Phosphatidylserine externalization during differentiation-triggered apoptosis of erythroleukemic cells

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Received 25.8.98; revised 12.11.98; accepted 25.11.98

Edited by G. Salvesen

Abstract

K562 erythroleukemia cells undergo apoptosis when induced to differentiate along the erythroid lineage with hemin. This event, characterized by DNA fragmentation, correlated with downregulation of the survival protein, BCL-x_L, and decrease in mitochondrial transmembrane potential ($\Delta\Psi_m$) that ultimately resulted in cell death. Reorientation of phosphatidylserine (PS) from the cells inner-to-outer plasma membrane leaflet and inhibition of the aminophospholipid translocase was observed upon hemin-treatment. Constitutive expression of BCL-2 did not inhibit hemin-induced alterations in lipid asymmetry or decrease in $\Delta\Psi_m$, and only moderately prevented DNA fragmentation. BCL-2, on the other hand, effectively inhibited actinomycin D-induced DNA fragmentation, the appearance of PS at the cells outer leaflet and the decrease in $\Delta\Psi_m$. The caspase inhibitor, z.VAD.fmk, blocked DNA fragmentation by both hemin and actinomycin D, but inhibited PS externalization only in the actinomycin D-treated cells. These results suggest that, unlike pharmacologically-induced apoptosis, PS externalization triggered by differentiation-induced apoptosis occurs by a mechanism that is associated with a decrease in $\Delta\Psi_m$, but independent of BCL-2 and caspases.

Keywords: apoptosis; differentiation; phosphatidylserine; BCL-2; BCL-x_L

Abbreviations: $\Delta\Psi_m$, mitochondrial membrane potential; NBD-PS, 1-oleoyl-2-[[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-phosphatidylserine; PI, propidium iodide; PS, phosphatidylserine; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbo-cyanine iodide

Introduction

One of the earliest manifestations of apoptosis, regardless of the initiating stimulus, is the redistribution of phospholipids in

the plasma membrane that leads to the exposure of phosphatidylserine (PS) at the cell surface.^{1–4} Although the biochemical mechanisms responsible for alterations in membrane lipid asymmetry are still not clear, it appears that caspases, a family of evolutionarily conserved cysteine proteases, play a role in the effector phase of apoptosis,^{3–6} because specific caspase peptide inhibitors block PS exposure in several model systems. Some studies suggest that proteolysis of fodrin, a caspase target and major component of the membrane-associated cytoskeleton, could be one of the sites where membrane lipid alterations are initiated.³

Caspase-independent mechanisms of PS exposure also exist. Elevation of intracellular Ca²⁺ levels can trigger PS exposure by activation of a Ca²⁺-dependent membrane protein that randomizes phospholipids across the membrane bilayer.^{7,8} This 'scramblase' is a membrane-spanning 37 kDa proline-rich polypeptide that is present in a variety of hematologic and nonhematologic cells.⁸ Its activation, combined with the Ca²⁺-dependent inactivation of aminophospholipid translocase, has also been implicated in apoptosis-dependent PS exposure.^{9–11} Ca²⁺-mediated PS exposure may also involve activation of calpain, which like caspases, can cleave fodrin.¹²

Similar to apoptosis, the differentiation of cells to mature phenotypes has been shown to coincide with the down-regulation of BCL-x_L¹³ and BCL-2^{14,15} DNA fragmentation, and loss of cell viability.¹⁶ These data suggest that restoration of differentiation may activate normal physiologic apoptotic pathways. Interestingly, other studies have suggested that erythroid development, from differentiation of RBC progenitors to senescence, is associated with alterations in the exposure of aminophospholipids at the cell surface.^{17–20}

In this study we investigated the pathways involved in the exposure of PS in differentiation-dependent apoptosis. Here we report that the enforced expression of BCL-2 in K562 cells had limited effects on hemin-induced PS exposure and mitochondrial membrane potential ($\Delta\Psi_m$), and only modestly delayed DNA fragmentation. The expression of BCL-2, on the other hand, suppressed these events in cells exposed to pharmacological inducers of apoptosis. Furthermore, the caspase inhibitor z.VAD.fmk effectively blocked both actinomycin D and hemin-induced DNA fragmentation. Unlike the effects on DNA fragmentation, however, the caspase inhibitor blocked PS exposure in actinomycin D-treated cells but not in hemin-treated cells. These results suggest that while PS exposure in hemin-induced differentiation is linked to apoptosis, it is associated with a disruption of mitochondrial membrane potential that occurs via a mechanism that is independent of BCL-2 and caspases.

