intracellular program for “silent” self-destruction called apoptosis (1, 2). If cell-death occurs by necrosis the cytotoxic cargo of granulocyte molecules is released, inducing tissue damage and chronic inflammation and stimulating the release of proinflammatory macrophage products to promote inflammation by other routes. The apoptotic program induces the morphological hallmarks of apoptosis, nuclear condensation and cell shrinkage, and shuts down the secretory potential of granulocytes (3). Changes to the molecular profile of the surface of apoptotic neutrophils target them for phagocytosis by macrophages (4, 5), without release of proinflammatory mediators from macrophages (6). Proinflammatory mediators and cytokines, such as granulocyte-macrophage colony-stimulating factor, lipopolysaccharide, C5a, or an hypoxic environment at the site of inflammation can prolong the functional life span of granulocytes by delaying apoptosis (7, 8) through increased expression of the anti-apoptotic proteins Bcl-XL (9) and Mcl-1 (10). The molecular mechanism triggering the execution phase of apoptosis in granulocytes is unknown but activation of tumor necrosis factor α and Fas (CD95) cell surface receptors increase expression of the proapoptotic proteins Bax and procaspase-3 (9–14). Both the p38 mitogen-activated protein kinase and p42/p44 mitogen-activated protein kinase, and the transcription factor nuclear factor-κB, regulate the granulocyte apoptotic program (15, 16), the signals transduced by these pathways converging to induce activation of procaspase-3 (17–20).

In mammalian cells the execution phase of apoptosis involves either the direct activation of procaspase-3 by caspase-8 (21), or indirect activation of procaspase-3 through the release of apoptosis-inducing factors, such as cytochrome c, from mitochondria (22–30). The proapoptotic Bcl-2 family member Bax is a soluble, monomeric, cytoplasmic protein (31) that inserts an hydrophobic C-terminal membrane-spanning domain into mitochondria (32, 33), inducing release of cytochrome c (31–35), triggering the activation of caspase-3 (36–38) and the execution phase of apoptosis. Bax dimerization (39), the addition of recombinant Bax to isolated mitochondria (36) or overexpression of Bax (40) has also been shown to induce the release of cytochrome c. The mechanism by which cytochrome c is translocated from mitochondria into the cytoplasm is controversial (41). However, once in the cytoplasm cytochrome c complexes with apoptosis-protective activating factor 1 (Apaf-1) and procaspase-9 (26) to form a protein complex the “apoptosome” (42). In the presence of dATP this complex induces the proteolytic cleavage and activation of procaspase-3 that triggers a downstream cascade of caspase activity (43). It has been reported that after differentiation and maturation neutrophils have a reduced number of phenotypically atypical mitochondria, obtaining ATP predominantly by glycolysis (10). Thus, whether mitochondria play a role in triggering neutrophil apoptosis remains to be established.

Here we show that peripheral blood neutrophils cultured in vitro at 15 °C for 20 h failed to induce the execution phase of apoptosis until warmed to 37 °C, when they showed a synchronous burst of apoptosis. In temperature-arrested neutrophils endogenous Bax showed peripheral binding to mitochondria but failed to induce activation of caspase-3 and apoptosis. On warming to 37 °C Bax inserted into the neutrophil membranes with concomitant proteolytic cleavage of procaspase-3 and induction of apoptosis. In both intact neutrophils and cell-free...
neutrophil extracts we show that the proteolytic cleavage of procaspase-3 is induced by translocation of cytochrome c into the cytoplasm. Analysis of plasma membrane events showed that externalization of phosphatidylserine and the inhibition of secretion were uncoupled from the activation of caspase-3, when the pan-caspase inhibitor benzoylcarboxyvalyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk) was added to 15 °C cultured neutrophils before warming them to 37 °C. Under these conditions the caspase inhibitor did not prevent Bax insertion into mitochondria when the cells were warmed from 15 to 37 °C. However, surprisingly, Bax insertion was inhibited if zVAD-fmk was added to neutrophils at the start of their incubation at 15 °C. This experimental model of apoptosis has provided insights into the molecular mechanisms that trigger the execution phase of neutrophil apoptosis.

EXPERIMENTAL PROCEDURES

Granulocyte Isolation and Culture—Neutrophils were purified on gradients of Percoll® from Amersham Pharmacia Biotech (Bucks, United Kingdom). They were cultured in Tuf-Tainer® Teflon® pots from Pierce & Warriner Ltd. (Chester, UK) at 5 × 10^6 cells/ml in growth medium containing: Iscove’s modified Dulbecco’s medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) autologous serum. Culture at 15 °C was in growth medium containing: 25 mM Hepes-NaOH, pH 7, 0.2% (w/v) endotoxin-free bovine serum albumin (BSA), and 20 μg/ml cycloheximide. These were all other chemicals were from Sigma. Neutrophil preparations were 98% pure with <2% eosinophil contamination (16). Granulocytes from atopic donors were used to purify eosinophils by a negative selection procedure (16). Incubations with zVAD-fmk from Ultralife Corp. (Vienna, Austria), was used at 1:200 dilution (5 μg/ml nigericin, in a high-potassium medium containing: 10 mM Hepes-NaOH, pH 7.4, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM MgCl₂, 5 mM EGTA, 1 mM dithiothreitol, supplemented with dATP (20 μM) and inhibitors where appropriate. Incubations were stopped by transferring samples to ice and adding 20 μl of 50 mM Tris-HCl, pH 8.0, 4 mM NaCl, 1% (v/v) deoxycholate, 1% (v/v) Nonidet P-40, 5 mM EDTA, containing protease inhibitor mixture (lysis buffer) for 30 min.

Isolation of Mitochondria—Rat liver was washed in 10 mM Pipes-NaOH, pH 7.2, 0.25 mM sucrose, 2 mM EDTA and protease inhibitor mixture then filtered through a stainless steel sieve (150 μm aperture; Endecots Ltd., London) to break cells (46). The mitochondria were isolated from a post-nuclear supernatant as described previously and washed in the assay dilution buffer described above (47).

