Regulation of membrane release in apoptosis

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Apoptosis is a fundamental process of cell regulation whereby cells execute one or more biochemical programs leading to cell death. Several mechanisms have been evaluated and suggested to play roles in the regulation of apoptosis, including the activation of phospholipase A₂ (PLA₂), usually measured as release of ³H-labelled arachidonic acid (AA) from prelabelled cells. The current study was aimed at examining the role of PLA₂ in regulating apoptosis in response to several inducers (such as vincristine and etoposide) in lymphoid cell lines. Cells were labelled with [³H]fatty acids and the released radioactivity was characterized. These studies indicated that the AA release assay did not reflect release of non-esterified fatty acid via activation of the PLA₂ pathway. Rather, studies using TLC and electron microscopy showed that AA release reflected a previously un-

suspected shedding of a heterogeneous population of membrane vesicles and fragments, probably as components of apoptotic bodies. Further studies demonstrated that this process is an integral part of apoptosis. Overexpression of Bcl-2 or the addition of caspase peptide inhibitor benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethane prevented the characteristic morphological changes of cell death, and completely inhibited the release of membrane vesicles and fragments. On the other hand, release of membrane vesicles and fragments was caused by various inducers of apoptosis, as measured by release of either ³H-labelled AA or palmitic acid. Thus the present study demonstrates that the release of membrane lipids during apoptosis defines a new assay for apoptosis and has allowed the investigation of the mechanisms regulating formation of apoptotic bodies.

INTRODUCTION

Apoptosis, or cell suicide, is a regulated process [1] during which cells take an active part in the orderly shut-down, packaging and dismantling of the cells. It differs from necrotic death, a toxic process in which the cell is a passive victim [2]. Interference with apoptosis is a potential factor in disorders of normal development, cell growth and differentiation [3]. Many cancers arise because abnormal cells fail to undergo apoptosis [1]. Understanding the process of apoptosis is also important because many chemotherapeutic agents, such as vincristine (VCR), dexamethasone, etoposide and actinomycin D, kill cancer cells by inducing apoptosis [4,5]. However, the essential features and sequence of events in apoptosis are not yet understood.

The mechanisms regulating apoptosis have come under intensive investigation. Two groups of endogenous regulators of apoptosis have been well studied, and they are conserved among species [6]. The first group of apoptotic regulators is caspases, a family of cysteine proteases represented by interleukin converting enzyme (ICE) [7]. These proteases are positive regulators of apoptosis, involved in the execution phase of apoptosis. Many substrates have been identified for these caspases, including poly(ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair. Monitoring the cleavage of PARP using Western-blot analysis is commonly used as a reliable marker of apoptosis [8].

The second group of regulators of apoptosis consists of a family of proteins represented by Bcl-2 [9]. Bcl-2 is a near-universal inhibitor of apoptosis, and overexpression of Bcl-2 prevents cells from undergoing apoptosis induced by chemotherapeutic agents, serum deprivation, γ -irradiation, oxidative damage and other inducers of apoptosis [1]. Bcl-2 appears to function upstream of

the caspases, inhibiting their activation [10], possibly by preventing the release of cytochrome *c* from mitochondria [11,12].

In addition to the above well-established regulators of apoptosis, lipid signalling molecules have been suggested to play an important role in apoptosis [5]. Ceramide, the central molecule of the sphingomyelin cycle, has been studied in signal-transduction processes [13], many of which lead to apoptosis. Arachidonic acid (AA) derived from the activation of phospholipase A₂ (PLA₂) has also been suggested to play an important role in apoptosis and cytotoxic responses [14–18]. Of the two main forms of PLA₂, cytosolic (cPLA₂) and secretory (sPLA₂) [19], sPLA₂ is secreted from cells, whereas cPLA₂ functions in the cytoplasm, where it is regulated by Ca²⁺ and phosphorylation and is believed to be involved in signal-transduction processes [20]. cPLA₂ specifically cleaves AA from the *sn*-2 position of phospholipid, whereas sPLA₂ is not specific with respect to the fatty acid in the substrate [21].

The release of [³H]AA has been used as a convenient assay for PLA₂ activity [22]. When ³H-labelled AA is incorporated into membrane lipids, PLA₂ activity can be measured by counting [³H]AA released into the medium. The intensity of released radioactivity is used as an indication of activity of PLA₂ and the PLA₂ signalling pathway. This AA-release method has been used extensively in investigating the involvement of PLA₂ in multiple processes, including phospholipid metabolism, host defence, signal transduction, and apoptosis. It should be noted, however, that in most of these studies the total radioactivity is quantified without specific assessment of the level of non-esterified AA.

