Surface exposure of phosphatidylserine during apoptosis of rat thymocytes precedes nuclear changes

Stuart, MCA; Damoiseaux, JGMC; Frederik, PM; Arends, JW; Reutelingsperger, CPM

Published in:
European Journal of Cell Biology

DOI:
10.1016/S0171-9335(98)80019-8

Publication date:
1998

Surface exposure of phosphatidylserine during apoptosis of rat thymocytes precedes nuclear changes

Marc C. A. Stuart[a], Jan G. M. C. Damoiseaux[b], Peter M. Frederik[1)a], Jan-Willem Arends[c], Chris P. M. Reutelingsperger[d]

[a] Electron Microscopy Unit, Department of Pathology, Maastricht University, Maastricht/The Netherlands
[b] Department of Immunology, Maastricht University, Maastricht/The Netherlands
[c] Department of Pathology, Maastricht University, Maastricht/The Netherlands
[d] Department of Biochemistry, Maastricht University, Maastricht/The Netherlands

Received July 7, 1997
Accepted December 18, 1997

Apoptosis – annexin V – ultrastructure

Cell surface exposure of phosphatidylserine (PS) during apoptosis serves recognition and removal of the dying cell by phagocytes. Loss of phospholipid asymmetry and PS exposure is investigated by immunocytochemistry and related to morphological changes. Loss of membrane asymmetry was determined on dexamethasone-treated rat thymocytes using the PS specific probe annexin V. Thymocytes incubated in the presence of dexamethasone were studied in time series during the execution of the apoptotic program. Thymocytes first start to expose PS at their cell surface. At this initial stage the barrier function of the plasma membrane remains intact. At a later stage the plasma membrane becomes leaky for compounds like propidium iodide and subsequently the cell disintegrates into apoptotic bodies. Microscopical evaluation of dexamethasone-treated thymocytes showed that the cells with an apoptotic morphology all bound annexin V. The cells with a normal viable morphology lacked annexin V binding except for those cells that started to shed small vesicles. These vesicles were positive for annexin V, indicating a local disturbance of the phospholipid asymmetry. The local exposure of PS is considered to be a very early event of apoptosis, preceding the full sequence of morphological changes at the ultrastructural level.

Introduction

Apoptosis entails gene-regulated processes of cell suicide, silent death and removal without inflammatory response. These apoptotic processes are characterized by a sequence of morphological alterations, which are clearly distinguishable from necrosis [13]. At the ultrastructural level, rapid blebbing of the cell membrane [22] and chromatin condensation [26] can be observed during the first stage of apoptosis. These changes are often but not always accompanied by nuclear convolution, followed by nuclear pycnosis and cell shrinkage. Also a number of biochemical changes takes place considered to be typical for apoptosis. The main biochemical feature of apoptosis is DNA cleavage at the internucleosomal sites, by calcium-activated endonucleases [2, 11, 32]. This results in the typical DNA laddering on agarose gels, which is considered the biochemical hallmark of apoptosis. However, this hallmark is not always present, some cell types show apoptotic morphology without demonstrable DNA laddering [5, 21]. Other biochemical processes underlying the morphological changes of apoptosis include the degradation of nuclear lamins and cytoskeletal proteins by activated proteases [18]. During execution of apoptosis the cell changes its phospholipid asymmetry of the plasma membrane by translocating phosphatidylserine (PS) to the outer leaflet where it functions as a tag on the dying cell for recognition and removal by phagocytes [9, 10]. Contrary to the apoptotic cell the viable cell retains PS exclusively in the inner leaflet by energy-dependent processes [8, 35].

With the use of annexin V, a phospholipid binding protein with high affinity for PS [1, 25, 27], flow cytometric studies indicated that during apoptosis PS exposure precedes morphological changes as visualized by light scatter parameters [14, 29]. Recently it was shown in dexamethasone-treated thymocytes, a prototypic model of apoptosis [6, 32], that disruption of mitochondrial potential marks irreversible commit-

[1)] Dr. Peter M. Frederik, Electron Microscopy Unit, Department of Pathology, Maastricht University, P. O. Box 616, 6200 MD Maastricht/The Netherlands.

ment to apoptosis and precedes internucleosomal DNA cleavage [33]. Furthermore, this model system showed that PS exposure occurs downstream of the mitochondrial changes [4]. Other phenomena like cytoplasmic vacuolization, chromatin condensation and oligonucleosomal DNA fragmentation also occur downstream of mitochondrial potential disruption [17].

In this study we investigated the loss of membrane phospholipid asymmetry in relation to morphological alterations of apoptotic thymocytes, using immunoelectron microscopy and flow cytometry. The aim of this approach was to correlate the (ultra)structural aspects of apoptosis to the loss of membrane phospholipid asymmetry in apoptotic cells.

