Constitutive Apoptosis in Human Neutrophils Requires Synergy between Calpains and the Proteasome Downstream of Caspases*

(Received for publication, July 2, 1998, and in revised form, August 26, 1998)

Birgit Knepper-Nicolai, John Savill, and Simon B. Brown‡

Division of Renal and Inflammatory Disease, Department of Medicine, University Hospital, Nottingham NG7 2UH, United Kingdom

Programmed cell death invariably requires the activation of proteolytic cascades that are not yet well defined but are initiated after apical caspase activation. We provide evidence that calpains and the proteasome function synergistically downstream of caspases to assist the constitutive apoptotic program of aging neutrophils, which plays an important role in resolution of inflammatory responses. Inhibitor studies indicated that “tethering” of preapoptotic senescent neutrophils to human macrophages required caspase activity. However, the development of morphological features characteristic of apoptosis, including nuclear morphology, PS exposure, surface protein shedding, and the capacity to be ingested by macrophages, required the downstream action of either calpains or the proteasome. Calpain activities were constitutively active in freshly isolated neutrophils and responsible for rearrangements in the protein composition and structure of the plasmalemmal cytoskeleton as they aged in culture and underwent apoptosis. This included a dissociation of protein(s) from F-actin, a candidate mechanism for increased susceptibility to cleavage, and a loss in immunodetectable α-actinin and ezrin, two actin-binding, membrane-anchoring proteins. These results clarify roles for different classes of proteases in a physiologically important form of constitutive apoptosis.

Proteases are increasingly recognized as playing a key role in effecting apoptosis and its associated morphology in cells destined to die programmed deaths (1–4). Although traditionally viewed as participating in self-contained catabolism, the importance of these proteases has strengthened with two major advances: first, the discovery of a novel family of cysteinyl proteases (5), the caspases, which cleave to the carboxyl side of aspartic residues and which are predominantly activated via autonomous cascades in the execution of an apoptotic program (6); and second, the realization that limited proteolysis results not only in a loss of function for many substrates (7) but also in a gain of function for others. Of relevance is the caspase-dependent activation of Gas2 (8), PAK-2 (9), and gelsolin (10, 11), which effect apoptotic morphology by disrupting the filamentous actin cytoskeleton, which may then undergo limited proteolysis (12, 13).

The first clear evidence that proteases were important in apoptosis came from the use of nonspecific protease inhibitors.

Darzynkiewicz and colleagues (14) showed that as cells underwent apoptosis they lost cellular protein and that both apoptosis and protein loss could be blocked with protease inhibitors (15). These studies have since been expanded with a plethora of inhibitors to implicate serine proteases (16–19), granzymes (20–23), calpains (12, 13, 24–26), cathepsins (26), caspases (27–32), and most recently the proteasome (33, 34) in mediating molecular aspects of apoptosis. Unfortunately the inhibitors used in these studies have frequently proved not to be as selective as first believed so that, with the exception of caspases, our understanding of the role for these proteases in regulating apoptosis has remained limited.

A case in point are calpains, for which several isoforms have now been identified and which are categorized as being either ubiquitous or tissue specific (3, 35, 36). Like the caspases they are cysteinyl proteases but without the constraints of a defined sequence-specific cleavage site within their target substrates other than accessibility. Calpains are also regulated by calcium and are implicated in the proteolysis of proteins involved in maintaining and regulating the cytoskeletal structure of the cell, particularly at the plasmalemmal interface (37, 38). It is because of these outcomes of calpain action, which have much in common with aspects of apoptosis, i.e. calcium fluxes and the disassembly of the cytoskeleton, that calpains are often postulated to have a role in apoptosis. But whether calpain activity is proximal, as has been suggested, or distal to initiation of the apoptotic process remains unclear. Certainly, inhibitors of calpains are known to prevent calpain activation, block calpain-dependent proteolytic events, and delay cell death in a number of experimental models (12, 13, 24–26, 39–41).

However, serious doubts have been raised as to whether calpains have any role in apoptosis. First, the cleavage of fodrin as a molecular marker of apoptosis is more than likely effected by a caspase distinct from CPP32 (41–43) than by a calpain (39). Second, inhibition of thymocyte apoptosis by the calpain inhibitor ALLN, which also inhibits the proteasome (44), can be reproduced with a specific inhibitor of the proteasome, i.e. lactacystin (33, 34). Defining the role of calpains in apoptosis has also been confounded by the use of experimental models in which apoptosis is drug or stress induced, in which case calpain activation might occur independently of processes necessary for apoptosis.

