Dissociation of Phagocyte Recognition of Cells Undergoing Apoptosis from Other Features of the Apoptotic Program*

Jianguo Zhuang‡, Yi Ren§, Roger T. Snowden‡, Huijun Zhu‡, Vladimir Gogvadze‡, John S. Savill§, and Gerald M. Cohen‡

From the ¤Medical Research Council Toxicology Unit, Centre for Mechanisms of Human Toxicity, University of Leicester, Hodkin Building, Lancaster Road, Leicester LE1 9HN and the §Division of Renal and Inflammatory Disease, Department of Medicine, University Hospital, Nottingham NG7 2UH, United Kingdom

Apoptosis is a programmed form of cell death characterized by biochemical and morphological changes affecting the nucleus, cytoplasm, and plasma membrane. These changes in various cellular compartments are widely regarded as mechanistically linked events in a single “program” in which activation of caspases and proteolysis of intracellular substrates represent a final common pathway leading to cell death. To date there has been very limited exploration of the linkage of this program to the plasma membrane changes, which bring about swift recognition, uptake, and safe degradation of apoptotic cells by phagocytes. Using the mitochondrial inhibitors antimycin A and oligomycin in human monocytic THP.1 cells triggered into apoptosis, we report the uncoupling of plasma membrane changes from other features of apoptosis. These inhibitors blocked increased plasma membrane permeability, externalization of phosphatidylserine, and recognition by two classes of phagocytes but not activation of caspase-3, cleavage of poly(ADP-ribose) polymerase and DNA fragmentation. Externalization of phosphatidylserine in apoptotic human leukemic U937 cells was also dissociated from caspase activation. Thus changes governing safe clearance of apoptotic cells may be regulated by an independent pathway to those bringing about caspase activation. This finding could have important consequences for attempts to manipulate cell death for therapeutic gain in vivo.

Apoptosis is a fundamentally important and programmed form of cell death with wide-ranging significance in health and disease (1, 2). Apoptotic cell death occurs in two phases, an initial commitment phase followed by an execution phase, which is characterized by a series of stereotypic changes including cell shrinkage, plasma membrane alterations, and condensation and fragmentation of chromatin (3). Internucleosomal cleavage of DNA, demonstrated as a DNA ladder by agarose gel electrophoresis, was originally considered as a biochemical hallmark of apoptosis (4), although it is now considered as a late event in apoptosis, which may be dissociated from early more critical changes (5). These DNA ladders are derived from large fragments of DNA of 30–50 and 200–300 kilobase pairs in length (6). A family of at least 10 interleukin-1β-converting enzymes (now known as caspases) (7) appear to be responsible for most of the biochemical changes associated with the execution phase of apoptosis (reviewed in Refs. 8–10). Caspases are cysteine proteases that have an absolute specificity for an aspartic acid in the P1 position of the substrate and cleave important cellular substrates including lamins, poly(ADP-ribose) polymerase (PARP)1 and DNA-dependent protein kinase (3, 8–10). Cleavage of intact PARP (116 kDa) to its 85-kDa signature fragment is observed in many different types of cells undergoing apoptosis and has also been proposed as a biochemical marker of apoptosis (11). Many if not all caspases are capable of cleaving PARP in vitro, but in intact cells it is likely that caspase-3 (CPP32) and caspase-7 (Mch3) are primarily responsible for the cleavage (8, 10, 12).

In vivo intact cells dying by apoptosis are usually swiftly recognized and safely cleared by phagocytes. This protects surrounding tissues from exposure to injurious contents leaking from dying cells, which is inevitable in accidental cell death by necrosis (1, 13). Currently, there is only limited understanding of the molecular mechanisms that render apoptotic cells recognizable to phagocytes. However, exposure of phosphatidylserine (PS), an anionic phospholipid normally confined to the inner leaflet of the membrane bilayer, serves as a recognition signal for yet to be characterized PS receptors on certain phagocyte populations (14–16). The mechanisms mediating PS exposure are complex but seem to involve activation of caspases (17, 18). Another mechanism by which phagocytes recognize cells undergoing apoptosis involves the phagocyte adhesion receptor CD36 co-operating with the α3β1 vitronectin receptor integrin to bind thrombospondin (TSP), which acts as a molecular bridge between the apoptotic cell and the macrophage (19).

We have studied the induction of apoptosis in two human tumor cell lines, THP.1 and U937. Using the mitochondrial inhibitors antimycin A and oligomycin, we now demonstrate for the first time that in THP.1 cells induction of the apoptotic phenotype, characterized by DNA fragmentation and the activation of caspases with ensuing cleavage of PARP, can be dissociated from cell surface changes that result in the phagocytic recognition of apoptotic cells by both the PS and CD36/α3β1/TSP mechanisms. Similar dissociation of externalization of phosphatidylserine from activation of caspases was also observed in U937 cells.