Results

Exposure of PS during erythroid differentiation of K562 cells

To study the relationship between erythroid differentiation and the appearance of PS at the cell surface, hemin-treated cells were analyzed for hemoglobin accumulation, PS expression, and propidium iodide (PI) staining. PS was detected by labeling cells with FITC-annexin V and flow cytometry analysis. As can be seen in Figure 1A, hemin treatment resulted in a progressive increase in the appearance of PS at the cell surface and concomitant loss in cell viability. As measured by both peroxidase activity (Figure 1B) and Western blot analysis (Figure 1B inset), hemin also induced hemoglobin expression characteristic of erythroid differentiation.

It has been previously demonstrated that once PS is externalized due to vesiculation induced by Ca^{2+} ionophores or amphipathic reagents, the aminophospholipid translocase corrects for alterations in lipid asymmetry by transporting PS back to the inner leaflet of the membrane.^{21,22} The translocation of an exogenously-supplied fluorescent PS analog from the outer to inner leaflet of cells is therefore an indirect way to measure endogenous aminophospholipid translocase activity. As shown previously²³ untreated K562 cells rapidly translocate NBD-labeled PS to the inner leaflet (Figure 2). In contrast, exposure to hemin resulted in a time and differentiation-dependent decrease in the ability of the cells to transport

lipid (Figure 2). As might be expected, this effect also coincided with the appearance of endogenous PS at the cell surface (Figure 1A).

Figure 2 Transport of NBD-PS in differentiating K562 cells. Cells were incubated in the absence (○) or presence of hemin for 7 days (●). The cells were then assessed for their ability to transport exogenously supplied NBD-labeled lipid

Figure 1 PS exposure and hemoglobin production during differentiation of K562 cells. K562 cells were treated with 50 μM hemin for indicated times. (A) PS exposure was determined by flow cytometric analysis of FITC-annexin V-labeled cells. (B) Cell lysates were incubated with 2,7-diaminofluorene and hydrogen peroxide for 15 min at 20°C and read at 600 nm. Insets: (A) The fraction of viable cells was determined as described in the Materials and Methods section. (B) Western blot analysis of globin expression. Equal loading was determined by estimating glyceraldehyde 3-phosphate dehydrogenase

DNA fragmentation and BCL-x_L expression during K562 differentiation

To determine whether the loss in cell viability observed upon hemin-induced differentiation (Figure 1A, inset) was due to apoptosis, cell cycle distribution was measured by flow cytometry in permeabilized PI-labeled cells. Analysis of hemin-induced apoptosis-associated DNA fragmentation showed an accumulation of cells in G₂M after 4 days (Figure 3). Indicative of DNA fragmentation of the G₂ cells, PI label was also distributed to the left of G₂/M suggesting that hemin induces K562 cell arrest in G₂. Although terminal differentiation normally occurs upon exit of the cell cycle and arrest in G₁, several differentiating agents are known to delay the cell cycle in G₂ and induce terminal differentiation in K562²⁴ and other cell lines.²⁵ Because BCL-x_L has been shown to protect hemin-treated cells from undergoing apoptosis,¹³ it is possible that its downregulation and concomitant apoptosis is

Figure 3 DNA fragmentation in hemin-treated cells. Control (A), and cells treated with hemin for 4 (B) and 7 days (C), were permeabilized, labeled with PI and analyzed by flow cytometry

associated with the exposure of PS and downregulation of the aminophospholipid translocase.⁹ Analysis of BCL-x_L in hemin-treated cells showed a progressive decrease in BCL-x_L expression (Figure 4A) that correlated with apoptosis as measured by DNA fragmentation (Figure 3). In contrast, BCL-x_L levels were not influenced by treatment of the cells with actinomycin D or staurosporine (Figure 4B). Levels of BCL-2 were low to undetectable in untreated K562 cells (Figure 4C) and did not change upon treatment with hemin (not shown).