While investigating the role of PLA₂ in regulation of apoptosis in lymphoid cells in response to chemotherapeutic agents, we found that the commonly used assay of AA release does not measure non-esterified AA; rather it measured release of AA

Abbreviations used: VCR, vincristine; Rb, retinoblastoma; PARP, poly(ADP-ribose) polymerase; PLA₂, phospholipase A₂; cPLA₂, cytosolic phospholipase A₂; sPLA₂, secretory phospholipase A₂; AA, arachidonic acid; ICE, interleukin converting enzyme; Z-DEVD-CH₂F, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethane; Z-YVAD-CH₂F, benzyloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethane.

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incorporated in intact lipids. Therefore this release does not indicate activation of cPLA₂; rather, it is a quantitative assay of membrane shedding and/or formation of membrane vesicles. Next, the regulation of this process by known regulators of apoptosis was examined. The results show that protein kinase C, caspases and Bcl-2 modulate the release of membrane lipids. These results suggest that the release of membrane vesicles and fragments is an integral part of apoptosis, possibly a reflection of the release of apoptotic bodies.

EXPERIMENTAL

Cell lines and reagents

Transfected pre-B-leukaemia cell line ALL-697 with vector (neo) or Bcl-2 overexpression vector (Bcl-2) were kindly provided by Dr. John Reed (Burnham Institute, La Jolla, CA, U.S.A.). T-cell leukaemia Molt-4 cells carrying a hygromycin vector or a murine Bcl-2 overexpression vector were also used [23]. HL60 cells were purchased from the A.T.C.C. (Rockville, MD, U.S.A.). Molt-4 and ALL-697 cell lines were maintained in RPMI 1640 medium supplemented with 25 mM Hepes buffer and 10% (v/v) fetal bovine serum. HL60 cells were maintained in RPMI 1640 medium supplemented with 25 mM Hepes buffer and 15% (v/v) fetal-bovine serum. All experiments were conducted under serum-free conditions. HL60 cells were also supplemented with ITS (5 µg/ml insulin + 5 µg/ml transferrin + 5 ng/ml sodium selenite). VCR, etoposide and melittin were purchased from Sigma. [³H]AA and [³H]palmitic acid were purchased from DuPont –NEN.

Measurement of release of radioactivity

Cells were seeded at 1.2×10^6 cells/ml in serum-free medium and prelabelled with 1.5 µCi/ml ³H-labelled non-esterified fatty acid overnight. Before treatment, the cells were washed twice with sterile PBS (Sigma) and seeded at 5×10^5 /ml in serum-free medium. The cells were treated for the indicated time, collected, suspended evenly, and transferred into one microtube. From the microtube a 200 µl sample was removed to a scintillation vial and counted for radioactivity. The value for ³H was regarded as the total incorporation of AA in the cell membrane (*A*). The cells remaining in the microtube were pelleted at 2000 *g* in a micro-centrifuge for 5 min. The supernatant (200 µl) was transferred to another scintillation vial and was counted for radioactivity as an indication of the release of radioactivity into the medium, giving the value *B*. The amount of release of radioactivity was expressed as percentage of *B* over *A*, the percentage of total radioactivity incorporated.

Lipid extraction and analysis

Lipids were extracted following the method of Bligh and Dyer [24]. The cells were pelleted and the lipid extracted using methanol/chloroform/water (2:2:1, by vol.). The lower phase was dried down under extra dry nitrogen and applied to different TLC systems as described in the text. To study the lipid content in the culture medium, cells were spun down at 2000 *g* for 5 min in a table-top centrifuge. The medium was transferred to a 50 ml conical tube, and lipids were extracted using the Bligh and Dyer [24] method.

Isolation of components released from the apoptotic cells

After the indicated treatments for 6 h, the medium was separated from the cells through low-speed centrifugation at 2000 *g* for 5 min. The components released from apoptotic cells were

isolated from the medium using ultracentrifugation at a force of 100000 *g* for 30 min. The small pellets at the bottom of the centrifuge tube were resuspended in PBS and were used for further studies.

Electron-microscopy studies

After the isolation of the released components from the apoptotic cells, the pellets were sent to the Electron Microscopy Virology Laboratory of Duke University Medical Center for further electron-microscopic analysis.

