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Regulation of phosphatidylserine exposure and phagocytosis of apoptotic T lymphocytes

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Abstract

In lymphocytes, an asymmetric distribution of phospholipids across the plasma membrane is maintained by an ATP-dependent translocase which specifically transports aminophospholipids from the outer to the inner leaflet of the bilayer. During apoptosis, this enzyme is down-regulated and a lipid flipsite, termed the scramblase, is activated. Together, these events lead to the appearance of phosphatidylserine (PS) on the cell surface. In DO11.10 T lymphocyte hybridoma cells undergoing apoptosis, the kinetics of PS externalization are paralleled by the development of PS-sensitive phagocytosis by macrophages. This parallel is also observed when PS externalization is effected directly by application of a Ca^{2+} ionophore, suggesting that PS externalization is not only necessary, but sufficient, to generate a recognition signal. The broad spectrum aspartate-directed cysteine protease (caspase) inhibitor zVAD-fmk blocks externalization of PS and terminal cell lysis after induction of apoptosis by anti-CD3 antibody, but is ineffective when apoptosis is induced in the same cells by treatment with glucocorticoid. These results suggest that apoptosis induced by glucocorticoid does not require the same zVAD-sensitive caspase steps which are required for Fas/FasL-dependent death induced by anti-CD3 antibody, and that the action of these proteases is also not required for PS externalization. Extracellular Ca^{2+} is required to complete the later stages of apoptosis in DO11.10 cells, and its removal restores normal transport of PS, suggesting that down-regulation of the aminophospholipid translocase and up-regulation of the scramblase are not effected by irreversible protease cleavage.

Keywords: apoptosis; T lymphocytes; phagocytosis; cell surface molecules

Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; zVAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-fluoromethylketone; CD3, cluster of differentiation 3; ICE, interleukin-1β-converting enzyme; PARP, poly (ADP-ribose) polymerase; NBD, 12-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino] dodecanoyl; FITC, fluorescein isothiocyanate

Introduction

Presentation of signals triggering recognition and engulfment of apoptotic cells is a fundamental feature of most forms of programmed cell death.[1] In a variety of cell types and regardless of the initiating stimulus, expression of phosphatidylserine (PS) on the cell surface is necessary for entombment of the dying cell.[2–8] Normally, PS is not present in the external leaflet of the plasma membrane, reflecting the asymmetric distribution of phospholipids between the two leaflets of the bilayer.[9] This lipid asymmetry is maintained by a P-type ATPase,[10] called the aminophospholipid translocase, which transports the anionic phospholipids PS and phosphatidylethanolamine from the outer to the inner leaflet of the bilayer.[11,12] However, the resulting asymmetric distribution can be randomized by activation of a second membrane protein, termed the scramblase, that catalyzes rapid, bidirectional transbilayer movement of all classes of phospholipids across the membrane.[13–16] Coordinated inhibition of the translocase and activation of the scramblase occur in T lymphocytes undergoing apoptosis.[17,18] These alterations in activity bring PS to the cell surface, prior to the characteristic DNA degradation, membrane blebbing and cell lysis that constitute later steps in the cell death pathway.[17]

A variety of stimuli induce apoptosis, acting through several different signaling pathways which eventually converge to a sequence of execution steps, the machinery for which is constitutively expressed and common to all cells.[19] A growing family of aspartate-directed cysteine proteases (caspases) with homology to interleukin-1β-converting enzyme (ICE) has been implicated in apoptosis.[20] One of these caspases, encoded by the *ced-3* gene, is required for apoptosis in the nematode *C. elegans*.[21] In vertebrates, *ced-3*/ICE-like proteases have been implicated in apoptosis induced by Fas ligand,[22–24] growth factor deprivation[25] and some forms of motor neuron death *in vivo*.[26] The *ced-3*/ICE-like caspases in mammals are a complex family, with at least ten distinct members in humans.[27] Of these, caspase-3 and -7 have the greatest sequence homology with *ced-3*, a high degree of substrate specificity, and rapid cleavage kinetics.[28–30] Specific caspase inhibitors such as the viral CrmA protein[25,28,31,32] and peptides that irreversibly bind to the protease active-site cysteine[22–25,30,33] can block apoptosis, suggesting a role for these enzymes in the signaling phase of apoptosis, prior to the decision to enter the common execution phase. At the same time, the identification of caspase targets such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP)[22,28] indi-

cate that these proteases may be actors in the execution phase of apoptosis as well.

