Histochem Cell Biol (1998) 110:553–558 © Springer-Verlag 1998

ORIGINAL PAPER

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Plasma membrane phospholipid asymmetry precedes DNA fragmentation in different apoptotic cell models

Accepted: 29 June 1998

Abstract Biochemical alterations occurring in many cell types during apoptosis include the loss of plasma membrane phospholipid asymmetry and nuclear DNA fragmentation. Annexin V staining detects phosphatidylserine translocation into the outer plasma membrane layer occurring during cell death, while the in situ tailing (IST or TUNEL) reaction labels the DNA strand breaks typical of apoptosis. To compare the time course of these processes we investigated methylprednisolone-induced apoptosis of rat thymocytes, topoisomerase inhibitor-induced apoptosis in the human histiocytic lymphoma cell line U937, and serum deprivation-induced apoptosis in the rat pheochromocytoma cell line, PC12. At all time points, FACS analysis and quantitative fluorescence light microscopy showed a higher proportion of annexin V-positive than IST-positive cells, with significantly different time courses in the apoptotic cell models investigated (Anova test). Results were confirmed by confocal microscopy. Our data indicate that the exposure of phosphatidylserine, a potential phagocyte recognition signal on the cell surface of apoptotic cells in vivo, precedes DNA strand breaks during apoptosis in different cell types.

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Introduction

The term apoptosis describes a special form of cell death, based on morphological criteria which are clearly distinct from those for necrosis (Kerr et al. 1972). Several biochemical features have been identified which are associated with apoptotic cell death. Among these, the internucleosomal cleavage of genomic DNA into fragments of multiples of about 180–200 bp is now widely used as a marker for apoptosis in many cell systems (Cohen et al. 1992; Wyllie 1980). Enzymatic terminal deoxynucleotidyl transferase (TdT) DNA-labeling techniques [in situ tailing reaction (IST) or TdT-mediated dUTP nick end labeling (TUNEL)] with incorporation of labeled nucleotides into fragmented DNA thus allow the investigation of apoptosis at the single-cell level (Gavrieli et al. 1992; Gold et al. 1994). Other biochemical events during the process of apoptosis include changes of the cell surface, which may also serve as recognition signals for phagocytes (Savill 1997; Savill et al. 1993). Of these, the loss of plasma membrane phospholipid asymmetry with exposure of phosphatidylserine (PS) at the outer leaflet of the bilayer is an early event during the course of apoptosis, which can be specifically detected using the Ca^{2+}-dependent phospholipid-binding protein, annexin V (Fadok et al. 1992a; Verhoven et al. 1995; Vermes et al. 1995). This plasma membrane change has been demonstrated in a variety of cell types in response to different apoptosis-inducing stimuli (van Engeland et al. 1996; Koopman et al. 1994; Martin et al. 1995; O'Brien et al. 1997). Importantly, PS exposure occurs in enucleated cells as well, indicating its independence from nuclear processes (Castedo et al. 1996). The aim of this study was to compare the time course and the quantitative correlation of the loss of plasma membrane phospholipid asymmetry with nuclear DNA fragmentation in different models of apoptotic cell death.

Materials and methods

Cell culture experiments

Freshly prepared thymocytes of Lewis rats (Charles River, Sulzfeld, Germany) were used for in vitro studies of cortisone-induced apoptosis. Cell suspensions were cultured at a density of 10^7 cells/ml in RPMI 1640 medium supplemented with 2.5% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Apoptosis was induced by 0.1 µg/ml methylprednisolone (Hoechst, Frankfurt, Germany). Topoisomerase inhibitor-induced apoptosis (4 µg/ml camptothecin; Sigma, Deisenhofen, Germany) was investigated in the human histiocytic lymphoma cell line, U937 (ATCC, Rockwell, USA). Cells were cultured at a density of 5×10^5 cells/ml in RPMI 1640 medium (10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin). The rat pheochromocytoma cell line, PC12, was cultured in DMEM medium supplemented with 4.5 g/l glucose, 1 mM glutamine, 10 U/ml penicillin, 10 µg/ml streptomyin, 10% horse serum, and 5% FCS. Following trypsination (0.05% trypsin, 0.02% EDTA) cells were seeded on 96-well microtiter plates at densities of 5000 and 50000 cells/well in DMEM medium in the absence of serum. When indicated, recombinant human nerve growth factor (NGF; Roche Diagnostics Boehringer Mannheim, Mannheim, Germany) was added at a concentration of 100 ng/ml.