Western-blot analysis

Cells were seeded at 5×10^5 /ml in serum-free medium. After the treatments indicated, 1×10^6 cells were collected and washed once with PBS. Cells were resuspended in the sample buffer and separated by SDS/6%-PAGE. After transferring to a nitro-cellulose membrane, the blots were blocked with 5% dry milk. Anti-PARP was applied in a 1:2000 dilution. The blots were developed using enhanced chemiluminescence (ECLTM; Amersham).

Statistical analysis

All the results were analysed using the Microsoft Excel T-test (paired two samples for means; **P* < 0.05; ***P* < 0.01). We also used two-factor analysis of variance followed by *post hoc* testing where necessary, as indicated in the Table legends.

RESULTS

Release of radiolabelled lipids during apoptosis

To investigate the possible involvement of cPLA₂ and the AA signalling cascade in apoptosis, we measured [³H]AA release during apoptosis of lymphoma cells induced by chemotherapeutic agents. Both VCR and etoposide can induce apoptosis in ALL-697 and Molt-4 cells [23]. Upon induction of apoptosis with VCR, in ALL-697 cells prelabelled with [³H]AA, there was an increase of radioactivity released from the cells into the medium as early as 3 h. About 30% of total radioactivity incorporated in the cell membrane was released into the medium by 6 h of VCR treatment. In contrast, only ≈ 8% of total radioactivity was released from the controls. By 24 h, apoptotic cells released ≈ 40% of total label into the medium, compared with ≈ 18% radioactivity released from control cells (Figure 1A). These results clearly show increased release of radioactivity in VCR-induced apoptosis.

We next determined if the radioactivity released from the cells represented the activation of cPLA₂. Since palmitate in the *sn*-2 position of phospholipid is a poor substrate for cPLA₂, we next used [³H]palmitic acid to label cells. The labelled cells were treated using the same duration and concentration of VCR as described above. The radioactivity was released starting at 3 h following treatment and mirrored the release seen with [³H]AA (Figure 1B). The data demonstrate that the same amount of radioactivity was released, despite palmitate being a poor substrate for cPLA₂, suggesting that ³H release was unlikely to be a result of activation of cPLA₂. These results raised two possibilities: either secreted PLA₂ is involved in apoptosis, which has no major preference for the fatty acid in the *sn*-2 position of its substrate, or the release of radioactivity is not a true reflection of release of non-esterified fatty acid (palmitic acid or AA), rather it is a measurement of release of membrane lipids from membranes that are ³H-labelled with either AA or palmitic acid.

Figure 1 Effects of VCR on the induction of release of $[^3\text{H}]$ AA and $[^3\text{H}]$ palmitic acid

ALL-697 cells were labelled overnight as described in the Experimental section. The unincorporated non-esterified fatty acid was washed off with sterile PBS. The cells were seeded at 5×10^5 cells/ml in serum-free medium and treated with vehicle control or with $1 \mu\text{M}$ VCR. The release of radioactivity was calculated as described in the Experimental section. (A) Release of radioactivity from cells labelled using $[^3\text{H}]$ AA. The result is the average for four independent experiments. (B) Release of radioactivity from cells labelled using $[^3\text{H}]$ palmitic acid. The results were the average for six independent experiments.

To examine the possibility that sPLA_2 is involved in VCR-induced apoptosis, we measured PLA_2 activity using an *in vitro* enzyme assay. VCR treatment did not increase PLA_2 activity above basal levels measured in the control cells. In addition, Western-blot analysis using antibodies selectively against either cPLA_2 or sPLA_2 did not show any changes in the levels of these proteins upon VCR treatment (results not shown). These results suggest that involvement of either cPLA_2 or sPLA_2 in VCR-induced apoptosis in this system is unlikely.

Characterization of the lipids associated with radioactivity

To determine the nature of the released radioactivity, we analysed the medium containing the radioactivity released from the cells treated with vehicle control or VCR. The medium was collected by centrifugation at 2000 g for 5 min after 6 h of treatment. The lipid was extracted at neutral conditions, basic conditions (1 M KOH in methanol), or acidic conditions (1 M methanolic HCl), the modified method of Bligh and Dyer [24] being used to avoid

Table 1 Composition of neutral lipids released from the cells

Cells were suspended at $5 \times 10^5/\text{ml}$. After 6 h treatments with control vehicle, $1 \mu\text{M}$ VCR, $10 \mu\text{M}$ etoposide and $2.5 \mu\text{M}$ melittin, each sample was collected and the medium was separated from the cells after a 2000 g centrifugation for 5 min. Total lipids from the medium were extracted following the methods of Bligh and Dyer [24], and were separated using a TLC system as indicated. The amount of each species of lipids was quantified using a liquid-scintillation counter. The results were expressed as the percentage of the total d.p.m. of each lipid species over the total d.p.m. This result was the representative of three experiments. The TLC solvent system was ethyl acetate/iso-octane/acetic acid/water (55:75:8:100, by vol.).