To extend our own studies on the role of calpains and eliminate the foregoing concerns, we have focused on the self-contained and constitutive apoptosis of senescent neutrophils, a physiological form of programmed cell death responsible for safe phagocytic removal of senescent neutrophils during resolution of inflammation (45–47). We report herein that calpains are involved in neutrophil apoptosis and that one of their functions is to modify the composition and association of actin-binding proteins with F-actin at the plasma membrane.
Calpains Mediate Neutrophil Apoptosis

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of analytical reagent grade and purchased from Sigma unless stated otherwise and include the anti-actin mAb1 AC-15 and the anti-α-actinin polyclonal antibody (catalog number A-2543). Percoll was obtained from Amersham Pharmacia Biotech; sodium citrate solution was from Pharma Hanel GmbH (Hannover, Germany); culture media (Hanks’ balanced salt solution, RPMI 1640 medium, and Iscove’s modified Eagle’s medium), and supplements (penicillin, streptomycin, glutamine, and fetal calf serum) were from Life Technologies, Inc.; FITC- and r-phyceroerythrin-conjugated anti-CD16 (mAb 3G8) was from Caltag Laboratories (Brad- sure Biologicals, Shepshed, UK); FITC-conjugated annexin V was from BioWhittaker UK Ltd. (Wokingham, UK); anti-ezrin mAb was from ICN Biomedicals Ltd. (Thame, UK); ALLN was from Boehringer Mannheim; zVAD was from Enzyme Systems Products (Dublin, CA); calpeptin and lactacystin were from Calbiochem; and CellProbe LY-Calpain enzyme substrate was from Coulter Electronics Ltd. (Luton, UK).

Granulocyte Isolation and Culture—Human neutrophils and monocytes were isolated from freshly drawn venous blood after citration, dextran sedimentation, and plasma-Percoll density gradient centrifugation as described previously (46, 48). Percoll density separation resulted in two distinct leukocyte layers, a neutrophil-enriched fraction (>95% purity), which contained eosinophils as the only major contaminant, and a mixed monocyte/lymphocyte fraction contaminated by neutrophils. The neutrophil fraction was washed free of plasma and incubated at 37 °C in Iscove’s modified Dulbecco’s medium supplemented with 10% autologous platelet-rich plasma, defined serum. Monocytes were further purified by counterflow centrifugation (J2-21, Beckman Instruments) to yield preparations of >95% purity (49). Monocyte-derived macrophages (Mø) were obtained by the standard technique of culturing adherent monocytes in Iscove’s medium plus 10% platelet-rich, plasma-derived serum (46, 51). Aged neutrophil cultures were also enriched for apoptotic versus nonapoptotic cells by counterflow centrifugation, which removed all nonviable cells as assessed by trypan blue staining.

The protease inhibitors ALLN (100 mM stock concentration), calpeptin (100 mM), lactacystin (10 mM), and zVAD (100 mM), all dissolved in Me2SO, were added to neutrophils cultured in Teflon-lined flat-bottom wells at concentrations defined elsewhere. For controls, Me2SO was used at an appropriate dilution. Neutrophils were routinely cultured for 3 × 106 neutrophils, in some experiments defined as apoptotic (50), as those with diminished calpain activity, were added to neutrophils cultured in Teflon-lined flat-bottom wells at concentrations defined elsewhere. For controls, Me2SO was used at an appropriate dilution. Neutrophils were routinely cultured for 1 h before the addition of any reagents.

Macrophage Recognition of Aged Neutrophils—Neutrophils, aged in culture and washed free of conditioned media, were resuspended in serum-free Iscove’s modified Dulbecco’s medium and added to a prewashed monolayer of monocyte-derived macrophages (Mø), as described (46, 47). Typically 5 × 105 neutrophils, in some experiments enriched for apoptotic cells by counterflow centrifugation, to a 1 × 106 Mø cells for 30 min at 37 °C before the Mø monolayer was washed free of noninteracting neutrophils with phosphate-buffered saline, fixed with 1% glutaraldehyde, and stored in inverted microscopy. Neutrophils were histochemically distinguished within macrophages by their endogenous myeloperoxidase activity (46). Alternatively, washed Mø cells were first treated with trypsin/EDTA to remove all non-ingested neutrophils before recovering the Mø cells by EDTA treatment at 4 °C (46). Myeloperoxidase staining was then performed on cytocentrifuge preparations of the harvested Mø cells.