Staining procedures

For double labeling procedures, 5×10^5 cells were suspended in 100 µl binding buffer (10 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) and incubated for 15 min on ice with 2.5 µg/ml annexin V–biotin (Roche Diagnostics Boehringer Mannheim). Cells were washed once with binding buffer and fixed for 18 h at 4° C at a density of 2×10^6 cells/ml in 5% formalin/PBS (Merck, Darmstadt, Germany). After washing twice with binding buffer, cells were incubated on ice with 2.5 µl streptavidin–tricolor (0.2 mg/ml; Caltag, Burlingame, USA; 10 min, light protected). After a washing step, cells were end-labeled using the in situ cell death detection kit according to the manufacturer's instructions (Roche Diagnostics Boehringer Mannheim) and our previous studies (Gold et al. 1994). In brief, 5 µl enzyme solution containing TdT was added to 45 µl label solution containing fluorescein-labeled nucleotides. The samples were light protected and placed in a rotating shaker (400 rpm) at 37° C for 60 min. Single labeling experiments with annexin V–fluorescein (Roche Diagnostics Boehringer Mannheim) and the IST reaction were performed following the manufacturer's instructions. Propidium iodide (PI; Sigma) staining was performed at a final concentration of 0.1 µg/ml.

Flow cytometry

Labeled cells were suspended in 300 µl binding buffer after pilot experiments did not show any differences from analysis carried out in PBS. Analyses were performed on a FACScan using Cell Quest software (Becton Dickinson, San Jose, USA). At each timepoint, 5000 cells were analyzed. Cell debris was excluded by setting appropriate light scatter gates. Quantitative analysis was done by setting region markers for positive cells in the appropriate channels.

Confocal scanning laser microscopy

Preparations of cells concentrated by cytocentrifugation were visualized by a Leica True confocal scanner (Leica, Heidelberg, Germany) as simultaneous scans (488 nm and 568 nm) on the excitation side with a double detection beam splitter and on the detector side with a RSP580 beam splitter and BP-FITC and LSP590 barrier filters. Images were photographed by an Imagecorder Plus (version 2.03, Focus Graphics, Belmont, USA).

Microscopic analyis of PC12 cells

For fluorescence light microscopy analysis on a Leica DM IL microscope (Leica, Heidelberg, Germany), frames were recorded by a CCD-RGB video camera system (model DEI-470; Optronics Engineering, Stemmer, Puchheim, Germany) and fed into a personal computer via a frame grabber card. Recordings and image analyses were carried out using the Optimas software, release 6.0 (Optimas, Borthell, USA). Blanks with fluorescent dyes in the absence of cells were recorded for each set of incubates and used for background correction. For cellular analysis, a frame from the center of a well was selected which contained, on average, 254±10 cells (mean±SD). Analyses were performed in quadruplicate.

Statistical analysis

Statistical analysis of data obtained by flow cytometry and fluorescence microscopy was performed using the two-way ANOVA test (Graph Pad Software, San Diego, USA) after square root transformation of the data, since this transformation showed the best approximation to a Gaussian distribution.

Results

Fixation procedures for double labeling

Different fixatives were compared (paraformaldehyde, formaldehyde, ethanol, methanol, ethanol/acidic acid, acetone) in combination with various detergents [Nonidet P40 (NP-40), saponin] in order to optimize fixation procedures that allow simultaneous detection of annexin V binding and DNA fragmentation (IST). The strongest annexin V+/IST+ double staining signal on apoptotic thymocytes could be achieved using 5% formaldehyde fixation over 18 h at 4° C. Additional treatment with a non-ionic detergent (0.025% NP-40) at different time points (5, 8 h) resulted in a stronger mean fluorescence intensity of the IST signal, but no difference in the absolute number of IST+ cells could be observed (data not shown). Moreover, the non-ionic detergents completely destroyed the annexin V binding signal.