Effect of constitutive BCL-2 expression on hemin-induced differentiation and apoptosis

Experiments in transgenic mice indicated that the effects of BCL-2 and BCL-x_L on the apoptotic pathway are similar.²⁶ The constitutive expression of both BCL-2 and BCL-x_L protect K562 cells from apoptosis in response to inhibitors of macromolecular synthesis²⁷ and okadaic acid.²⁸ Enforced expression of BCL-2 has also been shown to promote erythroid and myeloid differentiation in FDCP-mix cells.²⁹ Thus, differentiation and apoptosis could share a common pathway dependent upon a progressive loss of BCL-x_L protection. We therefore questioned whether constitutive expression of BCL-2 might uncouple apoptotic cell death from differentiation, and if this would affect the appearance of PS at the cell surface. K562 cells were transfected with the human BCL-2 gene. Clones expressing high levels of the BCL-2 protein (Figure 4C) were selected and analyzed. Differentiation-independent apoptosis was quantified by PI

Figure 4 Western blot analysis of (A) BCL-x_L expression in K562 cells during hemin-induced differentiation, (B) BCL-x_L expression in K562 cells during actinomycin D and staurosporine-induced apoptosis, and (C) BCL-2 and BCL-x_L in BCL-2 transfectants

staining and FACS analysis after treatment with actinomycin D and staurosporine. By 24 h, both agents induced extensive DNA fragmentation (Figure 5A) and PS externalization (Figure 5B) in the parent cells. Clones that constitutively expressed high levels of BCL-2, on the other hand, were protected against drug-induced apoptosis and PS externalization (Figure 5). The same BCL-2-dependent protection was also observed in cells cultured without fetal bovine serum for 4 days (data not shown).

In contrast to the results obtained with actinomycin D and staurosporine, DNA fragmentation was only slightly delayed when apoptosis/differentiation was triggered with hemin (Figure 6). PS exposure, on the other hand, did not decrease in the BCL-2 expressing clones.

Effect of the caspase inhibitor z.VAD.fmk

Genetic evidence indicates that BCL-2 and BCL-x_L act upstream of the caspases thereby preventing their activation.³⁰ In an attempt to dissociate apoptosis from PS exposure and differentiation, parental K562 cells were treated with actinomycin D and hemin in the presence of z.VAD.fmk, a broad spectrum caspase antagonist that blocks apoptosis. Incubation of K562 cells with z.VAD.fmk strongly inhibited DNA fragmentation induced by both compounds (Figure 7), suggesting that both differentiation-dependent and independent apoptosis are caspase-dependent events. In contrast, z.VAD.fmk inhibited PS exposure induced by actinomycin D, but had no effect on hemin-treated cells (Figure 7). These results suggest, therefore, that hemin-induced PS externalization, unlike actinomycin D-induced PS externalization, occurs via a caspase-independent mechanism.

$\Delta\Psi_m$ during hemin-induced differentiation and apoptosis

Several studies have shown that inhibitors of mitochondrial oxidative phosphorylation cause PS externalization subsequent to a decrease in $\Delta\Psi_m$,^{31,32} suggesting a correlation between these two events. To determine whether hemin can induce a decrease in $\Delta\Psi_m$, parent and BCL-2 transfectants were labeled with the $\Delta\Psi_m$ -sensitive fluorochrome, JC-1, which allows simultaneous analysis of mitochondrial mass (green fluorescence) and $\Delta\Psi_m$ (red fluorescence). Flow cytometry analysis of parental K562 cells treated with actinomycin D and hemin revealed a dissociation between mitochondrial mass and $\Delta\Psi_m$ by a decrease in mean red fluorescence without a corresponding increase in green fluorescence (Figure 8 and Table 1). Unlike the apparent complete protection against decreased $\Delta\Psi_m$ by actinomycin D-treatment in the BCL-2 transfectants, hemin-treated BCL-2 transfectants still exhibited a decrease in mean red fluorescence, consistent with the inability of BCL-2 to protect the cells against mitochondrial membrane damage. Thus, while both hemin and actinomycin D induced decreased $\Delta\Psi_m$, only the effect of actinomycin D was protected by BCL-2, a result similar to the hemin-dependent/BCL-2 independent exposure of PS in these cells (Figures 5 and 6).

