Caspase-mediated proteolysis during apoptosis: insights from apoptotic neutrophils

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Abstract Apoptosis is initiated by activation of caspases (interleukin 1β-converting enzyme homologues), which cause coordinated cleavage of several death substrates that function in structural or homeostatic pathways. The relationship between substrate cleavage and apoptosis is not yet known, nor is it clear whether cleavage of specific substrates is a critical requirement for apoptosis. The human neutrophil provides novel insights into the roles of proteolysis of specific substrates during apoptosis, since only a subset of caspase substrates are present in mature neutrophils. Of the death substrates we screened, PARP, the nuclear mitotic apparatus protein (NuMA), the 70 kDa subunit of the U1 small ribonucleoprotein (U1-70kDa) and the catalytic subunit of DNA-dependent protein kinase (DNA-PK$_{cs}$) were not detected in non-apoptotic neutrophils; in contrast, lamin B and fodrin were present in amounts similar to those found in other cells. Caspase-3 activity was absent in freshly isolated neutrophils, but was detected when neutrophils were aged in vitro, coincident with the onset of morphologic and biochemical apoptosis. The absence of PARP, NuMA, U1-70kDa and DNA-PK$_{cs}$ in non-apoptotic neutrophils suggests that these are not critical anti-apoptotic proteins, and that their fragments are not required components of the neutrophil apoptotic pathway. These studies highlight the conserved role of caspase activation in the apoptotic mechanism, and focus attention on several conserved structural substrates as potential transducers of the proteolytic signal in apoptosis.

Key words: Caspase; Proteolysis; Apoptosis

1. Introduction

Activation of caspases is a critical component of the common execution pathway in apoptotic cells [1,2], likely by inducing the specific cleavage and functional alteration of key downstream substrates [3,4]. In human cells, several substrates for these proteases have been defined including (i) enzymes functioning in DNA repair pathways (e.g. PARP [5–7] and DNA-PK$_{cs}$ [8,9]); (ii) proteins involved in homeostatic pathways (including D4-GDI [10], protein kinase C-δ [11] and sterol regulatory element binding proteins [12]; and (iii) structural proteins including the nuclear lamins A and B [13], non-erythroid spectrin (fodrin) [14–16], and the nuclear mitotic apparatus protein (NuMA) [17,18]. However, the mechanisms whereby cleavage of these substrates generates elements of the apoptotic phenotype, and whether the generation of specific substrate fragments is critical in this regard, remain unknown [3].

The human neutrophil is highly specialized for stimulus-induced degranulation and oxygen burst, but as a terminally differentiated cell, it preserves only a truncated repertoire of homeostatic pathways. Mature neutrophils undergo spontaneous apoptosis after isolation from blood [19–21]. To gain insight into the nature of proteolysis during neutrophil apoptosis, we addressed whether substrates for the caspases are present in these cells, and studied the kinetics and characteristics of their cleavage during apoptosis. Our studies demonstrate that mature neutrophils lack PARP, NuMA, U1-70kDa and DNA-PK$_{cs}$, indicating that fragments generated from these molecules are unlikely to be critical mediators of the apoptotic pathway in neutrophils. Several other caspase substrates (e.g. fodrin and lamin B) are present in neutrophils in amounts comparable to those found in HeLa cells, and are cleaved during apoptosis. The data demonstrate that caspase-mediated generation of specific substrate fragments is not a universal requirement during apoptosis of all cells. These data further suggest that, in dividing cells capable of homeostasis, caspases interrupt multiple homeostatic pathways by additively abolishing the function of several substrates.

2. Materials and methods

2.1. Isolation of neutrophils

Neutrophils (>99% viable as assessed by trypan blue exclusion) were isolated from fresh citrated normal human blood by dextran sedimentation and Percoll (Pharmacia, Piscataway, NJ) density gradient centrifugation as previously described [19,20]. Aging of neutrophils was performed in Iscove's medium supplemented with 10% autologous serum at 37°C.