Assessment of Apoptosis and FACS Analysis—Apoptosis was assessed either by microscopic examination of cytocentrifuge preparations fixed in methanol and stained with May Grunwald/Giemsa (46, 49) or by FACS analysis of immunofluorescently stained cells labeled with either an FITC-conjugated anti-CD16 mAb (3G8) (50) or an FITC-conjugated annexin V (52). Labeled cells were applied to a Becton Dickinson FACSscan flow cytometer, which automatically and simultaneously measures the fluorescence of individual cells identified by their size-dependent light-scattering properties. Shedding of the cell surface RIIC (anti-α-actinin polyclonal antibody) as well as the binding of annexin V to exposed PS are reliable markers for the terminal phase of neutrophil apoptosis of cultured neutrophils (12, 50, 52).

Flow Cytomtyzological Assessment of Intracellular Calpain Activity—Flow cytometry is the study of enzyme activities in intact viable cells by flow cytometry using cell-permeable quenched fluorogenic substrates. Calpain activity was determined using LY-Calpain according to the manufacturer’s instructions, except that neutrophils were maintained in Teflon wells until FACS analysis. Briefly, fresh or aged neutrophils, which may have been preincubated with calpeptin (100 μM), ALLN (100 μM), or Me2SO (0.1%), were washed with Hanks’ balanced salt solution and resuspended at 3 × 106/ml in Hanks’ balanced salt solution prewarmed to 37 °C. Aliquots (100 μl) were then removed and incubated at 37 °C with 50 μl of LY-Calpain or its control rhodamine 110 didepitate substrate for 10 min before stopping the reaction by transferring to precooled FACS tubes kept on ice. Samples were typically analyzed within 15 min of collection by flow cytometry.

Subcellular Fractionation, SDS-Polyacrylamide Gel Electrophoresis, and Protein Blotting—Subcellular fractionation, SDS-polyacrylamide gel electrophoresis, and immunodetection of transblotted protein to polyvinylidene difluoride membranes was performed as described previously (12). Protein was analyzed on the basis of equal numbers of extracted cells rather than on the amount of protein loaded.

RESULTS

Calpains Are Constitutively Active in Cultured Neutrophils and Differentially Sensitive to Inhibition by ALLN and Calpeptin—Using a quenched rhodamine 110 fluorogenic substrate for calpains, we were able to show by flow cytometry that calpains were constitutively active in freshly isolated quiescent neutrophils (Fig. 1A) and that the level of this activity, as measured by the mean peak fluorescence of rhodamine 110 released from a dipeptide substrate after its uptake and enzymatic hydrolysis, diminished as cells aged in vitro and underwent apoptosis (Fig. 1A). By counterlabeling cell samples with an r-phyceroerythrin-conjugated anti-CD16 mAb, we were able to further identify a CD16-low population of cells, previously defined as apoptotic (50), as those with diminished calpain activity (Fig. 1B). That calpains were constitutively active in fresh neutrophils and responsible for the hydrolysis of LY-Calpain was confirmed by the ability of calpeptin, and to a lesser extent ALLN, to inhibit substrate hydrolysis (Fig. 1C).

Selective Inhibition of Both the Calpains and the Proteasome Is Required to Inhibit Constitutive Apoptosis in Neutrophils—We next determined whether calpeptin, like ALLN, could also inhibit the constitutive apoptosis of neutrophils. Such studies (Fig. 2) revealed that calpeptin at 200 μM had no significant effect on neutrophil apoptosis (although a slight inhibition was found at lower concentrations), contrasting with the previously reported inhibitory effect of ALLN (12). However, when lactacystin was used at a concentration (10 μM) commonly used for the selective inhibition of the proteasome (33, 34, 53), it too was found to be ineffective, although higher concentrations did partially inhibit neutrophil apoptosis (Fig. 2C). Considering that ALLN inhibited neutrophil apoptosis, and that separately lactacystin and calpeptin were ineffective, we postulated that the effect of ALLN may be to target both the calpains and the proteasome. This was supported when calpeptin and lactacystin, used in combination, resulted in an inhibition of neutrophil apoptosis that was substantially greater than that seen for either ALLN or zVAD alone (Fig. 2) (12), in which zVAD at the concentrations used is a broad spectrum inhibitor of caspasps. This contrasted with combinations of calpeptin and zVAD (Fig. 2) or ALLN and zVAD (12), which showed no synergy at inhibiting neutrophil apoptosis and in which the combination of lactacystin and zVAD exhibited only partial synergy (Fig. 2F).