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‡ Supported by the European Science Foundation Program in Toxicology. Present address: Inst. of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino 142292 Russia.

§ Supported by the European Science Foundation Program in Toxicology. Present address: Inst. of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino 142292 Russia.

¶ To whom correspondence should be addressed: MRC Toxicology Unit, Centre for Mechanisms of Human Toxicity, University of Leicester, Hodkin Bldg., P.O. Box 138, Lancaster Road, Leicester LE1 9HN, UK. Tel.: 44-116-252-5589; Fax: 44-116-252-5616; E-mail: gmc2@le.ac.uk.

1 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; TPCK, N-tosyl-L-phenylalanyl chloromethyl ketone; Z-VAD.FMK, benzoyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone; PS, phosphatidylserine; TSP, thrombospondin; TNF-α, tumor necrosis factor-α; AIF, apoptosis-inducing factor.
Uncoupling of Phagocytic Recognition of Apoptotic Cells

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—Media and serum were purchased from Life Technologies, Inc. (Paisley, UK). All chemicals were obtained from Sigma Chemical Company (Dorset, UK) except for N-tosyl-l-phenylalanyl chloromethyl ketone (TPCK, Boehringer-Mannheim UK, Lewes, UK) and benzoylhexylcarbonyl-valinyl-alanyl-aspartyl (Ome) fluoromethyl ketone (Z-VAD.FMK, Enzyme Systems Inc., Dublin, CA). Both human mononuclear THP-1 cells and human leukemic U937 cells were maintained in suspension culture as described (20, 21). Apoptosis was induced in THP.1 cells by incubating cells for 4 h with either etoposide or TPCK, whereas U937 cells were incubated for either 3 h with tumor necrosis factor-a (TNF-a) in combination with cycloheximide or 5 h with etoposide. To assess the effects of the inhibitors on apoptotic stimuli, annexin V, oligomycin A, or oligomycin A-Z-VAD.FMK were added 5 min before the cells were treated with apoptotic stimuli in all experiments except for apoptosis induced by TNF-a in U937 cells, which were pre-incubated with Z-VAD.FMK for 30 min before exposure to TNF-a.

Flow Cytometric Analysis of Apoptosis—Apoptosis in THP.1 and U937 cells (0.5 × 106 cells) was assessed with Hoechst 33342 and propidium iodide (20, 21). Cells with a high blue fluorescence (Hoechst 33342), because of an increase in cell membrane permeability (22), exhibit an apoptotic morphology together with internucleosomal cleavage of DNA (20, 21). PS exposure in apoptotic cells was detected by incubating cells with fluorescein isothiocyanate-labeled Annexin V and propidium iodide (20, 21). Cells with a high blue fluorescence (Hoechst 33342) and a high red fluorescence (propidium iodide) were classified as apoptotic.

Western Blot Analysis—THP.1 and U937 cells (0.5 × 106) were prepared for SDS-polyacrylamide gel electrophoresis as described previously (25). Intact PARP (116 kDa) and its apoptotic 85-kDa signature fragment were detected with a rabbit polyclonal (318) or a mouse monoclonal antibody (C2-10) (from Dr. G. Poirier, Laval University, Quebec, Canada). Procaspase-3 and its catalytically active large subunit were detected with a rabbit polyclonal antibody raised against the p17 subunit of caspase-3 (from Dr. D. Nicholson, Merck Frosst, Quebec, Canada) as described (25).

Agarose gel electrophoresis was used to detect DNA laddering in whole cells (2 × 106) as described previously (5).

Phagocytosis Assay—Isolation and culture of macrophage mouse macrophages were essentially as described previously (26) except for minor modifications as follows. Inflammatory macrophages were elicited into the peritoneal cavity of 8–16-week-old Balb/c mice with 2% Brewer’s thioglycolate. Macrophages were harvested 4 days later by peritoneal lavage with balanced salt solution, plated in 96-well plates at 0.1 × 106 cells/well, and cultured overnight in complete medium (Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum) in the presence of 2% Brewer’s thioglycolate. Culture supernatants were harvested 24 h after the macrophages were harvested and plated into 96-well plates in Dulbecco’s modified Eagle’s medium containing 10% conditioned medium from L929 cells as a source of macrophage colony-stimulating factor. Bone marrow macrophages were used in the phagocytosis assay after being cultured for 5–7 days. For the phagocytosis assays, macrophage monolayers were washed, and THP.1 cells (0.5 × 106) in RPMI 1640 containing 5% fetal calf serum were added to each well. The plates were incubated for 1 h and then washed in ice-cold saline after treatment with trypsin to remove adherent macrophages. After cytopepin the macrophages onto microscope slides, cells were fixed with 2% glutaraldehyde and stained with Giemsa dye. Phagocytosis was evaluated by counting 500 macrophages/slide of four replicate slides in each experiment. Results were expressed as the percentages of macrophages that had phagocytosed THP.1 cells.