Discussion

Apoptosis is especially important in hematopoiesis, where cytokine survival factors control the emergence of particular cell lineages and the elimination of inappropriate or non-

Figure 5 DNA fragmentation and PS expression in BCL-2 expressing K562 clones. Cells were incubated with actinomycin D (1 μ g/ml) or staurosporine (1 μ M) for 24 h (solid bars) and 48 h (hatched bars). Cells were then labeled with PI (A) or fluorescent-labeled annexin V (B)

Figure 6 Hemin-dependent DNA fragmentation and PS-exposure in BCL-2 expressing cells. K562 cells were incubated with hemin for the indicated time and then labeled with PI (A) or fluorescent-labeled annexin V (B). (●) parent cells, (■) BCL-2 transfectants

Figure 7 Effect of caspase inhibitor on actinomycin D and hemin-induced apoptosis. Control cells (solid bars), z.VAD.fmk-treated cells (hatched bars)

functional cells. One of the early events of apoptosis is the breakdown of lipid asymmetry in the plasma membrane, which leads to the movement of phosphatidylserine to the cell surface.¹⁻⁴ This phospholipid, which is normally sequestered in the inner membrane leaflet, is recognized by macrophages *in vitro*, and seems to be an important determinant for the

clearance of apoptotic cells and the prevention of inflammation *in vivo*.^{1,2}

Random phospholipid scrambling and loss of PS transport capacity both appear to contribute to PS exposure in apoptosis and differentiation. Verhoven *et al*⁹ showed that apoptosis-associated PS exposure in a lymphocytic cell line activated with CD3 antibody coincided with the inability to transport aminophospholipids. It has been suggested that the inability to correct for alterations in the lipid asymmetry could be related to downregulation of the aminophospholipid translocase. However, even though downregulation of the translocase can be important in the failure to correct for PS exposure, it does not appear to be sufficient for scrambling.¹¹ In fact, the phospholipid scramblase, a 37 kDa transmembrane protein, has been shown to be primarily responsible for membrane lipid scrambling and PS externalization.⁷ Interestingly, this protein is present in a variety of tissues and cells, showing highest levels in platelets, and correlates with their activity upon activation.⁸

A variety of agents (hemin, retinoids, phorbol esters, transforming growth factor- β , dimethylsulfoxide, and butyrate) can induce myeloid cells to differentiate *in vitro*, a phenomenon that is usually associated with concomitant apoptosis. Although this may also be true for normal erythroid progenitors, it seems likely that cell cycle deregulation in transformed cell lines used to model this process *in vitro*, makes them more resistant and susceptible to differentiation and apoptosis, respectively. When K562 cells were induced to differentiate along the

Figure 8 JC-1 fluorescence in K562 cells incubated with hemin and actinomycin-D. K562 parent cells (top panels) and BCL-2 transfectants (bottom panels) were incubated with actinomycin D (left) and hemin (right) for 2 and 6 days, respectively. The cells were then labelled with JC-1 and analyzed by flow cytometry

Table 1 Mean JC-1 red and green fluorescence

	Control	Parent K562 Actinomycin	Hemin	Control	BCL-2 Actinomycin	Hemin
JC-1 red	45	29.8	29.4	45.5	48.9	34.1
JC-1 green	206	193	256	212	192	201
red/green	0.22	0.15	0.11	0.21	0.25	0.17

erythroid lineage with hemin, an increase in cell surface PS paralleling the synthesis of hemoglobin was observed. This was followed by DNA fragmentation, loss of $\Delta\Psi_m$, and cell death. Although the molecular mechanisms involved in differentiation-triggered apoptosis await identification, our data suggest that downregulation of BCL- x_L , an anti-apoptotic member of the BCL-2 family, is involved. These results are consistent with those obtained by Benito *et al*,¹³ who reported that differentiation-induced apoptosis in erythroleukemia cells exposed to hemin, retinoic acid or TGF- β is associated with downregulation of BCL- x_L . A similar result has been obtained with HL-60 cells where downregulation of BCL-2 is associated with apoptosis.^{14–16}