SDS-PAGE and Immunoblotting—Assay samples (40 μl), treated with ice-cold lysis buffer for 0.5 (v/v) were solubilized with 80 μl of 2 × SDS-PAGE sample buffer (46) at 95 °C for 10 min, then treated with 1 mM dithiothreitol, cooled, and treated with 10 mM iodoacetamide. The proteins were separated on 12% (w/v) polyacrylamide gels and electrophoretically transferred to nitrocellulose (46). The blots were probed with monoclonal antibodies to procaspase-3 (clone 19) and procaspase-7 (clone 51) from Transduction Laboratories (Lexington, KY) and used as a positive control. We also used an anti-human Bax antibody (clone B40), cytochrome c (clone 7H8.2C12), and polyclonal human Bax (13666E) all from PharMingen (San Diego, CA), at dilutions of 1:500–1:1000. A hybridoma supernatant to poly(ADP-ribose)polymerase (PARP), used at 1:500 dilution, was a gift from Saito Aouchi (Laboratory of Molecular Biology, Cambridge, UK). The horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgGs from Kirkegaard & Perry Labs (Gaithersburg, MD) were used at 1:1000 (v/v). The level of protein loading and nonspecific proteolysis were monitored by Ponceau S staining (46). Tubulin and actin were assayed with anti-bovine α-tubulin monoclonal antibody (236-10501) from Molecular Probes and a monoclonal antibody to actin; a gift from Simon Brown (Center for Inflammation Research, University of Edinburgh).

Confocal Microscopy—Neutrophils (10^6 cells in 100 μl of medium) were cytocentrifuged (300 rpm for 3 min) onto 1.5 × 22 × 22-mm glass coverslips and fixed in methanol-free 3% (w/v) p-formaldehyde/phosphate-buffered saline and processed for immunofluorescence microscopy as described previously (46). The fixed cells were permeabilized with 0.1% (w/v) Triton X-100 and nonspecific binding sites blocked for 1 h with 0.2% (w/v) fish skin gelatin and 20% (v/v) sheep serum in phosphate-buffered saline. A monoclonal antibody to mitochondrial heat shock protein 70 (mHSP70) from Affinity Bioreagents Inc. (Golden, CO) and a rabbit polyclonal antibody to ubiquinol-cytochrome c oxidoreductase (complex III) produced by Herman Schagger (University of Frankfurt-am-Main), were used at 1:200 dilution to stain mitochondria.

The monoclonal antibody to human Bax was used at 1:200 dilution. The secondary antibodies used at 1:400 dilution were Alexa® 488 (green) and Alexa® 568 (red) goat anti-rabbit (highly cross-adsorbed) and the nucleic acid stain TOPRO-3® (8 μM) from Molecular Probes. Cells were observed using a ×63 water immersion objective lens with a numerical aperture of 1.2 on a Leica TCS NT confocal laser scanning microscope system (Heidelberg, GMBH). Single optical sections of the images captured with Leica TCS software were digitally processed using Adobe Photoshop 5.02 and Paint Shop Pro 4.
RESULTS

Neutrophil Apoptosis Is Arrested at 15 °C—Neutrophils from peripheral blood can be maintained in culture at 37 °C for several hours in autologous serum before asynchronously undergoing apoptosis (Fig. 1A, closed squares). Apoptosis was quantified using annexin V-FITC binding to externalized phosphatidylserine and morphological counting of pyknotic nuclei (16). A synchronous commitment to apoptosis has previously been induced in cells by using cell-free systems (28, 47–49). Dividing cells, for example, blocked at cell cycle checkpoints provide an homogeneous cytosol (49). Although neutrophils are terminally differentiated (post-replicative) cells, we synchronized their commitment to the execution phase of apoptosis by exposure to low temperature.

Low temperature blocks intracellular pathways that rely on membrane fission and fusion, as exemplified by vesicular transport (50, 51). In neutrophils (Fig. 1A, open circles) or eosinophils (data not shown) cultured at 15 °C, apoptosis was arrested suggesting that a membrane-associated event required for apoptosis was inhibited. In contrast, culture of HL-60 promyelocytic leukemia cells at 15 °C induced apoptosis (data not shown) as described previously for these and many other dividing mammalian cells (52), suggesting that there may be cell-type or differentiation state-dependent pathways for apoptosis. The rate of neutrophil apoptosis at 37 °C was accelerated by treatment with cycloheximide (Fig. 1A, closed circles). In addition to preventing the translation of new proteins in the cytoplasm, cycloheximide can induce apoptosis through FADD-dependent mechanisms downstream of cell-surface Fas death receptors (53). However, when we cultured neutrophils at 15 °C with cycloheximide there was no increase in the rate of apoptosis (Fig. 1A, open circles). Treatment of 15 °C cultured neutrophils with tumor necrosis factor α (16) did not induce apoptosis (data not shown), suggesting that the block to the induction of the execution phase of apoptosis was downstream of these plasma membrane-associated events.

Temperature Shift to 37 °C Triggers Synchronous Apoptosis—The low temperature arrest of neutrophil apoptosis was reversed by re-warming neutrophils, cultured for 20 h at 15 °C, to 37 °C. There was reorganization of the cytoskeleton associated with cell polarization (shape-change), monitored by flow cytometry, that showed cytoskeletal integrity had been maintained at 15 °C (data not shown). This polarization of the neutrophils was followed by a burst of synchronous apoptosis (Fig. 1B, closed circles). The initial rate of apoptosis was 10-fold greater than the rate of constitutive apoptosis in neutrophils maintained at 37 °C (Fig. 1A, closed squares), and by 2 h after warming 80–90% of the cells were apoptotic (Fig. 1B, closed circles). Neutrophils maintained for a further 2 h at 15 °C showed no shape change and no increase in their rate of apoptosis (Fig. 1B, open circles). By culturing neutrophils in medium containing BSA and cycloheximide we removed experimental variables induced by serum factors and translation of mRNA into new protein.