	Composition (%)			
	Control	$1 \mu\text{M}$ VCR	$10 \mu\text{M}$ Etoposide	$2.5 \mu\text{M}$ Melittin
Phospholipid (loading area)	62	70	70	16
Monoacylglycerol	3	3	3	2
AA/diacylglycerol	5	5	7	69
Triacylglycerol	21	15	12	8

Table 2 Composition of phospholipids released from the cells

The lipids were extracted as described in Table 1. The solvent system was used as indicated. The amount of each species of lipids was quantified using a liquid-scintillation counter after the separation. The result was expressed as the percentage of the total d.p.m. of each lipid species over the total d.p.m. The TLC solvent system used was chloroform/methanol/acetic acid/water (50:25:8:4, by vol.). Abbreviations used: SM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; PE, phosphatidylethanolamine.

	Composition (%)			
	Control	$1 \mu\text{M}$ VCR	$10 \mu\text{M}$ Etoposide	$2.5 \mu\text{M}$ Melittin
SM	10	6	7	3
PC	31	36	34	3
PI	8	9	12	1
PA/PE/AA	47	46	45	89

the possible loss of any non-esterified fatty acids during the extraction process.

The amount of the major lipid species in the lipid extract was quantified using TLC techniques (Tables 1 and 2). Lipids were separately resolved in solvent systems for neutral lipids and phospholipids respectively [25]. We found that total non-esterified fatty acid accounted for less than 10 % of total radioactivity released from either VCR-treated cells or control cells. The majority of released radioactivity was incorporated in the form of both neutral lipids and phospholipids. Phosphatidylcholine, the major component of phospholipids, accounted for more than 30 % of the total released radioactivity. The major component of the neutral lipid species was triacylglycerol. The overall composition of lipids was similar to that of cell membrane. Another chemotherapeutic agent, etoposide, was also used to treat the cells labelled with $[^3\text{H}]$ AA. Radioactivity released from the cells treated with etoposide had a similar lipid composition (Tables 1 and 2).

To avoid the possibility that the lipid released from the treated cells might be lost in the lipid-extraction process, the lipid-extraction efficiency of the Bligh and Dyer [24] method was evaluated by comparing the radioactivity present in both organic and inorganic phases. On the basis of that comparison, more than 96 % of total radioactivity was present in the organic phase. Therefore the extracted lipids represent most of the radioactivity released from the apoptotic cells (results not shown).

Table 3 Radioactivity released from the cells was not in the form of non-esterified fatty acid, rather it could be collected by high-speed centrifugation

The medium (1 ml) from 6 h VCR-treated and control cells was collected as described in Figure 1. The medium was transferred into microtubes and spun in a TL-100 table-top ultracentrifuge at 10000 *g* for 30 min. The supernatant was separated from the pellet and counted for radioactivity separately in a liquid-scintillation counter. The results were expressed as percentages of the d.p.m. of supernatant and pellet respectively over the total d.p.m. (d.p.m. of supernatant plus that of pellet). The result is the mean $\pm$ S.E.M. for three independent experiments.

	Radioactivity (% of total)	
	Supernatant	Pellet
$[^3\text{H}]\text{AA}$ -labelled cells (control)	24.8 $\pm$ 4.4	75.2 $\pm$ 4.4
VCR-treated AA-labelled cells	20.0 $\pm$ 8.9	80.0 $\pm$ 8.9
$[^3\text{H}]\text{Palmitic acid}$ -labelled cells (control)	17.4 $\pm$ 3.7	82.6 $\pm$ 3.7
VCR-treated palmitic acid-labelled cells	12.5 $\pm$ 7.2	87.5 $\pm$ 7.2

Melittin, a short-chain polypeptide from bee venom [26], is an effective activator of sPLA₂ [27]. This polypeptide was used as the positive control for the above lipid analysis. Cells were labelled with $[^3\text{H}]\text{palmitic acid}$ overnight and suspended in serum-free medium after washing away unincorporated non-esterified fatty acid. After the cells were challenged with 2.5 μM melittin for 6 h, the lipids in the medium from the melittin-treated cells were extracted and the lipid profile was determined. In contrast with the lipids collected from the medium of VCR-treated cells, non-esterified fatty acid accounted for around 70 % of the total radioactivity released into the medium from the cells treated with melittin (Tables 1 and 2). These results using melittin demonstrate that the above lipid analysis could detect the activation of PLA₂ and the release of non-esterified fatty acid, and they provide further evidence against activation of PLA₂ in response to either VCR or etoposide.

