We have previously demonstrated that calpain is responsible for the cleavage of Bax, a proapoptotic protein, during drug-induced apoptosis of HL-60 cells (Wood, D. E., Thomas, A., Devi, L. A., Berman, Y., Beavis, R. C., Reed, J. C., and Newcomb, E. W. (1998) Oncogene 17, 1069–1078). Here we show the sequential activation of caspases and calpain during drug-induced apoptosis of HL-60 cells. Time course experiments using the topoisomerase I inhibitor 9-aminoo-20(S)-camptothecin revealed that cleavage of caspase-3 substrates poly(ADP-ribose) polymerase (PARP) and the retinoblastoma protein as well as DNA fragmentation occurred several hours before calpain activation and Bax cleavage. Pretreatment with the calpain inhibitor calpeptin blocked calpain activation and Bax cleavage but did not inhibit PARP cleavage, DNA fragmentation, or 9-amino-20(S)-camptothecin-induced morphological changes and cell death. Pretreatment with the pan-caspase inhibitor benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) inhibited PARP cleavage, DNA fragmentation, calpain activation, and Bax cleavage and increased cell survival by 40%. Interestingly, Z-VAD-fmk-treated cells died in a caspase- and calpain-independent manner that appeared morphologically distinct from apoptosis. Our results suggest that excessive or uncontrolled calpain activity may play a role downstream of and distinct from caspases in the degradation phase of apoptosis.

Recent work has demonstrated that a family of cysteine proteases, now termed caspases (1), related to the Caenorhabditis elegans protease CED-3 and the mammalian interleukin-1β-converting enzyme becomes activated during apoptosis and is necessary for several processes within the apoptotic pathway. Caspases are constitutively expressed and synthesized aszymogens that require proteolytic cleavage, either through a proteolytic cascade or by intermolecular autoproteolysis, to become activated (2). Once activated, caspases cleave their substrates in a highly sequence-specific fashion with a near absolute requirement for an aspartic acid in the P1 position (3). For example, caspase-3 cleaves its substrates at a conserved DXXD motif (2). Many caspase substrates have been identified and include nuclear proteins such as poly(ADP-ribose) polymerase (PARP) and retinoblastoma (RB) as well as structural proteins of the nucleus and cytoskeleton including lamins and gelsolin (4–7). It is believed that cleavage of some of the known caspase substrates leads to the characteristic changes in morphology and biochemistry observed in apoptotic cells (8). As an example, caspase-3 mediated cleavage of DNA fragmentation factor results in chromatin condensation and DNA fragmentation during apoptosis (9, 10).

Although caspases play a major part in the demise of cells that have been triggered to undergo apoptosis, there is evidence that other proteases including calpain may also be involved in this process. Calpain is a family of calcium-dependent cysteine proteases of which two isozymes, μ- and m-calpain, are ubiquitously expressed (11). These enzymes are heterodimeric and consist of an 80-kDa catalytic subunit and a 30-kDa subunit whose function is unclear (11). In contrast to caspases, calpain does not appear to have strict sequence requirements for substrate cleavage (11). Under normal physiological conditions these proteases engage in limited proteolysis of a number of different substrates found throughout the cell, including nuclear proteins such as p53 and cyclin D1 (12, 13), as well as proteins associated with the cytoskeleton and plasma membrane, including talin and N-methyl-D-aspartate receptors (14, 15). Unlike the caspases, which function only during apoptosis, calpain has been implicated in several processes involved in normal cellular metabolism and physiology (13–17), such as remodeling of the actin cytoskeleton during cell motility (16).

Uncontrolled or constitutive calpain activity has been observed in several pathological conditions including Alzheimer’s disease, muscular dystrophies, and tumorigenesis (18–21). Excessive calpain activation has also been observed in many instances of cell death (22–24). For example, proteolysis of several calmodulin-binding proteins by calpain occurs in neurons that have been exposed to neurotoxins (24). Other studies have demonstrated that calpain may play a role in the apoptotic death of a variety of different cell types (25–31). For instance, calpain-mediated cleavage of the cytoplasmic domain of the integrin β3 subunit in human umbilical vein endothelial cells contributes to disruption of signaling and detachment from the matrix resulting in apoptosis (30).