The accelerated rate of neutrophil apoptosis at 37 °C, following preincubation at 15 °C, depended on the period of time neutrophils had been cultured at 15 °C. It was not a cold-shock response, as previously shown for lymphocytes (54). We demonstrated this by maintaining cells at 15 °C for increasing periods of time before warming them to 37 °C and estimating the initial rate of apoptosis over a 1.5-h period as shown in Fig. 1B (closed circles). For the first 6 h in culture there was no increase in the initial rate of apoptosis (Fig. 1C). However, as the cells were cultured for longer periods at 15 °C there was an increase in the initial rate of apoptosis on warming (Fig. 1C). These data suggested that there was a time- and temperature-

FIG. 1. Human neutrophil apoptosis was arrested at 15 °C and warming to 37 °C induced a rapid and synchronous apoptosis. Neutrophils were cultured in medium supplemented with: A, 0.2% BSA and 20 μg/ml cycloheximide at 15 °C (●) and 37 °C (○), an incubation with 10% (v/v) autologous serum, without cycloheximide at 37 °C is also shown (■). Apoptosis was assessed by annexin V-FITC binding analyzed by flow cytometry. B, neutrophils cultured and analyzed as described in A were held at 15 °C for 20 h (○), and subsequently warmed to 37 °C (●). C, neutrophils were cultured at 15 °C as described in A and at 1-h intervals harvested and warmed to 37 °C for 1.5 h as described in B to assess the initial rate of apoptosis (single experiment). D, DNA, extracted from 5 × 10⁶ neutrophils cultured at 15 °C for 20 h and warmed to 37 °C as described in B, was separated in agarose gels and stained with ethidium bromide. A 1-kilobase ladder of DNA standards (Std) is shown. Annexin V-FITC binding was used to assess apoptosis and the percentage (%) apoptosis for each sample is shown.
FIG. 2. zVAD-fmk inhibited morphological apoptosis but not the externalization of phosphatidylserine that is inhibited by staurosporine but not okadaic acid. Effects of the global caspase inhibitor zVAD-fmk on the triggering of neutrophil apoptosis. A, neutrophils were cultured, as described in the legend to Fig. 1A, at 15 °C for 20 h (C, □) and warmed to 37 °C (E, ■) and apoptosis estimated by morphological counting. The neutrophils were treated with 100 μM zVAD-fmk for 15 min (□, ■) prior to warming to 37 °C and control neutrophils were mock treated with Me2SO (C, ○). B, DNA fragmentation in the 15 and 37 °C neutrophils shown in A was analyzed by TUNEL22 and flow cytometry. C, the externalization of phosphatidylserine at the surface of the neutrophil plasma membrane at 15 °C (□, ■) and 37 °C (○, ■) was estimated by annexin V-FITC binding for the samples shown in A above. D, staurosporine inhibited phosphatidylserine externalization. Apoptosis, assessed by morphological counting for neutrophils warmed from 15 °C (□) to 37 °C (○) was not inhibited by 2 μM staurosporine treatment of 15 °C cultured neutrophils (□) for 1 h prior to warming to 37 °C (■). E, however, the externalization of phosphatidylserine, assessed by annexin V-FITC-binding, at 15 °C (○), and after warming to 37 °C (■) was inhibited by treatment with staurosporine (□, ■). F, neutrophils were cultured at 15 °C for 20 h (□) and before warming to 37 °C (■) they were treated at 15 °C (□) for 1 h with the 1 μM okadaic acid then warmed to 37 °C (■) and showed no inhibition of the triggering of apoptosis assessed by annexin V-FITC binding.

15 °C Inhibits Bax-insertion and Arrests Neutrophil Apoptosis

15 °C Arrest Is Proximal to Caspase-3 Activation. To establish whether inhibition of neutrophil apoptosis at 15 °C (Fig. 2A, open circles) was upstream of caspase-3 activation and apoptosis at 37 °C (Fig. 2A, closed circles), 15 °C cultured neutrophils were treated with 100 μM zVAD-fmk (18) (Fig. 2A, open squares) for 15 min before warming to 37 °C. zVAD-fmk inhibited chromatin condensation and the formation of pyknotic nuclei when neutrophils were warmed from 15 to 37 °C (Fig. 2A, closed squares). DNA fragmentation at 37 °C (Fig. 2B, closed circles), indicative of caspase-3 activation, was also inhibited (Fig. 2B, closed squares) by zVAD-fmk. Phosphatidylserine was still translocated to the cell surface in the presence of zVAD-fmk (Fig. 2C, closed squares), although the kinetics of translocation were significantly different from the untreated neutrophils (Fig. 2C, closed squares). Phosphatidylserine externalization has been linked to caspase-3 activity (19, 55–57) so their uncoupling was surprising. However, two other plasma membrane hallmarks of apoptosis phagocytosis (57) and regulated secretion2 were also uncoupled from caspase-3 by zVAD-fmk. The protein kinase inhibitor staurosporine together with cycloheximide induces apoptosis in many cells (58). However, staurosporine did not stimulate apoptosis at 15 °C (Fig. 2D, open squares) nor did it accelerate the induction of morphological apoptosis when neutrophils were warmed to 37 °C (Fig. 2D, closed squares). Phosphatidylserine, detected on the cell surface by annexin-V-FITC after warming to 37 °C (Fig. 2E, closed circles), was not detected in the presence of staurosporine (Fig. 2E, closed squares), suggesting that translocation of this phospholipid to the cell surface may rely on a critical phosphorylation event. The inhibition of mitochondrial respiration also blocks phosphatidylserine externalization in apoptotic U937 and THP-1 cells, suggesting that this may be an energy-dependent event (57). Okadaic acid, an inhibitor of phosphatases 1 and 2A (46) had no effect on the induction of apoptosis in 15 °C cultured neutrophils warmed to 37 °C (Fig. 2F, closed squares). This suggested that caspase activation might not be modulated by phosphorylation events in this experimental model. A number of other agents that induce neutrophil apoptosis, such as the nuclear factor-xB inhibitor gliotoxin (16) and the phosphatidylinositol kinase inhibitor wortmannin, had no effect on the rate of apoptosis in 15 °C cultured neutrophils (data not shown). Our results suggest that the temperature-dependent arrest of apoptosis in neutrophils was not due to signal transduction nor gene transcription events.