With the use of annexin V it was demonstrated that PS exposure is a general feature of apoptosis regardless of the initiating stimulus and the cell type [12, 14, 19, 29]. Annexin V has successfully been used for ultrastructural localization of PS exposure on activated blood platelets [24]. Using this experimental approach it is possible to discriminate between local and general loss of phospholipid asymmetry of the plasma membrane. It also allows to visualize this phenomenon of apoptosis and to place it relative to morphological changes.

Materials and methods

Materials

Dexamethasone (40 mg/ml) was purchased from Pharmachemie (Haarlem, The Netherlands). Recombinant annexin V was prepared as described before [20]. Annexin V-FITC was obtained from Nexins Research BV (Hoeven, The Netherlands) as the APOPTEST®-FITC kit. Anti-annexin V antibodies were raised against human annexin V in rabbits. The polyclonal IgG fraction was affinity purified and used at final concentration of 7 μg/ml IgG. Protein A labeled with gold (10 nm) was purchased from Aurion (Wageningen, The Netherlands). All other reagents used were of analytical grade.

Thymocytes

Thymocytes were isolated from female inbred Lewis rats, 8–10 weeks of age, which were obtained from the Central Animal Facility of the Maastricht University. The animals were maintained under specific pathogen-free conditions until use and had free access to food and water. Single cell suspensions of thymus were prepared by pressing the tissue through a 100 mesh nylon gauze and rinsed with RPMI-1640 (Gibco, Paisley, Scotland, United Kingdom). The cells were washed once and resuspended in culture medium (RPMI-1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin) to a final concentration of 2.5×10^6 cells per ml. Cells were cultured in a humidified CO_2 incubator at 37 °C.

Induction of apoptosis

At T = 0 the cells were divided into 2 groups; In the experimental group 1 μM dexamethasone was added to the culture medium, whereas the control group was incubated in the culture medium only. At T = 0, 1, 2, 4, 6, 8, 12, and 24 hours samples were taken from both groups and placed on ice immediately for subsequent analysis by flow cytometry or immunoelectron microscopy.

Flow cytometric analysis

The thymocytes were pelleted by centrifugation (4 °C) and resuspended into Hepes buffer (137 mM NaCl, 2.68 mM KCl, 10 mM Hepes, 1.7 mM $MgCl_2$, pH 7.4) containing 3 mM calcium. Annexin V-FITC was added to a final concentration of 1 μg/ml and propidium iodide in a final concentration of 5 μg/ml (incubation for at least 10 min in the dark). Flow cytometry was performed on a FACSort (Becton Dickinson, San Jose, CA) equipped with an argon laser (excitation at 488 nm). The cells were run using the LYSYS II software package (Becton Dickinson), and 10000 events were acquired in list mode. From each cell forward scatter, side scatter, fluorescence channel 1 (annexin V-FITC) and fluorescence channel 3 (propidium iodide) were recorded. Analysis was performed off line using the same software.

Immunoelectron microscopy

Annexin V and calcium were added to the thymocyte samples to give final concentrations of 10 μg/ml and 3 mM, respectively (incubation for at least 10 min). The thymocytes were prepared for ultrastructural investigation and localization of annexin V by jet freezing and freeze substitution, as described previously [24]. In brief, thymocyte suspensions were vitrified by jetting them through a 50 μm platinum aperture disk into liquid ethane cooled to its melting point. After evaporation of the ethane under vacuum (at -100 °C), the vitrified cell suspension was freeze substituted in methanol containing 0.5% uranyl acetate. Freeze-substitution was started at -90 °C and subsequently the samples were warmed to -30 °C in four steps, each step with a duration of minimally eight hours. At -30 °C the substitution medium was replaced by pure methanol followed by infiltration with Lowicryl K4M. At -20 °C the cells were finally embedded and polymerized under UV light.

On ultrathin sections annexin V was localized by immunocytochemistry. The sections were incubated with polyclonal antibody against annexin V (7 μg/ml) followed by protein A-gold complex (diluted 1/20). The sections were stained with neutral uranyl acetate to enhance the contrast. The sections were examined in a Philips CM 10 electron microscope (Philips, Eindhoven, The Netherlands) operating at 80 keV.

Results

Annexin V-FITC, propidium iodide flow cytometry

Using annexin V-FITC and propidium iodide (PI) in 2 color flow cytometry, 4 subpopulations can be distinguished within the dexamethasone-treated thymocyte population. Double negative cells, annexin V single positive cells, annexin V propidium iodide double positive cells with normal amount of DNA (high PI signal) and annexin V propidium iodide double positive cells with a reduced amount of DNA (decreased PI signal) (Fig. 1A). The double negative population consists of normal viable thymocytes because there is no indication of cell damage and loss of membrane asymmetry and integrity as probed by the fluorescent markers. Cells single positive for annexin V are considered to be the cells which have lost only (part of) their membrane asymmetry. Double positive cells have lost membrane asymmetry as well as membrane integrity as indicated by annexin V staining and propidium iodide uptake, respectively. The double positive cells can be divided into cells having the normal amount of DNA as indicated by high propidium iodide signal and cells with a decreased amount of DNA, as indicated by a reduced propidium iodide signal.