Previously we have shown that the proapoptotic protein Bax is cleaved from its native 21-kDa form to an 18-kDa species by calpain during drug-induced apoptosis of HL-60 cells (32, 33). Here we extend these findings and show that calpain is activated secondary to caspase activation during apoptosis. Time course experiments demonstrate that calpain activation occurs after cleavage of caspase substrates and DNA fragmentation. Experiments with HL-60 cells revealed that inhibition of calpain by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: PARP, poly(ADP-ribose) polymerase; RB, retinoblastoma; COX II, cytochrome oxidase (subunit II); Z-VAD-fmk, benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone; 9-AC, 9-amino-20(S)-camptothecin; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-propionate; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propane sulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
pain activity by the calpain inhibitor calpeptin failed to block caspase activity and apoptosis, whereas pretreatment of cells with the pan-caspase inhibitor benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) blocked both caspase and calpain activity and partially inhibited cell death. Thus, in this apoptosis model there appears to be a hierarchy of protease family activation during the degradation phase of the apoptotic program.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HL-60 cells were cultured in 5% CO2 and 95% humidified air atmosphere at 37 °C in complete RPMI 1640 medium (Cellgro) containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 2 mM glutamine (all from Life Technologies, Inc.). Cells were split every 3 days to ensure logarithmic growth.

**Time Course of Drug-induced Apoptosis and Inhibitor Studies**—For the time course experiments, 3 x 10^5 cells were cultured in 6-well dishes (Nunclon) in 3 ml of medium for 36 h at 37 °C before the addition of the drug 9-amino-20(S)-camptothecin (9-AC; Ref. 32) or its diluent (20% dextrose and 0.9% NaCl in sterile water). Cells were harvested at the appropriate time points, washed with ice-cold PBS, and subjected to lysis and protein extraction for the purposes of immunoblotting and in vitro fluorogenic assays. For the inhibitor studies, cells cultured as described above were pretreated with either 10 μM Z-Leu-Nle-H (calpeptin; Calbiochem) for 30 min or 100 μM Z-VAD-fmk (Enzyme Systems Products) for 2 h at 37 °C. Control cultures were pretreated for the same time periods with an equivalent volume of inhibitor vehicle (Me2SO; Sigma). Cells were then left untreated, treated with 9-AC diluent, or challenged with 4.0 mM 9-AC. At the appropriate time intervals, the samples were removed from each culture condition, diluted 1:5 with trypan blue, and counted (at least 50 cells were counted from each sample). The percentage of cells scoring positive for uptake of the dye was calculated using the following formula: (number of trypan blue positive cells/number of trypan blue positive cells + number of trypan blue negative cells) x 100. To photograph the cells, the entire culture volume from each culture condition at each time point was diluted 5-fold with trypan blue and allowed to equilibrate for 10 min at 25 °C. Cells were then washed once with PBS, resuspended in PBS at 1 x 10^5 cells/ml, and plated into 24-well plates (Nunclon) for light microscopy.

**RESULTS**

**Time Course of Caspase Activation and DNA Fragmentation with 9-AC Treatment of HL-60 Cells**—Previous studies suggested that drug treatment of HL-60 cells activated both caspases and calpain (33). To delineate well characterized caspase-dependent events during apoptosis relative to the timing of calpain activation and Bax cleavage, HL-60 cells were treated with the topoisomerase I inhibitor 9-AC, a camptothecin analog (32), to induce apoptosis. Whole cell protein lysates were generated for immunoblotting with antibodies against the caspase substrates PARP and RB, both of which have been shown to be proteolytically cleaved in HL-60 cells during apoptosis (4, 5). The DNA repair enzyme PARP is a substrate for caspases-2, -3, -7, and -9 (37), which proteolyze it from its full-length form of 116 kDa to an apoptosis-specific 85-kDa fragment (4). Another caspase target is RB, which has been shown to be cleaved to a 48-kDa fragment in a caspase-dependent manner in HL-60 cells during etoposide-induced apoptosis (5). As shown in Fig. 1A, PARP cleavage was detected by 2 h after the introduction of 9-AC, with complete conversion of the full-length 116-kDa protein to the 85-kDa fragment by 4 h. Also illustrated in Fig. 1A is RB cleavage, which occurred after 4 h of 9-AC treatment as indicated by the appearance of the 48-kDa cleavage product (5). In addition to caspase activation detected by proteolytic cleavage of PARP and RB, we observed DNA fragmentation by 4 h of 9-AC treatment as illustrated by the characteristic ladder formation (Fig. 1B). Pretreatment of HL-60 cells with the pan-caspase inhibitor Z-VAD-fmk (10 μM) inhibited DNA fragmentation in cells treated with 9-AC, whereas pretreatment with the calpain-specific inhibitor calpeptin (100 μM) did not inhibit this process (Fig. 1B). These results demonstrate the requirement for caspase-3-like proteases in the pathway leading to DNA fragmentation in HL-60 cells treated with 9-AC.