Although these results suggest that differentiation and apoptosis utilize overlapping pathways, the two responses can be dissociated by enforced overexpression of BCL- x_L in K562 cells¹³ or BCL-2 in HL-60 cells,²⁷ preventing cell death without blocking differentiation. Interestingly, overexpression of BCL-2 fails to block apoptosis induced by macromolecular synthesis inhibitors in HL-60 cells, even though it does block differentiation-associated cell death.²⁷ Therefore, drug-induced and differentiation-associated cell death in myeloid cells may occur via distinct pathways that are selectively targeted by BCL-2 or BCL- x_L in a cell type-specific fashion. Indeed, some studies have suggested that erythroid differentiation could be a specialized form of

apoptosis.³³ Based on this hypothesis, differentiation-related apoptosis could share some steps of the apoptotic pathway, but still be regulated by other mechanisms.

Several studies have documented the involvement of caspases in PS externalization and apoptosis.^{3,6} Our data support the notion that caspase activation is essential for PS exposure in K562 cells treated with actinomycin D, and argue against a requirement for caspases in hemin-induced PS exposure. These results are consistent with data obtained with a human monocytic leukemia cell line U937, where it was shown that high concentrations of caspase inhibitor prevented both apoptosis and PS externalization. At low inhibitor concentrations, however, PS exposure was not inhibited while other manifestations of apoptosis were still suppressed.⁵

It has recently been shown that mitochondrial changes distinguished by a disruption in $\Delta\Psi_m$ is a feature of early apoptosis that is largely independent of the inducing stimulus.^{31,34,35} In the experiments presented here, we show that overexpression of BCL-2 effectively inhibited apoptosis induced by actinomycin D, but did not inhibit the effect on $\Delta\Psi_m$ or exposure of PS induced by hemin. It has been suggested that BCL-2 might be physically linked to the mitochondrial membrane and inhibit apoptosis by direct action on the pore complex.^{36,37} Although it is unclear why BCL-2 was not able to protect against hemin-induced apoptosis, the arrest of cells in G₂/M might suggest that signal transduction pathways involving tyrosine kinases are involved.^{25,38}

Because the clearance and removal of apoptotic cells is critical to the maintenance of homeostasis, multiple systems that control the appearance of PS at the cell surface are likely operative. Redundant mechanisms that regulate PS exposure could be the consequence of evolutionary pressure to ensure that membrane lipid asymmetry remains intact in cells that lack BCL-2 or are resistant to caspase-dependent events. In the case of hemin-induced differentiation/apoptosis, it seems likely that PS exposure follows differentiation because of breakdown of the mitochondrial membrane by a mechanism that is independent of BCL-2.

Materials and Methods

Cell culture

The human erythroleukemia cell line, K562 (ATCC CCL-243), was maintained in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin (50 U/ml). Apoptosis was induced by incubating the cells with actinomycin D (1 μ g/ml) or staurosporine (1 μ M) for 24 or 48 h at 37°C. Differentiation was induced with hemin (50 μ M) for the indicated time (see Figures). The medium was changed every 72 h except when cells were incubated with the caspase inhibitor z.VAD.fmk (100 μ M), in which case the medium and inhibitor were replaced every 48 h.

Cell transfection

K562 cells were transfected by electroporation using a Cell Porator (Gibco-BRL) in 0.4 cm electrode gap cuvettes. 10⁷ cells were washed

free of serum and resuspended in 1 ml of RPMI-1640. Cells were transfected with the pCI-BCL-2 plasmid vector (provided by Dr. John Reed, The Burnham Institute, La Jolla, CA, USA). Electroporation was carried out at 275 V and 1600 μ F. Cells were selected in increasing concentrations of G-418 (Gibco-BRL) up to 0.8 mg/ml. Cells were cloned by limiting dilution and tested for the expression of BCL-2 by Western blotting.