In vivo, the process of apoptosis functionally culminates in the phagocytosis of dying cells, with later stages such as degradation of nuclear DNA only occurring in cells sequestered in phagocytic vacuoles.[34–36] The redistribution of PS early in the apoptotic process[4,17] and its requirement for phagocytosis[2,6] raises the question of whether the appearance of PS on the cell surface is sufficient, as well as necessary, to signal recognition. Here, we confirm the relationship between exposure of PS and the susceptibility of cells to phagocytosis, and present evidence that in DO11.10 T lymphocyte hybridoma cells the former process is sufficient to trigger the latter. That PS becomes exposed early in the apoptotic process raises the question of whether caspases are involved in the mechanisms regulating PS distribution, and if so, whether they act during the triggering phase leading to the decision to enter the common death pathway, or as effectors in the execution steps following this decision. Using the broad spectrum caspase inhibitor zVAD-fmk, we show that activation of caspases is required for apoptosis in DO11.10 cells when the inducing stimulus is occupancy of the T cell receptor, but not when apoptosis is induced by glucocorticoid. Redistribution of PS is similarly sensitive to zVAD in cells induced by anti-CD3, indicating that this step is downstream of these caspase-dependent events, but is insensitive in cells induced by glucocorticoid, suggesting that PS exposure does not itself depend on activation of caspases and that the translocase and scramblase are not substrates of these proteases. We also show that alterations in translocase and scramblase activity are reversed by removal of extracellular Ca^{2+}, suggesting that regulation of these proteins does not occur by direct cleavage by caspases, or by other proteolytic activities.

Results

PS is not normally present in the outer leaflet of the plasma membrane of DO11.10 T lymphocyte hybridoma cells, as evidenced by their failure to bind the PS-specific probe annexin V (Figure 1, upper inset). However, 4 h after treatment with anti-CD3 antibody to induce apoptosis, a population of DO11.10 cells appears which binds fluoresceinated annexin V, indicating surface exposure of PS (Figure 1, lower inset). These cells retain their membrane integrity, since they are not labeled by the impermeant DNA fluorophore YO-PRO-3 iodide; they also retain the forward and side light scatter characteristics of ininduced cells, indicating that PS reaches the surface prior to the cell shrinkage and zeiosis stages of the apoptotic program (data not shown). The number of these cells in the population soon reaches a peak, then falls by about 8 h of induction as the responsive cells shrink, fragment, and lyse (see Figure 7, below). The appearance of cells displaying PS on their surface parallels precisely the appearance of cells with inactivated aminophospholipid translocase, the enzyme responsible for clearing PS from the cell surface, and activated scramblase, which randomizes phospholipids between the inner and outer leaflets of the bilayer[17] (see Figure 5, below).

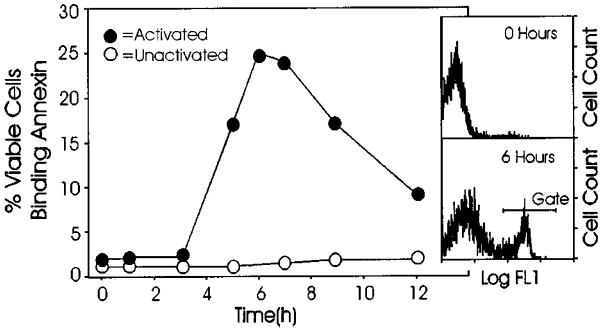

Figure 1 Annexin V binding to apoptotic DO11.10 cells. YO-PRO-PI-negative DO11.10 cells, either stimulated with anti-CD3 antibody or left unstimulated as controls, were stained with annexin V-FITC and 5000 cells analyzed by flow cytometry. Cells within the indicated gate were considered positive for staining with annexin

Exposure of PS on the surface of apoptotic cells is required for phagocytosis by macrophages.[2,6] The appearance of a functional phagocytosis signal on DO11.10 cells treated with anti-CD3 was measured as their rate of uptake by macrophages as a function of time after addition of antibody (Figure 2A). A small increase in the rate of phagocytosis was measurable after about 1 h of treatment (Figure 2A). This low level of interaction does not involve PS, since it is insensitive to the addition of PS vesicles; it is also relatively invariant over time after its first appearance, and is observed well before intermediate apoptotic signaling events, such as the synthesis of Fas and FasL, are completed.[37] This low level of recognition is then supplanted by a much larger increase in the rate of apoptotic cell phagocytosis. The new recognition process is blocked by PS vesicles and is low until about 4 h after induction of apoptosis, at which time it begins to rise dramatically. The increase in the rate of PS-sensivite uptake (Figure 2B) correlates well with the time course of PS appearance and the modulation of transbilayer lipid movements which underlie it.[17]

The correlation between PS appearance and the development of a functional phagocytosis signal suggests that the appearance of PS may not only be necessary, but also sufficient, to trigger recognition. However, recognition of apoptotic cells involves several cell surface molecules[6] and the extended time between anti-CD3 administration and the exposure of PS (4 h) leaves open the possibility that some of these other surface components are not normally exposed and must be inserted into the membrane prior to PS exposure. To determine whether this is the case, cytosolic Ca^{2+} levels were elevated using extracellular Ca^{2+} and high levels of the Ca^{2+} ionophore, A23187. In other cell types, this treatment brings PS immediately to the cell surface by inhibiting the aminophospholipid translocase and activating the phospholipid scramblase.[13,15,16] As shown in Figure 3, similar Ca^{2+}-induced changes in lipid movements can be induced in DO11.10 cells. Immediately upon treatment with Ca^{2+} and ionophore, the rate of transport of a fluorescent PS analog (NBD-PS) from the surface of the cell into the cell interior is

dramatically reduced in 95% of the cells, indicating that the aminophospholipid translocase which mediates this transport is blocked (Figure 3A). At the same time, transbilayer movement of NBD-PC to the cell interior, which is diagnostic of the nonspecific scramblase, is activated in all cells, as indicated in Figure 3B. As would be expected, this combination of events results in the immediate appearance of PS on the cell surface, as judged by annexin binding (Figure 3C).