Protease Inhibitors Coordinate Affect Surface and Nuclear Changes of Apoptosis—In the data presented in Fig. 2, apoptosis was quantified by the well established criterion of typical morphological changes apparent on examination of Giemsa-

1 The abbreviations used are: mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; Me2SO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; PS, phosphatidylserine; ALLN, acetyl-leucyl-leucyl-norleucinal; zVAD, benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone.

2 Data for the effect of ALLN plus zVAD on neutrophil apoptosis in Table I of the article by Brown et al. (12) should have read 18.9 ± 2.2% compared with 21.7 ± 4.0% for ALLN and 21.2 ± 1.3% for zVAD alone and where control values for apoptosis were 44.5 ± 10.0%.
Calpains Mediate Neutrophil Apoptosis

FIG. 1. Neutrophils possess calpain activity that is diminished with apoptosis. A, freshly isolated (●) and 20-h aged (▲) neutrophils (47% apoptotic) were assessed for intracellular calpain activity with varying concentrations of a cell-permeable, fluorescently quenched rhodamine 110 substrate (LY-Calpain) according to the manufacturer’s instructions (Coulter Electronics). The degree of hydrolysis was determined from the linear arithmetic mean of the peak fluorescence output (FL1-H). B, apoptotic cells were further distinguished by their shedding of CD16 (FL2-H). C, calpain-dependent hydrolysis of LY-Calpain in fresh neutrophils (i, ii) was assessed by preincubating cells for 2 h with either calpeptin at 100 μM (ii) or ALLN at 100 μM (iii) relative to a control rhodamine 110 dipeptide substrate (i).

FIG. 2. Constitutive apoptosis of neutrophils is dependent on several proteases, including calpains, caspases, and the proteasome. Freshly isolated human neutrophils were cultured with a number of protease inhibitors for 18 h, whereupon apoptosis was determined after morphological examination of Giemsa-stained cytospins. Typically 500 counts were made from duplicate slides of the same experiment for a minimum of six separate experiments. All concentrations are reported in μM. E, lactacystin was fixed at 10 μM, whereas calpeptin was varied from 0 to 200 μM. F, zVAD, calpeptin, and ALLN were fixed at 100 μM, whereas lactacystin was used at 10 μM. All experiments are normalized to the 0.1% Me 2SO control (42 ± 6% apoptosis), and all error bars represent the 95% confidence interval in an unpaired t test. Note that data for the effect of ALLN plus zVAD on neutrophil apoptosis in Table I by Brown et al. (12) should have read 18.9 ± 2.2%, which compares with 21.7 ± 4.0% for ALLN and 21.2 ± 1.3% for zVAD alone, where control values for apoptosis were 44.5 ± 10.0%.

stained cytospin preparations (46, 54). It was also determined by PS exposure as evidenced by annexin V-labeled cells after flow cytometric analysis. A direct comparison between the Giemsa counts of Fig. 2, irrespective of the inhibitors used, with the percentage of cells that labeled positive for annexin V, as assessed by FACS, revealed a good correlation between the two methods (Table I). This suggested that the inhibitors, either alone or in combination, were unable to disrupt the tight correlation between nuclear morphology and PS exposure. Note that data for the effect of ALLN plus zVAD on neutrophil apoptosis found for control aged cultures (r = 0.9709; p < 0.0001; n = 12), emphasizing earlier reports (12, 52). Similarly, and again irrespective of the protease inhibitors used, we observed good correlation between nuclear apoptotic morphology and the shedding of both CD16 and CD43 (Table I) (12). These results demonstrated that although the protease inhibitors modulated constitutive neutrophil apoptosis, they did not dissociate nuclear morphological changes from a number of cell surface phenomena typical of apoptosis and further confirms that the neutrophils were not activated by the various inhibitor treatments.