Forward-, side scatter flow cytometry

Since morphological alterations can also be determined globally by measuring the forward and side scatter by flow cytometry, this can be used to estimate the change in size of a population as defined by the fluorescent markers. The 4 different subpopulations of thymocytes as defined by annexin V and propidium iodide (Fig. 1A) are clearly different with respect to forward and side scatter profiles (Fig. 1B–E). The normal viable cells have the highest forward scatter, the annexin V single positive cells have intermediate forward scatter and high side scatter, the double positive cells with normal amount

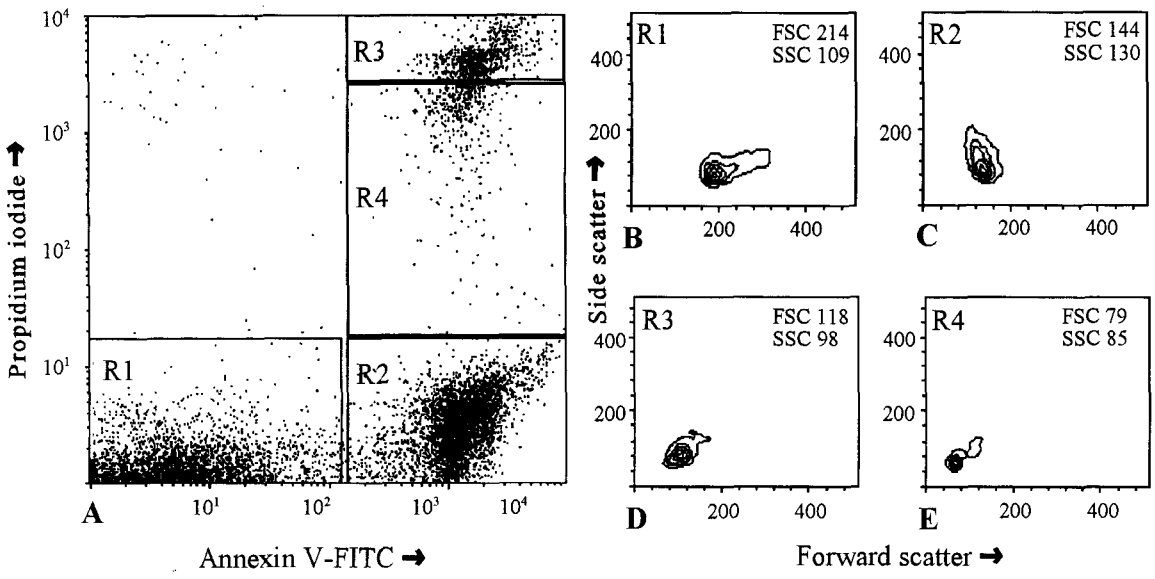

Fig. 1. A. Dot plot of annexin V-FITC fluorescence versus propidium iodide (PI) fluorescence. Four subpopulations were made on the basis of annexin V-FITC binding and propidium iodide uptake of the thymocytes (area R1 "normal" cells, negative for both annexin V-FITC and propidium iodide, R2 apoptotic cells, positive for annexin V-FITC but negative for propidium iodide, R3 cells with an irreversibly damaged membrane positive for both fluorescent markers, with a normal amount of DNA (high PI) and R4 apoptotic bodies, with a reduced amount of DNA (decreased PI signal). The thymocytes were cultured for four hours in the presence of 1 μM dexamethasone. – **B–E.** Each subpopulation (R1–R4) presented by forward vs. side scatter with their mean forward scatter (FSC) and side scatter (SSC) values. **B.** R1 "normal" cell population having the highest forward scatter. **C.** R2 apoptotic cell population with the typical high side scatter. **D.** R3 irreversible damaged cell population positive for both the fluorescent markers with a reduced forward and side scatter. **E.** R4 the apoptotic bodies with a low forward and side scatter.

of DNA have a lower forward and side scatter, whereas the double positive cells with reduced amount of DNA have the lowest forward and side scatter.

Kinetics

Using the 2 fluorescent markers we resolved in time the relation between loss of membrane asymmetry and membrane integrity upon treatment of the thymocytes with dexamethasone.

Fig. 2A gives the kinetics of the population distribution following dexamethasone stimulation. Twenty percent of the population had lost membrane phospholipid asymmetry with intact membrane integrity after 2 hours of incubation with dexamethasone. Six hours later this reached a maximum of 52 %. The percentage of cells with lost membrane integrity and a normal amount of DNA starts to increase after four hours of dexamethasone treatment and peaks at 12 hours. The population of cells with lost membrane asymmetry and a reduced amount of DNA starts to increase after six hours of dexamethasone treatment and is still inclining after 24 hours. These cells have severely lost membrane integrity enabling the extrusion of DNA fragments into apoptotic bodies. After 24 hours of dexamethasone treatment nearly all cells had changed the structure of their plasma membrane as indicated by annexin V and propidium iodide. About 40 % of thymocytes in the control group lost membrane asymmetry after culturing for 24 hours (Fig. 2B). About 20 % of the control thymocytes subsequently lost their membrane integrity. This is the result of death by neglect representing natural occurring apoptosis in the thymus.