This immediate (<5 min) exposure of PS on the cell surface limits the time available for the development of components of the recognition signal other than PS itself. To determine whether a functional phagocytosis signal appeared under these circumstances, ionophore-treated cells were presented to macrophages. As shown in Figure 3D, these cells were recognized by macrophages as quickly as uptake could be measured (the 30 min incubation required for the phagocytosis assay itself). Recognition was sensitive to inhibition by PS vesicles, and the level of recognition was complete at the earliest time, with no changes over several hours of incubation

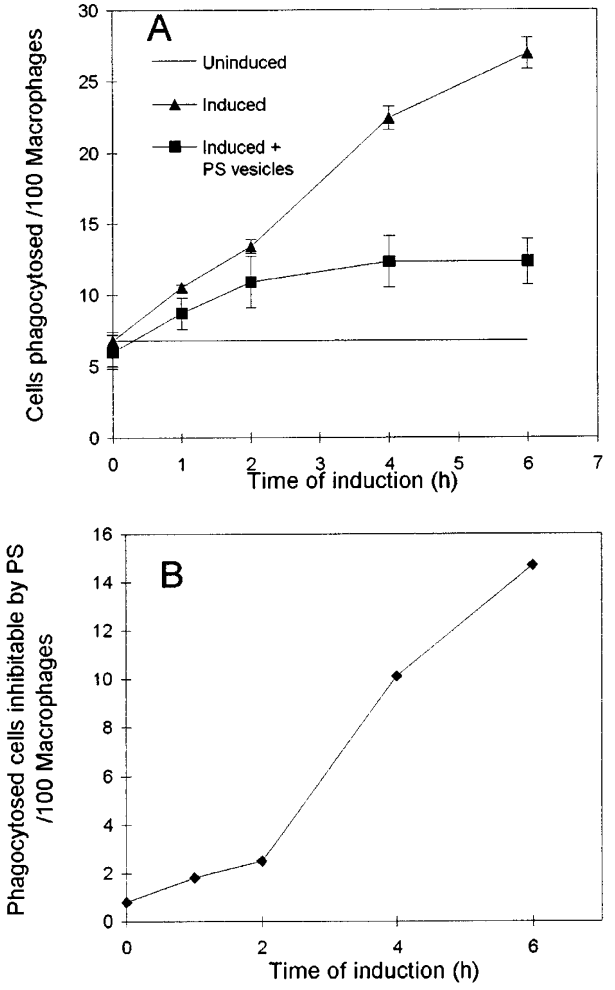

Figure 2 Phagocytosis of apoptotic DO11.10 cells by elicited mouse peritoneal macrophages. DO11.10 cells were incubated in the presence or absence of anti-CD3 antibody. At various times samples were taken, presented to peritoneal macrophages in the presence or absence of PS vesicles, and the number of lymphocytes phagocytosed in 30 min was counted. Values in (**B**) are the difference between the values in (**A**) for induced lymphocytes phagocytosed in the presence and absence of PS vesicles