In order to investigate further the regulation of membrane-lipid release during apoptosis, we chose to label cells with $[^3\text{H}]\text{palmitic acid}$ rather than $[^3\text{H}]\text{AA}$ in all subsequent experiments. Palmitate is the major fatty-acyl species in membrane lipids, and it can better reflect the release of major lipid species from apoptotic cells. Moreover, release of non-esterified AA in response to activation of cPLA₂ could complicate the study, whereas palmitate in the *sn*-2 position, a poor substrate for cPLA₂, is devoid of this problem. Finally, $[^3\text{H}]\text{palmitic acid}$ is much less expensive than $[^3\text{H}]\text{AA}$.

Next we isolated most of the radioactivity through differential speed centrifugation. After cells were labelled with $[^3\text{H}]\text{palmitate}$ and treated with VCR for 6 h; the cells were then pelleted out following a low-speed centrifugation (2000 *g*). The supernatant was collected and a small aliquot of medium was counted for radioactivity in a scintillation counter to calculate the total radioactivity present in the medium. The remaining supernatant was centrifuged at 10000 *g* for 30 min. The pellet formed after high-speed centrifugation was counted for radioactivity. Comparing the counts from the medium and the pellet after this centrifugation, we found that more than 80 % of the radioactivity present in the medium was recovered in the pellet (Table 3). Non-esterified fatty acid is soluble and cannot be pelleted, supporting our observation that not much non-esterified fatty acid was released from the cells. Since most of the radioactivity was associated with the pellet, the components released from the apoptotic cells by high-speed centrifugation could be further characterized.

Figure 2 Electron-microscopic studies of the components released from the apoptotic cells

The cells were seeded at $5 \times 10^5/\text{ml}$, and treated with 1 μM VCR. After 6 h treatment, the components were isolated by the method described in the Experimental section. These components were further processed by the Electron Microscopy Virology Laboratory of Duke University Medical Center for electron-microscopic observation.

To characterize the components released from the apoptotic cells, 50 ml of 5×10^5 ALL-697 cells/ml were suspended in serum-free medium. After treatment with VCR or vehicle control for 6 h, the cells were pelleted out at low speed (2000 *g*). The components released to the medium were pelleted by centrifugation at 10000 *g* for 30 min. The amount of protein, RNA and DNA were measured in the pellets from the VCR-treated cells and the control cells. We found that, in addition to lipid molecules, significant amounts of DNA, RNA and proteins were present in the pellets from the treated cells as compared with control cells (results not shown). Therefore these results suggest that what were released into the medium were cellular fragments.

In order to characterize these cellular fragments released into the medium, we next performed electron-microscopic studies. For this purpose, VCR-treated cells were pelleted at 2000 *g*, the medium was spun at high speed (10000 *g* for 30 min) and prepared for electron-microscopic studies. We found that what was released from the VCR-treated cells was a heterogeneous mixture of cellular remnants. There were fragments of cell membranes containing cellular organelles, such as mitochondria (Figure 2). Some cellular fragments contained vesicles and some

Table 4 Membrane lipid release was induced by several inducers of apoptosis in ALL-697 cells

Cells were labelled as described. Inducers of apoptosis (etoposide, C₂-ceramide, actinomycin D, okadaic acid, staurosporine and VCR) were applied and [³H]palmitate release was measured after 6 h treatments. The results are expressed as percentage of ³H release from cells undergoing apoptosis over untreated cells. This result is the mean ± S.E.M. for three independent experiments **P* < 0.05 based on paired Student *t*-test.

	Membrane lipid release (% of that for untreated cells)
Untreated cells	100
VCR (1 μ M)	299 ± 30*
Etoposide (10 μ M)	290 ± 23*
C ₂ -ceramide (20 μ M)	281 ± 15*
Okadaic acid (20 nM)	289 ± 27*
Staurosporine (100 nM)	401 ± 25*
Actinomycin D (5 nM)	295 ± 19*

Figure 3 Z-DEVD-CH₂F ('DEVD') but not Z-YVAD-CH₂F ('YVAD') could prevent VCR-induced PARP cleavage

The anti-caspase polypeptide Z-DEVD-CH₂F (CPP32 inhibitor) and Z-YVAD-CH₂F (ICE inhibitor) were used together with inducers of apoptosis. Their effects on PARP cleavage were assessed. Cells (1×10^6) were treated with VCR together with different doses of Z-DEVD-CH₂F or Z-YVAD-CH₂F. PARP cleavage was assessed using a rabbit anti-(human PARP) antibody.

contained condensed chromatin. This observation demonstrates that, upon induction of apoptosis, the fractions that contained the radiolabelled fatty acid were parts of cellular fragments which could be collectively described as 'apoptotic bodies'.

