Review Article

Annexin V-Affinity Assay: A Review on an Apoptosis Detection System Based on Phosphatidylserine Exposure

Manon van Engeland,1 Luc J.W. Nieland,1 Frans C.S. Ramaekers,1 Bert Schutte,1* and Chris P.M. Reutelingsperger2

1Department of Molecular Cell Biology and Genetics, Maastricht University, The Netherlands
2Department of Biochemistry, Maastricht University, The Netherlands

Received 24 April 1997; Accepted 23 September 1997

Apoptosis is a programmed, physiological mode of cell death that plays an important role in tissue homeostasis. Understanding of the basic mechanisms that underlie apoptosis will point to potentially new targets of therapeutic treatment of diseases that show an imbalance between cell proliferation and cell loss. In order to conduct such research, techniques and tools to reliably identify and enumerate death by apoptosis are essential. This review focuses on a novel technique to detect apoptosis by targeting for the loss of phospholipid asymmetry of the plasma membrane. It was recently shown that loss of plasma membrane asymmetry is an early event in apoptosis, independent of the cell type, resulting in the exposure of phosphatidylserine (PS) residues at the outer plasma membrane leaflet. Annexin V was shown to interact strongly and specifically with PS and can be used to detect apoptosis by targeting for the loss of plasma membrane asymmetry. Labeled annexin V can be applied both in flow cytometry and in light microscopy in both vital and fixed material by using appropriate protocols. The annexin V method is an extension to the current available methods. This review describes the basic mechanisms underlying the loss of membrane asymmetry during apoptosis and discusses the novel annexin V-binding assay. Cytometry 31:1–9, 1998. © 1998 Wiley-Liss, Inc.

Key terms: annexin V; phosphatidylserine; flow cytometry; programmed cell death; membrane asymmetry
MOLECULAR BIOLOGY OF APOPTOSIS

A cell is continuously exposed to multiple opposing "death" and "survival" triggers. To date, little is known about the molecular events controlling this balance of signals. Multiple triggers of apoptosis are known, such as withdrawal of growth factors, DNA damage, Fas ligand binding, application of chemotherapeutic agents, etc (3,9). Although these triggers initiate a cascade of events that finally results in cell death by apoptosis, they differ in the length of the so-called "trigger phase," i.e., the lag time between exposure to the trigger and the time of the first morphological signs of apoptosis. The duration of this phase depends on the cell type, type of the trigger and growth conditions of the cell. All the different induction mechanisms seem to converge to the activation of a proteolytic cascade, involving interleukin converting enzyme (ICE) and the ICE-like family of proteases recently renamed caspase family (34,37). Some genes are reported to govern this initial phase of apoptosis (49,63), such as the bcl2 gene family that is supposed to protect cells against apoptosis by opposing the effect of the bax gene family. Both gene products have been shown to form either homo- or heterodimers and the eventual cellular decision is supposed to be determined by the relative quantities of both gene products (for reviews, see refs. 23,60,63). The activation of the proteolytic cascade seems to determine the beginning of the so-called "execution phase" of the apoptotic process, which is relatively short and shows little variation in duration (17). The activation of the caspase family of proteases results in the breakdown of cellular proteins such as the nuclear matrix, the cytoskeleton, and the poly-ADP-ribose polymerase, either directly or through activation of other cellular proteases such as calpain or proteasomes (22,30). At this stage of the process apoptotic cells also display PS at their outer membrane. However, the molecular mechanisms underlying this process are to date poorly understood.