2.2. Fractionation of neutrophil contents and preparation of lysates

Fractionation for immunoblotting was performed as previously described [22], with minor modifications. Neutrophils were washed twice in ice-cold phosphate buffered saline, resuspended at 5×10^7 cells/ml in relaxation buffer (10 mM PIPES pH 7.4, 100 mM KCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 1 mM ATP supplemented with antipain, pepstatin A, chymostatin, leupeptin, and PMSF), and incubated at 30 bar of nitrogen for 20 min in a cavitation bomb (Parr Instruments, Moline, IL) on ice. Rapid depressurization produced almost total lysis of cells, with preservation of multilobed nuclei. The crude lysate was spun at $500\times g$ for 10 min, and the supernatant ('cytosol fraction') was further spun at $14\,000\times g$ for 5 min to remove contaminating granules. Removal of granules from the cytosolic fraction was essential since their presence leads to non-specific proteolysis, which destroyed all substrates we investigated even when the sample was immediately boiled in 2% SDS (data not shown). The pellet ('nuclear fraction') was washed three times in relaxation buffer ($500\times g$ for 10

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min), and resuspended in a volume equal to that of the cytosol. For apoptosis reconstitution experiments, neutrophils were cavitated at 4–8×10^8 cells/ml to achieve protein concentrations of 10–12 mg/ml. For cleavage assays employing the fluorometric amino-4-methylcoumarin tetrapeptide substrate Ac-DEVD-AMC, whole neutrophils were aged for various times and then washed twice in PBS, resuspended in 10 mM PIPES pH 7.3, 10 mM KCl, 3 mM NaCl, 1.25 mM EGTA, and 3.5 mM $MgCl_2$ with protease inhibitors, sonicated, and spun at $14\,000\times g$ for 5 min to remove granules.

2.3. HeLa lysate preparation

Confluent HeLa cells were harvested and lysed in buffer containing 1% Nonidet P-40, 20 mM Tris pH 7.4, 1 mM NaCl, 1 mM EDTA, and protease inhibitors. Where indicated, apoptosis was induced by irradiation with UVB as previously described [8,23].

2.4. Immunoblotting

Samples were electrophoresed on either 10% polyacrylamide gels containing 0.087% bisacrylamide or 15% polyacrylamide gels containing 0.39% bisacrylamide, and transferred to nitrocellulose. Proteins were immunoblotted with monospecific patient sera recognizing PARP, U1-70kDa, and NuMA, or monoclonal antibodies recognizing DNA-PK_{cs} (gift from T. Carter, St. Johns University, Jamaica, NY), lamin B (Matritect, Cambridge, MA), actin (Sigma, St. Louis, MO), and fodrin (Chemicon International, Temecule, CA), or rabbit polyclonal antibodies raised against caspase-3 (gift from Don Nicholson, Merck Frosst, Canada). Immunoblotted substrates were visualized using enhanced chemiluminescence (Pierce, Inc., Rockford, IL). Negligible amounts of these substrates were detected in red cells and non-neutrophil lymphoid cells (amounts equivalent to their contamination of neutrophil samples were blotted (data not shown)).

2.5. Fluorogenic substrate assays

A continuous fluorometric assay for caspase-3 using the substrate Ac-DEVD-AMC (amino-4-methylcoumarin) was used as described [6]. Reaction mixtures containing 100 μM Ac-DEVD-AMC and neutrophil cytosols were monitored continuously in a spectrofluorometer at an excitation wavelength of 380 nm and emission wavelength of 460 nm at 25°C.

2.6. Apoptosis reconstitution system

S3 HeLa nuclei were used in in vitro apoptosis reconstitution experiments as described [24], with the following modifications. S3 HeLa cells were harvested by centrifugation, washed twice with PBS, once with NB (10 mM PIPES pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 10 μM cytochalasin B, plus protease inhibitors), and resuspended at 1×10^8 cells/ml in NB. After swelling on ice for 20 min, cells were lysed using a Dounce homogenizer (20 strokes). Nuclei were obtained by layering the homogenate over 30% sucrose in NB, centrifuging at $800\times g$ for 10 min, washing the pellet once in NB, and resuspending at 1×10^8 nuclei/ml. Nuclei were either used immediately or stored at −70°C in the following buffer: 10 mM PIPES pH 7.4, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 50% glycerol, 5 mM EGTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, and protease inhibitors. To perform the reconstitution experiments, 5 μl of S3 HeLa nuclei were combined with 140 μg of cytosol, and the reaction volume was made up to 50 μl by adding buffer containing 10 mM HEPES/KOH pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 40 mM β-glycerophosphate, 1 mM dithiothreitol, 2 mM ATP, 10 mM phosphocreatine, 50 μg/ml creatine kinase and protease inhibitors. Samples were incubated at 37°C for 2 h in the presence or absence of increasing concentrations of Ac-DEVD-CHO and Ac-YVAD-CHO (Merck Research Laboratories, Rahway, NJ). Nuclei were then immunoblotted for substrates as described above.