**In Vitro Kinetics of Caspase-3-like Activity with 9-AC Treatment of HL-60 Cells**—The fluorogenic peptide Ac-DEVD-AMC has been shown to be a suitable substrate for caspase-3 in vitro DNA Fragmentation Assay—The following protocol was adapted from McGahan et al. (36). HL-60 cells, cultured as described above, were treated with either 4.0 μM 9-AC or 9-AC diluent for the time course experiments, or cells were pretreated with either 10 μM calpeptin or 100 μM Z-VAD-fmk and then challenged with 4.0 μM 9-AC for 9-AC treatment. At the appropriate time points, the supernatants were resuspended in 20 μl of lysis buffer (20 mM EDTA, 100 mM Tris, pH 8.0, and 0.8% sodium laureyl sarcosine; all from Sigma). After complete resuspension, 10 μl of a 1 mg/ml RNase/T1 mixture mix (Ambion) was added, and the lysates were incubated at 50 °C with constant rotation. The recovered DNA (10 μl/sample) was electrophoresed on 1.0% agarose gels containing 1 μg/ml ethidium bromide in TAE buffer (40 mM Tris acetate, pH 8.0, and 2 mM EDTA) to visualize DNA fragmentation.

**Trypan Blue Exclusion Assay**—Loss of membrane integrity was determined by the inability of cells to exclude the vital dye trypan blue. For the time course experiments, cells were cultured as described above and either left untreated or were pretreated with Z-VAD-fmk (100 μM for 2 h), calpeptin (10 μM for 30 min), or both calpeptin and Z-VAD-fmk. Cells were then left untreated, treated with 9-AC diluent, or challenged with 4.0 mM 9-AC. At the appropriate time intervals, the samples were removed from each culture condition, diluted 1:5 with trypan blue, and counted (at least 50 cells were counted from each sample). The percentage of cells scoring positive for uptake of the dye was calculated using the following formula: (number of trypan blue positive cells/number of trypan blue positive cells + number of trypan blue negative cells) x 100. To photograph the cells, the entire culture volume from each culture condition at each time point was diluted 5-fold with trypan blue and allowed to equilibrate for 10 min at 25 °C. Cells were then washed once with PBS, resuspended in PBS at 1 x 10^5 cells/ml, and plated into 24-well plates (Nunclon) for light microscopy.

**In Vitro Fluorogenic Caspase-3 Cleavage Assay**—Whole cell protein lysates (10 μg) generated as described above, from 9-AC-treated cells or diluent-treated control cells were incubated with 25 μM caspase-3 substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) (Calbiochem) (34) for 1 h at 37 °C in the presence of a caspase reaction buffer containing 100 μM HEPES, pH 7.4, 10% sucrose, 5 mM dithiothreitol, and 0.1% CHAPS (all from Sigma) (35). For the inhibition studies, 10 μM caspase-3 inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) (Calbiochem) (34) was added simultaneously with the substrate. All reactions were carried out in 96-well plates (Dynatech) with proteolysis of the fluorescent peptides measured using a fluorescent plate reader (Perkin-Elmer LS50B) with filter settings at 380 nm for excitation and 460 nm for emission. All reactions were accompanied by an AMC standard curve.
To confirm the immunoblot results for PARP cleavage, which demonstrated caspase-3-like activity after 2 h of 9-AC treatment, we analyzed the ability of protein extracts, taken from 9-AC or 9-AC diluent-treated HL-60 cells at successive time points, to hydrolyze Ac-DEVD-AMC. At 2 h following drug challenge there was a 4-fold increase in Ac-DEVD-AMC hydrolysis compared with lysates made from diluent-treated cells (Fig. 2). This increase in activity coincided with the initiation of PARP cleavage (Fig. 1A). Peak values for Ac-DEVD-AMC hydrolysis were achieved by 8 h after introduction of drug to the cultures, at which point there was a 20-fold increase in caspase-3-like activity compared with control lysates (Fig. 2). From 8 to 12 h, this activity progressively declined, although there was still a 15-fold increase in activity at 12 h compared with control lysates (Fig. 2). The addition of 1.0 μM caspase-3 inhibitor Ac-DEVD-CHO (34) to the lysates derived from drug-treated cells at each time point reduced the hydrolysis of Ac-DEVD-AMC to levels that were indistinguishable from those derived from control diluent-treated cells (Fig. 2).