Immunoelectron microscopy

Using immunoelectron microscopy we determined the relation between annexin V labeling and morphological alterations, such as chromatin condensation and blebbing. From the kinetics of the plasma membrane changes we chose to analyze thymocytes that were incubated for one, six and eight hours with dexamethasone. The results from the ultrastructural observation and immunolocalization of annexin V are presented in Fig. 3. From control incubation, the majority of thymocytes appear structurally unchanged, as judged by the chromatin and cytoplasm morphology (not shown). From the dexamethasone-treated groups most "unchanged" thymocytes with normal chromatin were annexin V negative (Fig. 3a), even after 8 hours of incubation with dexamethasone. After one hour of incubation with dexamethasone some cells showed blebbing; pinching off microvesicles from the cell membrane (Fig. 3b). On these cells with signs of blebbing no ultrastructural features of apoptosis could be detected, and no annexin V label was present on the cell membrane. We never found protrusions from the cells to be positively labeled with gold particles. The blebs, however, were positive for annexin V (Fig. 3c). On all cells with clear morphological characteristics of apoptosis (condensation of chromatin) annexin V labeling was observed (Fig. 3d). Finally the cells will lose membrane integrity. Morphologically the cells appear with massive blebbing and the plasma membrane becomes separated from the cytoplasm (Fig. 3e). This is followed by a complete degradation of the cell, where the border between nucleus and cytoplasm becomes faint (not shown). It should be mentioned that all different types of cells, as shown in Fig. 3, were found

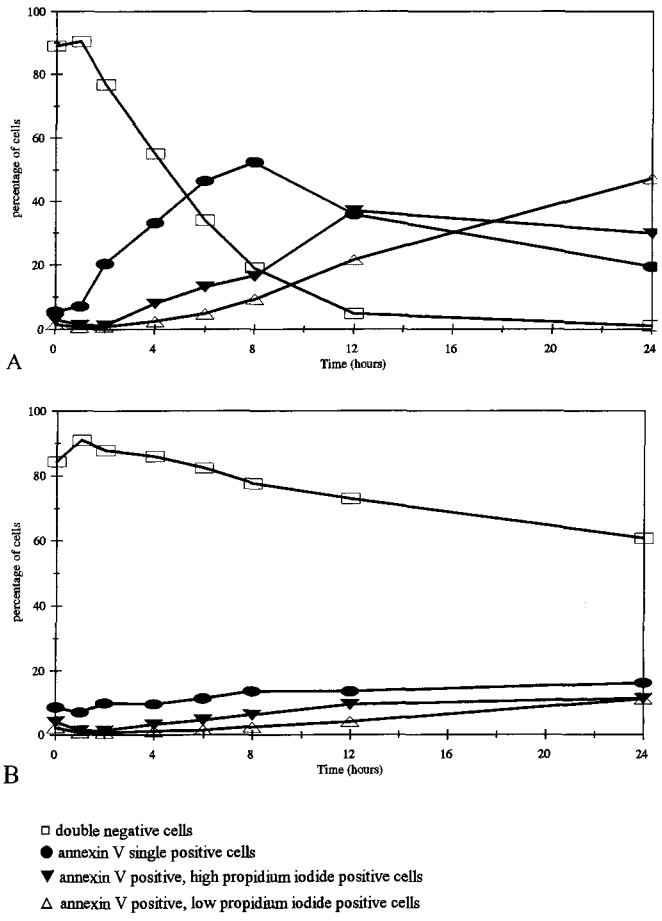

Fig. 2. Kinetics of the apoptotic process of thymocytes after incubation with dexamethasone (**A**), and in control thymocytes (**B**). Number of thymocytes in each subpopulation (Fig. 1A) as the percentage of the total cell population, at each time point. At each time point 10000 events were acquired, the mean values of two independent experiments are presented.

intermingled in each thymocyte preparation, as to be expected on basis of the flow cytometric data, because no sorting or other separation was performed. The relative amount of each cell population changed in time as demonstrated in Figure 2. It was not possible to compare the electron microscopic and flow cytometric changes, because the criteria for division into the categories are not the same for the two techniques. Some background label is present on the nucleus of the thymocytes probably due to non-specific interactions with protein A-gold particles. On incubations without primary antibody similar background label was found.

Discussion

Using dexamethasone-treated thymocytes as a model for apoptosis we were able to distinguish several stages during the apoptotic process on the basis of biochemical and morphological alterations of the cells. The flow cytometric data were used to determine the kinetics of the apoptotic process in order to relate the ultrastructural observations to the time sequence of the apoptotic process. On the basis of the two fluorescent probes, annexin V-FITC and propidium iodide, the thymocyte population could be divided into 4 distinct subpopulations, as was shown for other hemopoietic cell types [14, 19, 29]. The subpopulation of cells which are annexin V positive and propidium iodide negative is considered to comprise the apoptotic cells, which have changed membrane asymmetry while membrane integrity remains intact. DNA fragmentation proved the presence of apoptosis under the conditions used in the experiments (data not shown).