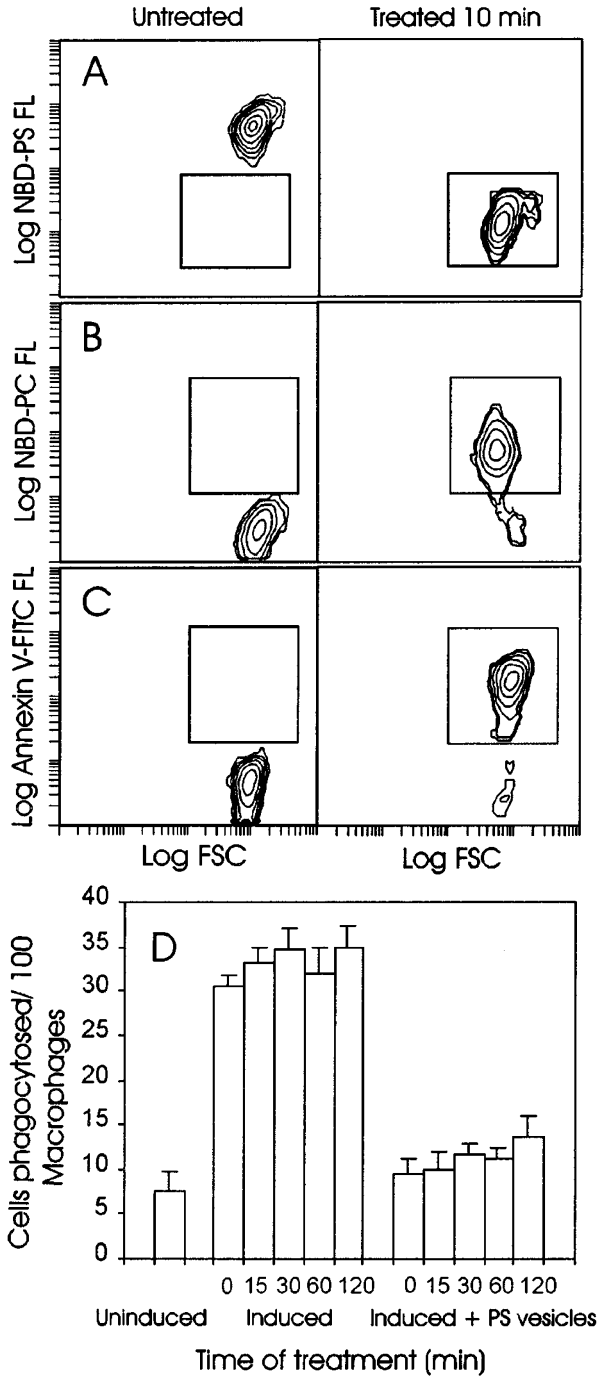

Figure 3 Alteration of translocase and scramblase activity in, and phagocytosis of DO11.10 cells treated with Ca^{2+} and the Ca^{2+} ionophore A23187. NBD-PS uptake (translocase activity), NBD-PC uptake (scramblase activity) and annexin V binding was analyzed in untreated cells and cells treated for 10 min with 10 μg/ml A23187 (**A, B** and **C**). At various times after Ca^{2+} and A23187 treatment cell samples were presented to peritoneal macrophages in the presence or absence of PS vesicles, and the number of lymphocytes phagocytosed in 30 min was counted (**D**)

thereafter. Together, these results suggest that the exposure of PS during apoptosis is not only necessary, but sufficient to trigger phagocytosis, and that any other required components of the signal are already present on the cell surface when PS rearrangement takes place.

The generality and functional importance of PS exposure in the apoptotic program raises the issue of the signaling pathway that leads to the changes in lipid movements. One potentially important event is the activation of caspases, which have been shown to be essential for the progress of Fas/FasL-induced apoptosis in lymphoid cells.[22–24] To investigate the role of these enzymes in DO11.10 cells, the effects of the cell permeant, wide-spectrum caspase inhibitor zVAD-fmk on apoptosis were examined, using several different agents to induce apoptosis. To monitor the overall progress of apoptosis, cell lysis, which occurs *in vitro* in the absence of engulfment, was measured. As shown in Figure 4A, anti-CD3 induces progressive cell lysis, culminating at about 8 h. This progression requires the action of zVAD-sensitive caspases since addition of inhibitor prevents cell lysis over the same time course (Figure 4A). Apoptosis can also be induced in DO11.10 cells by treatment with PMA and low levels of A23187 in the presence of extracellular Ca^{2+}.[38] As shown in Figure 4B, the kinetics of apoptotic cell lysis after this treatment are similar to those seen using anti-CD3, and death is similarly sensitive to zVAD-fmk. As shown in Figure 4C, apoptosis is also induced in DO11.10 cells by exposure to glucocorticoid. In this case, the final stages of cell lysis occur a little more slowly and less synchronously than in the case of anti-CD3- or PMA/ionophore-induced death. More importantly, cell death in this case is not blocked by zVAD-fmk (Figure 4C). Lack of an effect cannot be due to the inability of the cells to take up the inhibitor, or to the absence of a target, since zVAD-fmk effectively blocks the effects of the other two inducers of apoptosis in the same cells.

These experiments indicate that zVAD-sensitive caspase activity is required to complete anti-CD3- and PMA/ionophore-induced, but not glucocorticoid-induced death. Apoptosis induced by all three agents includes translocase inhibition and scramblase activation as early events[17] (and see Figure 6 below). To determine whether the caspase requirement for lysis, a late event in apoptosis, extends to these early events as well, the effect of zVAD-fmk on the alterations in lipid movement were examined. Aminopho-