**Time Course of Calpain Activation with 9-AC Treatment of HL-60 Cells**

Under normal conditions calpain activation is a controlled process leading to limited cleavage of its substrates (11, 39). Autolysis of the 80- and 30-kDa calpain subunits is believed to be an irreversible process that results in activation of calpain but is thought to occur in vivo only under extreme conditions such as during necrosis and apoptosis (39). Autolysis of the 30-kDa subunit has been used previously to indicate calpain activation during apoptosis (26). As shown in Fig. 3, the 30-kDa subunit of calpain underwent autolysis beginning at 8–10 h, with a significant increase in the amount of calpain subunit breakdown products observed at 12 and 24 h. In HL-60 cells, Bax has been shown to be cleaved by calpain from its native 21-kDa form to an 18-kDa fragment upon treatment with 9-AC (32, 33). As illustrated in Fig. 3, Bax cleavage was initiated at 10 h, and by 24 h more than 50% of the 21-kDa Bax had been converted to the 18-kDa form as determined by densitometric analysis. Bax normally resides in the cytosol, but upon treatment of cells with apoptotic stimuli, it rapidly translocates to the mitochondria where it inserts into the outer membrane as a 42 kDa homodimer (40–42). We have shown that calpain activity within mitochondria-enriched fractions is responsible for Bax cleavage (33). To determine whether Bax
cleavage at the mitochondria is the result of a selective proteolytic event as opposed to a general degradative process occurring during the late stages of apoptosis, we examined the status of another mitochondrial membrane protein, the electron transport chain component COX II (43). As shown in Fig. 3, COX II remained intact over the 24-h time interval of drug treatment, suggesting that Bax cleavage is not the result of a bulk degradation of mitochondrial membrane proteins. Together with the results presented in Fig. 1, these experiments show that caspase-3-like proteases become activated after 2 h of 9-AC treatment and attain peak activity by 8 h, the time at which calpain autolysis/activation is first detected.

The Pan-caspase Inhibitor Z-VAD-fmk Blocks the Activation of Both Caspases and Calpain—Because we have demonstrated that calpain appeared to be active downstream of the caspases, we sought to determine whether calpain activation was dependent upon caspase activation. The pan-caspase inhibitor Z-VAD-fmk blocks the processing of caspases-2, -3, -6, and -7, suggesting that its target is a caspase(s) at or near the initial stage of the apoptotic program (3). When HL-60 cells were pretreated with this caspase inhibitor at 100 \( \mu \text{M} \) prior to 9-AC treatment, PARP cleavage was effectively blocked (Fig. 4A). Autolysis of the 30-kDa calpain subunit was also completely blocked, whereas cleavage of Bax was inhibited by more than 80% in the presence of Z-VAD-fmk (Fig. 4A). In contrast, pretreatment with the calpain inhibitor calpeptin at 10 \( \mu \text{M} \) did not block PARP cleavage (Fig. 4B). Although calpeptin was unable to completely inhibit autolysis of the 30-kDa calpain subunit, it did inhibit calpain activation by ~60% (Fig. 4B). Calpeptin pretreatment also resulted in a 65% reduction in Bax cleavage (Fig. 4B). Despite the fact that calpeptin was highly effective at blocking calpain activation and Bax cleavage, it did not provide any protection against 9-AC-induced apoptosis (see Fig. 5). As illustrated in Fig. 4C, pretreatment with a combination of calpeptin and Z-VAD-fmk gave similar results to cultures treated with Z-VAD-fmk alone. The ability of Z-VAD-fmk to effectively block caspase function as well as calpain activation suggests that calpain activation may be a caspase-mediated event.