Membrane lipid release is neither cell-type-specific nor inducer-specific

We next wanted to evaluate if this membrane lipid release was inducer-specific. To this end we evaluated the effect of several well-known inducers of apoptosis on the release of the membrane lipids into the medium, using released [³H]palmitate as a convenient assay. Staurosporine, ceramide, etoposide, actinomycin D and doxorubicin were used as inducers of apoptosis in [³H]palmitic acid-labelled ALL-697 cells. In all instances there was ≈ 200 – 300% increase in radioactivity released from the treated cells over that released from the control cells (Table 4). To verify that these chemotherapeutic agents induced cell death through apoptosis, we evaluated cleavage of the death substrate PARP, a reliable indicator of apoptosis. Western blots showed that PARP was cleaved in response to treatment by these chemotherapeutic agents (Figure 3), confirming the activation of apoptotic pathways in this model by these agents.

In order to evaluate if the release of membrane vesicles and fragments is a cell-specific phenomenon, release of [³H]palmitate was also evaluated in several cell lines, including Molt-4 T cells and HL60 cells. These cells were labelled with [³H]palmitic acid similar to studies in ALL-697 cells. Upon induction of apoptosis

Table 5 ³H-labelled-membrane-lipid release in the HL60 macrophage cell line on induction of apoptosis

HL60 cells were seeded in serum-free medium supplemented with ITS (see the text). VCR (1 μ M), etoposide (10 μ M) and C₂-ceramide (20 μ M) were applied, and [³H]palmitate release was measured after 6 h treatments. The results were expressed as percentage of ³H release from cells undergoing apoptosis over untreated cells. This result is the mean ± S.E.M. for three independent experiments. **P* < 0.05.

	Membrane lipid release (% of total)
Untreated cells	100
VCR (1 μ M)	140 ± 10*
Etoposide (10 μ M)	167 ± 9*
C ₂ -ceramide (20 μ M)	202 ± 10*

by either VCR or etoposide, there was an increase of radioactivity released as compared with the untreated control cells [Table 5 and Table 7 (below)]. These data suggest that release of [³H]palmitate is a general phenomenon in apoptosis.

Mechanisms regulating the release of membrane vesicles and fragments

In an effort to understand the mechanisms involved in membrane release, we evaluated the effects of several known regulators of apoptosis. We initially investigated the effects of Bcl-2 on [³H]palmitate release. ALL-697 cells carrying a retroviral vector overexpressing human Bcl-2 protein (Bcl-2) or the control vector (neo) were used for this purpose [23]. Both Bcl-2 and neo cells were labelled with [³H]palmitic acid and treated with either C₂-ceramide, etoposide or VCR. Neo cells showed extensive release of radioactivity, whereas overexpression of Bcl-2 prevented the release of [³H]palmitic acid (Table 6). Similar results were also observed in the Molt-4 cells transfected with murine Bcl-2 expression vector (Table 7).

Activation of protein kinase C has been shown to protect cells from apoptosis initiated by several inducers [28]. To study the effect of protein kinase C activation on the release of radioactivity, the protein kinase C activator PMA was used in combination with VCR. ALL-697 cells labelled with [³H]palmitic acid were treated with VCR with and without 100 nM PMA for 6 h. The radioactivity released from the cells labelled with [³H]palmitic acid was inhibited by 50% in the presence of PMA (Table 8). PMA treatment alone did not induce release of radioactivity. The effect of staurosporine, an inhibitor of protein kinase C, was also evaluated. Staurosporine alone induced [³H]palmitate release, consistent with its role as a well-recognized inducer of apoptosis.