A Low Intracellular pH Inhibits the Triggering of Apoptosis—Acid conditions at sites of inflammation inhibit neutrophil apoptosis (59), but conversely, low pH has also been implicated in triggering apoptosis in many cells (60, 61). The pH of freshly isolated neutrophils was 7.1, equivalent to the “set point” for resting cells in culture when measured by accumulation of carboxy-seminaphthorhodafluor-1 (SNARF-1), a fluorescent probe whose emission changes with pH (61–63). The pH of neutrophils incubated at 15 °C for 20 h was 6.8–7.0 (Fig. 3A, open circles). However, following the induction of apoptosis (assayed by annexin-V-binding and morphology, Fig. 3, B and...
Acid pH arrested the triggering of apoptosis following a shift in temperature from 15 °C to 37 °C. A, neutrophils were cultured at 15 °C for 20 h then either maintained at an external pH of 7.2 at 15 °C (∇) or 37 °C (●) for 1.5 h, or clamped at pH 6.2 in a high-potassium buffer containing nigericin to maintain a pH of 6.2 when cultured at 15 °C (△) or warmed to 37 °C (●) for 1.5 h or exposed to an external pH of 6.2, in the absence of any added nigericin and potassium, at 15 °C (◇) or 37 °C (▲) for neutrophils exposed to an external pH of 6.2 (∆, ▲) or clamped with nigericin and potassium (pH 6.2 N). B, apoptosis for the samples shown in A was assessed by annexin V-FITC binding at 15 °C (∇, △, ◇, ▲) and 37 °C (●, ▲) for neutrophils exposed to an external pH of 7.2 (∆, ▲) or warmed to 37 °C (∇, ▲) for neutrophils exposed to an external pH of 6.2 (∆, ▲) or clamped with nigericin and potassium (pH 6.2 N). D, apoptosis estimated by annexin-V-FITC binding for neutrophils cultured at 15 °C for 20 h and either held at 15 °C (◇) or warmed to 37 °C (●) and their pH dropped to 6.2 (△, ▲) or 6.2 (∆, ▲) in a high-potassium buffer containing nigericin.

37 °C for 2 h, proteolytically cleaved procaspase-3 in a time-dependent manner (Fig. 4B, upper panel, lanes 1–11) that correlated with estimates of apoptosis by counting of pyknotic nuclei and annexin V-FITC binding (Fig. 2, A and C). Neutrophils held for a further 2 h at 15 °C showed no proteolytic processing of procaspase-3 (Fig. 4B, lower panel, lanes 1–11).

Proapoptotic Events Can Be Detected in Neutrophil Cytosols—To identify the molecular events leading to the proteolytic cleavage and activation of neutrophil procaspase-3, cytosols were prepared from cultures of neutrophils maintained at 15 °C for 20 h. The neutrophils were homogenized in buffered sucrose and fractionated using a two-step ultracentrifugation procedure (see “Experimental Procedures”) to minimize damage to organelles, particularly secretory granules. Elastase, a secretory granule marker, sedimented with membrane fractions (data not shown), while procaspase-3 remained in the cytosol (Fig. 5A, lane 1). Neutrophils cultured at 15 °C and warmed to 37 °C for 1.5 h before preparing the cytosol did not contain procaspase-3 (Fig. 5A, lane 2). The proteolytic cleavage of procaspase-3, observed in unbroken 15 °C cultured neutrophils warmed to 37 °C (Fig. 4B, lanes 5–11), had presumably been triggered by apoptosis-inducing factors released from the membranes into the cytoplasm before the cells had been homogenized. Significantly, when cytosols from 15 °C cultured neutrophils were warmed to 37 °C in the presence of dATP, a cofactor involved in the activation of the apoptosisosome (42), procaspase-3 was not proteolytically cleaved (Fig. 5A, lane 5) and was present in an amount comparable to the zVAD-fmk-treated controls (Fig. 5A, lane 6). These data suggested that apoptosis-inducing factors were missing from the cytosol and had been removed with the membrane fraction. The fraction of cytosol from the small number of contaminating apoptotic cells did not catalyze a significant rate of proteolytic cleavage of procaspase-3.

Mitochondria have been shown to play a key role in the control and amplification of apoptotic signals (23, 42). Cytosols from Xenopus laevis eggs only trigger apoptosis when membrane fractions enriched in mitochondria are added (25, 27, 48). When rat liver mitochondria were added to cytosols isolated

C), by increasing the temperature to 37 °C the pH dropped to 6.4 (Fig. 3A, closed circles) as apoptosis progressed (Fig. 3B, closed circles), consistent with previous measurements of acidic pH during apoptosis (60, 61). However, clamping neutrophils at pH 6.2, with nigericin and high K+ (Fig. 3A, squares) did not trigger apoptosis at 15 °C (Fig. 3B, open squares, and C) or at 37 °C (Fig. 3B, closed squares, and C). This suggested that acid pH alone was not a sufficient trigger for apoptosis as previously suggested (60, 61). Neutrophils cultured in growth medium buffered at pH 6.2, in the absence of nigericin (Fig. 3A, triangles), did not induce apoptosis at 15 °C (Fig. 3B, open triangles, and C) nor on warming to 37 °C (Fig. 3B, closed triangles, and C). This in vitro response to low pH appears to mimic the arrest of neutrophil apoptosis at inflammatory foci where the pH has dropped below 7 (59). When the pH of 15 °C cultured neutrophils was clamped at 7.2, the rate of apoptosis was significantly greater when the cells were warmed to 37 °C (Fig. 3D, squares) than in cells clamped at pH 6.2 (Fig. 3D, closed triangles). However, apoptosis was still not as efficient as in the untreated cells (Fig. 3D, closed circles). This result is, however, consistent with reports that suggest an alkaline pH transient is necessary to trigger the execution phase of apoptosis (64).

