# Phosphatidyl serine exposure during apoptosis precedes release of cytochrome *c* and decrease in mitochondrial transmembrane potential

Geertrui Denecker, Hans Dooms, Geert Van Loo, Dominique Vercammen, Johan Grooten, Walter Fiers, Wim Declercq, Peter Vandenabeele*

*Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology and University of Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium*

Received 6 October 1999; received in revised form 2 December 1999

Edited by Marc Van Montagu

**Abstract Time kinetics of phosphatidyl serine (PS) exposure were compared to other apoptotic parameters following different apoptotic stimuli. Our data indicate that anti-Fas treatment of L929sAhFas cells results in rapid exposure of PS, which precedes decrease in mitochondrial transmembrane potential ($\Delta\Psi_m$) and release of cytochrome *c*, indicating that PS exposure occurs independently of these mitochondrial events. Also during TNF-, etoposide- or staurosporine-mediated apoptosis in PC60 RI/RII cells, PS-positive cells were observed before they had a decreased $\Delta\Psi_m$. However, during growth factor depletion-induced death of 32D cells, both phenomena seemed to occur at the same time.**

**© 2000 Federation of European Biochemical Societies.**

*Key words:* Apoptosis; Phosphatidyl serine; Mitochondrial transmembrane potential

## 1. Introduction

Apoptosis is a multistep process characterized by several morphological and biochemical alterations [1]. Following apoptotic trigger, a chain of events leads to cell shrinkage, membrane blebbing, nuclear condensation, loss of membrane integrity and enzymatic DNA degradation. It is well established now that caspase-dependent proteolysis is crucial in the apoptotic process [2]. Activation of caspases is initiated by receptor-mediated oligomerization of procaspases [3] and/or by release of mitochondrial caspase-activating factors [4–6]. The mitochondrial release of proapoptotic factors is regulated by members of the Bcl-2 protein family [6,7].

Another important hallmark of the apoptotic process is loss of aminophospholipid asymmetry of the plasma membrane [8]. Phosphatidyl serine (PS), which is confined to the inner membrane leaflet in normal healthy cells, becomes exposed at the outer plasma membrane of apoptotic cells. Exposure of PS is an early event, as it precedes cell membrane permeabilization, cell shrinkage and nuclear condensation [9]. PS exposure has been implicated in the recognition of apoptotic cells by phagocytes and results in rapid engulfment of the apoptotic cell [10]. Furthermore, PS at the outside of platelets promotes the assembly of the blood coagulation system [11]. The aminophospholipid asymmetry of PS in viable cells results from an active inward transport of PS by aminophospholipid translocase [12,13] and bidirectional transport of phospholipids (PLs) by scramblase [11,14]. Exposure of PS during apoptosis is associated with a loss of aminophospholipid translocase activity and with a non-specific flip-flop of PLs [15,16].

Besides exposure of PS, also disruption of the mitochondrial transmembrane potential ($\Delta\Psi_m$) has been reported to be an early event in the apoptotic process [1]. $\Delta\Psi_m$ results from an asymmetric distribution of protons on either side of the inner mitochondrial membrane and is the driving force for oxidative phosphorylation. Collapse of $\Delta\Psi_m$ during apoptosis has been observed in many different cell types, irrespective of the apoptotic stimulus. The mitochondrial intermembrane fraction contains several proapoptotic proteins released during the apoptotic process, such as cytochrome *c* [4], procaspase-2, -3 and -9 [17,18], as well as apoptosis-inducing factor (AIF) [19]. The latter, recently identified as a flavoprotein with homology to bacterial oxido-reductases, can cause chromatin condensation in isolated nuclei as well as release of cytochrome *c* and caspase-9 from purified mitochondria. When overexpressed, AIF also induces collapse of $\Delta\Psi_m$, exposure of PS and hypoploidy [6,19]. The functional correlation between decrease in $\Delta\Psi_m$ and release of these proapoptotic factors is not clear. In this respect, it has been reported that release of cytochrome *c* can occur without loss of $\Delta\Psi_m$ [20].

It has been reported that during dexamethasone-induced apoptosis in splenocytes or thymocytes, cells first exhibit a decreased $\Delta\Psi_m$ followed by PS exposure [21,22]. Other reports mention that PS exposure only slightly precedes or coincides with collapse of $\Delta\Psi_m$ in 1-β-D-arabinofuranosylcytosine-induced apoptosis in leukemic blast cells, in anti-Fas-induced apoptosis in Jurkat cells and in etoposide- or *N*-tosyl-L-phenylalanine chloromethylketone-elicited apoptosis in THP-1 cells [23–25]. Therefore, we examined the time course of these two early apoptotic phenomena, viz. exposure of PS and decrease in $\Delta\Psi_m$ in different cell lines after various apoptotic stimuli. Time kinetic studies were performed on anti-Fas-treated L929sAhFas cells, on TNF-, staurosporine (STS)- and etoposide-stimulated PC60 RI/RII cells and on growth factor-depleted 32D cells.

