Actin is cleaved during constitutive apoptosis

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Proteases play an important role in the programme of cell death by apoptosis but little is known of the substrates cleaved, particularly in constitutive models of this type of cell death. Neutrophils spontaneously undergo apoptosis in culture without requiring external stimuli. During this process we found biochemical and immunochemical evidence for the cleavage of membrane-associated actin, a component of the cytoskeleton that links polymerized actin to the plasma membrane. Cleavage occurred at a single site at the N-terminus, between residues Val[43]-Met[44], a site devoid of a consensus motif for cleavage by cysteine proteases of the interleukin-1β-converting enzyme (ICE)-family. Whereas actin cleavage and nuclear/cell surface markers of apoptosis were co-ordinately diminished by zVAD-fmk, an inhibitor of the ICE-like family of proteases, only acetyl-leucyl-leucylnormethional, an inhibitor of calpains, was capable of completely inhibiting actin cleavage. Our results suggest that actin is not a direct substrate for the ICE-like family of proteases. By disabling the cytoskeleton, actin cleavage may be an important component in the capacity of apoptosis to reduce the injurious potential of neutrophils.

INTRODUCTION

Apoptosis is a physiological and programmed form of cell death which is now widely recognized as being of critical importance in health and disease [1–3]. However, although highly characteristic morphological changes allowed the definition of apoptosis over 20 years ago [4], it is only very recently that insights have been gained into the biochemical mechanisms which underlie the stereotypical ultrastructural features of this mode of cell death. Accumulating evidence points to the importance of intracellular protease activation in cells dying by apoptosis [5–14]. However, to date there is very limited knowledge of the identity of the protein substrates known to be cleaved during apoptosis [15–28], or of how this may result in the structural changes so characteristic of apoptosis [26–28]. Furthermore, the majority of studies have relied on cellular systems in which apoptosis must be induced by potentially noxious stimuli such as staurosporine, ceramide or glucocorticoids. Arguably, it can be difficult to assess whether cleavage of a particular protein substrate is truly part of the endogenous cell death programme, or is an unrelated consequence of the lethal stimulus.

Neutrophils isolated from normal human peripheral blood constitutively undergo apoptosis when cultured under favourable conditions (physiological pH, presence of serum etc.) [29]. Constitutive apoptosis in neutrophils therefore represents a useful system in which to study the biochemical consequences of this type of cell death, since no external stimuli are needed and there is negligible cell death by necrosis over a 24 h period in culture. Strikingly, as the neutrophil undergoes apoptosis it changes from a motile cell type capable of polarization, pseudopod formation, chemotaxis and degranulation, into a spherical apoptotic cell incapable of any of these functions but nevertheless possessing an intact plasma membrane and able to survive for many hours in culture before eventual disintegration [30–32]. Furthermore, and by contrast with some other cell types, neutrophils typically do not fragment into apoptotic bodies, an event which in tumour cells can be suppressed by agents which inhibit the functions of the actin-based cytoskeleton [33]. Taken together, these observations suggest that disruption of cytoskeletal elements might be a consequence of intracellular protease activation during constitutive apoptosis in neutrophils [34]. Recent work on stimulus-induced apoptosis [20] or cell-free systems [13] has suggested that cytoskeletal elements may be subject to degradation by calpains [34–37] or proteases of the interleukin-1β-converting enzyme (ICE) family [13], but few studies have examined the cytoskeleton in constitutive apoptosis [38].

In this report we present the results of studies on membrane-associated proteins in neutrophils undergoing constitutive apoptosis. We present evidence that during constitutive apoptosis actin, a major component of the cytoskeleton, is cleaved at a single major site which lies between Val[43] and Met[44] in an N-terminal region of the molecule which lacks consensus sites for cleavage by cysteine proteases of the ICE family. A clear link between actin cleavage and apoptosis was demonstrated by the capacity of the calpain-selective inhibitor acetyl-leucyl-leucylnormethional (ALLN) to co-ordinately inhibit both events. We conclude that proteolytic cleavage of actin is a hitherto undemonstrated event in the programme of constitutive apoptosis which is likely to have important consequences for cell structure and function.