Glucocorticoid-induced thymocyte apoptosis

Figure 1a depicts a flow cytometric histogram of double-labeled thymocytes early in the course of apoptosis (3 h of methylprednisolone treatment). In comparison to the corresponding negative controls, a clear annexin V staining signal was observed at this time point, whereas no IST signal could be detected. A later stage of apoptosis is shown in a representative dot-plot of double-stained apoptotic thymocytes in Fig. 1b. After 5 h of methylprednisolone treatment, 21.1% of the cells show both annexin V and IST positivity. A significant amount (19.5%) of the cells are annexin V positive without a tailing signal. Only very few IST+/annexin– cells can be observed at this time point. The results of time kinetic studies of double-stained apoptotic thymocytes are shown in Fig. 2. At all time points the number of Annexin V+ cells was higher than that of IST+ cells, with significantly different time

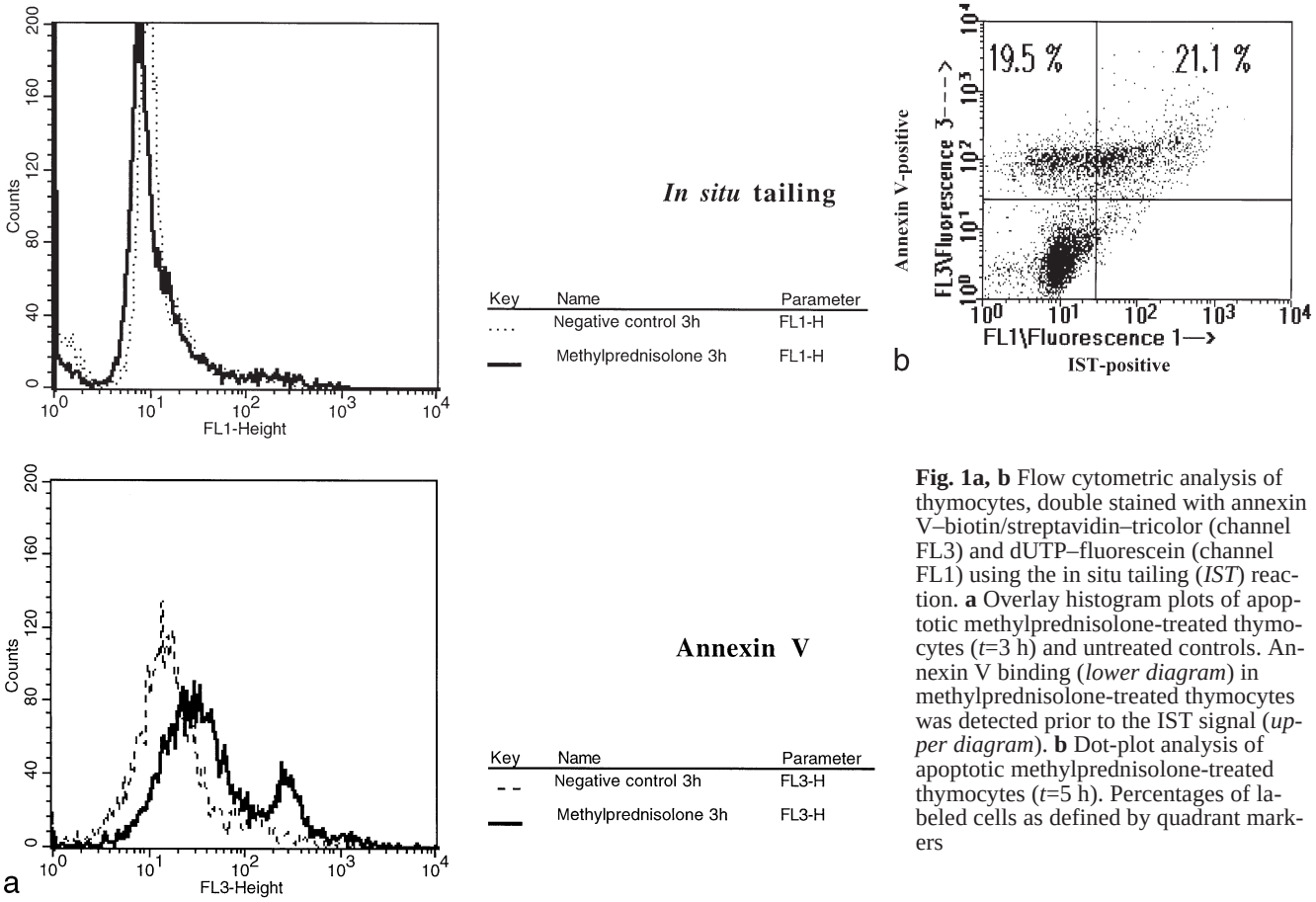

Fig. 1a, b Flow cytometric analysis of thymocytes, double stained with annexin V–biotin/streptavidin–tricolor (channel FL3) and dUTP–fluorescein (channel FL1) using the in situ tailing (*IST*) reaction. **a** Overlay histogram plots of apoptotic methylprednisolone-treated thymocytes (*t*=3 h) and untreated controls. Annexin V binding (*lower diagram*) in methylprednisolone-treated thymocytes was detected prior to the IST signal (*upper diagram*). **b** Dot-plot analysis of apoptotic methylprednisolone-treated thymocytes (*t*=5 h). Percentages of labeled cells as defined by quadrant markers

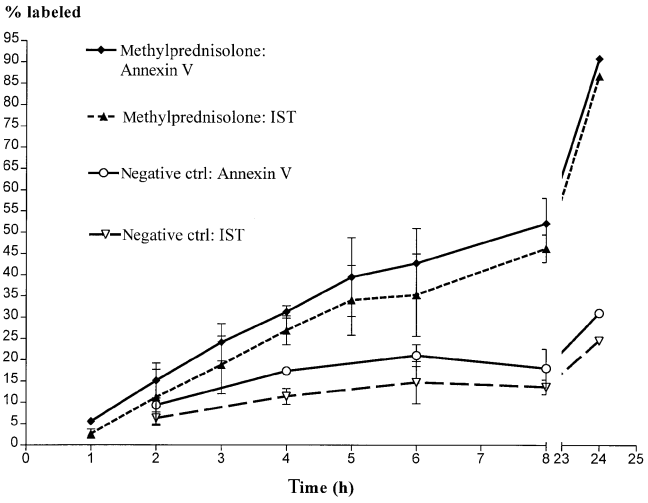