## 2. Materials and methods

### 2.1. Cell cultures

L929sAhFas is a murine fibrosarcoma cell line transfected with the human Fas (hFas) receptor [26]. hFas cDNA was kindly provided by Dr. S. Nagata (Osaka Bioscience Institute, Osaka, Japan). Cells were grown in DMEM medium, supplemented with 5% fetal calf serum, 5% newborn calf serum, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml) and L-glutamine (2 mM). Hybridoma PC60.21.14.4 (PC60), derived from a fusion between an IL-2-dependent murine

*Corresponding author. Fax: (32)-9-264 5348.
E-mail: peter.vandenabeele@dmb.rug.ac.be

0014-5793/00/$20.00 © 2000 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(99)01702-0

CTL line B6.1SF.1 and a rat thymoma (C58.NT)D [27], was kindly provided by Dr. M. Nabholz (Swiss Institute for Experimental Cancer Research, Épalinges, Switzerland). Transfection of these cells with TNF-RI and TNF-RII was described previously [28]. Cells were grown in RPMI 1640 medium, supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), L-glutamine (2 mM), sodium pyruvate (1 mM) and β-mercaptoethanol ($2\times10^{-5}$ M). The IL-3-dependent murine myeloid leukemic cell line 32D was maintained in RPMI 1640 medium, supplemented with 10% fetal calf serum, 10% WEHI-3B cell-conditioned medium as a source of murine IL-3, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), L-glutamine (2 mM), sodium pyruvate (1 mM) and β-mercaptoethanol ($2\times10^{-5}$ M).

### 2.2. Cytokines and reagents

Anti-hFas antibody (clone 2R2) was purchased from Cell Diagnostica (Münster, Germany). Recombinant human TNF was produced in *Escherichia coli* and purified in our laboratory. The specific biological activity of TNF was $9.4\times10^7$ IU/mg, based on a standardized cytotoxic assay on WEHI 164 cl 13 cells [29]. The TNF standard was obtained from the National Institute for Biological Standards and Control (Potters Bar, UK). STS was purchased from Roche Diagnostics (Basel, Switzerland) and was stored as a solution in DMSO. Carbonyl cyanide *m*-chlorophenylhydrazone (mClCCP) and etoposide were purchased from Sigma Chemical (St. Louis, MO, USA); the latter was solubilized in ethanol or DMSO. Propidium iodide (PI; Becton-Dickinson, Sunnyvale, CA, USA) was prepared as a 3 mM stock solution in phosphate-buffered saline and was used at 30 μM. The fluorescent marker chloromethyltetramethylrosamine (CMTMros) was purchased from Molecular Probes (Eugene, OR, USA); it was prepared as a 1 mM stock solution in DMSO and was used at 0.05 μM. Annexin V (FITC conjugate; PharMingen, San Diego, CA, USA) was used at 1 μg/ml.

### 2.3. Induction of apoptosis

L929sAhFas cells for FACS analysis were kept in suspension by seeding $2\times10^5$ cells the day before in uncoated 24 well tissue culture plates. PC60 RI/RII cells or 32D cells ($1\times10^5$) were cultured from the day before in 24 well tissue culture plates, the latter in the presence of 10% WEHI-3B cell-conditioned medium. The next day, anti-Fas (250 ng/ml) was added to L929sAhFas cells and TNF (100 ng/ml), STS (2 μM) or etoposide (50 μg/ml) to PC60 RI/RII cells. 32D cells were washed three times and resuspended in the presence or absence of WEHI-3B cell-conditioned medium. Cell samples were taken at regular time intervals and analyzed on a FACScalibur flow cytometer (Becton-Dickinson, Sunnyvale, CA, USA).

### 2.4. Cytofluorometric analysis of $\Delta\Psi_m$, cell membrane alterations and hypoploidy

To measure the decrease in $\Delta\Psi_m$, the fluorogenic probe CMTMros was used [30]. Cells were incubated at 37°C for 15 min in the presence of CMTMros (0.05 μM) and then left on ice for 10 min before analysis on a FACScalibur flow cytometer. CMTMros fluorescence was excited with an argon ion laser (250 mW) at 488 nm and detected between 545 and 615 nm. Exposure of PS at the cell surface was analyzed with annexin V [31]. Cells were washed in HEPES buffer (10 mM HEPES NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) and incubated for 5 min on ice with annexin V (1 μg/ml; excitation at 488 nm and detection between 515 and 550 nm). The loss of cell membrane integrity was determined on all cells by means of a PI exclusion method [28,32]. Each sample was incubated with 30 μM PI, 3–10 min before analysis on a FACScalibur flow cytometer. The dye was excited with the 488 nm line of an argon ion laser and fluorescence emission was measured at 610 nm. The percentage of cells containing hypoploid DNA was determined on all cells by PI staining of cells after one freeze-thaw cycle to permeabilize the cells.

### 2.5. Cytochrome c release

Cells were treated with anti-Fas (250 ng/ml) and were washed in cold PBS-A. They were permeabilized with 100 μl 0.02% digitonin dissolved in cell-free system buffer (220 mM mannitol, 68 mM sucrose, 2 mM $MgCl_2$, 2 mM NaCl, 2.5 mM $H_2KPO_4$, 0.5 mM EGTA, 0.5 mM sodium pyruvate, 0.5 mM L-glutamine and 10 mM HEPES NaOH pH 7.4) and were left on ice for 1 min. After centrifugation, the supernatant was dissolved in Laemmli buffer; it was analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using a monoclonal antibody to cytochrome *c* (PharMingen, San Diego, CA, USA) and ECL-based detection.