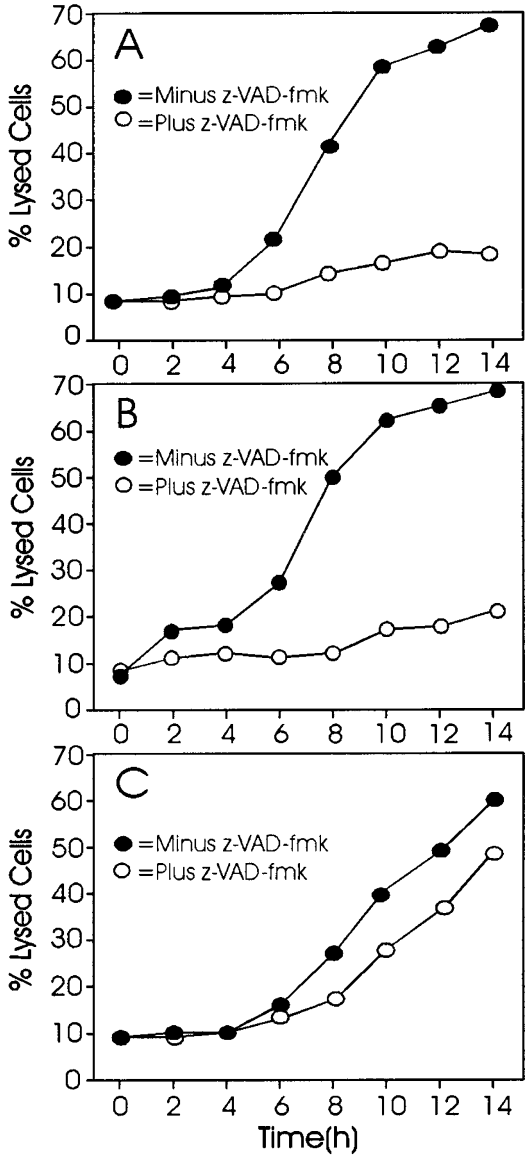

Figure 4 Effects of zVAD-fmk on lysis of apoptotic DO11.10 cells. DO11.10 cells were incubated in either medium alone or medium containing 10 μM zVAD-fmk; apoptosis was induced by anti-CD3 antibody (**A**), PMA and ionophore (**B**) or dexamethasone (**C**). Lysis was monitored by flow cytometry by the inability of cells to exclude the membrane impermeable dye YO-PRO-PI at the specified times after induction

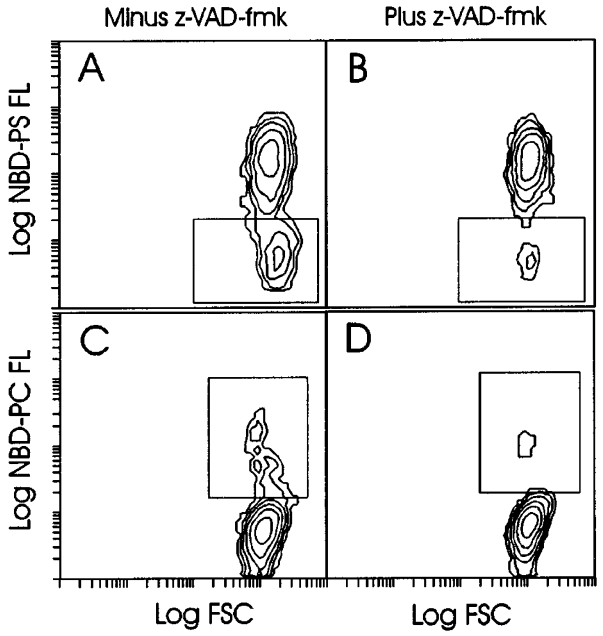

Figure 5 Effects of zVAD-fmk on lipid movements in apoptotic DO11.10 cells induced by anti-CD3. Uptake of NBD-PS (translocase activity) or NBD-PC (scramblase activity) was monitored 6 h after induction in the absence (**A** and **C**) or presence (**B** and **D**) of 10 μM zVAD-fmk

spholipid translocase activity was determined by measuring the level of internalization of NBD-PS; the results for induction by anti-CD3 are shown in Figure 5. In the absence of inhibitor, intact cells of normal size in which translocase is inactivated appear as a discrete population with reduced NBD-PS internalization (Figure 5A). The appearance of this population is blocked in the presence of zVAD-fmk (Figure 5B). Similarly, the appearance of cells in which NBD-PC is translocated to the cell interior by the activated scramblase (Figure 5C) is also blocked by zVAD-fmk (Figure 5D).

These results imply that the z-VAD-sensitive step occurs at or upstream of PS redistribution during anti-CD3-induced apoptosis, and raise the question of whether caspases are also required for PS redistribution in glucocorticoid-treated cells, where cell lysis proceeds in the presence of inhibitor. As shown in Figure 6, the translocase is inactivated (Figure 6A) and the scramblase activated (Figure 6C) in glucocorticoid-induced cells just as in anti-CD3-induced cells. However, in glucorticoid-induced cells both NBD-PS and NBD-PC movement occur normally in the presence of zVAD-fmk (Figure 6B and D) and annexin labeling confirms the externalization of endogenous PS (data not shown). Verification that cell death occurring in the presence of zVAD-fmk is apoptosis was obtained by staining with acridine orange and analyzing DNA degradation. As shown in Figure 6F, glucocorticoid-induced and zVAD-fmk treated cells with degraded chromatin are identified as a discrete subpopulation with reduced fluorescence compared to the normal population of cells in G_1/G_0 of the cell cycle (Figure 6E). These results indicate that caspase activity is not required for PS exposure in apoptosis induced by dexamethasone, and suggest that the translocation and scramblase are not direct targets of proteases inhibitable by zVAD-fmk.

Extracellular Ca^{2+} has been shown necessary for PS exposure on several other types of apoptotic cells.[39] Similarly, addition of EGTA to DO11.10 cells at any time following induction with anti-CD3 prevents subsequent redistribution of PS as well as further cell shrinkage and lysis (Figure 7C and D) compared to untreated cells (Figure 7A and B). When EGTA was added at 6 h after induction