Neutrophil Apoptosis Is Correlated with Procaspase-3 Cleavage—Procaspase-3 cleavage is required for neutrophil apoptosis (65), but was not detected by immunoblotting in freshly isolated human neutrophils (Fig. 4A, lane 2). However, treatment of neutrophils with diisopropyl fluorophosphate, a serine protease inhibitor, before solubilization at 0 °C in a nondenaturing lysis buffer and SDS sample buffer for PAGE, prevented nonspecific proteolytic cleavage of procaspase-3 (Fig. 4A, lane 3). Neutrophils were treated with diisopropyl fluorophosphate before isolating cytosols, but while this was not absolutely necessary for 15 °C cultured neutrophils (Fig. 4A, lanes 4 and 5), diisopropyl fluorophosphate treatment did allow detection of endogenous procaspase-7 and procaspase-8 by immunoblotting. We have been unable to detect procaspase-9 (data not shown).

Neutrophils cultured at 15 °C for 20 h, then warmed to
from 15 °C cultured neutrophils, supplemented with dATP then incubated at 15 °C for 2 h, immunoblotting revealed no significant proteolytic processing of procaspase-3 (Fig. 5B, lanes 1–5). The degree of proteolytic processing was compared with 15 °C cytosols pretreated with zVAD-fmk (Fig. 5B, lane 6) and with 15 °C cytosols without added mitochondria (Fig. 5B, lane 8). In cytosols containing dATP and mitochondria permeabilized with Triton X-100, to release apoptosis-inducing factors, an efficient proteolytic cleavage of procaspase-3 was observed by 2 h at either 15 °C or 37 °C (Fig. 5B, lanes 7 and 14). Cytosol from 15 °C neutrophils warmed to 37 °C with rat liver mitochondria, and dATP induced proteolytic cleavage of procaspase-3 in a time-dependent manner (Fig. 5B, lanes 9–12). The protease activity was inhibited by zVAD-fmk (Fig. 5B, lane 13) and was dependent upon the addition of mitochondria at 37 °C (Fig. 5B, lane 15).

The involvement of membrane-associated events in the triggering of neutrophil apoptosis was also suggested by analysis of the rate of apoptosis as a function of temperature (Fig. 5C). There was a sharp decline in the rate of apoptosis below 20 °C and an Arrhenius plot of this data (not shown) (66) showed that the temperature dependence was biphasic. As the temperature drops to 15 °C reduction in the fluidity of the membrane lipid may affect the behavior of membrane-associated proteins (50, 51, 66). Mitochondrial anion channels have also been shown to respond to lowered temperature by changing their probability of being open, a parameter that is also affected by changes in pH (67). Thus, in our cell-free assay low temperature maintained the segregation of apoptosis-inducing factors within mitochondria, preventing apoptosis activation.

**Cytochrome c Induces Procaspase-3 Cleavage**—When horse heart cytochrome c and dATP were added to cytosol from 15 °C cultured neutrophils and incubated at 15 or 37 °C for 1 h, proteolytic cleavage of procaspase-3 was induced (Fig. 5D, lanes 2 and 4). The kinetics of procaspase-3 proteolytic cleavage were dependent on the concentration of cytochrome c, being complete between 10 and 100 ng of cytochrome c/80 μg of cytosol protein after 1 h at 37 °C. Treatment of the neutrophil cytosol with 100 μM zVAD-fmk prior to the addition of cytochrome c prevented proteolytic cleavage of procaspase-3 at 15 and 37 °C (Fig. 5D, lanes 3 and 5). Since an efficient proteolytic cleavage of procaspase-3 occurred at 15 °C the arrest of apoptosis was unlikely to be a consequence of the failure of the apoptosome proteins to undergo conformational changes at low temperature (68).

**Poly(ADP-ribose)polymerase Is Cleaved by 15 °C Cytosols**—Neutrophil cytosols containing cytochrome c and dATP produced active caspase-3 that processed HL-60 PARP (116 kDa), not present in mature neutrophils (17), to an 85-kDa polypeptide fragment after a 1.5-h incubation at 15 °C (Fig. 6, lower panel, lane 3). This proteolytic cleavage was inhibited by zVAD-fmk (Fig. 6, lower panel, lane 4). There was no apparent proteolysis of procaspase-3 under these conditions (Fig. 6, upper panel, lane 3). There was variability between cytosol preparations, and under similar conditions procaspase-3 was fully processed (see Fig. 5D, lane 2). At 37 °C there was complete proteolytic cleavage of procaspase-3 (Fig. 6, upper panel, lane 5) with concomitant proteolytic cleavage of PARP (Fig. 6, lower panel, lane 5); again this was inhibited by zVAD-fmk (Fig. 6, lower panel, lane 6). No proteolytic processing of neutrophil procaspase-3 or HL-60-PARP by endogenous proteases was detected in the absence of cytochrome c (Fig. 6, upper panel, lane 1, and lower panel, lane 7, respectively).