Immunocytochemistry at the EM level strongly suggests that before the apoptotic stage with chromatin condensation and an overall loss of membrane asymmetry, PS exposure is confined to focal areas on blebs on the cell membrane. It can be postulated that these blebs are shed as microvesicles from the plasma membrane. These early (local) changes in membrane architecture take place well before nuclear changes and overall PS exposure as was reported by Martin et al. [19]. Recently it was shown that CD95 ligation induces PS exposure in Jurkat cytoplasts indicating that this part of the apoptotic machinery may run in the absence of a nucleus [16]. In the flow cytogram the small blebs attached to cells may contribute to a slight increase in the annexin V signal, but apparently not enough to identify them as a separate (intermediate) population. We showed that during the course of apoptosis loss of asymmetry of the plasma membrane can be divided into two stages. Firstly (micro)vesicles are pinched off and only the membranes of these vesicles have lost membrane asymmetry. This phenomenon of focal PS exposure is reported here for dexamethasone-treated thymocytes and may be part of apoptosis in general [9, 19]. The second stage is characterized by drastic morphological changes pertinent to apoptosis and a loss of phospholipid asymmetry over the whole plasma membrane, which still retains its barrier function. In Fig. 4 the different morphological stages of apoptosis are schematically drawn at the background of a flow cytometer diagram.

Martin and co-workers [19] reported on the basis of flow cytometric and light microscopic studies that annexin V binding to cells stimulated to activate the apoptotic program emerged well before morphological changes took place. In conjunction with this study these early apoptotic cells likely commenced the process of blebbing with locally exposed PS. Ultrastructural inspection of dexamethasone-treated thymocytes also revealed rarely cells with a normal morphology and locally exposed PS. This indicates that once the Bcl-2 checkpoint is trespassed the dexamethasone-treated thymocyte enters the first stage, where membrane blebbing occurs. Early reduction in mitochondrial potential [33] is probably also a feature of this first stage. After the first stage the thymocytes progress very quickly to the second stage of altered morphology with an overall loss of membrane asymmetry and then more slowly to the third stage of lost membrane integrity. Recently other investigators also observed, both biochemical and morphological, apoptotic events well before membrane integrity was lost [7, 30]. The relative kinetics of these different stages likely depend on the type of cell and the stimulus of apoptosis. With annexin V as a discriminative parameter studies will be facilitated to get further insight into the kinetics of the early apoptotic process and to elucidate the question whether the point of no return is beyond or below the early changes of the plasma membrane structure characterized by a local exposure of PS [see also 15, 16].

The mechanism by which PS is exposed to the outside of the plasma membrane is not clear at the moment. Probably all eukaryotic cells carry in their membranes a translocase activity, that maintains an asymmetric phospholipid distribution