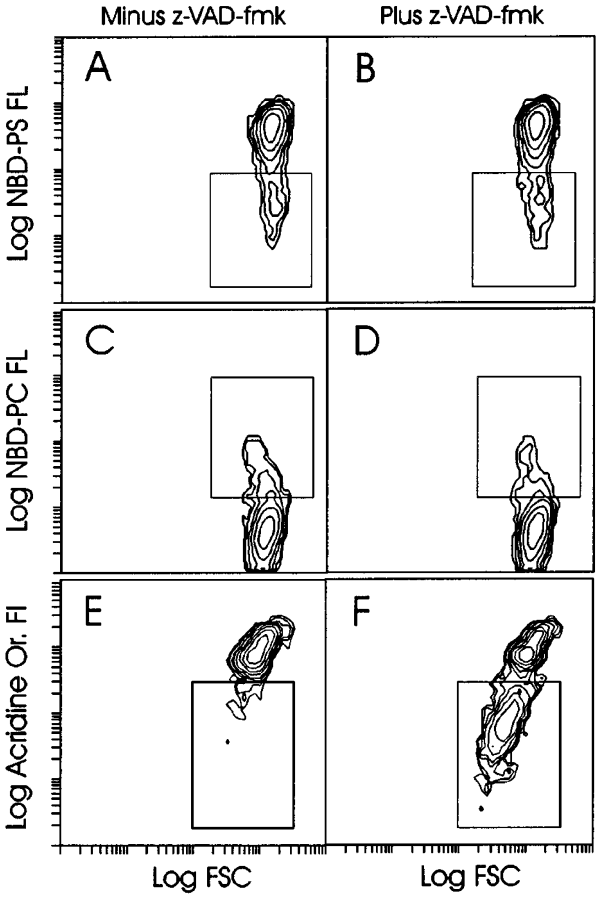

Figure 6 Effects of zVAD-fmk on lipid movements and DNA degradation in apoptotic DO11.10 cells induced by dexamethasone. Uptake of NBD-PS (translocase activity) or NBD-PC (scramblase activity) was monitored 7 h after induction in the absence (**A** and **C**) or presence (**B** and **D**) of 10 μM zVAD-fmk. Chromatin degradation was monitored at the same time by flow cytometry of acridine orange stained, uninduced control cells (**E**) and dexamethasone-induced, zVAD-fmk treated cells (**F**)

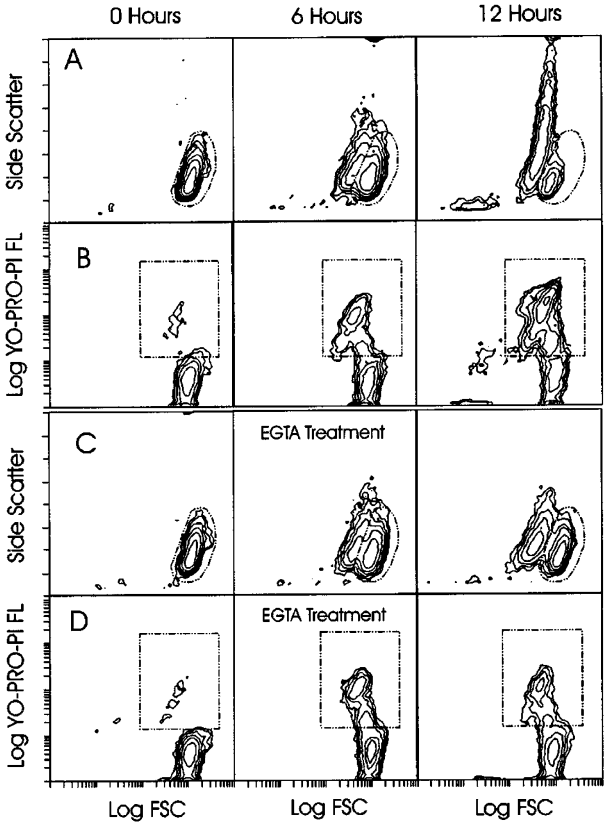

Figure 7 Effects of EGTA on cell shrinkage and lysis of DO11.10 cells induced by anti-CD3. Shown is flow cytometric analysis of anti-CD3-induced control cells (**A** and **B**) and induced cells treated at 6 h with 2 mM EGTA (**C** and **D**). Cell size and density was monitored at various times via forward and side scatter (**A** and **C**) while loss of membrane integrity was determined using YO-PRO-PI (**B** and **D**)

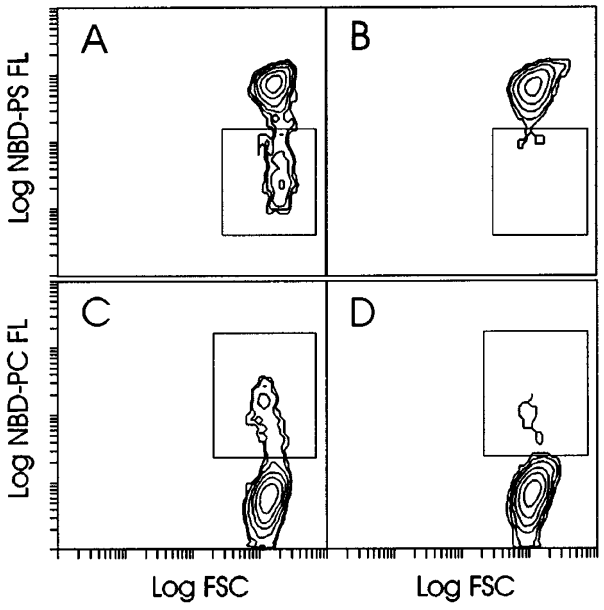

Figure 8 Effect of removal of extracellular Ca^{2+} on lipid movements in apoptotic DO11.10 cells. Uptake of NBD-PS (translocase activity) or NBD-PC (scramblase activity) was monitored 6 h after stimulation with anti-CD3 in untreated control cells (**A** and **C**) or cells treated for 15 min with 2 mM EGTA (**B** and **D**)