Fig. 2 Time kinetics of annexin V/IST double-labeled thymocytes after induction of apoptosis with methylprednisolone or control (*ctrl*) thymocytes, which spontaneously undergo apoptosis in vitro. Time courses of apoptotic annexin V+ and IST+ cells (mean±SD of two independent experiments) are significantly different ($P<0.05$, Anova test). Note that quantitative analysis of labeled cells was performed by setting region markers for positive cells in the appropriate channels

courses of annexin+ and IST+ cells ($P<0.05$). Similar results could be obtained comparing the time courses of single-stained cells (data not shown). In order to assess the proportion of necrotic or late apoptotic cells with loss of plasma membrane integrity, time kinetic studies using annexin V–FITC/PI double staining were performed (data not shown). PI-positive (necrotic or late apoptotic) cells could be observed after 3 h of cortisone treatment (1.8%), rising to 8.8% after 6 h.

Confocal scanning laser microscopy

Since the fixation procedures used for the double labeling experiments had not been established before, confirmation of the data obtained by FACS analysis was sought by confocal microscopy. Apoptotic thymocytes (culture with methylprednisolone for 5 h) were used for confocal microscopy after flow cytometry. Annexin V–biotin/streptavidin–tricolor labeling of apoptotic thymocytes yielded an inhomogeneous distributed plasma membrane signal (Fig. 3A). IST–FITC single-labeled apoptotic thymocytes showed a strong, crescent-shaped nuclear signal, morphologically characteristic of apoptosis (Fig. 3B). Double-stained annexin V+/IST+ thymocytes are depicted in Fig. 3C, with a proof of correct localization of the staining signal and an exclusion of non-