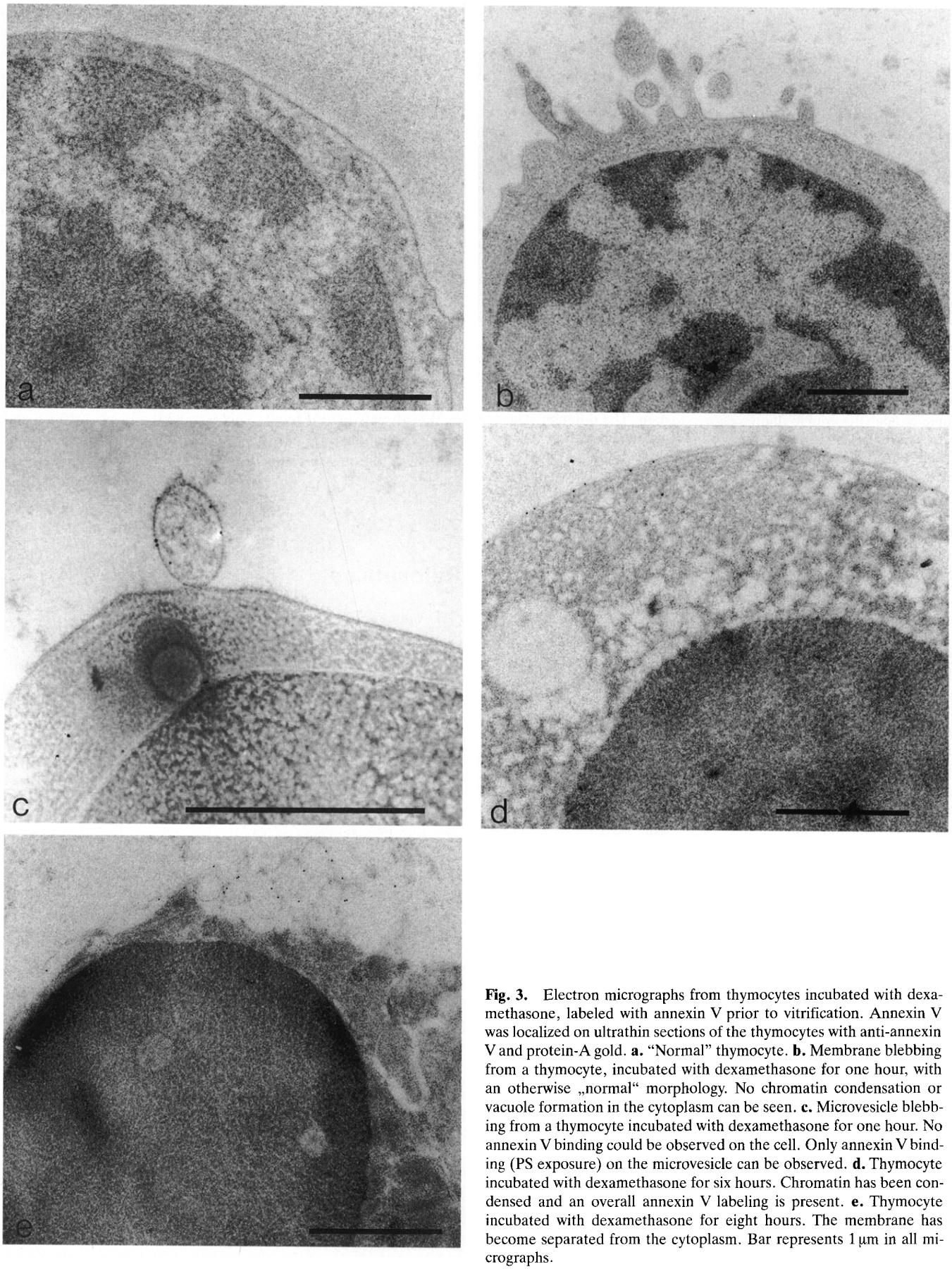

Fig. 3. Electron micrographs from thymocytes incubated with dexamethasone, labeled with annexin V prior to vitrification. Annexin V was localized on ultrathin sections of the thymocytes with anti-annexin V and protein-A gold. **a.** "Normal" thymocyte. **b.** Membrane blebbing from a thymocyte, incubated with dexamethasone for one hour, with an otherwise „normal" morphology. No chromatin condensation or vacuole formation in the cytoplasm can be seen. **c.** Microvesicle blebbing from a thymocyte incubated with dexamethasone for one hour. No annexin V binding could be observed on the cell. Only annexin V binding (PS exposure) on the microvesicle can be observed. **d.** Thymocyte incubated with dexamethasone for six hours. Chromatin has been condensed and an overall annexin V labeling is present. **e.** Thymocyte incubated with dexamethasone for eight hours. The membrane has become separated from the cytoplasm. Bar represents 1 μm in all micrographs.

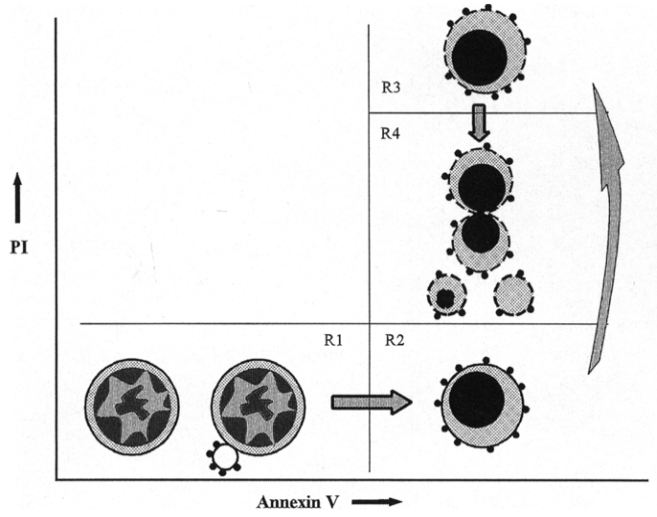

Fig. 4. Schematic sequence of events during dexamethasone-induced apoptosis in rat thymocytes at the background of an annexin V, propidium iodide (PI) double fluorescence cytogram. The different stages are marked R1–R4 as in the flow cytometer diagram with in R1 the viable cells and the cells with early loss of membrane phospholipid asymmetry at the side of the blebs. R2 the apoptotic cells with an overall loss of membrane asymmetry and drastic morphological alterations typical for apoptosis. R3 cells with lost membrane integrity and R4 with the apoptotic bodies. The • indicate phosphatidylserine exposure on the outside of the cell membrane. The dashed lines indicate loss of membrane integrity.

over the two leaflets of their membranes, by translocating PS and phosphatidylethanolamine to the leaflet facing the cytosol (for a review see [8]). As was shown for erythrocytes, inhibition of this translocase is not sufficient to get PS in the outer leaflet of the plasma membrane [34]. This requires another mechanism, which is regulated by cytosolic calcium levels as was shown for agonist-stimulated blood platelets [31] and apoptotic T lymphocytes [28]. Both studies demonstrated with fluorescent phospholipids a translocation of phospholipids over the plasma membrane. Recently Bassé et al. [3] described a 37 kDa membrane protein with a phospholipid translocase activity.