with anti-CD3, at a time when cells with inactive translocase and active scramblase were present in the population (Figure 8A and C), these cells returned to their normal pattern of transporting NBD-PS but not NBD-PC (Figure 8B and D) lost the ability to bind annexin V (data not shown). Similar results were obtained after induction with dexamethasone (data not shown). These results imply that PS exposure requires external Ca^{2+} in both activation- and glucocorticoid-induced apoptosis in DO11.10 cells. Moreover, the alteration in activity of both the translocase and scramblase is reversible, implying that neither of these enzymes is a direct target for proteases activated during apoptosis in DO11.10 cells.

Discussion

Cells differentiate to many different cell types in order to carry out differing specific functions required at various stages in the life of the organism. However, these various cell types all retain a capacity to continue differentiation in response to a variety of stimuli via a common program leading to cell death. Given the range of circumstances under which this common pathway is invoked, it is not surprising that there are many different signaling pathways through which cells enter it. Eventually, however, these signaling pathways necessarily converge, given that the final morphological features and molecular consequences of the execution phase are common to all cells. Distinguishing whether an event is part of an idiosyncratic upstream signaling pathway or part of the common execution phase requires distinguishing the two phases from each other. One way to distinguish the two phases is in a single cell system inducible by several stimuli.

Some properties of the final common pathway are clear. It does not require gene transcription or mRNA translation, for example, and the necessary molecular machinery is ubiquitously distributed in all cell types.[19] Proteolytic degradation of selected cellular constituents is a common theme,[40] leading to destruction of cytoskeletal architecture, inactivation of the DNA repair machinery, and disassembly of an ordered chromatin array, among other events. *In vivo*, some of these degradative steps are likely to be functionally continuous with the processes leading to the ultimate measure of cell death, cell removal. In *C. elegans*, for example, a nuclease is required for removal of the apoptotic body.[41] The gene for this nuclease must be expressed in an engulfing cell, while in mammals, activation of nucleases occurs in the apoptotic cell itself. Activation of these nucleases in the apoptotic or phagocytic cell is probably functionally equivalent, because in both cases digestion of the apoptotic cell DNA occurs after the dying cell has been recognized as such and phagocytosed by a living cell.[35,36] In this sense, expression of signals for recognition by phagocytes is the death knell of apoptosis.

Degradation of the apoptotic cell occurs in a phagocytic vacuole because recognition of apoptotic cells is an early event in the common pathway. The machinery for recognition of apoptotic cells is complex. In the recognition of apoptotic blood cells by macrophages, integrin[42] and lectin-like[43] components, CD14 recognized by the antibody 61D3,[44,45] and accessory components such as CD36 and thrombospondin[46] have all been implicated in the recognition process. However, with respect to the signal presented on the apoptotic cell surface, PS is one central, universal component regardless of cell type[5–8] or inducing stimulus[4] (plus data herein), clearly identifying this event as part of the common execution pathway. The mechanisms which bring PS to the outer leaflet of the plasma membrane have the potential to be not only necessary, but also sufficient to trigger recognition. As shown here, the appearance of PS on the surface of DO11.10 cells, whether naturally as part of the apoptotic program or artificially by elevation of cytosolic Ca^{2+}, correlates temporally with development of recognition by macrophages. These results suggest that other components of the recognition signal are present on the surface of nonapoptotic cells, but are ignored by the receptors on phagocytic cells until they are joined by PS from the cell interior. While the nature and assembly of complex PS recognition signals will be an important subject of further investigations, the centrality of PS appearance implies that understanding the regulation of PS redistribution is the key to understanding the timely appearance of a recognition signal.

Proteolytic enzymes, and caspases in particular, are essential effectors of apoptosis. In mammalian cells, however, caspases participate at multiple points in the apoptotic process, and it is not always obvious whether any particular point is part of the idiosyncratic pathway upstream of the decision to die, or part of the common execution pathway following the decision. The finding that zVAD blocks PS redistribution in DO11.10 hybridoma cells

induced by anti-CD3 implies that there is a caspase step upstream of PS exposure in this case.[47,48] This conclusion is consistent with the fact that the zVAD-sensitive caspase-8 is part of the Fas/FasL signaling pathway deployed by T lymphocyte hybridomas activated via the T cell receptor.[37] The loss of this sensitivity to zVAD in the same cells under the same conditions when apoptotic death is induced by glucocorticoid therefore implies that this particular caspase-sensitive step is not part of the common pathway induced by both stimuli. Even so, late caspase-dependent steps, such as caspase-3 cleavage of PARP do occur during thymocyte apoptosis triggered by glucocorticoid[49] in keeping with the multiple roles played by caspases in apoptosis.

The failure of zVAD to block PS redistribution in glucocorticoid-induced DO11.10 cells argues that the translocase and scramblase are unlikely to be direct targets of the zVAD-sensitive caspases activated by anti-CD3 treatment. However, caspases are not uniformly zVAD-sensitive[50] and are not the only proteases proposed as participants in apoptosis.[51,52] Thus neither sensitivity to zVAD nor the lack thereof are conclusive tests of whether the scramblase and translocase are themselves protease targets. That the inactivation of the translocase and activation of the scramblase are both reversible provides a stronger argument that some nonproteolytic regulatory mechanism must intervene between the redistribution of PS and the protease-dependent steps of the apoptotic pathway in these cells.