## 3. Results

### 3.1. Surface exposure of PS in apoptotic L929sAhFas cells precedes collapse of $\Delta\Psi_m$, release of cytochrome c, loss of membrane integrity and appearance of hypoploid DNA

L929sAhFas cells die apoptotically when exposed to anti-Fas agonistic antibodies, characterized by caspase activation, extensive membrane blebbing, hypoploid DNA pattern and nuclear condensation [26]. Simultaneous two-parameter analysis was performed in order to examine the time sequence of exposure of PS in relation to the disruption of $\Delta\Psi_m$ during anti-Fas-induced apoptosis in L929sAhFas cells. Both phenomena have been reported to occur at an early stage in the apoptotic process [33]. To measure the extent of PS exposure, cells were labeled with annexin V, which specifically binds PS in a calcium-dependent way. $\Delta\Psi_m$ was measured with CMTMros. The accumulation of CMTMros in the mitochondria depends on active mitochondria with an intact $\Delta\Psi_m$; this is an irreversible process, as the chloromethyl group reacts with intramitochondrial thiols forming a thioester bond. To confirm that CMTMros-based measurements were indeed due to a decrease in $\Delta\Psi_m$, L929sAhFas cells were pretreated with 100 μM mClCCP, an uncoupling agent of mitochondrial oxidative phosphorylation. After 30 min incubation with mClCCP before staining with CMTMros, the cells shifted to a weaker fluorescence intensity (data not shown).

Control cells did not bind annexin V-FITC, while anti-Fas-treated cells displayed rapid staining, which was already detectable after 10 min and which was maximal 60 min after addition of anti-Fas (Fig. 1A). Although a small portion of the cells showed an early drop in $\Delta\Psi_m$ ($\Delta\Psi_m^{low}$/PS-negative cells, lower left quadrant), the majority of the cells first became annexin V-positive with a normal $\Delta\Psi_m$ ($\Delta\Psi_m^{high}$/PS-positive cells, upper right quadrant) before losing $\Delta\Psi_m$ ($\Delta\Psi_m^{low}$/PS-positive cells, lower right quadrant) (Fig. 1A). To avoid misinterpretation of annexin V or CMTMros staining, exposure of PS and loss of $\Delta\Psi_m$ were measured on the cell population still sharing the scatter characteristics of viable cells. Indeed, all cells with the scatter profile of shrunken dead cells showed enhanced annexin V and diminished CMTMros staining, as a consequence of permeabilization of their cellular and mitochondrial membranes, respectively (data not shown).

We also investigated the time kinetics of other apoptotic parameters, viz. loss of cell membrane integrity (measured with PI) and appearance of a hypoploid DNA pattern (measured with PI on permeabilized cells) compared to surface exposure of PS and decrease in $\Delta\Psi_m$. The latter phenomena occurred clearly before the cells lost their plasma membrane integrity and started to fragment their nuclear DNA, eventually leading to a hypoploid DNA content (Fig. 1B).

Finally, we compared the surface exposure of PS and release of cytochrome *c* from the mitochondria after anti-Fas stimulation. Release of cytochrome *c* in the cytosol was determined by digitonin treatment, which under proper conditions only affects the outer cell membranes and leaves the membranes of organelles intact. As shown in Fig. 1C, exposure of PS clearly precedes the release of cytochrome *c* after