RESULTS

Antimycin A and Oligomycin Inhibit Apoptosis Assessed by PS Exposure and Hoechst Staining But Not by Decreased Mitochondrial Membrane Potential (∆Ψm) —Treatment of THP.1 cells with etoposide, a DNA topoisomerase II inhibitor, or TPCK, a chymotrypsin-like serine protease inhibitor, induces apoptosis, assessed by an increase in Hoechst 33342 fluorescence (20, 25, 27). Increase in Hoechst fluorescence results from an increased cell permeability of apoptotic cells compared with normal cells (22). In this study we have also assessed apoptosis using two other flow cytometric methods, i.e. externalization of PS and the percentage of cells with decreased ∆Ψm. Externalization of PS is considered to be a general feature of apoptosis induced by many different stimuli and is measured by binding of annexin V, a PS-binding protein (15, 23). A reduction in ∆Ψm has also been reported to be an early event in the induction of apoptosis in many different systems (28, 29). Incubation of THP.1 cells with etoposide (25 μM) or TPCK (75 μM) resulted in a time-dependent induction of apoptosis, as assessed by both PS exposure and decreased ∆Ψm (Table I). Although a decreased ∆Ψm has been proposed to regulate the externalization of PS (29), in our studies both of these changes demonstrated a similar time dependence irrespective of the apoptotic stimulus (Table I). Thus induction of apoptosis in THP.1 cells is accompanied by an increased Hoechst 33342 fluorescence, externalization of PS, and a decreased ∆Ψm.

To investigate the possible relationship between reduction in mitochondrial ∆Ψm and other features of apoptosis such as externalization of PS, we used antimycin A and oligomycin, inhibitors of the mitochondrial respiratory chain and ATP synthase, respectively (30). Both antimycin A and oligomycin caused a concentration-dependent inhibition of apoptosis induced by either etoposide or TPCK as assessed either by PS exposure or Hoechst 33342 staining, whereas they had little effect on the decreased ∆Ψm (Fig. 1). Neither antimycin A nor oligomycin alone induced apoptosis assessed by PS exposure, Hoechst 33342 staining, or DNA fragmentation (data not shown). Thus these experiments dissociated those features of the apoptotic phenotype assessed by PS exposure and Hoechst 33342 staining from those measured by the changes in ∆Ψm.

Z-VAD.FMK (50 μM), a cell membrane-permeable inhibitor of caspases, inhibited apoptosis induced by both etoposide and TPCK, as assessed by all three methods (Fig. 1), supporting a role for caspases in the execution phase of apoptosis in THP.1 cells in agreement with our previous observations (20, 25, 27). These results demonstrated that in this model of apoptosis the activation of caspases occurred upstream of PS exposure, increased Hoechst 33342 staining, and a decrease in ∆Ψm.

Dissociation of PS Externalization from Caspase-3 Activation and PARP Cleavage—During the execution phase of apoptosis, caspases are activated and cleave cellular substrates, such as PARP (3, 8–12). Therefore we wished to examine whether the mitochondrial inhibitors differentially affected the activation of caspases and the cleavage of PARP. Using Western blot analysis, control cells contained almost entirely intact PARP (116 kDa) (Fig. 2A, lane 1). Induction of apoptosis by either TPCK or etoposide was accompanied by the cleavage of PARP to its 85-kDa signature fragment (Fig. 2A, lanes 2 and 6), which was completely blocked by Z-VAD.FMK (50 μM) (Fig. 2A, lanes 5 and 9), further supporting the involvement of caspases in the
execution phase of apoptosis in THP.1 cells. Neither oligomycin nor antimycin A blocked proteolysis of PARP to its 85-kDa fragment following incubation with either TPCK (Fig. 2A, lanes 3 and 4) or etoposide (Fig. 2A, lanes 7 and 8). The cleavage of PARP to its 85-kDa signature fragment, which occurs at a DEVD↓G sequence separating the amino-terminal DNA binding domain and carboxy-terminal catalytic domain of the enzyme (31), suggested the activation of caspase-3 (CPP32) and/or caspase-7 (Mch3). We therefore examined the activation of caspase-3 using an antibody raised against its p17 large subunit (LS) of caspase-3. Results are representative of three experiments.