3. Results and discussion

Previous studies have demonstrated that PARP and DNA-PK are absent from mature neutrophils, although these proteins are present in the promyelocyte cell line, HL-60 [25,26]. To determine whether absence of caspase substrates is a general feature of neutrophils, freshly isolated human neutrophils or neutrophils incubated in vitro for 24 h were disrupted by nitrogen cavitation, and separated into cytosolic and nuclear fractions prior to immunoblotting. As a standard, we used control and UVB-irradiated (apoptotic) HeLa cell lysates, since the caspase substrates and their apoptotic fragments have been previously defined in these cells [8,27]. Neither the intact forms of PARP, NuMA, U1-70kDa and DNA-PK_{cs}, nor their characteristic apoptotic fragments were present in freshly isolated neutrophils (Fig. 1, top 3 panels, lanes 3 and 5, and data not shown). In contrast, fodrin and lamin B were present in freshly isolated neutrophils in amounts equivalent to HeLa cells (Fig. 1, lower 2 panels, lanes 3 and 5). While fodrin was cleaved upon aging to generate 150 and 120 kDa apoptotic fragments similar to those found in apoptotic HeLa cells (Fig. 1, 4th panel), no fragments of lamin B were detected (data not shown). However, the biochemical amount of lamin B was decreased in both irradiated HeLa cells and 24 h aged neutrophils (Fig. 1). Interestingly, HL-60 cells, which are promyelocytes that differentiate into neutrophils in response to retinoic acid or DMSO [28], possessed PARP [25] and all the other caspase substrates in amounts comparable to HeLa cells (data not shown).

We have previously demonstrated that PARP, U1-70kDa, and DNA-PK_{cs} are cleaved during apoptosis with similar kinetics and at similar sites by proteases of the caspase-3 subfamily [29]. To address whether the absence of these substrates from freshly isolated, mature neutrophils might be caused by premature activation of the caspase-3 family in these cells, we examined the presence and activity of caspases in fresh and aged neutrophils. Neutrophils were lysed by sonication, and after removal of granules by centrifugation, supernatants were immunoblotted with antibodies against precursors of caspases-1, -3 and -7. The precursors of caspases-1, 3 and 7 were detected (Fig. 2, and data not shown); levels of caspase-7 precursor were much lower than the other caspases (data not shown). An enzymatic assay that quantitates the cleavage of the fluorogenic peptide substrate Ac-DEVD-AMC was used to measure caspase-3 activity on aging neutrophil lysates. Ac-DEVD-AMC cleavage activity increased in a time-dependent manner from zero at 0 h to 3.2 U/mg at 24 h (Fig. 2, lowest panel), which is similar to the increase in activity seen in apoptotic HeLa cells (data not shown). This activity was inhibited by Ac-DEVD-CHO (500 nM) but not by Ac-YVAD-CHO (500 nM), confirming that the activity was due to caspase-3 or a closely related homologue (data not shown).

The caspases are all synthesized as inactive precursors which are cleaved at Asp-X sites during apoptosis, generating a large and small subunit which together constitute the active protease [2,30]. Since the Ac-DEVD-AMC cleavage is mediated by a protease with caspase-3-like inhibition characteristics, we immunoblotted caspase-3 in aging neutrophils at 8 h intervals, to address whether caspase-3 precursor processing occurred during neutrophil aging. A time-dependent decrease in the 32 kDa caspase-3 precursor was observed, with 85% being lost by 24 h; in contrast, the amount of actin remained constant (Fig. 2, middle and lower panels). Consistent with other studies, the active p17 fragment of caspase-3 could not be well visualized by immunoblotting with this antibody [31]. In all experiments, at least 45% of neutrophils aged in vitro for 24 h displayed chromatin condensation and loss of nuclear lobulation on Wright-Giemsa staining (data not shown), features characteristic of apoptosis in neutrophils. Therefore,