MATERIALS AND METHODS

Neutrophil isolation and culture

Human neutrophils were isolated from freshly drawn venous blood following treatment with citrate, dextran sedimentation and plasma–Percoll density gradient centrifugation as described previously [29,30]. Isolated neutrophils ($> 98\%$ pure, with eosinophils as the major contaminant) were incubated at 37 °C in Iscoves modified Dulbecco's medium containing antibiotics [29] and supplemented with 10% (v/v) autologous platelet-rich plasma-derived serum for 18–28 h to induce apoptosis. The protease inhibitors ALLN and carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk), both at 100 mM in DMSO, were each added to the 'calmed' neutrophil cultures to a final concentration of 100 μM.

Abbreviations used: ALLN, acetyl-leucyl-leucylnormethional; ICE, interleukin-1β-converting enzyme; mAb, monoclonal antibody; pAb, polyclonal antibody; zVAD-fmk, carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone.

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Assessment of apoptosis and flow cytometric analysis

Apoptosis was assessed by either microscopic examination of cytocentrifuge preparations fixed in methanol and stained with May Grunwald–Giemsa [29,30] or by flow cytometric analysis of immunofluorescent-stained cells. Labelling was either with FITC-conjugated anti-CD16 monoclonal antibody (mAb) 3G8 [39], or with biotinylated annexin-V binding detected with a mouse anti-biotin mAb followed by a rhodamine isothiocyanate-conjugated rabbit anti-mouse polyclonal antibody (pAb) [40]. After fluorescent labelling of the cells with conjugated antibodies, samples were applied to a Becton-Dickinson FACScan flow cytometer which automatically and simultaneously measures the fluorescence of individual cells identified by their size-dependent light-scattering properties.

Subcellular fractionation

Cultured neutrophils were harvested by centrifugation at 200 ***g*** and at 4 °C, followed by several washes with ice-cold PBS before resuspension in ice-cold lysis buffer (10 mM Hepes, pH 7.4/1 mM EDTA/1 mM benzamidine/10 μM pepstatin A/1 mM σ-phenanthroline/10 μM leupeptin/10 μM antipain/1 mM phenylmethylsulphonyl fluoride) at 20×10^6 cells/ml and sonication using five 10 s pulses at an amplitude of 4–6 microns, peak to peak, and at a nominal frequency of 20 kHz (MSE; 100 watt Ultrasonic Disintegrator). The cell lysate was centrifuged sequentially at 500 ***g*** (15 min), 10000 ***g*** (30 min), and 100000 ***g*** (90 min) and the pellets were fractionated further by extraction with 20 volumes of 2% (v/v) Triton X114 in PBS.

Partial purification of Triton X114-extractable proteins

Each Triton X114 extract of a 100000 ***g*** pellet was diluted with 10 volumes of 25 mM Tris/HCl, pH 7.5, containing 0.05% (v/v) Triton X100 and was applied to a Mono Q (HR5/5) column pre-equilibrated with the same buffer containing 0.02 M NaCl and subjected to FPLC (Pharmacia). The proteins were eluted with a linear gradient of NaCl (0.02 M–0.40 M) over 30 min at a flow rate of 1.5 ml/min, fractions were collected at 1.5 min intervals. Eluted fractions were typically dried by rotary evaporation and stored at −80 °C until required. Alternatively, the eluted fractions were concentrated by centrifugal filtration (Ultrafree-MC 10000 NMWL filter unit, prewashed with 0.4 M glycine, pH 6.8) and the retentate was washed with 1% (w/v) glucose in 50 mM Tris/HCl, pH 6.8, before removal and analysis by SDS/PAGE.

Purification of platelet actin

Actin was purified from an acetone-dried powder of freshly isolated human platelets in accordance with published procedures [41,42]. Briefly, after extraction of the acetone-dried powder ×2 with 10 ml of G-buffer/g of powder [42] for 1 h at 4 °C (G-buffer: 2 mM Tris/0.2 mM $CaCl_2$/0.2 mM dithiothreitol/0.2 mM ATP, pH 8.0), the clarified supernatant was applied to a DEAE–cellulose column (DE-52, Sigma) equilibrated with G-buffer and then washed with increasing concentrations of NaCl (0, 0.15, 0.25, and 1.0 M) in a stepwise manner. The eluate from the 0.15–0.25 M NaCl wash was concentrated to a 2 ml volume and applied to a Superose-12 (Pharmacia) gel-filtration column equilibrated with buffer G (4 applications of concentrated eluate at 0.5 ml per loading). Active fractions, identified by immunoblotting of dot-blots with the β-actin specific mAb AC-15, were pooled and the actin was polymerized overnight at 4 °C in the presence of added $MgCl_2$ (2 mM) and KCl (100 mM). After centrifugation at 100000 **g**, the pellet was dissolved in buffer G, reclarified, and was found, by SDS/PAGE, to yield a 43 kDa band that was greater than 90% pure when stained with Coomassie Blue. This material was then applied to a MonoQ anion-exchange column and eluted as described for the purification of neutrophil membrane-associated proteins.