Calpain Inhibition Interferes with Exposure of an N-terminal Actin Epitope and Cleavage of α-Actinin and Ezrin—We have previously reported that ALLN, but not zVAD, was capable of completely inhibiting the cleavage of membrane-associated actin from preparations of both aged and apoptotic neutrophils (12). Because the scissile site was found to be located within the DNase I binding loop of β-actin between residues Val33 and Met44, we probed for differences between nonapoptotic and apoptotic cells using the mAb AC-15, which recognizes the immediate N terminus of β-actin. By flow cytometry, we observed that the mAb AC-15 was unable to immunodetect β-actin within freshly isolated, permea-fixed neutrophils but that a bimodal distribution occurred in aged cultures (Fig. 3A). The percentage of cells that stained positive for β-actin correlated well with the percentage of cells found to be apoptotic by Giemsa staining (p < 0.001), and epifluorescent microscopic examination of aged samples counterstained with propidium iodide confirmed that apoptotic cells were those binding AC-15 (Fig. 4B). The finding that fresh neutrophils did not immunostain at all (Fig. 4A) suggested that the immediate N-terminal epitope of actin recognized by AC-15 was sterically hindered, presumably via protein-protein interactions.

Intriguingly the percentage of cells that stained positive for β-actin in aged cultures was not reduced by ALLN treatment (Fig. 3B) despite the ability of this agent to inhibit both apoptosis and actin cleavage in aged cultures (12). This suggested that, in contrast to control aged cultures, some nonapoptotic cells in ALLN-treated cultures would stain positive for β-actin, which was confirmed by epifluorescent microscopy (Fig. 4C). These results contrasted with those obtained for calpeptin, in
Calpains Mediate Neutrophil Apoptosis

Data are expressed as mean ± 95% confidence intervals for n separate experiments. The percentage of cells staining positive with trypan blue remained <2% in all experiments and was highest for control cultures.

Table I
Effect of protease inhibitors on constitutive apoptosis of neutrophils cultured for 24 h in vitro

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Giemsa staining (n)</th>
<th>Annexin V binding (n)</th>
<th>CD16 shedding (n)</th>
<th>CD43 shedding (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>47.9 ± 11.2 (14)</td>
<td>46.5 ± 7.8 (14)</td>
<td>51.3 ± 9.7 (14)</td>
<td>46.7 ± 8.4 (8)</td>
</tr>
<tr>
<td>ALLN (100 µM)</td>
<td>24.5 ± 6.8 (12)</td>
<td>25.0 ± 9.6 (12)</td>
<td>25.0 ± 11.3 (12)</td>
<td>23.3 ± 5.1 (8)</td>
</tr>
<tr>
<td>zVAD (100 µM)</td>
<td>22.8 ± 5.4 (8)</td>
<td>21.8 ± 3.1 (8)</td>
<td>24.4 ± 12.5 (6)</td>
<td>21.4 ± 8.8 (6)</td>
</tr>
<tr>
<td>Calpeptin (100 µM)</td>
<td>41.6 ± 8.1 (6)</td>
<td>43.8 ± 10.2 (6)</td>
<td>41.7 ± 9.6 (4)</td>
<td>42.7 ± 8.9 (4)</td>
</tr>
<tr>
<td>Lactacystin (10 µM)</td>
<td>45.7 ± 3.9 (5)</td>
<td>42.7 ± 5.4 (5)</td>
<td>47.9 ± 5.4 (4)</td>
<td>46.1 ± 2.5 (4)</td>
</tr>
<tr>
<td>Calpeptin (100 µM) + lactacystin (10 µM)</td>
<td>10.4 ± 2.1 (4)</td>
<td>9.0 ± 15.2 (4)</td>
<td>12.5 ± 8.0 (4)</td>
<td>9.0 ± 6.6 (4)</td>
</tr>
<tr>
<td>zVAD (100 µM) + ALLN (100 µM)</td>
<td>23.2 ± 3.8 (4)</td>
<td>20.1 ± 4.1 (4)</td>
<td>23.9 ± 6.9 (4)</td>
<td>21.6 ± 7.9 (4)</td>
</tr>
<tr>
<td>zVAD (100 µM) + calpeptin (100 µM)</td>
<td>24.5 ± 4.2 (4)</td>
<td>24.5 ± 5.0 (4)</td>
<td>22.5 ± 5.7 (4)</td>
<td>23.6 ± 8.5 (4)</td>
</tr>
<tr>
<td>zVAD (100 µM) + lactacystin (10 µM)</td>
<td>15.6 ± 2.7 (4)</td>
<td>12.7 ± 9.8 (4)</td>
<td>18.8 ± 11.7 (4)</td>
<td>14.8 ± 7.5 (4)</td>
</tr>
</tbody>
</table>