**Release of Cytochrome c from Neutrophil Mitochondria Occurs at 37 but not 15 °C**—Cytochrome c induced the proteolytic cleavage of endogenous neutrophil procaspase-3 but there was the possibility that this was only a property of the cell-free assay, particularly since we were unable to detect procaspase-9, a component of the apoptosome (42). To monitor translocation of cytochrome c from mitochondria into the cytoplasm of intact neutrophils, warmed from 15 to 37 °C, we separated post-nuclear supernatants into membrane and cytosol fractions. Cytochrome c was immunoprecipitated from the fractions with a monoclonal antibody to a native cytochrome c epitope and identified by immunoblotting with an anti-cytochrome c antibody that recognized SDS-denatured cytochrome c. Neutrophil cytochrome c (Fig. 7, lane 3) co-migrated with cytochrome c from both horse heart and HeLa cells (Fig. 7, lanes 1 and 2). In neutrophils held at 15 °C, cytochrome c remained with the membrane fraction (Fig. 7, lanes 4 and 5), but on warming to 37 °C cytochrome c was translocated to the cytosol (Fig. 7, lanes 7 and 8). Thus neutrophils released endogenous cytochrome c from mitochondria into the cytoplasm, an event that correlated with the onset of apoptosis.
The Translocation of Bax to Membrane Fractions—How the mitochondrial membrane is permeabilized to release cytochrome c during apoptosis is not known (68, 69). However, while the opening or formation of the putative membrane channels may be temperature-sensitive, movement of cytochrome c through the channels may not be sensitive to reduced temperature (30). Bax is highly expressed in neutrophils (9–14) and can form membrane pores (39) so we examined the subcellular distribution of Bax during neutrophil apoptosis. Immunoblotting showed that Bax was present in the postnuclear supernatants of 15 °C cultured neutrophils (Fig. 8, upper panel, lane 1). Sedimentation of the membranes by ultracentrifugation (Fig. 8, upper panel, lane 2) showed that Bax partitioned into the cytosol (Fig. 8, upper panel, lane 3) with procaspase-3 (Fig. 8, lower panel, lane 3). This is consistent with their localization in freshly isolated neutrophils (data not shown), murine thymocytes, splenocytes, and HL-60 cells (33, 35). In 15 °C cultured neutrophils warmed to 37 °C for 3 h, procaspase-3 was proteolytically cleaved (Fig. 8, lower panel, lanes 4–6) and Bax had translocated to the membrane fraction (Fig. 8, upper panel, lane 4). This location suggested that Bax had inserted its hydrophobic membrane-spanning domain into mitochondria (40). In some experiments membrane-associated Bax was processed to an 18-kDa fragment (data not shown), a cleavage product identified in a number of other apoptotic cells (70, 71).

Bax insertion into mitochondria and the release of cytochrome c in many experimental models is insensitive to zVAD-fmk (28). In subcellular fractions from neutrophils cultured for 18 h at 15 °C and treated with 100 μM zVAD-fmk for 2 h, before warming to 37 °C, Bax had translocated to the membrane fraction (Fig. 8, upper panel, lane 11). Under these conditions...
Proteolytic processing of HL-60 PARP in heterologous cell-free assays. HL-60 nuclei were incubated with 15 °C neutrophil cytosol and dATP and proteolytic cleavage of procaspase-3 triggered by the addition of cytochrome c (200 ng). Samples were taken at 0 h (lane 2) and at 1.5 h after incubation at 15 °C (lanes 3 and 4), without zVAD-fmk treatment (lanes 1–3, 5 and 7), with 100 μM zVAD-fmk treatment (lanes 4 and 6), and after incubation at 37°C (lanes 1 and 5–7). Samples were solubilized as described in the legend to Fig. 5B and the immunoblots probed with monoclonal antibodies to procaspase-3 (upper panel) and PARP (lower panel).

The release of endogenous neutrophil cytochrome c from membrane fractions into the cytosol. Neutrophils were cultured at 15 °C as described in the legend to Fig. 1A. Samples were solubilized in a nondenaturing lysis buffer for immunoprecipitation (46) with monoclonal antibodies to a native epitope on cytochrome c. After SDS-PAGE and electroblotting to nitrocellulose, cytochrome c was identified with a monoclonal antibody to the SDS-denatured form of cytochrome c. Lane 1 contains 50 ng of horse heart cytochrome c (C). Lane 2 shows an immunoprecipitation of cytochrome c from 0.5 × 10⁶ HeLa cells (H). Lanes 3–8 show cytochrome c immunoprecipitated from: in lane 3, a post-nuclear supernatant (P) of 15 °C cultured neutrophils (7.5 × 10⁶ cells); lane 4, 15 °C cultured neutrophil membrane fraction (M); lane 5, 15 °C cultured neutrophil supernatant (S) fraction; lane 6, post-nuclear supernatants (S) of 15 °C cultured neutrophils warmed to 37 °C for 2 h (apoptosis by annexin V-FITC 85%); lane 7, 37 °C membrane fraction (M); lane 8, 37 °C supernatant (S) fraction.

Subcellular localization of Bax. Neutrophils (3 × 10⁶ cells) were held at 15 °C for 18 h. 10⁶ cells were incubated at 15 °C for 5 h (lanes 1–3); 10⁶ cells were cultured for a further 2 h at 15 °C before warming to 37 °C for 3 h (lanes 4–6) and 10⁶ cells were treated for 2 h at 15 °C with 100 μM zVAD-fmk before warming to 37 °C for 3 h (T18: lanes 10–12). One sample of 10⁶ freshly isolated neutrophils was treated with 100 μM zVAD-fmk at 15 °C for 20 h and then warmed to 37 °C for 3 h (lanes 7–9). Post-nuclear supernatants (post-nuclear supernatants: lanes 1, 4, 7, and 10) were fractionated into membrane (M: lanes 2, 5, 8, and 11) and cytosol (C: lanes 3, 6, 9, and 12) by ultracentrifugation, the membrane fractions being re-homogenized to their original volume in buffer. The fractions were immunoblotted with antibodies to Bax (upper panel) and procaspase-3 (lower panel).

procaspase-3 was still present in the cytosol (Fig. 8, lower panel, lane 12). However, when freshly isolated neutrophils were treated with 100 μM zVAD-fmk at 15 °C for 20 h prior to warming to 37 °C, Bax remained in the cytosol fraction (Fig. 8, upper panel, lane 8) with procaspase-3 (Fig. 8, lower panel, lane 9). The Bax present in the membrane fraction (Fig. 8, upper panel, lane 8) can be accounted for by the apoptotic cells (12%) present. These results suggest that during incubation at 15 °C, caspase activity may prepare Bax for translocation from the cytosol to mitochondria and insertion into the mitochondrial membrane on warming to 37 °C. Caspase-8, a potential candidate for the indirect activation of Bax, can be detected in neutrophil cytosol. However, procaspase-8 was not proteolytically cleaved during either incubations at 15 °C or during the warming of 15 °C cultured neutrophils to 37 °C in the presence of zVAD-fmk (data not shown).