SDS/PAGE and protein blotting

SDS slab gels were prepared and run according to the method of Laemmli [43] except that the running conditions were modified to include 1 mM thioglycolic acid in the cathode buffer and the anode buffer was diluted two-fold. Each sample was prepared on the basis of equal numbers of extracted cells rather than on protein content. A typical $200 \times 200 \times 1.5$ mm-gel was run at 10 mA overnight (approx. 20 h) and the separated proteins were visualized by silver staining [44]. Transfer of the separated proteins to PVDF membranes was performed at 1 mA/cm² for 8 h at 4 °C, according to the method of Towbin et al. [45] and, where required, were visualized by Amido Black staining. PVDF-blotted membranes, when required for sequence analysis, were washed exhaustively with distilled/deionized water and the band of interest were excised and sequenced directly using an ABI 473 Automated Sequencer. Alternatively, the membranes were prepared for immunoblotting by blocking in 4% (w/v) milk powder/PBS (Marvel, 99% fat-free) for 1 h before the appropriate primary antibody was added; incubation was for a minimum of 4 h and all steps were performed at 4 °C. Blots were then washed with ice-cold PBS before probing with a peroxidase-conjugated secondary pAb of appropriate specificity and detection with 4-chloro-1-naphthol.

RESULTS AND DISCUSSION

We compared membrane-associated proteins of freshly isolated, non-apoptotic neutrophil populations with those of neutrophils actively undergoing apoptosis after 20 h in culture (38 ± 2%

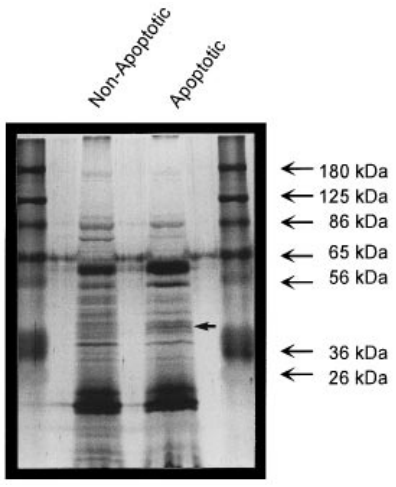

Figure 1 SDS/PAGE analysis of microsomal membrane preparations from normal and apoptotic neutrophils

The particulate membrane fraction (100000 ***g*** pellet) from preparations of fresh (non-apoptotic) and 24 h aged (apoptotic) neutrophil cultures was extracted with Triton X114 and the proteins were resolved by SDS/PAGE (7.5% gel) under denaturing conditions before fixing and silver staining as described in the Materials and methods section. Molecular-mass markers (Sigma, SDS-7B) are shown on the right (kDa). The arrow within the Figure indicates a protein band to be sequenced in later experiments

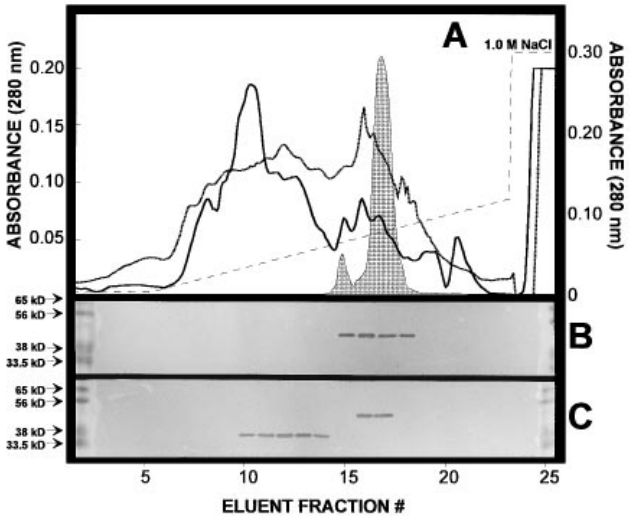

Figure 2 Mature actin is cleaved from membrane fractions of apoptotic neutrophils