Fig. 3. Calpeptin and ALLN have strikingly different effects on the immunodetection of β-Actin with AC-15 in aged neutrophils. Fresh and aged (A) or protease inhibitor-treated (B) neutrophils, fixed with Permafix (OrthoDiagnóstics Ltd.), were immunofluorescently labeled with β-actin-specific mAb AC-15 followed by an FITC-conjugated sheep anti-mouse polyclonal antibody and analyzed by flow cytometry. Neutrophil cultures were aged in vitro for 24 h at 37°C with 0.1% Me₂SO (aged control) and either calpeptin, zVAD, and ALLN at 100 µM or lactacystin at 10 µM. In four separate experiments the percentage of control aged cells staining positive with AC-15 correlated well with the percentage of cells found to be apoptotic by Giemsa (p < 0.05).

which, despite an inability to inhibit apoptosis, detection of β-actin with AC-15 was wholly blocked (Fig. 3B). The finding that lactacystin had no effect on its own suggests that the proteasome had no direct bearing on the immunodetection of β-actin. Furthermore, the loss in β-actin immunodetection after a combined lactacystin and calpeptin treatment was consistent with the degree to which apoptosis was inhibited.

Because these data suggested that early in the program of apoptosis calpains might disrupt association of the N terminus of actin with the cell membrane, we examined actin-binding, plasma membrane-anchoring proteins that can associate with the lateral face of F-actin. These proteins were thought to be likely candidates for masking the actin epitope recognized by AC-15. For this purpose we chose α-actinin, which also binds L-selectin (55), and ezrin, which binds CD43 (56), because L-selectin (57) and CD43 (12, 57) are both shed from senescent neutrophils undergoing apoptosis.

Western blot analysis for α-actinin in the microsomal membrane (P100) fraction of inhibitor-treated or untreated neutrophils revealed that in control aged preparations, in which apoptosis was 56%, there was no α-actinin to be found (Fig. 5A). This did not reflect a redistribution of α-actinin from the plasma membrane to the cytosol, because analysis of the supernatant after a 100,000 × g centrifugation revealed no specific immunodetectable protein (data not shown). Hydrolysis of α-actinin was apparently blocked by zVAD, calpeptin, and the combination of calpeptin and lactacystin, partially inhibited by ALLN, but unaffected by lactacytin alone. This same pattern of results was found for ezrin (Fig. 5B).

Inhibition of Calpains and the Proteasome in the Aging Neutrophil Promote Cell-Cell Interactions with Mφ Cells but Not Their Ingestion—Considering the variable inhibitory effects of the protease inhibitors on morphological apoptosis, we sought to examine what effect they may also have on the uptake of treated neutrophils by Mφ cells. Mφ recognition of protease inhibitor-treated neutrophils (Fig. 6) revealed that zVAD, calpeptin, and lactacystin all affected the level of Mφ recognition and ingestion of neutrophils according to the degree to which they also inhibited apoptosis. That is, Mφ recognition was proportionately dependent on the number of apoptotic cells found within the treated culture. This was found irrespective of whether the recognition assay was scored in situ or from cytosin preparations after EDTA/trypsin treatment. However, this was not what was observed for ALLN or for the combination of lactacystin and calpeptin, in which the number of neutrophils interacting with Mφ cells was found to be greater than that expected based on the level of apoptosis. This discordance for both treatments was completely abrogated when the Mφ monolayer was trypsinized to remove all adherent and non-ingested neutrophils before histochemical detection. These data indicated that the adhesion or “tethering” of aged, non-apoptotic neutrophils to Mφ cells was dependent on the dual inhibition of both calpains and the proteasome but that, regardless of the inhibitors used, ingestion was tightly correlated with nuclear morphology and PS exposure.