Microvesicles, with a random distribution of phospholipids, attached to a cell, with otherwise intact membrane asymmetry, might be originating from the same cell or become secondarily attached and thus originate from an other cell which is disintegrating. If microvesicles with lost membrane asymmetry originate from a cell with more or less intact membrane asymmetry, the mechanism of loss of membrane asymmetry might be due to this local difference in PS exposure. The ultrastructural data strongly suggest that blebbing is accompanied by local exposure of PS on the blebs that are formed. A similar conclusion has been drawn in the study of platelet activation [24]. During activation, blood platelets shed membrane vesicles [23], and we observed also microvesicles with lost membrane asymmetry trapped in the open canalicular system of platelets with otherwise intact membrane asymmetry. From their location it is very unlikely that these microvesicles originate from other platelets [24], and we postulate that the PS exposure is initially restricted to the shedded vesicles.

Further evidence of focal PS exposure can be found from the ratio of total phospholipids between the in- and outside of the plasma membrane. In most cells the amount of phospholipids in the inner leaflet of the bilayer equals the amount in the outer leaflet of the bilayer, although the various phospholipid species are asymmetrically distributed over inner and outer leaflet. However, microblebs on the plasma membrane with a diameter of 100–200 nm have 10–2.5 % (respectively) more phospholipids in the outer membrane leaflet than in the inner one. The formation of (micro)vesicles requires a local increase of the lipid surface in the outer bilayer leaflet to induce the curvature needed to form the microvesicle. Apparently this net lipid transfer to the outer leaflet is accompanied by PS exposure as was demonstrated on the sections. This is probably a very fast process because we never found protrusions still attached to the cell positively labeled for annexin V.

In conclusion, morphological evidence is presented that the formation of blebs with lost membrane asymmetry is a very early sign from cells who are predestined to undergo the full sequence of apoptosis. The formation of blebs is an early apoptotic event that is locally expressed on the plasma membrane and this event can be detected by immunocytochemistry at the ultrastructural level. This is a new and powerful technique to demonstrate early plasma membrane alterations with annexin V binding as a discriminative parameter.

References

[1] Andree, H. A., C. P. Reutelingsperger, R. Hauptmann, H. C. Hemker, W. T. Hermens, G. M. Willems: Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. J. Biol. Chem. **265,** 4923–4928 (1990).

[2] Arends, M. J., R. G. Morris, A. H. Wyllie: Apoptosis: The role of the endonuclease. Am. J. Pathol. **136,** 593–608 (1990).

[3] Bassé, F., J. G. Stout, P. J. Sims, T. Wiedmer: Isolation of an erythrocyte membrane protein that mediates Ca^{2+}-dependent transbilayer movement of phospholipids. J. Biol. Chem. **271,** 17205–17210 (1996).

[4] Castedo, M., T. Hirsch, S. A. Susin, N. Zamzami, P. Marchetti, A. Macho, G. Kroemer: Sequential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. J. Immunol. **157,** 512–521 (1996).

[5] Cohen, J. J., R. C. Duke, V. A. Fadok, K. S. Sellins: Apoptosis and programmed cell death in immunity. Annu. Rev. Immunol. **10,** 267–293 (1992).

[6] Cohen, J. J., R. C. Duke: Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. J. Immunol. **132,** 38–42 (1984).

[7] Desjardins, L. M., J. P. MacManus: An adherent cell model to study different stages of apoptosis. Exp. Cell Res. **216,** 380–387 (1995).

[8] Diaz, C., A. J. Schroit: Role of translocase in the generation of phosphatidylserine asymmetry. J. Membrane Biol. **151,** 1–9 (1996).

[9] Fadok, V. A., D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton, P. M. Henson: Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J. Immunol. **148,** 2207–2216 (1992).

[10] Fadok, V. A., J. S. Savill, C. Haslett, D. L. Bratton, D. E. Doherty, P. A. Campbell, P. M. Henson: Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. J. Immunol. **149,** 4029–4035 (1992).

[11] Hockenbery, D.: Defining apoptosis. Am. J. Pathol. **146,** 16–19 (1995).

[12] Homburg, C. H. E., M. Dehaas, A. E. G. K. Vondemborne, A. J. Verhoeven, C. P. M. Reutelingsperger, D. Roos: Human neutrophils lose their surface Fc gamma RIII and acquire annexin V binding sites during apoptosis in vitro. Blood **85,** 532–540 (1995).

[13] Kerr, J. F. R., G. C. Gobé, C. M. Winterford, B. V. Harmont: Anatomical methods in cell death. In: L. M. Schwart, B. A. Osborne (eds.): Methods in cell biology. Vol. 46, Cell death. pp. 1–27. Academic press. London 1995.