(**A**) Trace chromatographs showing the elution profile of proteins from membrane soluble extracts of fresh (———) and apoptotic (-----) neutrophils (2×10^8 cells for each) resolved by anion-exchange chromatography (see the Materials and methods section). The proteins were eluted with an NaCl gradient (– – –). Also shown is the elution profile for β-actin purified from human platelets (shaded peaks) with its presence in fractions 15–18 confirmed by dot-blots of probing with the β-actin specific mAb, AC-15 (results not shown). Aged but non-apoptotic cells were removed from the apoptotic population by counterflow centrifugation before Triton X114 extraction. (**B**) A Western-blot analysis of fractions 2–24, probed with a rabbit pAb to actin, confirmed that a single 43 kDa band found in fresh neutrophil preparations was cleaved to generate a membrane-associated species of 38 kDa in aged populations, shown in (**C**), fractions 10–14, which was consistent with that found by silver-staining of fraction 12 (see Figure 3**A**). All gels were 12% (w/v) acrylamide. Molecular-mass markers (kD = kDa) are shown to the left of the Western blots.

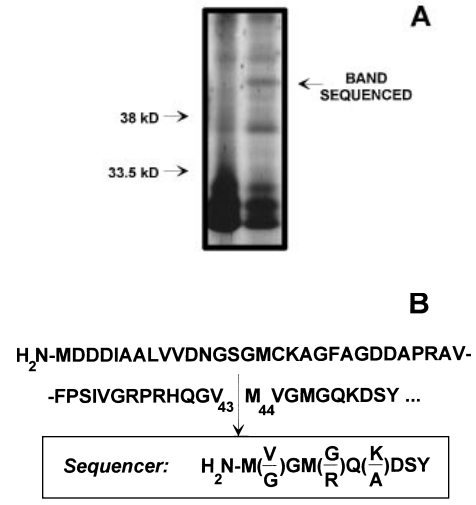

Figure 3 N-terminal amino acid sequence of β-actin, identifying the site of cleavage in constitutive apoptosis

(**A**) Shown is a small region of a silver-stained SDS-polyacrylamide gel to allow comparison of eluted proteins from fraction 12 of both fresh (left lane) and apoptotic (right lane) preparations. Of particular interest is a protein band of approx. 38 kDa (marked with an arrow on the right) which was sequenced in a subsequent experiment as an actin fragment (shown in **B**). Molecular-mass markers (kD = kDa) are shown on the left. (**B**) After Triton X114 extraction, anion-exchange chromatography and SDS/PAGE (12% gel) of apoptotic neutrophils (4×10^8), a 38 kDa protein was identified on a PVDF transfer-blot by Amido Black staining of fraction 12. This band was excised and automated sequencing (Applied Biosystems 473) was performed. The first 10 residues sequenced (boxed), with an initial yield of 5 pmol for methionine on the first cycle, are shown and are compared with a sequence data-bank match (GenBank) for the first 53 amino acid residues of β-actin.

apoptosis, means $\pm$ S.E.M., $n = 7$). Contamination of preparations by low percentages (5%) of necrotic neutrophils or eosinophils was eliminated by the use of counterflow centrifugation [32] in the purification of apoptotic neutrophils. Proteins were resolved by anion-exchange chromatography of Triton X114-solubilized material from plasma-membrane enriched preparations, followed by SDS/PAGE under denaturing conditions. Although few gross differences were observed, a consistent finding was the appearance, in apoptotic cells, of a 38 kDa polypeptide (Figure 1). Mono-Q separation, immunoblotting and N-terminal micro-sequencing provided incontrovertible evidence that a proteolytic fragment of actin occurred, with cleavage consistently observed between Val^{43} and Met^{44} (Figures 2 and 3). Indeed, immunoblotting with a pAb confirmed the 38 kDa band as the only detectable form of actin other than native actin (43 kDa) that associated with plasma membrane preparations (Figures 2C and 3).

While the foregoing data might have been an artefact of cell lysis, albeit selective for actin and restricted to apoptotic cells, strong evidence of actin cleavage *in vivo* came from microscopic examination of neutrophils snap-fixed with methanol and stained with the anti-actin mAb, AC-15. This mAb recognizes the N-terminus of β-actin (Sigma) and consistently revealed a significant reduction in the level of fluorescent staining for apoptotic cells relative to freshly prepared neutrophils (Figure 4A). However, this did not reflect a general loss of F-actin in neutrophil populations undergoing apoptosis, since no reduction in staining with phalloidin-FITC was observed by the sensitive technique of flow cytometry (Figure 4B). Since the N-terminal fragment of actin recognized by mAb AC-15 would be small in molecular weight and highly soluble because of the presence of a significant number of charged residues (13/43), it is more than likely that the relevant peptide was extracted during the multiple washing steps involved in staining after fixation.