The role of caspases in the apoptotic process was next examined by studying the effects of caspase inhibitors on the [³H]fatty acid-release assay. Small polypeptides which mimic caspase substrate cleavage sites are known to be competitive inhibitors of caspase activation in apoptosis. Benzylloxycarbonyl-Asp-Glu-Val-Asp-fluoromethane (Z-DEVD-CH₂F) is modelled after the P₁-P₄ amino acids of the caspase 3 (apopain/CPP32) substrate cleavage site, and benzylloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethane (Z-YVAD-CH₂F) is modelled after the caspase 1 (ICE) family protease substrate cleavage site [29]. We investigated the involvement of these two proteases in etoposide- and VCR-induced apoptosis by determining the effects of these two specific inhibitors on the cleavage of PARP. Cells were treated with VCR in the presence of Z-DEVD-CH₂F or Z-YVAD-CH₂F. Whole

Table 6 Effects of Bcl-2 protein on ^3H -labelled-membrane-lipid release in the ALL-697 cell line

Human Bcl-2-transfected cells were used together with their vector controls. The cells were labelled, and [^3H]palmitate release was measured after 6 h treatments with inducers of apoptosis. The data are expressed as described in the Experimental section. Results are the means for four independent experiments $\pm$ S.E.M. * $P < 0.05$.

	^3H -labelled-membrane-lipid release (% of total)	
	Neo cells	Bcl-2 cells
Control	8.1 $\pm$ 2.0	5.8 $\pm$ 0.2
VCR (1 μM)	21.3 $\pm$ 3.2	5.6 $\pm$ 0.6*
Etoposide (10 μM)	21.7 $\pm$ 3.0	5.6 $\pm$ 0.6*
C ₂ -ceramide (10 μM)	17.7 $\pm$ 0.6	9.6 $\pm$ 0.2*
C ₂ -ceramide (20 μM)	22.2 $\pm$ 0.9	10.7 $\pm$ 1.4*

Table 7 Effects of Bcl-2 protein on ^3H -labelled-membrane-lipid release in the Molt-4 cell line

Murine Bcl-2-transfected cells were used together with their vector controls. The cells were treated with VCR (1 μM) or etoposide (10 μM) for 6 h. The results are expressed as described in Experimental section. Results are means $\pm$ S.E.M. for three independent experiments. Means for the effect of VCR or etoposide were compared using two-factor analysis of variance ($P < 0.05$), and specific sources of differences were identified with subsequent *post hoc* testing. *VCR versus control or etoposide versus control, $P < 0.05$; †Bcl-2 versus Molt4 cells, $P < 0.05$.

	^3H -labelled-membrane-lipid release (% of total)		
	Control	VCR (1 μM)	Etoposide (10 μM)
Molt-4 cells	7.2 $\pm$ 0.5	15.0 $\pm$ 1.4*	13.9 $\pm$ 1.7*
Molt-4 Bcl2 cells	4.8 $\pm$ 0.7	5.0 $\pm$ 0.6†	4.9 $\pm$ 0.6†

cell protein extract was evaluated for PARP cleavage. Western-blot analysis showed that, in ALL-697 cells, Z-DEVD-CH₂F (100 μM) effectively inhibited PARP cleavage, whereas Z-YVAD-CH₂F (100 μM) did not (Figure 2).

The effects of the increased doses of Z-DEVD-CH₂F and Z-YVAD-CH₂F on [^3H]palmitate release were also evaluated in VCR-treated (Table 9) and etoposide-treated cells (results not shown) simultaneously. Consistent with the Western-blot analy-

Table 8 Effects of activation of protein kinase C on [^3H]palmitate release

ALL-697 cells were labelled with [^3H]palmitic acid. The effects of VCR treatment on [^3H]palmitate release were assessed in the presence of an activator of protein kinase C, PMA (100 nM) or with an inhibitor of protein kinase C, staurosporine (100 nM). Results are means $\pm$ S.E.M. for four independent experiments. *, PMA + VCR versus VCR alone, $P < 0.05$ based on paired Student *t* test.

Treatment	Membrane lipid release (% of total)
Control	10.2 $\pm$ 0.7
VCR (1 μM)	32.7 $\pm$ 3.0
Staurosporine (100 nM)	10.1 $\pm$ 0.9
PMA (100 nM)	41.2 $\pm$ 1.9
VCR + PMA	17.7 $\pm$ 2.3*

Table 9 Activation of caspases is required for the ^3H -labelled-membrane-lipid release

Z-DEVD-CH₂F (inhibitor of caspase 3), but not Z-YVAD-CH₂F (inhibitor of caspase 1) could inhibit VCR-induced [^3H]palmitate release. The cells were labelled with [^3H]palmitic acid. ^3H release was measured as described in the Experimental section. Results are means $\pm$ S.E.M. for four independent experiments, * $P < 0.05$.