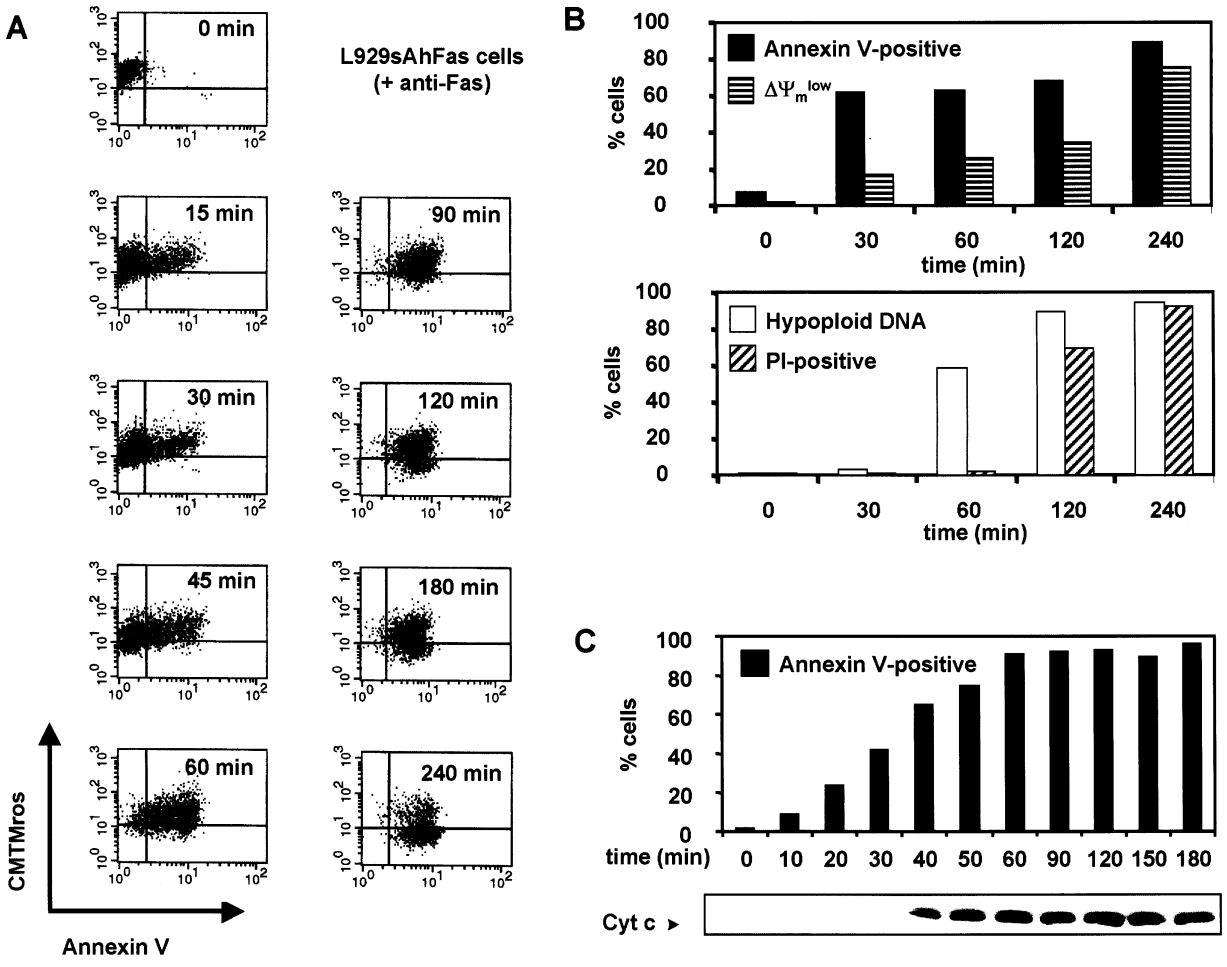



Fig. 1. Time kinetics of anti-Fas-induced exposure of PS, decrease in $\Delta\Psi_m$, cytochrome *c* release, loss of membrane integrity and hypoploid DNA in apoptotically dying L929sAhFas cells. Cells were incubated at $2\times10^5$ cells/ml with hFas (500 ng/ml). (A) Dot plots simultaneously stained with annexin V and CMTMros at different time intervals. (B) Time kinetic analysis of exposure of PS (percentage of annexin V-positive cells; black bars) and decrease in $\Delta\Psi_m$ (percentage of cells with decreased CMTMros staining; horizontally hatched bars), compared to the percentage of hypoploid cells (white bars) and loss of membrane integrity (percentage of PI-positive cells; diagonally hatched bars). (C) Time course of exposure of PS compared to release of cytochrome *c* from the mitochondria. Results are representative of three independent experiments.

anti-Fas treatment. Apparently, releasable cytochrome *c* does not gradually accumulate in the cytosol, but is suddenly, and almost maximally, released during a short period of time, namely 40–50 min after addition of anti-Fas. Although the time of maximal cytochrome *c* release may vary from experiment to experiment, rapid accumulation of releasable cytochrome *c* in the cytosol of anti-Fas-stimulated cells is a recurrent observation.

### 3.2. Comparison of surface PS exposure and decrease in $\Delta\Psi_m$ during apoptosis in PC60 RI/RII cells

We investigated whether the sequence of apoptotic events described above was also valid for other cells and stimuli, where the apoptotic process is usually less rapid as compared to anti-Fas-treated L929sAhFas cells, which die within 3 h [34]. PC60 RI/RII cells die apoptotically when stimulated with TNF [28]. A time kinetic was performed on TNF-treated PC60 RI/RII cells, which were labeled simultaneously with annexin V and CMTMros. Also here, analysis was performed only on the cell population with scatter characteristics of viable cells. The viable $\Delta\Psi_m^{high}$/PS-negative cells moved to the final apoptotic $\Delta\Psi_m^{low}$/PS-positive stage through the intermediate $\Delta\Psi_m^{high}$/PS-positive stage, since no PS-negative cells were detectable with lowered $\Delta\Psi_m$ (Fig. 2A). The $\Delta\Psi_m^{high}$/PS-positive cells represented only a minor population (less than 15%) of the total cell culture. Most probably, these cells rapidly proceeded to a stage with a completely altered scatter profile once $\Delta\Psi_m$ had dropped. In a next set of experiments, we examined whether this sequence of apoptotic events also occurred after induction of apoptosis with STS or etoposide. In agreement with the results obtained after TNF treatment, we observed that PC60 RI/RII cells first externalized PS and then shifted to the final PS-positive/$\Delta\Psi_m^{low}$ stage (Fig. 2B,C). This indicates that also under these conditions, exposure of PS occurred before loss of $\Delta\Psi_m$.