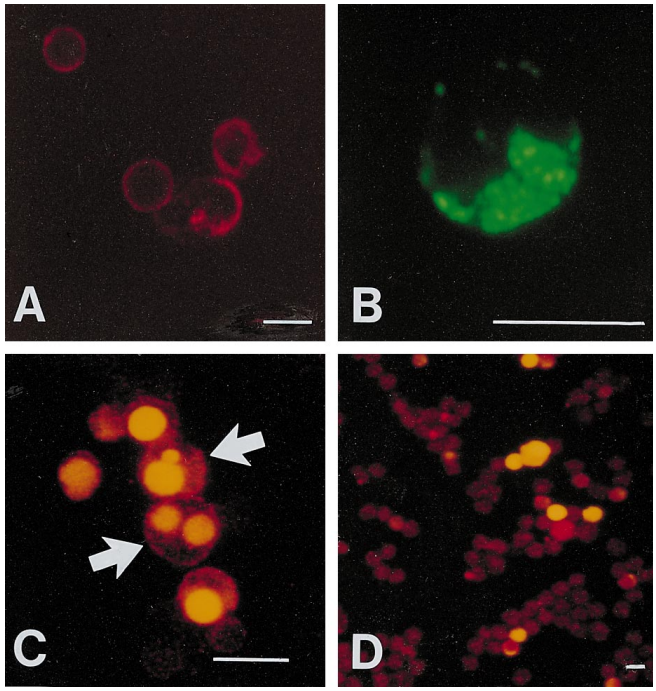

Fig. 3A–D Confocal microscopy images of apoptotic thymocytes (incubated with methylprednisolone for 5 h). *Scale bars* represent 10 µm. **A** Annexin V/streptavidin–tricolor plasma membrane staining of externalized phosphatidylserine. **B** dUTP–fluorescein-labeled DNA strand breaks using IST. **C** Annexin V/IST double-labeled apoptotic thymocytes. Note that both fluorescein and tricolor were simultaneously excited. Due to limitations of the available filter combinations, the fluorescein emission at high wavelengths could not be blocked completely and thus results in an orange color. *Arrows* indicate cells with apoptotic bodies. **D** Annexin V/IST double-labeled apoptotic thymocytes. Note the higher prevalence of cells which are positive for annexin only. Limitations of filter combinations are the same as in **C**

specific staining signals. Figure 3D shows a higher prevalence of annexin V single-labeled cells after 5 h of methylprednisolone treatment.

Topoisomerase inhibitor-induced apoptosis of the U937 cell line

As another model for apoptotic cell death, we investigated camptothecin-induced apoptosis in the human histiocytic lymphoma cell line, U937. Time kinetic studies with single-stained (annexin V, IST) U937 cells are shown in Fig. 4. As in apoptotic thymocytes, the annexin V signal exceeded the IST positivity ($P<0.01$) in topoisomerase inhibitor-induced apoptosis of U937 cells. Of note, untreated U937 negative controls showed high standard deviations in their annexin V signals (data not shown).

Serum deprivation-induced apoptosis of the PC12 cell line

As a non-lymphoid cell model, we extended our studies to the rat pheochromocytoma cell line, PC12, which

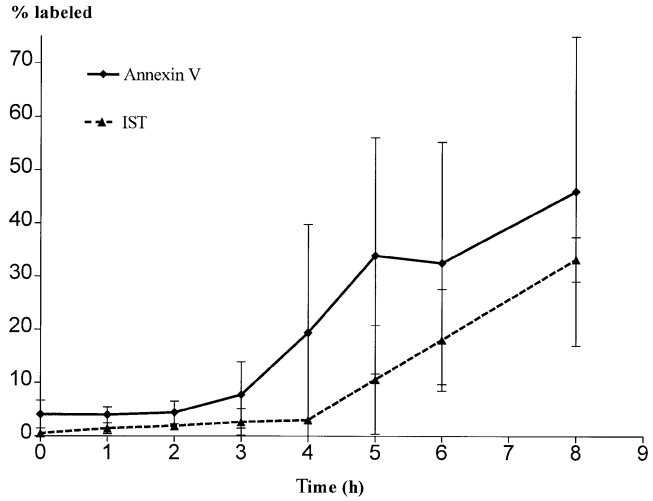

Fig. 4 Time kinetics of annexin V or IST single-labeled monocytic U937 cells after induction of apoptosis with camptothecin (4 µg/ml). Time courses of apoptotic annexin V+ and IST+ cells (mean±SD of two independent experiments) are significantly different ($P<0.01$, Anova test)