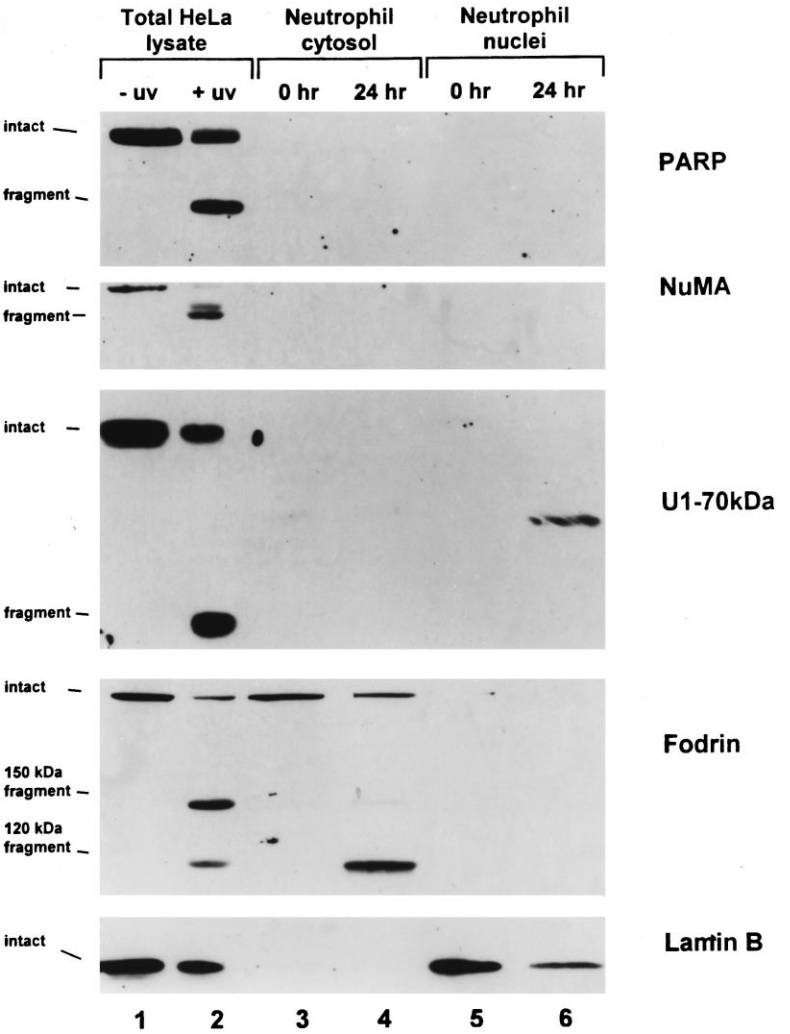

Fig. 1. Several caspase substrates are absent in neutrophils. Lanes 1, 2: Total cell lysate from control or apoptotic HeLa cells was prepared as described, and were electrophoresed and immunoblotted with antibodies against PARP, NuMA, U1-70kDa, fodrin, and lamin B. Lanes 3–6: Freshly isolated neutrophils or neutrophils that had been aged in vitro for 24 h were separated into cytosolic and nuclear fractions as described in Section 2. Equivalent cell numbers were loaded in all lanes. Similar results were obtained in three separate experiments. Neutrophils lack PARP, NuMA, and U1-70kDa, but contain fodrin and lamin B in amounts similar to those found in equal numbers of HeLa cells.

freshly isolated neutrophils (i) contain the caspase-3 precursor; the amount of this decreases with aging and apoptosis, and (ii) acquire Ac-DEVD-AMC cleavage activity with caspase-3-like inhibition characteristics with aging.