### 3.3. Comparison of surface PS exposure and decrease in $\Delta\Psi_m$ during apoptosis in growth factor-depleted 32D cells

Finally, we investigated the time kinetics of PS exposure and decrease in $\Delta\Psi_m$ during apoptosis induced by growth factor withdrawal of 32D cells. In contrast to the other apoptotic systems studied, no cells accumulated in the $\Delta\Psi_m^{high}$/PS-

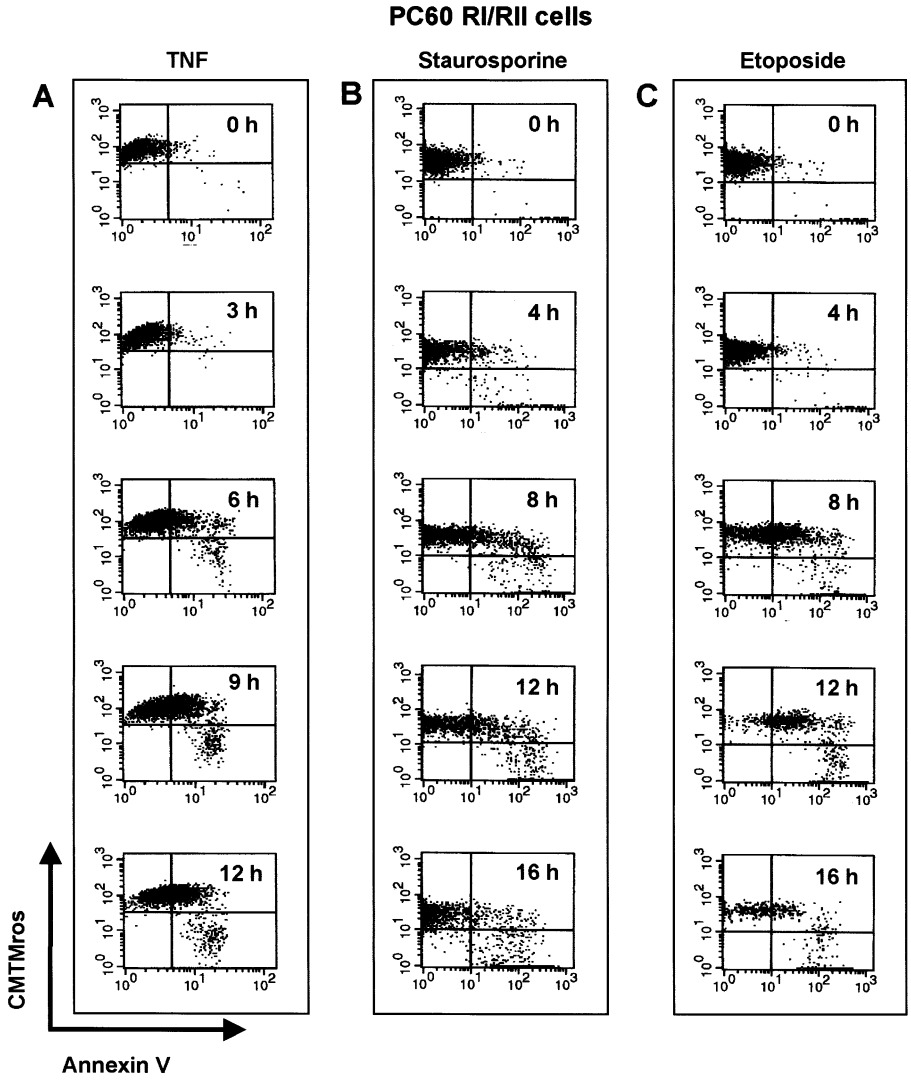



Fig. 2. Time kinetic analysis of TNF-induced exposure of PS compared to decrease in $\Delta\Psi_m$ in apoptotically dying PC60 RI/RII cells. Cells were cultured in the presence of 100 ng/ml TNF (A), 50 μg/ml etoposide (B) or 2 μM STS (C). Dot plots are shown from cells that were stained simultaneously with annexin V and CMTMros at different time intervals. Results are representative of three independent experiments.

positive transition stage. Neither were cells found in the other transition stage ($\Delta\Psi_m^{low}$/PS-negative) (Fig. 3). Hence, cells dying from growth factor withdrawal become immediately and simultaneously $\Delta\Psi_m^{low}$/PS-positive. These observations might be explained either by simultaneous occurrence of both apoptotic phenomena or, more likely, by a process whereby the cells remain in the $\Delta\Psi_m^{high}$/PS-positive transition stage for a short time.

## 4. Discussion

Anti-Fas and TNF initiate cell death through the *extrinsic* apoptotic pathway, using death domain-containing receptors, adaptors and recruitment of the initiator procaspase-8 [35]. Recently, two different pathways of anti-Fas-mediated apoptosis have been described [36]. In type-I cells, a large amount of active caspase-8 is generated at the death-inducing signaling complex (DISC), which directly leads to activation of downstream caspases. In type-II cells, only a small amount of DISC is formed, resulting in minor caspase-8 activation. In this type of cells, propagation and amplification of the apoptotic signal by mitochondrial factors is required. The apoptogenic process of the mitochondria in death domain-initiated signaling is probably induced by caspase-8-mediated cleavage of Bid, a proapoptotic member of the Bcl-2 family [5]. The C-terminal part of Bid translocates to the mitochondria, where it induces release of cytochrome *c*, a cofactor in Apaf-1-induced activation of procaspase-9 [5,37]. STS, etoposide and growth factor depletion initiate cell death through the intrinsic apoptotic pathway, initiated by formation of the apoptosome complex, integrating Apaf-1, cytochrome *c*, ATP and the initiator procaspase-9. Both cell death pathways converge on the proteolytic activation of the short prodomain effector caspases, such as caspase-3, -6 and -7 [38].