	^3H -labelled-membrane-lipid release (% of total)	
	Z-DEVD-CH ₂ F	Z-YVAD-CH ₂ F
Control	8.0 $\pm$ 1.5	8.0 $\pm$ 1.5
VCR (1 μM)	20.1 $\pm$ 0.9	20.1 $\pm$ 0.9
100 μM inhibitor alone	7.5 $\pm$ 0.5	7.0 $\pm$ 0.4
10 μM inhibitor + VCR	13.7 $\pm$ 0.7*	17.9 $\pm$ 2.3
50 μM inhibitor + VCR	9.7 $\pm$ 0.7*	17.5 $\pm$ 2.0
100 μM inhibitor + VCR	6.7 $\pm$ 0.4*	19.2 $\pm$ 0.8

sis of PARP cleavage, we found that, with increased Z-DEVD-CH₂F concentration, [^3H]palmitate release decreased in VCR-induced apoptosis. On the other hand, Z-YVAD-CH₂F up to 100 μM did not inhibit [^3H]palmitate release (Table 9).

DISCUSSION

The present study originated with the evaluation of the role of PLA₂ in the regulation of apoptosis. The results showed that neither AA nor palmitate was released as non-esterified fatty acid. Instead, the label was recovered in membrane lipids when centrifuged at 10000 *g*. This led to the conclusion that, during apoptosis, there is release of various ^3H -labelled membrane lipids in cellular components, indicating that the lipids were part of membrane fragments and cellular organelles.

The release of [^3H]lipid-containing membrane components appears to be a general feature of the apoptotic process, as it was found to occur in several cell lines and to be induced by various established inducers of apoptosis. Moreover, the release of these components was inhibited by Bcl-2, a negative regulator of apoptosis, by activation of protein kinase C, and by inhibition of caspase activity in apoptotic cells.

One major implication of these results relates to the release of incorporated [^3H]AA. The assay of AA release has been widely used as an indication of activation of PLA₂. This method has served as the basis for many conclusions concerning the involvement of PLA₂ in cell growth, differentiation and apoptosis based on the assumption that it accurately reflects changes in PLA₂ activity. However, it should be noted that only in some cases were HPLC or TLC analysis applied to confirm that the released label was indeed the non-esterified AA. Our studies suggest that results based on AA release without further lipid analysis could generate misleading conclusions concerning the activity of PLA₂ in apoptosis. By using [^3H]palmitic acid instead of [^3H]AA to label cells, we found that release of membrane vesicles and fragments is part of apoptosis, perhaps a reflection of the release of apoptotic bodies formed in apoptotic cells. We suggest that measuring the level of release of [^3H]palmitate incorporated into membrane lipids can be a simple and quantitative assay for apoptosis. It can also be used to study regulation of apoptosis.

It should not be concluded that the traditional assay of AA release can not be used to measure PLA₂ activity in other

signalling processes. Reciprocally it should not be concluded that PLA₂ activity is not involved in the apoptotic process at all in other systems. We and others have found that cPLA₂ is involved in tumour-necrosis-factor- α -induced apoptosis in the murine fibroblast L929 cell line, upstream of ceramide formation [30]. However, we believe that the traditional AA-release measurement should be coupled with either HPLC or TLC studies to confirm that the measurement of radioactivity is a true reflection of non-esterified AA rather than total lipid species labelled with [³H]AA.

Our studies also indicate that the release of membrane vesicles and fragments is a general phenomenon in multiple cell lines and can be induced by multiple inducers of apoptosis. More importantly, the regulation of this event by the regulators of apoptosis, including Bcl-2, protein kinase C and caspases, strongly implies that the release of membrane vesicles and fragments is a general event in apoptosis, possibly as part of the execution phase of apoptosis. Therefore it is of importance to understand the cell-biological aspect of this phenomenon and its physiological significance in apoptosis.

Apoptotic bodies have been well described morphologically and well accepted for many years. However, the regulation of the generation of apoptotic bodies is still unclear. We suggest that the release of membrane vesicles and fragments could well be part of apoptotic-body formation, as part of the execution machinery for cells to dismantle themselves during the apoptotic process. Thus the present study provides a novel biochemical approach that is simple yet quite powerful to study the regulation of apoptotic body formation. It has also allowed the study of formation of apoptotic bodies at a biochemical level.

In conclusion, these results show that: (1) the traditional assay of AA release is not a reliable measurement of PLA₂ activity in chemotherapeutic-agent-induced apoptosis in leukemia cell lines; (2) palmitate release correlates closely with several parameters of apoptosis and is regulated by all known regulators of apoptosis that we tested; (3) release of membrane vesicles and fragments during apoptosis is a highly regulated process that depends on caspase activation and occurs downstream of the site of activation of Bcl-2.

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