table I

| Treatment | Peritoneal macrophages | Bone marrow macrophages |
|-----------|------------------------|-------------------------|
| Control   | 3.1 ± 0.9              | 1.7 ± 0.5               |
| TPCK      | 25.6 ± 5.3             | 19.7 ± 2.8              |
| TPCK + Z-VAD.FMK | 4.4 ± 1.1              | 3.8 ± 1.1               |
| TPCK + oligomycin | 4.9 ± 1.5             | 1.1 ± 0.5               |
| TPCK + antimycin A | 4.5 ± 0.5              | 2.4 ± 0.8               |
| Etoposide | 13.0 ± 2.3             | 10.7 ± 0.6              |
| Etoposide + Z-VAD.FMK | 5.2 ± 3.0           | 2.2 ± 0.3               |
| Etoposide + oligomycin | 3.9 ± 1.6              | 1.4 ± 0.5               |
| Etoposide + antimycin A | 3.8 ± 0.5              | 1.0 ± 0.1               |

Fig. 1. Antimycin A and oligomycin cause a concentration-dependent inhibition of externalization of PS and increase in Hoechst 33342 fluorescence but not of the reduction in ΔΨm in THP.1 cells were incubated for 4 h with either etoposide (25 μM) (A) or TPCK (75 μM) (B) either alone or in the presence of the indicated concentrations of antimycin A (1–30 μM) or oligomycin (0.1–5 μg/ml) or in the presence of Z-VAD.FMK (50 μM). The percentage of apoptotic cells was then measured flow cytometrically using an increase in externalization of PS (open bars), an increase in Hoechst 33342 fluorescence (filled bars), or a decrease in ΔΨm (diagonally hatched bars). The data represent the means (± S.E.) of at least three experiments.
cleosomal cleavage of DNA in THP.1 cells. THP.1 cells were incubated with etoposide (25 μM) in the presence or absence of oligomycin (Oligo, 5 μg/ml), antimycin A (Ant, 30 μM), and Z-VAD.FMK (Z-VAD, 50 μM) for 4 h. 2 × 10⁶ cells were loaded per lane and subjected to conventional agarose gel electrophoresis to detect internucleosomal cleavage of DNA. Results are representative of three experiments.

Increased externalization of PS but not other features of the apoptotic phenotype, we wished to assess whether they also interfered with phagocytic recognition of apoptotic THP.1 cells. Peritoneal and bone marrow macrophages phagocytose apoptotic cells utilizing receptors for PS or thrombospondin (i.e. αvβ3 and CD36), respectively (13, 14, 19, 26). Exposure of THP.1 cells to the two pro-apoptotic stimuli, etoposide and TPCK, resulted in an increase in the percentage of cells phagocytosed by both peritoneal macrophages and bone marrow-derived macrophages (Table II). Z-VAD.FMK, antimycin A, and oligomycin all inhibited the etoposide- and TPCK-induced increases in recognition and phagocytosis by both peritoneal and bone marrow-derived macrophages (Table II). Thus cells displaying many features of apoptosis including DNA fragmentation, processing of caspase-3, and PARP cleavage do not display characteristic cell surface changes, which result in phagocytic recognition and engulfment.

**Dissociation of Cell Membrane Changes from Caspase Activation during Induction of Apoptosis in U937 Cells**—To determine whether the phenomenon of dissociation of externalization from PS from other features of the apoptotic phenotype was cell type-specific, we extended the study to human leukemic U937 cells. These cells were treated with two commonly used apoptotic stimuli, etoposide (25 μM) or TFN-α (10 ng/ml) in the presence of cycloheximide (0.9 μM) (17). Apoptosis was induced by both these stimuli, as assessed by externalization of PS, increase in Hoechst 33342 fluorescence, and increase in cells with a decreased ΔΨm (Fig. 4A). Both oligomycin (5 μg/ml) and antimycin A (30 μM) largely blocked the apoptotic changes induced by either TNF-α or etoposide as assessed either by PS exposure or Hoechst 33342 staining but had little effect on the decreased ΔΨm (Fig. 4A). Control U937 cells contained almost entirely intact PARP (116 kDa) and its cleaved 85-kDa fragment.