[14] Koopman, G., C. P. M. Reutelingsperger, G. A. M. Kuijten, R. M. J. Keehnen, S. T. Pals, M. H. J. Vanoers: Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood **84,** 1415–1420 (1994).

[15] Kroemer, G., P. Petit, N. Zamzami, J. L. Vayssiere, B. Mignotte: The biochemistry of programmed cell death. FASEB J. **9,** 1277–1287 (1995).

[16] Kroemer, G., N. Zamzami, S. A. Susin: Mitochondrial control of apoptosis. Immunology today **18,** 44–51 (1997).

[17] Marchetti, P., M. Castedo, S. A. Susin, N. Zamzami, T. Hirsch, A. Macho, A. Haeffner, F. Hirsch, M. Geuskens, G. Kroemer: Mitochondrial permeability transition is a central coordinating event of apoptosis. J. Exp. Med. **184,** 1155–1160 (1996).

[18] Martin, S. J., D. R. Green: Protease activation during apoptosis: Death by thousand cuts. Cell **82,** 349–352 (1995).

[19] Martin, S. J., C. P. M. Reutelingsperger, A. J. McGahon, J. Rader, R. C. A. A. van Schie, D. M. LaFace, D. R. Green: Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of Bcl-2 and Abl. J. Exp. Med. **182,** 1545–1556 (1995).

[20] Maurer Fogy, I., C. P. M. Reutelingsperger, J. Pieters, G. Bodo, C. Stratowa, R. Hauptmann: Cloning and expression of cDNA for human vascular anticoagulant, a Ca^{2+}-dependent phospholipid-binding protein. Eur. J. Biochem. **174,** 585–592 (1988).

[21] Oberhammer, F., G. Fritsch, M. Schmied, M. Pavelka, D. Printz, T. Purchio, H. Lassmann, R. Schulte-Hermann: Condensation of the chromatin at the membrane of an apoptotic nucleus is not associated with activation of an endonuclease. J. Cell Sci. **104,** 317–326 (1993).

[22] Sanderson, C. J.: Morphological aspects of lymphocyte mediated cytotoxicity. Adv. Exp. Med. Biol. **146,** 3–21 (1982).

[23] Sims, P. J., E. M. Faioni, T. Wiedmer, S. J. Shattil: Complement proteins C5b-9 cause release of membrane vesicles from the surface that are rich in the membrane receptor for coagulation factor Va and express prothrombinase activity. J. Biol. Chem. **263,** 18205–18212 (1988).

[24] Stuart, M. C. A., E. M. Bevers, P. Comfurius, R. F. A. Zwaal, C. P. M. Reutelingsperger, P. M. Frederik: Ultrastructural detection of surface exposed phosphatidylserine on activated blood platelets. Thromb. Haemostasis **74,** 1145–1151 (1995).

[25] Tait, J. F., D. Gibson, K. Fujikawa: Phospholipid binding properties of human placental anticoagulant protein-I, a member of the lipocortin family. J. Biol. Chem. **264,** 7944–7949 (1989).

[26] Thomas, N., P. A. Bell: Glucocorticoid-induced cell-size changes and nuclear fragility in rat thymocytes. Mol. Cell. Endocrinol. **22,** 71–84 (1981).

[27] Vanheerde, W. L., P. G. de Groot, C. P. M. Reutelingsperger: The complexity of the phospholipid binding protein annexin V. Thromb. Haemostasis **73,** 172–179 (1995).

[28] Verhoven, B., R. A. Schlegel, P. Williamson: Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. J. Exp. Med. **182,** 1597–1601 (1995).

[29] Vermes, I., C. Haanen, H. Steffens-Nakken, C. P. M. Reutelingsperger: A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled annexin V. J. Immunol. Methods **184,** 39–51 (1995).

[30] Weis, M., J. Schlegel, G. E. Kass, T. H. Holmstrom, I. Peters, J. Eriksson, S. Orrenius, S. C. Chow: Cellular events in Fas/APO-1-mediated apoptosis in JURKAT T lymphocytes. Exp. Cell Res. **219,** 699–708 (1995).

[31] Williamson, P., E. M. Bevers, E. F. Smeets, P. Comfurius, R. A. Schlegel, R. F. A. Zwaal: Continuous analysis of the mechanism of activated transbilayer lipid movement in platelets. Biochemistry **34,** 10448–10455 (1995).

[32] Wyllie, A. H.: Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature. **284,** 555–556 (1980).

[33] Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, J. L. Vayssiere, P. X. Petit, G. Kroemer: Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J. Exp. Med. **181,** 1661–1672 (1995).

[34] Zwaal, R. F. A., P. Comfurius, E. M. Bevers: Mechanism and function of changes in membrane-phospholipid asymmetry in platelets and erythrocytes. Biochem. Soc. Trans. **21,** 248–253 (1993).

[35] Zwaal, R. F. A., A. J. Schroit: Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood **89,** 1121–1132 (1997).