We compared the time kinetics of surface PS exposure to a decrease in $\Delta\Psi_m$ in different cell lines, namely L929sAhFas, PC60 RI/RII and 32D. In this way, we were able to compare both extrinsically induced apoptosis (Fas, TNF-RI and TNF-RII) and intrinsically induced apoptosis (STS, etoposide and growth factor depletion). We demonstrated that during anti-Fas-induced apoptosis in L929sAhFas cells, PS exposure occurs before decrease in $\Delta\Psi_m$ and release of cytochrome *c* from

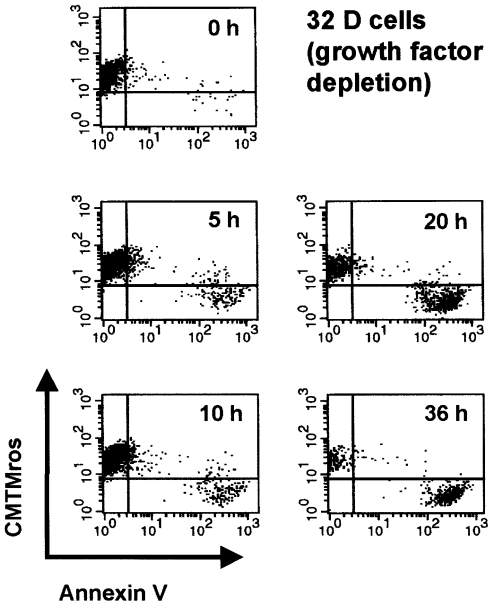



Fig. 3. Time kinetic analysis of growth factor depletion-induced exposure of PS compared to decrease in $\Delta\Psi_m$ in 32D cells. Cells were cultured in the presence or absence of WEHI-3B supernatant as a source of IL-3. Plots are shown from cells that were stained simultaneously with annexin V and CMTMros at different time intervals after growth factor withdrawal. Results are representative of three independent experiments.

the mitochondria. Both phenomena occur before cells exhibit late apoptotic features, such as DNA hypoploidy and loss of cell membrane integrity. Also in PC60 RI/RII cells exposed to TNF, STS or etoposide, exposure of PS occurred before a decrease in $\Delta\Psi_m$. However, under these conditions, there was only a small accumulation of PS-positive/$\Delta\Psi_m^{high}$ cells. Most probably, cells proceeded rapidly to the final apoptotic stage, once they lost their $\Delta\Psi_m$. It should be noted that the time lag between induction of surface PS exposure and decrease in $\Delta\Psi_m$ is similar (1 h) in anti-Fas-treated L929sAhFas and TNF-stimulated PC60 RI/RII cells, although maximal induction of apoptosis in the latter cells takes much longer (16 h vs. 3 h). This suggests that for two cell types with different apoptotic kinetics, the time required by the processes between surface PS exposure and decrease in $\Delta\Psi_m$ is similar. In growth factor-depleted 32D cells, there was almost no accumulation in the PS-positive/$\Delta\Psi_m^{high}$ stage. This observation is in agreement with previous reports on the simultaneous occurrence of both apoptotic events during ara-C-induced apoptosis in leukemic blast cells [23]. Also during apoptosis induced by anti-Fas in Jurkat T cells, only few cells were detected in the transition stages between viable $\Delta\Psi_m^{high}$/PS-negative cells and final apoptotic $\Delta\Psi_m^{low}$/PS-positive cells ([24] and our own unpublished results).

The mechanism by which PS is exposed on the outer cell membrane leaflet is still under investigation. Two enzymes have been proposed to play an important role in this process. The PL scramblase is a plasma membrane protein which facilitates the rapid mobilization of PS to the cell surface upon elevation of internal $Ca^{2+}$ concentration [14]. The extent to which PS migrates to the plasma membrane outer leaflet is correlated with the expression level of scramblase [39]. The ATP-dependent aminophospholipid translocase belongs to the subfamily of the P-type ATPases and mediates the inward transport of PS. Down-modulation of this enzyme during apoptosis has been implicated in exposure of PS at the outer cell membrane [15]. Both mitochondria and caspases have been suggested to play a role in the signaling pathway to PS exposure. First, it has been shown that exposure of PS on the cell surface is blocked by caspase inhibitors [40]. Also in PC60 RI/RII cells, TNF-induced exposure of PS and decrease in $\Delta\Psi_m$ are inhibited by zVAD-fmk, a broad-spectrum caspase inhibitor, or by CrmA expression in these cells (W.D. and G.D., unpublished results). As CrmA is an inhibitor of caspase-8 [41], these observations favor a mechanism whereby at least one initial step after receptor clustering, viz. activation of procaspase-8, is shared by the signaling pathways leading to PS exposure and decrease in $\Delta\Psi_m$. We demonstrated that also during etoposide- and STS-induced apoptosis PS exposure preceded a decrease in $\Delta\Psi_m$, similar to the events following TNF-RI or Fas triggering. Recently, it was shown that administration of either etoposide or STS results in the activation of procaspase-8 [42,43]. Hence, caspase-8 activation may occur in drug-induced PS exposure. However, as both zVAD-fmk and CrmA prevent initiation of the apoptotic process, it is not clear whether caspases are directly implicated. Secondly, several reports described that exposure of PS occurs after mitochondrial damage: release of cytochrome *c* or decrease in $\Delta\Psi_m$ in different apoptotic systems takes place before exposure of PS [20–22]; both microinjection of AIF and cytochrome *c* in cells have been reported to induce PS exposure [19,44]. These results are not necessarily in contradiction with ours, as it might be that in these systems post-mitochondrial activation of effector caspases induces activation of caspase-8 at a later stage of the apoptotic process, which in turn can lead to PS exposure. Furthermore, it is not unlikely that both signaling pathways, leading to mitochondrial damage and exposure of PS, are independent events. Depending on the cellular system used, PS exposure can be observed before, simultaneously or after mitochondrial damage.