To address the macromolecular specificity of the Ac-DEVD-AMC cleavage activity in neutrophils, we studied the cleavage of the nuclear substrates PARP, U1-70kDa, and DNA-PK_{cs} by incubating S3 HeLa cell nuclei (which have all these substrates; Fig. 3, lane 1) with cytosols from freshly isolated and aged human neutrophils (which lack these substrates; Fig. 3, lanes 3 and 4). After incubating S3 HeLa nuclei with neutrophil cytosol for 2 h, reactions were stopped by addition of 2% SDS and boiling, and samples were immunoblotted. Fresh neutrophil cytosol failed to induce cleavage of DNA-PK_{cs}, PARP, or U1-70kDa in HeLa nuclei (Fig. 3, lane 5). In contrast, after incubating with 24 h aged neutrophil cytosol, these proteins were cleaved to yield fragments identical to those seen in apoptotic HeLa cells (compare Fig. 3, lanes 2 and 6). To confirm that the proteolysis of DNA-PK_{cs}, U1-70kDa, and PARP was indeed mediated by a caspase-3-like protease, rather than other protease activity generated in the aging neutrophil, we examined the inhibitory effects of Ac-DEVD-CHO and Ac-YVAD-CHO on substrate cleavage by aged neutrophil cytosol. Ac-DEVD-CHO is a potent inhibitor of caspase-3 ($K_i = 0.35$ nM) in contrast to Ac-YVAD-CHO, which inhibits this enzyme only at high concentrations ($K_i = 10\,000$ nM) [6,32]. Ac-DEVD-CHO was a potent inhibitor of cleavage of DNA-PK_{cs}, U1-70kDa, and PARP by aged neutrophil cytosols ($IC_{50} = 0.6$–2 nM), while Ac-YVAD-CHO was markedly less potent ($IC_{50} = 20\,000$–$50\,000$ nM) (Fig. 3). Consequently, the inhibition profile of aged neutrophil cytosol is consistent with caspase-3, or a closely related homologue, being responsible for cleavage of substrates in proteolysis reconstitution system. The small amount of caspase-7 present in fresh neutrophils makes it likely that the majority of this cleavage activity is due to caspase-3.

These studies demonstrate that the caspase substrates PARP, NuMA, U1-70kDa and DNA-PK_{cs} are not detectable by immunoblotting of mature neutrophils, although they are

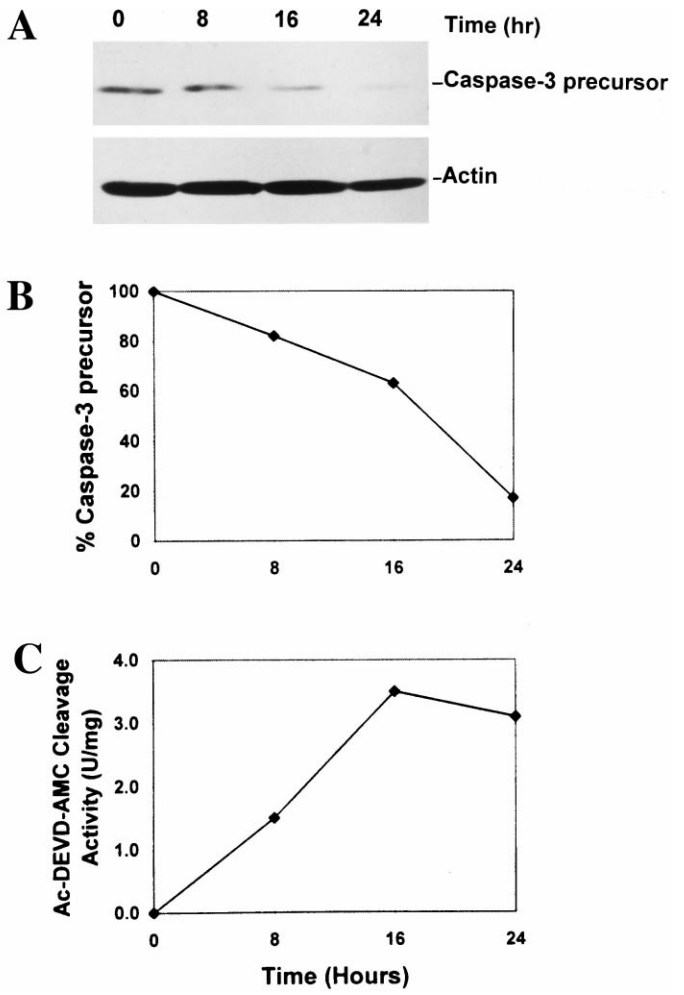

Fig. 2. Aging neutrophils show progressive loss of caspase-3 precursor (A and B) and increasing Ac-DEVD-AMC cleavage activity (C). A, B: Equal protein amounts of freshly prepared neutrophils, or neutrophils aged for 8, 16, or 24 h were immunoblotted with antibodies to actin or caspase-3 precursor as described in Section 2. Densitometric scanning of the data in (A) showed that the amount of actin remained constant over 24 h, but the caspase-3 precursor levels decreased to 17% of the zero time values (B, and data not shown). C: Neutrophils extracts were incubated with 100 μM of the fluorogenic substrate Ac-DEVD-AMC at 25°C as described in Section 2. The cleavage activity of neutrophil cytosol increased with aging, and corresponded with the decreased caspase-3 precursor levels. A,B,C: All experiments were repeated three times with identical results. THP-1 cell cytosol was used as a reference standard for migration of the caspase-3 precursor.