**DISCUSSION**

In this paper we present data demonstrating the dissociation of cell membrane changes leading to phagocyte recognition of cells undergoing apoptosis from other features of this programmed form of cell death. The mitochondrial inhibitors, antimycin A and oligomycin, not only inhibited surface changes of apoptosis, such as PS exposure and recognition by PS-dependent elicited murine peritoneal macrophages but also inhibited surface changes recognized by murine bone marrow-derived macrophages, which use the αvβ3 thrombospondin recognition mechanism (Table II). However, antimycin A and oligomycin did not inhibit caspase-3 activation, cleavage of PARP, DNA degradation, and reduction in ΔΨm (Figs. 1–4). These data imply that the surface changes of apoptosis relate to an independent pathway of events, which may be differentially regulated from the activation of "effector" caspases, which are believed to trigger the nuclear and some of the cytoplasmic changes of apoptosis.

**FIG. 3.** Neither antimycin A nor oligomycin inhibits internucleosomal cleavage of DNA in THP.1 cells. THP.1 cells were incubated with etoposide (25 μM) in the presence or absence of oligomycin (Oligo, 5 μg/ml), antimycin A (Ant, 30 μM), and Z-VAD.FMK (Z-VAD, 50 μM) for 4 h. 2 × 10⁶ cells were loaded per lane and subjected to conventional agarose gel electrophoresis to detect internucleosomal cleavage of DNA. Results are representative of three experiments.

**FIG. 4.** Dissociation of PS externalization from caspase activation in apoptotic U937 cells. Apoptosis was induced in U937 cells following incubation for either 3 h with TNF-α (10 ng/ml) in the presence of cycloheximide (0.9 μM) (lanes 2–5) or 5 h with etoposide (25 μM) (lanes 6–9). U937 cells were incubated either alone (lane 1) or with the apoptotic stimuli in the presence of antimycin A (30 μM) (lanes 3 and 7) or oligomycin (5 μg/ml) (lanes 4 and 8). Cells were also incubated with the apoptotic stimuli in the presence of the caspase inhibitor Z-VAD-FMK, the concentration of which was either 2 or 20 μM in the case of TNF-α or etoposide, respectively (lanes 5 and 9). A, the percentage of apoptotic cells was then measured flow cytometrically using either increase in externalization of PS (open bars), increase in Hoechst 33342 fluorescence (filled bars), or decrease in ΔΨm (diagonally hatched bars). The data represent the mean (± S.E.) of at least three experiments except for ΔΨm, which represents the mean of two determinations. B, cellular proteins were resolved on SDS-10% polyacrylamide gels, transferred onto nitrocellulose membrane, and probed with a mouse monoclonal antibody (C2-10) to detect both intact PARP (116 kDa) and its cleaved 85-kDa fragment.
It has become a widely held view that PS exposure and associated changes, such as increased plasma membrane permeability to Hoechst dye, represent "early" features of a single, coordinately regulated common pathway leading to cell death, whereas DNA degradation is a "late" feature. However, temporal dissociation of membrane changes including PS exposure from other features of apoptosis could also imply that mechanisms regulating the ability of dying cells to be recognized by phagocytes are part of a parallel process, which is potentially independent from other features of the program. This interpretation of the data is strongly supported by the effects of antimycin A and oligomycin reported here. Future studies will need to characterize the pathway by which dying cells display "eat me" signals. The effects of Z-VAD.FMK reported here support previous data implicating caspase activation in externalization of PS (17, 18), although effector caspases such as caspase-3 appear not to be involved.

The blockade of this putative "recognition pathway" by antimycin A and oligomycin also provides important clues for its further dissection. A critical role for the mitochondrial release of proteins, such as apoptosis-inducing factor (AIF) and cytochrome c, has been proposed in initiating the apoptotic program including DNA fragmentation and activation of caspase-3 (34, 35). Bcl-2 may prevent apoptosis in part by blocking the mitochondrial release of both AIF and/or cytochrome c (34, 36, 37). Although antimycin A and oligomycin may affect targets other than their known mitochondrial ones, our results suggest that their interference of mitochondrial function prevents the mitochondrial release of some factor(s), other than cytochrome c or AIF, that directly or indirectly results in exposure of PS.

Lastly, our findings could have very important consequences for attempts to manipulate apoptosis for therapeutic gain. In disorders characterized by unscheduled cell loss by apoptosis, such as neurodegenerative disease, potential therapies will not only need to block intracellular pathways leading to engagement of effector enzymes but will also need to interfere with those governing recognition, lest potentially rescued cells are undesirably removed by phagocytes. Conversely, there may be potential for selective triggering of the surface changes of apoptosis in "undesirable" cells (such as cancer cells) so that these are removed by innate mechanisms.

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