Our results indicate that exposure of PS at the cell surface is an early event, which might have important implications. First, as the externalization of PS is involved in recognition of apoptotic cells by phagocyting cells [10], phagocytosis might already be initiated before other apoptotic features become apparent. This rapid recognition by phagocytes or neighboring cells, followed by uptake and degradation of apoptotic cells, might protect the surrounding tissues from exposure to cells in late apoptotic stages and might also explain the apparent absence of full apoptotic cells in vivo [8]. It was also demonstrated that thymocytes, during glucocorticoid-induced apoptosis, die without DNA fragmentation and become TUNEL-positive only after being phagocytosed by macrophages [45].

Loss of asymmetric distribution of PS and other components of the membrane lipid bilayer might initiate another signal transduction at an early stage and might be part of the commitment of the cell to death. Since PS is an essential cofactor in cell membranes for activation of protein kinase C (PKC) [46], which is recruited to the membrane by interacting with diacylglycerol, it is possible that decrease in PS in the inner membrane is accompanied with loss of PKC activity. A role for PKC in protecting against cell death has been described in different apoptotic systems. This protection has been associated with the PKC-induced maintenance of Bcl-2 levels [47]. Recently, a PS-specific phospholipase $A_1$ (PS-

$PLA_1$) was cloned, which acts specifically on PS and 1-acyl-2-lysophosphatidylserine (lyso-PS) to hydrolyze fatty acids at the sn-1 position of these PLs [48]. It is likely that PS-$PLA_1$ is involved in regulating PS/lyso-PS-dependent reactions; however, the physiological role of PS-$PLA_1$ is still unknown.

Our results demonstrate that exposure of PS constitutes an early event in the apoptotic process, which does not require loss of $\Delta\Psi_m$ and release of cytochrome *c*. This suggests that phagocytes recognize apoptotic cells before mitochondrial dysfunction occurs.

*Acknowledgements:* Research was supported by the Interuniversitaire Attractiepolen, the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (Grant 9005097N) and the European Community Biomed Program (Grant BMH4-CT96-0300). G.D. was supported by the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen and Kom op tegen Kanker. G.V.L. and D.V. are a fellow and a postdoctoral researcher with the Vlaams Instituut voor de Bevordering van het Wetenschappelijk-technologisch Onderzoek in de Industrie, respectively. P.V. is a research associate with the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen. The authors thank A. Meeus and W. Burm for technical assistance in tissue culture.