The Translocation of Bax to Neutrophil Mitochondria—We followed the translocation of Bax from the cytoplasm to mitochondria in apoptotic neutrophils by confocal microscopy. Immunofluorescence staining of neutrophils with rabbit preimmune sera and Alexa dye-tagged secondary antibodies showed nuclear staining in freshly isolated neutrophils (Fig. 9A) and no significant cytoplasmic staining. Anti-Bax polyclonal antibodies also showed nuclear staining (Fig. 9B), but similar staining in HeLa cells and Bax-deficient tumor cells suggested that this staining was nonspecific (72). In fresh preparations of neutrophils the few constitutively apoptotic neutrophils showed Bax staining of large cytoplasmic structures (Fig. 9B, arrow). These cytoplasmic structures were also stained with polyclonal antibodies to ubiquinol cytochrome c oxidoreductase (complex III) that co-localized with mitochondrial staining (Fig. 9C), whose expression was increased in many preparations of 15 °C cultured neutrophils. Significantly, staining of 15 °C cultured neutrophils with mHSP70 (Fig. 9D) and Bax (Fig. 9E) showed co-localization (yellow staining) in merged images (Fig. 9F) of mitochondria in apoptotic neutrophils by confocal microscopy. Immunofluorescence staining of neutrophils with rabbit preimmune sera and Alexa dye-tagged secondary antibodies showed nuclear staining in freshly isolated neutrophils (Fig. 9A) and no significant cytoplasmic staining. Anti-Bax polyclonal antibodies also showed nuclear staining (Fig. 9B), but similar staining in HeLa cells and Bax-deficient tumor cells suggested that this staining was nonspecific (72). In fresh preparations of neutrophils the few constitutively apoptotic neutrophils showed Bax staining of large cytoplasmic structures (Fig. 9B, arrow). These cytoplasmic structures were also stained with polyclonal antibodies to ubiquinol cytochrome c oxidoreductase (complex III) that co-localized with mitochondrial staining (Fig. 9C), whose expression was increased in many preparations of 15 °C cultured neutrophils. Significantly, staining of 15 °C cultured neutrophils with mHSP70 (Fig. 9D) and Bax (Fig. 9E) showed co-localization (yellow staining) in merged images (Fig. 9F) of mitochondria in apoptotic neutrophils by confocal microscopy.
cytoplasmic structures that were present in cells that showed none of the pyknotic nuclear morphology associated with neutrophil apoptosis. Co-localization analysis, on single optical sections using Leica TCS software, confirmed the subcellular co-localization of Bax and mtHSP70 seen in the merged fluorescent micrographs (data not shown). Bax appeared therefore to translocate to mitochondria at 15 °C, without triggering apoptosis. The binding of Bax to mitochondria without insertion of its hydrophobic membrane-spanning domain would be consistent with Bax being peripherally associated with the mitochondrial membrane and redistributing to the cytosol fraction during homogenization and washing of the membrane fraction (Fig. 8, lower panel, lane 2). After warming the 15 °C cultured neutrophils to 37 °C for 3 h, mtHSP70 staining (Fig. 9G) and Bax staining (Fig. 9H) were co-localized in the merged micrographs (Fig. 9L) with a small number of large, but discrete, cytoplasmic structures. Similar cytoplasmic structures have been identified in HeLa cells treated with staurosporine to induce apoptosis (72) and in cells overexpressing Bax (37, 38) as aggregates of mitochondria. Not all mitochondria stained for Bax and some Bax-stained structures did not stain with mtHSP70. There was therefore a differential response by mitochondria to apoptotic signals and Bax may also translocate to other membrane compartments. Neutrophils cultured at 15 °C for 20 h then warmed to 37 °C for 3 h showed no staining with rabbit preimmune sera (Fig. 9J).

After culture of neutrophils at 15 °C for 20 h with zVAD-fmk and warming to 37 °C for 3 h there was no significant Bax staining of neutrophil mitochondria that showed non-apoptotic nuclear morphology (Fig. 9K). This was consistent with the immunoblotting data (Fig. 8, upper panel, lanes 7–9) that showed no insertion of Bax into mitochondria. However, neutrophils cultured at 15 °C for 18 h, then treated for 2 h with zVAD-fmk before warming to 37 °C for 3 h, did show significant Bax and mtHSP70 staining (Fig. 9L). This was consistent with immunoblotting data that showed that Bax had translocated to the membrane fraction (Fig. 8, upper panel, lanes 10–12). While the nuclear morphology was significantly different from the nuclei of freshly isolated neutrophils under these conditions, the pyknotic nuclear morphology characteristic of apoptotic neutrophils was not detected and there was no fragmentation of DNA under these conditions (see Fig. 2, A-C).
15 °C Inhibits Bax-insertion and Arrests Neutrophil Apoptosis

We have shown for the first time that culturing neutrophils at 15 °C reversibly arrests the induction of apoptosis, with subsequent warming to 37 °C triggering a burst of synchronous apoptosis. The molecular consequences of temperature reduction on cells are poorly understood. However, between 10 and 20 °C there is a reduction in membrane lipid fluidity, a decrease in the rate of protein translation, and an inhibition of vesicular trafficking and neutrophil respiratory burst activity (67, 50, 51). Our results suggest that the arrest of apoptosis in neutrophils cultured at 15 °C may be due to the failure of the procaspotic protein Bax to undergo the conformational changes necessary for it to insert into mitochondria. Once in the membrane Bax triggers the release of cytochrome c and the subsequent activation of caspase-3 that induces the execution phase of neutrophil apoptosis.