present in HL-60 neutrophil precursor cells. In contrast, mature neutrophils do contain intact lamin B and fodrin, which is cleaved during apoptosis to generate signature apoptotic fragments. The absence of several caspase substrates from neutrophils is unrelated to caspase activity, since Ac-DEVD-AMC cleavage activity is absent in freshly isolated cells, and is generated only with neutrophil aging. The failure to detect several homeostatic proteins in non-apoptotic neutrophils indicates that cells can tolerate significant decreases in the functions that these proteins serve without becoming apoptotic. Interestingly, cells deprived of functional NuMA form micronuclei that resemble the multilobed neutrophil nucleus, as well as nuclei undergoing apoptosis, in which NuMA cleavage occurs coincidently with apoptotic body formation [18]. We propose that the multilobed neutrophil nucleus may reflect the pre-apoptotic loss of NuMA in these cells.

The absence of several caspase substrates from neutrophils also provides insights into the potential functional relevance of cleavage of these and other substrates during apoptosis of different cells. While it has previously been proposed that fragments generated by cleavage of caspase substrates might have critical roles in generating the apoptotic phenotype, the lack of fragments of PARP, DNA-PK$_{cs}$, U1-70kDa and NuMA in neutrophils undergoing apoptosis makes it less likely that fragments of these molecules have a critical role in the execution phase of apoptosis. This conclusion is supported by the previous observation that PARP-knockout mice, which cannot generate fragments of this molecule, have no defects in apoptosis [33]. Interestingly, neutrophil apoptosis is characterized by activation of caspases and cleavage of fodrin, lamin B, and D4-GDI [34]; these changes occur concomitantly with typical internucleosomal cleavage of DNA, and morphologic changes of apoptosis. Since the cleavage of these cytoskeletal and homeostatic substrates coincides with the onset of apoptosis, the altered function of these proteins (or the generated fragments) may play a critical role in neutrophil apoptosis. In this regard, it is of interest that gelsolin (which is present in neutrophils) has recently been demonstrated to be cleaved by caspases during apoptosis, and that the fragments generated appear to have pro-apoptotic properties [35].

It has been proposed that the critical end-point of the apoptotic process is the rapid termination of independent life, culminating in efficient phagocytosis and degradation of the corpse. Several studies suggest that proteolysis during apoptosis alters the function of several pathways to render the surface of apoptotic cells identifiable by phagocytes, and the apoptotic cell powerless to resist these changes by crippling homeostatic pathways [14,19,20,29,36,37]. In terminally differentiated, post-mitotic cells like neutrophils, which have downregulated several pathways and the machinery for their homeostasis, rendering the surface of cells pro-phagocytic (by alteration of membrane skeletal structure) may be a critical initiator of neutrophil clearance by macrophages. In this regard, it is of interest that fodrin cleavage (which may occur due to caspase [14–16,38] or calpain activity [39]) has been linked with the redistribution of phosphatidylserine (PS) from the inner to the outer surface of the plasma membrane bilayer; both fodrin proteolysis and PS redistribution characterize the early apoptotic state [14,36,40,41]. This exteriorized PS appears to be one of the signals by which apoptotic neutrophils are recognized and cleared by phagocytic cells [36]. Previous studies have demonstrated that neutrophils from mice expressing bcl-2 in the neutrophil lineage are efficiently cleared by phagocytic cells upon aging, although they do not undergo apoptosis [42]. Since several proteases appear capable of cleaving fodrin, and one of these may be active under non-apoptotic circumstances, it would be of interest to determine if fodrin is cleaved, and PS is exteriorized in aged neutrophils from these bcl-2 transgenic mice.