Ample evidence now exists to implicate proteases as key mediators of cell death and which may act also within a cascade of activities. This certainly seems to be the case for members of the recently characterized ICE-like family of proteases, many of which have been shown to be activated during apoptosis. Another protease implicated in apoptosis by its association with membrane blebbing and cytoskeletal rearrangements, characteristic of many apoptotic models, is calpain [46]. Further evidence is provided by the effects of calpain inhibitors upon apoptosis in various systems [18,34,35,47,48], and the observation that fodrin, a known target of calpain, is cleaved in apoptosis [20].

Inclusion of the protease inhibitors ALLN (calpain) and zVAD-fmk (ICE-like proteases) in neutrophil cultures for 24 h was found to inhibit constitutive apoptosis by 51% and 52% respectively, judged by typical nuclear condensation on May Grunwald–Giemsa-stained cytospins (Table 1). In both instances this was closely correlated with three cell surface changes known to be associated with neutrophil apoptosis; downregulation of CD16 and CD43 [39], and display of annexin-V binding sites. The latter is believed to be a reliable marker of the phosphatidylserine exposure, which is characteristic of apoptosis [40,49]. In stark contrast was the effect of each inhibitor on actin cleavage, where Western blotting for actin with a pAb revealed that only ALLN was effective in blocking proteolysis of membrane-associated actin (Figure 5).

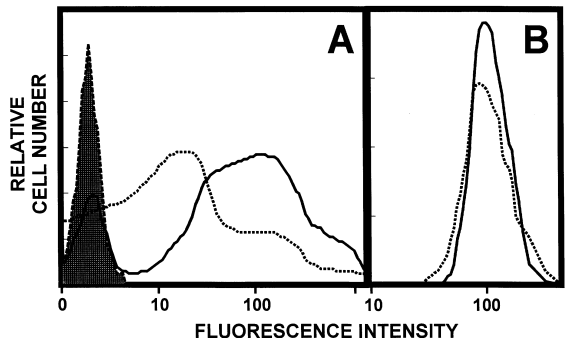

Figure 4 Flow cytometric evidence of cleavage of actin without a change in F-actin content

'Smoothed' staining profiles from a representative experiment. (**A**) Populations of fresh (————) and apoptotic (-----) neutrophils were fixed with ice-cold 70% ethanol and washed with PBS containing 0.2% (w/v) BSA. The preparations were then incubated with a mouse anti-actin mAb, AC-15 (an IgG_1), followed by an FITC-conjugated rabbit anti-mouse pAb to allow fluorescent detection by flow cytometry. Also shown is a control sample of fresh neutrophils incubated in the presence of an irrelevant IgG, followed by an FITC-conjugated rabbit anti-mouse pAb (shaded peak). Note the marked reduction in staining with mAb, AC-15; a 6.7-fold reduction in the intensity of staining was observed within this experiment as assessed by mean peak-channel fluorescence. (**B**) Fresh (————) and apoptotic (— — — — — —) neutrophils were washed free of conditioned media, fixed with Permeafix (Ortho Diagnostic Systems Inc., Raritan, NJ, U.S.A.) at 1 ml/10×10^6 cells for 30 min at 4 °C, washed with PBS and stained for F-actin with phalloidin-FITC as described elsewhere [50], before flow cytometric analysis. Note that the staining patterns are superimposed, indicating no reduction in F-actin content.

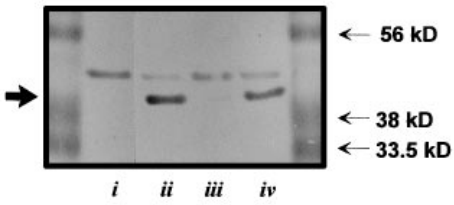

Figure 5 Effect of the protease inhibitors ALLN and zVAD-fmk on actin cleavage

Western-Blot analysis for the cleavage of actin. Particulate fractions (100 000 ***g*** pellet) were prepared from 2×10^7 cells for cultures of fresh (i), aged (ii), aged + 100 μM ALLN (iii), and aged + 100 μM zVAD-fmk (iv) cells. Each pellet was resuspended and boiled in Laemmli dissolving buffer and the proteins were separated by SDS/PAGE before trans-blotting onto nitrocellulose membranes. The blots were probed with a pAb raised against actin purified from chicken back muscle (Sigma product A2668). Note that the actin cleavage product (bold arrow) is selectively diminished by ALLN compared with zVAD-fmk. Molecular-mass markers (kD = kDa) are shown on the right.