Bax is a soluble protein located in the cytoplasm of freshly isolated neutrophils (10–12). Our immunofluorescence studies on Bax localization in neutrophils cultured at 15 °C have shown that in addition to its cytoplasmic localization, Bax was also associated with mitochondria that showed signs of aggregation; both observations reported for many cell lines cultured at 37 °C (73). However, in neutrophils cultured at 15 °C Bax failed to undergo the conformational changes necessary for insertion of its C-terminal membrane-spanning domain into mitochondria (33). Bax was readily washed from membranes isolated from neutrophils cultured at 15 °C suggesting a peripheral association with the membrane fraction. The N-terminal region of the Bax molecule may bind to and mask its C-terminal domain in the cytoplasm and the removal of the N-terminal domain leads to an autoactivation and constitutive insertion of the mutant protein into mitochondria (72). The cytoplasmic components that normally facilitate this unmasking of the C-terminal membrane-spanning domain and the conformational changes to neutrophil Bax are unknown. However, in HeLa cells, for example, the procaspotic protein Bid, can induce the insertion of Bax into membranes (22, 72) and may be one of a number of cytoplasmic factors that play a role in the release of cytochrome c from mitochondria (29).

In many models of apoptosis the insertion of Bax into mitochondria and the subsequent release of cytochrome c are not inhibited by the pan-caspase inhibitor zVAD-fmk, while proteolytic cleavage of procaspase-3 is inhibited (28). Treatment of neutrophils, cultured at 15 °C for 18 h, with ZVAD-fmk prior to triggering apoptosis by warming to 37 °C, also failed to inhibit Bax translocation to neutrophil membranes under conditions where procaspase-3 was not proteolytically cleaved, consistent with other models of apoptosis. However, when freshly isolated neutrophils were treated with ZVAD-fmk at the beginning of the culture period at 15 °C, Bax failed to insert into mitochondria when the cells were warmed to 37 °C. Assuming the fidelity of ZVAD-fmk for its caspase targets, these experiments suggested that caspase-mediated events were necessary for Bax insertion (74) but were not in themselves a sufficient trigger for the induction of Bax insertion into mitochondria at 15 °C. Whatever the role the caspases play in preparing Bax, or in activating a putative effector protein necessary for the activation of Bax binding and insertion once the neutrophils are warmed to 37 °C, we saw no evidence that Bax was proteolytically cleaved at 15 °C. Activation of an effector protein and Bax binding at 15 °C would provide an explanation for the synchronization of apoptosis we observe on warming the cells to 37 °C after culture at 15 °C. Direct proteolytic cleavage of p21 Bax does not appear to be involved in the translocation of Bax to mitochondria (70, 71). However, caspases have been shown to activate the calcium-activated cysteine protease, calpain, that cleaves p21 Bax to p18 Bax at the mitochondrial membrane (70, 71). While this proteolytic processing by calpain augments the homodimerization of Bax and the release of cytochrome c it is a relatively late apoptotic event concomitant temporally with the cleavage of many other caspase-3 substrates and with DNA fragmentation (75, 76).

The conformational changes necessary for neutrophil Bax to insert into the mitochondrial membrane were not only inhibited by low temperature, but also by clamping neutrophils at an acidic pH during warming to 37 °C, a treatment that was dominant over the effect of temperature reduction. In FL5.12 cells and D1 thymocyte cell lines transient increases in cytoplasmic pH have been correlated with pH-dependent conformational change in Bax, an effect that is also inhibited by acid pH (39, 64, 73). This observation may provide an explanation for the inhibition of neutrophil apoptosis observed at inflammatory foci where an acid environment has developed (59). Our data are also consistent with the observation that tumor necrosis factor/cycloheximide-induced cytochrome c release is inhibited by ZVAD-fmk (30) and that caspase-8 may trigger the release of cytochrome c (77). However, our preliminary data (not shown) have suggested that procaspase-8, like procaspase-3 is not proteolytically cleaved at 15 or at 37 °C in the presence of ZVAD-fmk, under conditions where Bax insertion occurs.

How cytochrome c is translocated across the outer membrane of mitochondria is not known (41, 68, 78, 79). Bax insertion may affect membrane channels by inducing permeability changes that result in the release of cytochrome c (78, 80–82). Many cells that obtain their ATP by oxidative phosphorylation can still release cytochrome c from mitochondria at low temperature (30, 81). It was possible that extended culture at low temperature would lead to collapse of the inner mitochondrial membrane potential (ΔΨm) and trigger the opening of the mitochondrial permeability transition pore in neutrophils, an event that correlates with the insertion of Bax and the induction of apoptosis (27, 41, 68). In granulocytes, however, ATP is obtained predominantly by glycolysis and this may allow their mitochondria to use pyruvate to maintain their ΔΨm at low temperature. Apoptosis triggered by warming 15 °C cultured neutrophils to 37 °C was not inhibited by preincubation with 50 μM bongkrekic acid (data not shown), an inhibitor that blocks the permeability transition pore (37, 77). This experiment suggested that the collapse of the membrane potential might be an event triggered downstream of caspase-3 activation (28).

The Bax-dependent release of cytochrome c from mitochondria in 15 °C cultured neutrophils, treated with ZVAD-fmk just before warming to 37 °C to inhibit caspase-3, triggered the activation of apoptosis inducing activities that led to the arrest of both secretion and phagocytosis (57). The externalization of phosphatidylserine on the cell surface was also induced and this may trigger recognition and phagocytosis of granulocytes by macrophages (55). We have established that mitochondria play a role in triggering neutrophil apoptosis. Our 15 °C cytotoxic isolates will now enable us to investigate not only the ZVAD-fmk sensitive activity that is required for Bax insertion into mitochondria, but also the ZVAD-fmk insensitive activities triggered during cytochrome c release, that lead to the apoptotic changes associated with the neutrophil plasma membrane. Finally, the differential response of granulocytes and proliferating cells to temperature reduction suggests that therapeutic targets specific for triggering of neutrophil apoptosis may possibly be identified in this clinically relevant cellular model of apoptosis.

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