DISCUSSION

With the exception of the caspase family (2, 4, 5), the role and importance of intracellular proteases in apoptosis remain poorly defined and often controversial. Insofar as any result with protease inhibitors must be interpreted with caution, we
have found evidence that caspases (zVAD), calpains (calpeptin and ALLN), and the proteasome (lactacystin and ALLN) all play a role in the constitutive apoptosis of neutrophils in vitro.

The interplay between these proteases, however, was not simple. Indeed, our results demonstrate that in aging neutrophils a high degree of synergy existed between calpains and the proteasome, which acted downstream of caspases, to effect the constitutive apoptotic program of neutrophils.

First we showed that calpains were constitutively active in fresh isolated neutrophils as assessed by the in situ hydrolysis of a synthetic calpain substrate, although we could not exclude the possibility that the conditions for substrate cell loading were responsible for its activation. This activity, nevertheless, was blocked with the cell-permeable inhibitors calpeptin and, to a lesser extent, ALLN. However, only ALLN, not calpeptin, was able to inhibit the constitutive apoptosis of se-
Calpains Mediate Neutrophil Apoptosis

The current data establish that calpains are present in neutrophils and that their inhibition, in conjunction with that of the proteasome, results in a significant suppression of constitutive neutrophil apoptosis. However, the data provide no indication as to how calpains, or for that matter the proteasome, can affect this inhibition. Nevertheless, although calpain activity was apparently diminished once neutrophils had undergone apoptosis, this was shown to be responsible for a number of phenomena associated with the apoptotic phenotype. Furthermore, possible roles for calpain and the proteasome were suggested by the ability of both calpeptin and ALLN to wholly inhibit the immunodetection of an epitope within the immediate N terminus of β-actin that became unmasked as neutrophils underwent apoptosis, and each inhibitor impaired the loss and degradation of α-actinin and ezrin, two actin-binding, membrane-anchoring proteins. Because the anti-actin mAb used in these studies recognized an epitope adjacent to a previously characterized site of cleavage within F-actin, it may be speculated that exposure of the AC-15 epitope in the N terminus of actin and degradation of α-actinin and ezrin may be linked. Furthermore, the scissile site was previously found to be devoid of a consensus site for caspase cleavage, and ALLN, not zVAD, was able to inhibit this cleavage (12), a finding we have since validated with the use of calpeptin (data not presented).

Taken together, these results suggest that calpains are responsible for the dissociation of actin-binding proteins, including α-actinin and ezrin, from filamentous β-actin. This results in the unmasking of an immediate N-terminal epitope recognized by the mAb AC-15, rendering it susceptible to subsequent proteolysis. It is tempting to speculate further that the breakdown in cytoskeletal protein associations at the plasmalemmal interface direct the shedding of L-selectin, which binds α-actinin, and CD43, which binds ezrin. Recently, ezrin was shown to first dephosphorylate and then translocate, without degradation, from the plasma membrane to the cytoplasm after Fas-induced death in L cells (61).

In conclusion, we have presented evidence that calpains and the proteasome function synergistically to complete the constitutive apoptotic program of senescent neutrophils, a physiological form of cell death important in resolution of inflammation. Most importantly, these two proteolytic activities appear to function downstream of caspases in mutually redundant pathways where the loss of both activities inhibits the characteristic phenotypic changes seen in apoptosis. This is supported, and not excluded, by recent reports in which both the calpains and the proteasome were separately implicated as key effectors of thymocyte apoptosis. In the absence of combined inhibitor studies and a wider range of criteria to assess apoptosis, other than DNA fragmentation and nuclear morphology, it remains a distinct possibility that thymocytes also require synergy between calpain and proteasomal activities.

REFERENCES
Constitutive Apoptosis in Human Neutrophils Requires Synergy between Calpains and the Proteasome Downstream of Caspases
Birgit Knepper-Nicolai, John Savill and Simon B. Brown

doi: 10.1074/jbc.273.46.30530

Access the most updated version of this article at http://www.jbc.org/content/273/46/30530

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 61 references, 31 of which can be accessed free at http://www.jbc.org/content/273/46/30530.full.html#ref-list-1