Loss of Membrane Integrity Is Partially Inhibited by Z-VAD-fmk but Not by Calpeptin—The culmination of apoptosis in HL-60 cells treated with 9-AC is the breakdown of the plasma membrane reflected by the inability of cells to exclude the vital dye trypan blue. As shown in Fig. 5, control cells and cells treated with protease inhibitors alone displayed a small amount (~5%) of membrane integrity loss that remained essentially unchanged over the 20-h observation period. Cells treated with 9-AC alone remained impermeable to the dye for up to 6 h, at which point less than 10% of the cells were stained (Fig. 5). However, after 6 h there was a steady progression in the number of cells that exhibited plasma membrane damage such that by 20 h, over 70% of the cells stained positive for the dye (Fig. 5). Cells that were pretreated with calpeptin before the addition of 9-AC showed virtually the same kinetics for loss of membrane integrity as cells treated with 9-AC alone (Fig. 5). Similar to cells treated with 9-AC alone, less than 10% of the cells pretreated with Z-VAD-fmk displayed loss of membrane integrity up to 6 h (Fig. 5). Although Z-VAD-fmk-pretreated cells became permeable to the dye over the next several hours, the overall levels of membrane integrity loss were reduced relative to cells treated with 9-AC. Indeed, by 20 h there was a 40% reduction in the number of Z-VAD-fmk-pretreated cells that had lost membrane integrity (Fig. 5). Cells pretreated with a combination of Z-VAD-fmk and calpeptin displayed essentially the same kinetics and levels of membrane integrity loss as cells treated with Z-VAD-fmk alone (Fig. 5). These results indicate that the effects of Z-VAD-fmk plus calpeptin were not additive in terms of inhibiting cell death and that Z-VAD-fmk alone afforded considerable protection from cell death even up to 20 h of 9-AC treatment.

Pretreatment with Z-VAD-fmk, but Not Calpeptin, Results in Nonapoptotic Death—The breakdown of the plasma membrane in 9-AC-treated cells was preceded by a defined series of morphological changes associated with apoptosis. Control, diluent-treated cells (Fig. 6A–C) and cells treated with calpeptin, Z-VAD-fmk, or calpeptin plus Z-VAD-fmk in the absence of 9-AC displayed no change in morphology or plasma membrane integrity throughout the 20-h time course interval (data not shown). In contrast, treatment of cells with 9-AC resulted in an early onset (2 h) of membrane blebbing that progressed to cellular condensation and formation of apoptotic bodies by 6 h (Fig. 6, D and E). These changes were followed several hours (12 h) later by disruption of the plasma membrane as measured...
Survival of HL-60 cells was assessed by measuring loss of membrane integrity with the uptake of the vital dye trypan blue. Taken together these results suggest that the presence of Z-VAD-fmk and 9-AC exhibit some blebbing at later time points, but do not condense in size. However, these cells eventually lose membrane integrity and become permeable to trypan blue. Taken together these results suggest that the 9-AC-induced apoptotic death observed in HL-60 cells with or without calpeptin differs from the 9-AC-mediated death observed in the presence of Z-VAD-fmk.

DISCUSSION

Previous reports have demonstrated a role for calpain in the apoptotic death of several cell types including primary cultures of thymocytes, neurons, human umbilical vein endothelial cells, and neutrophils as well as in permanently established cell lines (25–31, 35, 44). Recent data obtained in our laboratory indicated that calpain activity was present in HL-60 cells triggered to undergo apoptosis with the chemotherapeutic agent 9-AC (33). In this study we wanted to determine the timing of calpain activation relative to that of the caspases. Second, we wanted to determine what role calpain may play in our model of drug-induced apoptosis of HL-60 cells. The data presented here indicate a sequential relationship between activation of caspases, the primary death effector proteases, and activation of calpain. Our data suggest that there is a caspase-dependent activation of calpain during the degradation stage of apoptosis.