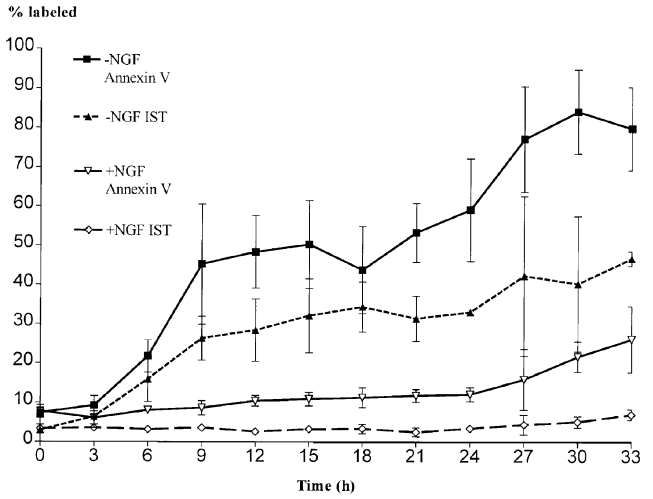

Fig. 5 Time kinetics of annexin V or IST single-labeled rat pheochromocytoma PC12 cells. Apoptosis was induced in serum-free culture medium and controls were treated with nerve growth factor (NGF). Time courses of apoptotic annexin V+ and IST+ cells (mean±SD, $n=4$) are significantly different ($P<0.0001$, Anova test)

shares close resemblance to the phenotype of sympathetic neurons when terminally differentiated by NGF. After serum deprivation, PC12 cells undergo internucleosomal DNA fragmentation, which can be prevented by NGF (Batistatou and Greene 1993). Time kinetic studies with single-stained, serum-deprived PC12 cells analyzed by fluorescence microscopy are shown in Fig. 5. In the absence of NGF, the number of apoptotic PC12 cells, labeled by annexin V–fluorescein or IST, increased as early as 3 h of cell culture compared to the NGF-protected cells. At all time points annexin V-positive cells exceeded cells with an IST signal, with significantly different time courses ($P<0.00001$). A consistent rise in the number of late apoptotic/necrotic cells with loss of plasma

membrane integrity, as detected by PI labeling, was only observed at ≥21 h of serum-deprived cell culture when compared to NGF-treated cells, with 37.3±4.8% PI-positive cells at 33 h (data not shown). Over 24 h of culture, the basal levels of annexin V and IST labeling in the NGF-treated control populations remained constant, with a slow rise only observed after prolonged incubation (Fig. 5).

Discussion

The loss of plasma membrane asymmetry and the occurrence of DNA fragmentation are early processes which precede the breakdown of plasma membrane integrity and the loss of cell viability during apoptosis (Koopman et al. 1994; Martin et al. 1995; Wyllie 1980). Since these two prominent biochemical events form the basis of different assays widely used for the quantitative analysis of apoptotic cells, the aim of this study was to investigate their temporal relationship and correlation on the single cell level. Our data indicate that during apoptosis induced by various stimuli in different lymphoid and neuronal cells, PS exposure on the outer membrane leaflet, as detected by annexin V, precedes the in situ tailing (IST or TUNEL) signal which reflects DNA fragmentation.

The optimization of the fixation/permeabilization procedures that allow simultaneous detection of annexin V binding and DNA fragmentation on apoptotic methylprednisolone-treated thymocytes proved to be essential. In fact, standard fixation procedures (4% paraformaldehyde/0.025% NP-40; Gold et al. 1994) resulted in abolition of the annexin V-binding signal. On peripheral blood mononuclear cells (PBMC) these fixation conditions resulted in the loss of the FACS-staining signal for different cellular antigens, e.g., FAS (CD95), CD1a, CD2, CD7, CD23, CD25, CD29, CD38 (R. Gold, S. Jung, G. Rothe, personal communication). Similar observations were reported with PBMC from HIV-infected individuals, where a permeabilization step with 70% ethanol for the in situ nick translation assay resulted in a drastic cell membrane alteration/destruction and a subsequent reduction of cell surface staining, particularly of CD19 (Lecoeur et al. 1997). Optimization of annexin V/IST double-staining procedures in our study was achieved by labeling apoptotic thymocytes with biotinylated annexin V prior to fixation with 5% formalin over 18 h at 4° C. In all cases the use of non-ionic detergents completely abrogated annexin V binding and was not necessary to obtain a sufficient IST signal in apoptotic thymocytes. In addition, the confirmation of an earlier increase of annexin V binding in the single-labeling studies under optimized conditions (Gold et al. 1994; Koopman et al. 1994; Vermes et al. 1995) indicates that the conditions used for the double staining are sufficient to detect differences between these two assays. In U937 cells we occasionally observed a weaker IST signal which could be improved by adding non-ionic detergent in the single-labeling studies. Because of the annexin V incubation prior to fixation, it seems unlikely that annexin V positivity in the early stages of apoptosis is due to PS binding inside the cells. In accord with this, annexin V binding in single-labeling experiments without prior fixation was comparable to the results of the double-staining experiments. Conversely, the results of annexin V/IST double-labeling experiments indicate that the higher percentage of annexin V-positive cells is not merely due to loss of cells, e.g., during the process of fixation. Staining with the cell-impermeable DNA dye, PI in apoptotic thymocytes as well as in PC12 cells proved that late apoptotic or necrotic cells with a loss of plasma membrane integrity did not contribute significantly to the amount of annexin V-positive cells in the early stages of apoptosis.