Western blot analysis

Cells were lysed with hypotonic buffer (10 mM Tris-HCl, 1 μ M PMSF, pH 8.1) and proteins were separated by SDS-PAGE electrophoresis in 11% acrylamide gels. After transferring to nitrocellulose, the blots were blocked with 3% milk solution in TBS (50 mM Tris, 150 mM NaCl, pH 7.5) and incubated for 2 h with mouse anti-human BCL-2 (clone 124, Dako) or anti-BCL-x_L (YTH-2H12, Trevigen, Inc., Gaithersburg, MD, USA) monoclonal antibody at 20°C at 0.2 μ g/ml and 1 μ g/ml, respectively. Horseradish peroxidase-conjugated goat anti-mouse IgG was used as the detection antibody. Blots were developed by enhanced chemiluminescence (ECL; Amersham). Expression of globin was determined with rabbit anti-human hemoglobin (Cappel, Durham, NC, USA) at a dilution of 1:500 overnight at 4°C and detected as described above using horseradish peroxidase-conjugated goat anti-rabbit IgG.

Determination of cell surface PS, apoptosis and $\Delta\Psi_m$

The expression of cell surface PS was detected by labeling cells with annexin V which has been shown to have high affinity and specificity to the lipid in the presence of Ca²⁺.^{4,39} Cells expressing PS were detected and quantified by flow cytometry using FITC-Annexin V and propidium iodide (PI) according to the manufacturer's instructions (Trevigen). Apoptosis was measured by flow cytometry of propidium iodide (PI)-labeled DNA in permeabilized cells. Briefly, cells were washed twice with TBS containing 0.1% bovine serum albumin and the pellet was resuspended in 1 ml of hypotonic PI solution (50 μ g/ml PI, 0.1% sodium citrate, 0.1% Triton X-100, 20 μ g/ml ribonuclease A) for 30 min at 4°C. The fraction of dead cells was determined by PI staining and verified by trypan blue dye exclusion. $\Delta\Psi_m$ was determined by monitoring the decrease in red fluorescence by flow cytometry after labeling the cells with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide; 1 μ M)⁴⁰ for 30 min at 37°C. For each experiment, hemin, actinomycin D-treated, and control cells were analyzed for both red and green fluorescence after JC-1 labeling. The ratio of mean fluorescence was calculated from the mean red and green fluorescence for the entire sample population.²⁵ Fluorescence was quantified with a Coulter Epics Profile flow cytometer employing EPICS Elite software. FACS analysis data are representative of three independent experiments.

Determination of hemoglobin

Hemoglobin was estimated by determining latent peroxidase activity as described previously.⁴¹ Briefly, samples were prepared by sonication in Tris buffer (10 mM containing 1 μ M PMSF, pH 8.1) for 1 min at 4°C. After removing nuclei by centrifugation, 8 μ l of 2,7-diaminofluorene (5 mg/ml in 90% acetic acid) was added to 50 μ l of cell lysate. 50 μ l of urea (6 M) was added and the reaction was started by the addition of 10 μ l of hydrogen peroxide (1.5%). The samples were then incubated for 15 min at 20°C and absorbance was read at 600 nm. A standard curve generated from known concentrations of hemoglobin was linear within the range of interest.

Transport of NBD-PS

Cells ($2 \times 10^5/\text{ml}$) were mixed with NBD-labeled PS (100 ng of lipid/ $10 \mu\text{l}$ ethanol/ml) and incubated at 15°C . Aliquots were removed at the indicated times and centrifuged through bovine serum albumin (BSA) to remove residual NBD-PS present in the cells outer leaflet. The remaining NBD-PS that was transported to the cells inner leaflet (BSA inaccessible) was quantified in detergent solubilized samples (1% Triton X-100) by fluorescence λ_{ex} 478 nm; λ_{em} 530 nm). The reported data are means $\pm$ S.D. of a representative experiment.

Acknowledgements

This work was supported in part by National Institutes of Health Grants CA-69676 (D.J.M.) and DK-41714 (A.J.S.). We thank Karen Ramirez for her invaluable assistance and advice on flow cytometry.

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