The foregoing data demonstrate that actin cleavage occurs during constitutive apoptosis, and that these two events are co-ordinately regulated. Previous work has emphasized that neutrophils undergoing apoptosis selectively lose certain 'injurious' functions normally requiring an intact cytoskeleton, including the capacity for shape change, chemotaxis and degranulation [32]. Although the cells remain intact, on ultrastructural examination they are seen to be spherical in shape demonstrating a lack of organized cytoskeletal elements [29]. Our finding of actin cleavage during constitutive apoptosis provides a new candidate for the mechanism of cytoskeletal dysfunction during cell death, particularly that occurring late in the programme when apoptotic cells cease membrane blebbing and contract into spheres ready for phagocytic clearance. However, the precise role of actin cleavage in the structural changes which occur in cells undergoing apoptosis will require careful study, since there is some evidence that additional cytoskeletal elements may be cleaved (S. B. Brown, unpublished work). For example, a recent report [20] demonstrates that fodrin, a spectrin-like, multifunctional protein present in the cortical cytoskeleton of many eukaryotic cells, undergoes proteolysis in response to a number of death-inducing agents. It was suggested that fodrin cleavage by calpains may be involved in plasma membrane blebbing, an early feature of apoptosis in some cell types but not in neutrophils [20].

Further work will be required also to define the mechanism of actin cleavage during constitutive apoptosis. While the inhibitory effects of ALLN on both events suggests that a calpain-like protease may be involved, we feel that data obtained with protease inhibitors should be interpreted with great caution. Nevertheless, it is notable that the Val^{43}-Met^{44} cleavage shown in our studies does not correspond to a consensus site for cleavage by cysteine proteases of the ICE family, which have been implicated very strongly in the programme of apoptosis [8,10–14]. Furthermore, the sequence of the 43 N-terminal amino acids of actin bears no such site, implying that the described cleavage site did not arise by molecular ticketing after putative initial cleavage by an ICE-like protease. This contrasts with two recent reports [27,28], which concluded that isolated actin may be cleaved by ICE or ICE-like proteases *in vitro*. Cleavage of purified actin with purified ICE or death-activated cell lysates must be interpreted with caution. Furthermore, the cleavage product of actin in extracts of PC12 cells by purified ICE [28] may not be inconsistent with our own results. Indeed, in the absence of an N-terminal sequence for either experiment it remains a distinct possibility that the 41 kDa actin fragments seen after treatment with cell extracts and with purified ICE were not the same [28]. This opens up the possibility of upstream regulation of a

Table 1 Effect of ALLN and zVAD-fmk on constitutive apoptosis in neutrophils cultured for 24 h *in vitro*

Data is expressed as the means ± the 95% confidence intervals for eight experiments except for the combined inhibitor study where $n = 4$. n.d., not determined.

Culture conditions	Apoptosis (%) Giemsa staining	CD16 shedding	CD43 shedding	Annexin-V binding
Control (aged)	44.5 ± 10.0	47.3 ± 9.1	44.0 ± 8.0	44.0 ± 11.4
Aged + ALLN (100 μM)	21.7 ± 4.0	27.4 ± 2.2	26.1 ± 2.8	25.6 ± 5.4
Aged + zVAD-fmk (100 μM)	21.2 ± 1.3	22.2 ± 2.9	20.3 ± 2.6	20.6 ± 2.9
Aged + ALLN + zVAD-fmk (each 100 μM)	8.9 ± 2.2	n.d.	n.d.	n.d.

proteolytic cascade. Furthermore, our findings with the broad spectrum inhibitor of ICE-like proteases, zVAD-fmk, imply that the actin cleavage event observed in constitutive apoptosis is unlikely to be directly dependent on such proteases. The identity and regulation of the protease(s) bringing about the Val^{43}-Met^{44} cleavage of actin will require further examination. However, the data presented here are an important addition to studies on apoptosis indicating key roles for proteases which are not members of the ICE family. We speculate that actin cleavage may play an important role in the previously observed disablement of cytoskeletal function associated with apoptosis in the neutrophil, thereby reducing the potential for damaging functions such as granule exocytosis and immobilization of the cell so that it can be cleared by phagocytosis.

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