There is accumulating evidence that apoptotic material constitutes an important immunogen in systemic lupus erythematosus (SLE), and that several caspase substrates (PARP, U1-70kDa, DNA-PK$_{cs}$, lamin B and NuMA) are targets of the autoantibody response in SLE [8,23,27,29]. In the setting of immune complex-initiated inflammatory responses (character-

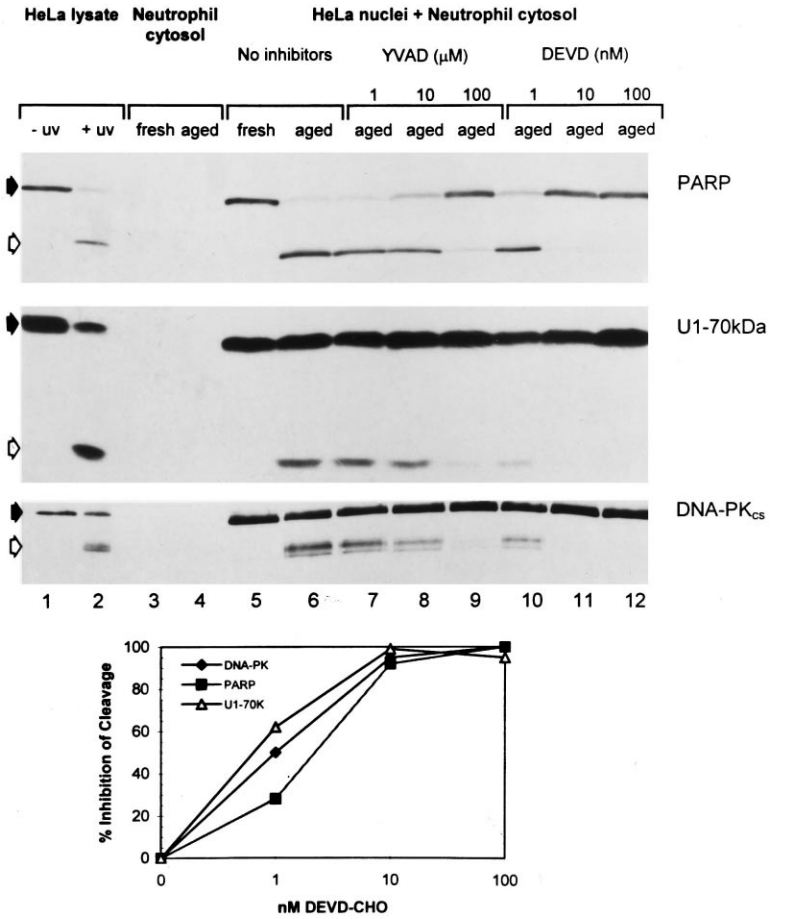

Fig. 3. Neutrophils acquire Ac-DEVD-CHO-inhibitable caspase activity with aging. Top panel: Control samples (lanes 1–4) contained nuclei prepared from control (lane 1) or apoptotic (lane 2) S3 HeLa cells, or cytosols prepared from freshly isolated neutrophils ('−Age', lane 3) or cytosol from neutrophils aged in vitro for 24 h ('+Age', lane 4). The apoptosis reconstitution samples consisted of S3 HeLa nuclei and either fresh neutrophil cytosol (lane 5) or aged cytosol (lanes 6–12) incubated together for 2 h at 37°C. Samples in lanes 7–12 also contained the indicated concentrations of Ac-YVAD-CHO or Ac-DEVD-CHO. All samples were immunoblotted with antibodies to PARP, U1-70kDa and DNA-PK_{cs} as described in Section 2, and equal protein amounts were electrophoresed in each lane. Note that the substrates are present in the S3 HeLa nuclei, but are absent from the neutrophil cytosol (consistent with the data shown in Fig. 1). Lower panel: The data in lanes 6, 10, 11 and 12 were scanned densitometrically and plotted. The IC_{50} for inhibition of cleavage of PARP, U1-70kDa and DNA-PK_{cs} by Ac-DEVD-CHO in this reconstitution system was 0.6–2.0 nM; the IC_{50} for Ac-YVAD-CHO was 20–50 μM (not shown graphically). Results shown are representative of two separate experiments.

istic of the lupus flare), a large number of neutrophils are recruited, and subsequently become apoptotic. This large source of apoptotic material, while rich in nucleosomes, contains few of the defined caspase substrates, making neutrophils an unlikely source of antigen to drive the ongoing immune response to these molecules in SLE.

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