HL-60 cells treated with the topoisomerase I inhibitor 9-AC exhibited caspase-3-like activity as early as 2 h after treatment, with peak activity at 8 h (Figs. 1 and 2). This initiation of caspase activity was coincident with membrane blebbing (Fig. 6D), but preceded DNA fragmentation (Fig. 1B) and condensation in cell size (Fig. 6, E and F), all of which occurred in cells still displaying intact plasma membranes (Fig. 5). In comparison with the early activation of caspases, calpain activation and subsequent cleavage of Bax took place several hours after the initiation of morphological changes and DNA fragmentation (Fig. 3). Indeed, the kinetics of calpain activity paralleled the initiation and progressive loss of membrane integrity (Figs. 3 and 5). These results taken together indicate a sequential activation of proteases consisting of an early activation of caspases followed by a 6-h increase in caspase function, which at its apex coincided with calpain activation.

Activation of both caspases and calpain has been reported in one other lymphoid cell line, U937, during tumor necrosis factor-induced apoptosis (35). Similar to the results described here, calpain in U937 cells appeared to be active following the initiation of caspase activity (35). However, in U937 cells, calpeptin proved to be more effective than Z-VAD-fmk at inhibiting several late apoptotic events including DNA and nuclear fragmentation, plasma membrane blebbing, and formation of apoptotic bodies (35). Unlike HL-60 and U937 cells, the neuroblastoma cell line SH-SY5Y was found to simultaneously activate caspases and calpain during staurosporine-induced apoptosis (26, 44). Interestingly, in SH-SY5Y cells caspases and calpain cleave some of the same target substrates, such as nonerythroid α-spectrin (α-fodrin) and calcium/calmodulin-dependent protein kinase IV, to produce caspase-specific and calpain-specific fragments (26, 44). This situation is not observed in HL-60 cells because α-spectrin is cleaved early during apoptosis in a caspase-dependent fashion, whereas Bax is cleaved by calpain late in apoptosis strengthening the notion of a temporal relationship between the activation of the two types of proteases in our model system. The differences reported between cell lines regarding the timing of calpain activation and the ability of calpain to effect some apoptotic changes may reflect cell lineage-specific requirements for calpain activity during apoptosis.

As we noted, caspase activity was at its highest at 8 h after 9-AC treatment, which was coincident with the onset of calpain activation. It is tempting to speculate that in some cell types, such as lymphoid cell lines, calpain activity may be dependent upon caspase function. Under normal circumstances calpain activity is modulated by the presence of its endogenous inhibitor calpastatin (11). Calpastatin is generally thought to reside in the cytosol where it complexes with calpain, resulting in its inactivation (11). Cleavage of calpastatin by caspases has been observed in both Fas-induced apoptosis of Jurkat cells and in

\[2 \text{ D. E. Wood, unpublished observations.} \]
staurosporine-induced apoptosis of SH-SY5Y cells, resulting in a reduction of this inhibitor’s effectiveness in vitro (45). At present, we have been unable to identify calpastatin in HL-60 cells by Western blotting thereby precluding a determination of whether caspase-mediated cleavage of calpastatin occurs in this cell line. However, the reduction in the activity of calpastatin by caspase cleavage could provide a mechanism for the uncontrolled calpain activity that is observed during the latter stages of apoptosis.

Pretreatment of HL-60 cells with the calpain inhibitor calpeptin did not prevent caspase-3-like function, DNA fragmentation, morphological changes, or plasma membrane damage associated with 9-AC-induced apoptosis. In marked contrast, pretreatment with the pan-caspase inhibitor Z-VAD-fmk resulted in the inhibition of caspase-3-like function, DNA fragmentation, and morphological changes. In addition, Z-VAD-fmk treatment reduced cell death by 40% (Fig. 5). However, HL-60 cells pretreated with Z-VAD-fmk eventually died in a process that by morphological criteria did not resemble the apoptotic changes observed in cells treated with 9-AC alone (Fig. 6). These results are similar to previously reported data indicating that inhibition of caspase activity with Z-VAD-fmk results in a nonapoptotic, necrotic-like death (46–49). In addition to blocking caspase activation and function, calpain activation measured by the autolysis of the 30-kDa calpain subunit 8314 indicating that inhibition of caspase activity with Z-VAD-fmk may play a role that is downstream and distinct from caspases in the degradation phase of apoptosis.

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