As demonstrated in the human histiocytic lymphoma U937 cell line, early PS exposure on the cell surface prior to DNA fragmentation could also be detected in topoisomerase inhibitor-induced apoptotic cell death. We extended our studies to another apoptotic cell model, i.e., serum-free cell cultures of the rat pheochromocytoma PC12 cell line. This cell line serves as a model system in studies of neuronal cell death after deprivation of neurotrophic factors (Batistatou and Greene 1993). Following serum deprivation, PC12 cells undergo apoptosis, which can be prevented by the addition of NGF. The detection of an earlier increase of annexin V binding on a non-lymphoid, adherent cell line by an independent quantitative method confirms that this phenomenon is of general biological relevance. Using camptothecin-treated plant cells as well as the the human promyelocytic leukemia cell line HL-60, O'Brien et al. (1997) could demonstrate annexin V binding prior to a TUNEL signal. These data, however, were not extended to other modes of apoptosis induction and no double-labeling experiments were performed. In a comparative study of different cytofluorometric methods in the quantitative detection of apoptosis on PBMC from HIV-infected individuals, no difference in the apoptosis rate could be found using characteristics of modified morphology (forward scatter/sidescatter), altered membrane permeability (7-aminoactinomycin D staining), DNA fragmentation (in situ nick translation) or PS exposure (annexin V) (Lecoeur et al. 1997). However, these PBMC were cultured over 24 h prior to analysis and no time kinetic studies were performed.

Although nuclear changes are prominent events during apoptosis, membrane and cytoplasmic alterations characteristic of apoptotic cell death have also been shown in enucleated cytoplasts (Schulze-Osthoff et al. 1994). Morever, since the externalization of PS has also been described in the absence of a cell nucleus and independently from DNA fragmentation (Castedo et al. 1996), our results underscore the value of annexin V binding for the detection of apoptosis. In addition, a recent report described different types of endonuclease activity in CLL lymphocytes as compared to HL-60 cells, leading to blunt or 3′-recessed DNA strand breaks with inefficient labeling by the IST/TUNEL assay using TdT (McKenna et al. 1998). Therefore, a combined approach using annexin V/IST single or double labeling could prove to be of advantage in these cell types. In addition,

morphological studies should always be included to confirm apoptotic cell death since both annexin V and IST have a certain potential to detect necrotic cells as well (Gold et al. 1994; Vermes et al. 1995). This is not surprising, in view of the recent findings that apoptosis and necrosis may be induced by the same stimulus, with the final mode of cell death depending on the intensity of the stimulus and the energy status of the cell (Bonfoco et al. 1995; Leist et al. 1997).

PS exposure on the outer cell surface is a widespread event during apoptosis in various cell types in vitro as well as in animal models in vivo (Martin et al. 1995; Van den Eijnde et al. 1997). Importantly, this membrane change is one of the major mechanisms by which some apoptotic cell types are recognized by phagocytes (Fadok 1992b; Savill 1997; Savill et al. 1993), although the potential PS receptors still remain to be characterized (Fukasawa et al. 1996; Platt et al. 1996; Sambrano and Steinberg 1995). Recognition and subsequent removal by phagocytes determines the fate of the apoptotic cell in vivo and, in the case of apoptotic inflammatory cells, this removal ultimately leads to the resolution of the inflammatory infiltrate (Gold et al. 1997; Savill 1997). Thus, the early externalization of PS prior to DNA fragmentation might help to terminate inflammation by apoptosis followed by phagocytosis of the dying yet intact cell.

Acknowledgements This work was supported by the Deutsche Forschungsgemeinschaft (Go 459/8-1). The authors gratefully acknowledge the assistance of Dr. I. Haubitz in the statistical analysis. They are indebted to Dr. Klaus V. Toyka for critically reading the manuscript and to Dr. H. Thoenen for providing PC12 cells.

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