The nature of the implied nonproteolytic regulatory mechanism remains unspecified. The requirement for external Ca^{2+} to effect PS redistribution suggests that Ca^{2+} influx across the plasma membrane is essential. However, recent evidence that cytosolic Ca^{2+} chelators do not have the same inhibitory effects as externally applied EGTA suggests that Ca^{2+} acts at the cell surface.[39] Notably, CTL-induced apoptosis of target cells, which occurs very rapidly, also requires external Ca^{2+}.[53] Understanding the role of Ca^{2+} in these processes may be an important step to understanding the regulation of PS redistribution which leads to cell removal.

Materials and Methods

Animals

Male CBA/J mice, 4–6 weeks of age, were maintained on food and water *ad libitum* in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Cell culture

DO11.10 cells were maintained in Eagle's Medium with glutamine at 8% CO_2 or in Dulbecco's Minimal Essential Medium (DMEM) containing glutamine and sodium pyruvate (GIBCO BRL, Gaithersburg, MD, USA) at 5% CO_2. In both cases, the medium was supplemented with 10% fetal bovine serum and cells were grown at 37°C.

Initiation of apoptosis

Activation-induced apoptosis was initiated by transferring DO11.10 cultures in log phase growth ($\sim 10^6$ cells/ml) to flasks with immobilized anti-CD3 monoclonal antibody (1452C11; hamster anti-mouse; Boehringer Mannheim Corp., Indianapolis, IN, USA). Glucocorticoid- and phorbol ester-induced apoptosis was initiated by direct addition of dexamethasone (Sigma Chemical Co., St. Louis, MO, USA) to the culture flask to 5 μM or PMA (Sigma) to 10 nM and A23187 (Sigma) to 0.1 μg/ml, respectively, when cells were in log phase at a density of $\sim 10^6$/ml.

Caspase inhibition

DO11.10 cells were preincubated at 37°C for 30 min with 10 μM carbobenzoxy-valyl-alanyl-aspartyl-fluoromethylketone (zVAD-fmk) (Enzyme Systems Products, Dublin, CA, USA) before induction of apoptosis. Following induction, cultures were replenished with 10 μM zVAD-fmk every 3 h until the end of the experiment.

Phagocytosis assays

Inflammatory peritoneal macrophages were prepared and plated onto coverslips as previously described.[6] Untreated and treated DO11.10 cells (10^6) in 150 μL of DMEM were overlayed onto triplet coverslips incubated at 37°C in 5% CO_2. At various times, coverslips were washed three times with ice-cold phosphate-buffered saline (PBS; 7.4 mM Na_2HPO_4, 2.6 mM NaH_2PO_4, 137 mM NaCl, 10 mM KCl), fixed with 1.8% formaldehyde, stained with Diff-Quik (Baxter) and phagocytosed cells enumerated by microscopy as previously described,[6] selecting random fields and counting 300 macrophages per coverslip.

PS vesicles

A film of brain PS (Avanti Polar Lipids, Inc.) was dried under a stream of nitrogen, followed by 4 h under vacuum, then hydrated in PBS to 150 μM lipid and sonicated in a bath-type sonicator.[31] Fifty μL (7.5 nmol) of these vesicles was added to 10^6 DO11.10 cells in 100 μL of DMEM, and the mixture overlayed onto macrophages.

Flow cytometric analysis of lipid movements and cell death

Aminophospholipid translocase activity, determined by the internalization of fluorescent 12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] dodecanoyl (NBD)-PS, and scramblase activity, determined by the internalization of NBD-phosphatidylcholine (PC), was monitored in normal and apoptotic DO11.10 cells as described previously.[13] Annexin V-FITC (Nexins Research B.V., Maastricht, The Netherlands) labeling was performed on 1 ml aliquots from normal or apoptotic DO11.10 cultures according to the Apoptest^{TM}-FITC kit (product #A-600) protocol sheet and cells analyzed within 10 min. Cells which had lysed were defined as those which had lost the ability to exclude membrane-impermeant dyes, and were determined by adding to 1 ml cell samples the membrane impermeable dye YO-PRO-3 iodide (Molecular Probes Inc., Eugene, OR, USA) to 0.5 μM within 1 min before cytometric analysis. The membrane permeable DNA chromophore acridine orange (Sigma) was used at 5 μM directly on 1 ml aliquots of normal or apoptotic cells that were analyzed within 10 min for chromatin degradation. All samples were analyzed on an Epics-Profile II flow cytometer (Coulter Electronics, Hialeah, FL, USA) tuned to 488 nm. YO-PRO-3 iodide emissions were measured through

a 635-nm bandpass filter; NBD-phospholipid fluorescence emissions, FITC annexin V fluorescence and acridine orange staining were detected using a 525-nm bandpass filter. Epics II workstation software was used for statistical analysis of 5000 cells from each sample. All experiments presented are representative of at least three separate experiments.

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