## References

[1] Kroemer, G., Dallaporta, B. and Resche-Rigon, M. (1998) Annu. Rev. Physiol. 60, 619–642.
[2] Nicholson, D.W. and Thornberry, N.A. (1997) Trends Biochem. Sci. 22, 299–306.
[3] Yang, X., Chang, H.Y. and Baltimore, D. (1998) Mol. Cell 1, 319–325.
[4] Liu, X., Kim, C.N., Yang, J., Jemmerson, R. and Wang, X. (1996) Cell 86, 147–157.
[5] Li, H., Zhu, H., Xu, C.J. and Yuan, J. (1998) Cell 94, 491–501.
[6] Susin, S.A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M. and Kroemer, G. (1996) J. Exp. Med. 184, 1331–1341.
[7] Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T., Jones, D.J. and Wang, X. (1997) Science 275, 1129–1132.
[8] Savill, J. (1997) Br. Med. Bull. 53, 491–508.
[9] Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M. and Green, D.R. (1995) J. Exp. Med. 182, 1545–1556.
[10] Fadok, V.A., Bratton, D.L., Frasch, S.C., Warner, M.L. and Henson, P.M. (1998) Cell Death Differ. 5, 551–562.
[11] Bevers, E.M., Comfurius, P., van Rijn, J.L., Hemker, H.C. and Zwaal, R.F. (1982) Eur. J. Biochem. 122, 429–436.
[12] Devaux, P.F. (1988) FEBS Lett. 234, 8–12.
[13] Tang, X., Halleck, M.S., Schlegel, R.A. and Williamson, P. (1996) Science 272, 1495–1497.
[14] Zhou, Q., Zhao, J., Stout, J.G., Luhm, R.A., Wiedmer, T. and Sims, P.J. (1997) J. Biol. Chem. 272, 18240–18244.
[15] Verhoven, B., Schlegel, R.A. and Williamson, P. (1995) J. Exp. Med. 182, 1597–1601.
[16] Bratton, D.L., Fadok, V.A., Richter, D.A., Kailey, J.M., Guthrie, L.A. and Henson, P.M. (1997) J. Biol. Chem. 272, 26159–26165.
[17] Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., Prévost, M.-C., Alzari, P.M. and Kroemer, G. (1999) J. Exp. Med. 189, 381–393.
[18] Mancini, M., Nicholson, D.W., Roy, S., Thornberry, N.A., Peterson, E.P., Casciola-Rosen, L.A. and Rosen, A. (1998) J. Cell Biol. 140, 1485–1495.
[19] Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M. and Kroemer, G. (1999) Nature 397, 441–446.
[20] Bossy-Wetzel, E., Newmeyer, D.D. and Green, D.R. (1998) EMBO J. 17, 37–49.
[21] Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D., Macho, A., Hirsch, T., Susin, S.A., Petit, P.X., Mignotte, B. and Kroemer, G. (1995) J. Exp. Med. 182, 367–377.
[22] Castedo, M., Hirsch, T., Susin, S.A., Zamzami, N., Marchetti, P., Macho, A. and Kroemer, G. (1996) J. Immunol. 157, 512–521.
[23] Backway, K.L., McCulloch, E.A., Chow, S. and Hedley, D.W. (1997) Cancer Res. 57, 2446–2451.
[24] Hirata, H., Takahashi, A., Kobayashi, S., Yonehara, S., Sawai, H., Okazaki, T., Yamamoto, K. and Sasada, M. (1998) J. Exp. Med. 187, 587–600.
[25] Zhuang, J., Ren, Y., Snowden, R.T., Zhu, H., Gogvadze, V., Savill, J.S. and Cohen, G.M. (1998) J. Biol. Chem. 273, 15628–15632.
[26] Vercammen, D., Brouckaert, G., Denecker, G., Van de Craen, M., Declercq, W., Fiers, W. and Vandenabeele, P. (1998) J. Exp. Med. 188, 919–930.
[27] Conzelmann, A., Corthésy, P., Cianfriglia, M., Silva, A. and Nabholz, M. (1982) Nature 298, 170–172.
[28] Vandenabeele, P., Declercq, W., Vanhaesebroeck, B., Grooten, J. and Fiers, W. (1995) J. Immunol. 154, 2904–2913.
[29] Espevik, T. and Nissen-Meyer, J. (1986) J. Immunol. Methods 95, 99–105.
[30] Macho, A., Decaudin, D., Castedo, M., Hirsch, T., Susin, S.A., Zamzami, N. and Kroemer, G. (1996) Cytometry 25, 333–340.
[31] Koopman, G., Reutelingsperger, C.P., Kuijten, G.A., Keehnen, R.M., Pals, S.T. and van Oers, M.H. (1994) Blood 84, 1415–1420.
[32] Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F. and Riccardi, C. (1991) J. Immunol. Methods 139, 271–280.
[33] Kroemer, G. (1998) Cell Death Differ. 5, 547.
[34] Vercammen, D., Vandenabeele, P., Beyaert, R., Declercq, W. and Fiers, W. (1997) Cytokine 9, 801–808.
[35] Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J.D., Zhang, M., Gentz, R., Mann, M., Krammer, P.H., Peter, M.E. and Dixit, V.M. (1996) Cell 85, 817–827.
[36] Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.-J., Krammer, P.H. and Peter, M.E. (1998) EMBO J. 17, 1675–1687.
[37] Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cell 91, 479–489.
[38] Sun, X.M., MacFarlane, M., Zhuang, J., Wolf, B.B., Green, D.R. and Cohen, G.M. (1999) J. Biol. Chem. 274, 5053–5060.
[39] Zhao, J., Zhou, Q., Wiedmer, T. and Sims, P.J. (1998) J. Biol. Chem. 273, 6603–6606.
[40] Vanags, D.M., Pörn-Ares, M.I., Coppola, S., Burgess, D.H. and Orrenius, S. (1996) J. Biol. Chem. 271, 31075–31085.
[41] Van de Craen, M., Van Loo, G., Declercq, W., Schotte, P., Van den brande, I., Mandruzzato, S., van der Bruggen, P., Fiers, W. and Vandenabeele, P. (1998) J. Mol. Biol. 284, 1017–1026.
[42] Boesen-de Cock, J.G., Tepper, A.D., de Vries, E., van Blitterswijk, W.J. and Borst, J. (1998) J. Biol. Chem. 273, 14255–14261.
[43] Johnson, V.L., Cooper, I.R., Jenkins, J.R. and Chow, S.C. (1999) Exp. Cell Res. 251, 175–184.
[44] Brustugun, O.T., Fladmark, K.E., Døskeland, S.O., Orrenius, S. and Zhivotovsky, B. (1998) Cell Death Differ. 5, 660–668.
[45] Nakamura, M., Yagi, H., Ishii, T., Kayaba, S., Soga, H., Gotoh, T., Ohtsu, S., Ogata, M. and Itoh, T. (1997) Eur. J. Immunol. 27, 999–1004.
[46] Nishizuka, Y. (1992) Science 258, 607–614.
[47] Itano, Y., Ito, A., Uehara, T. and Nomura, Y. (1996) J. Neurochem. 67, 131–137.
[48] Nagai, Y., Aoki, J., Sato, T., Amano, K., Matsuda, Y., Arai, H. and Inoue, K. (1999) J. Biol. Chem. 274, 11053–11059.