PLASMA MEMBRANE CHANGES DURING APOPTOSIS

Viable cells maintain an asymmetric distribution of different phospholipids between the inner and outer leaflets of the plasma membrane (6). This is particularly true for both choline-containing phospholipids, phosphatidylcholine and sphingomyelin, which are primarily located in the viable cells outer leaflet, and for the aminophospholipids phosphatidylethanolamine and PS, which are found at the cytoplasmic face. This asymmetry of the plasma membrane was first described for erythrocytes and platelets (42,45) and later also found to hold true for the nucleated cell types (15,64). The plasma membrane lipid asymmetry in viable cells is thought to be maintained by so called "flipases", membrane proteins that facilitate the translocation of lipid molecules from one leaflet to the other (24). Seigneur et Devaux (47) were the first to demonstrate that the erythrocyte membrane contains activities that translocate specifically aminophospholipids from one leaflet to the other. Such an activity was later also found in membranes of platelets (10) and nucleated cell types (31,64,66). Connor et al. (12) provided evidence for the Mg$^{2+}$/ATP-dependence of this process.

This flipase activity now appears to be ubiquitously present and contributes significantly to the PS asymmetry in membranes of enucleated and nucleated cell types (for recent reviews, see 16,68). Other proteins that show phospholipid translocase activity are a recently cloned bovine enzyme, which is a member of the subfamily of P-type adenosine triphosphates described by Tang et al. (54), and the multidrug-resistance protein encoded by the mdr2 gene (reviewed in ref. 21). So far, however, the identity of this translocase has not been resolved satisfactorily, and it is still unclear whether the activity of aminophospholipid translocation is mediated by a single or by a group of proteins.

In addition to the potency of maintaining PS asymmetrically at the inner membrane leaflet by flipase activity, cells also possess the ability to translocate PS to the outer leaflet of the plasma membrane. This was first demonstrated for platelets (4,5) and erythrocytes (11). Cell surface exposure of PS in these cells is of importance in catalyzing coagulation by activated platelets and elimination through the reticuloendothelial system of senescent erythrocytes (8). Later it became clear that nucleated cell types may also expose PS at their cell surface under controlled conditions. Fadok and coworkers (19,20) showed that leukocytes during apoptosis expose PS on the outer leaflet of the plasma membrane. This cell surface exposed PS functions as a tag for specific recognition by macrophages and for phagocytosis of the dying cell.

To date, the molecular machinery responsible for cell surface exposure of PS remains unidentified. Activities of scramblases, which are responsible for the inward-out translocation of PS by scrambling the aminophospholipids over the inner and outer membrane leaflet, have been demonstrated to be responsible for PS exposure in platelets and erythrocytes (16,68). Others claim a role for fodrin (nonerythroid spectrin) in maintaining an asymmetric phospholipid composition of the cytoplasmic membrane by anchoring PS at the cytofacial membrane. As a consequence of fodrin degradation by ICE-like proteases, PS would be expected during apoptosis (59).

ANNEXIN V AND DETECTION OF PS EXPOSURE IN APOPTOTIC CELLS

Investigations into the exposure of PS at the outer membrane leaflet of cells is facilitated by the finding that annexin V specifically binds to PS in the presence of calcium (2,53). Annexin V was first reported by Bohn and colleagues (25), who isolated the protein from human placenta and called it placental protein 4 (PP4), and by Reutelingsperger et al. (44), who isolated it from the umbilical cord by virtue of its anticoagulant activity and called it vascular-anticoagulant-α. After cloning and sequencing of the human annexin V cDNA, the protein got its name because of its homology with the family of...
annexin proteins (26,27,36). Successful expression in bacterial systems gave a source of recombinant protein that catalyzed numerous investigations exposing biological, biochemical and physicochemical properties of annexin V, including its in vitro anti-phospholipase, anti-coagulant, anti-kinase, Ca\(^{2+}\)-channeling and phospholipid binding activities (for recent reviews, see refs. 43,52,58). However, the phospholipid binding properties of annexin V appear central in most considerations and are regarded as the key activity elaborated by the organism to serve the as yet unknown role. The availability of biotin- or FITC-labeled annexin V facilitated studies on PS exposure at the outer membrane leaflet, and provides not only a useful tool to measure the PS exposition of platelets (13,55) and erythrocytes (53), but also in nucleated cells undergoing programmed cell death. (28,35,57,62).

Verhoven and coworkers (61) recently showed a scramblase to become active during apoptosis of lymphocytes. Using fluorescently labeled annexin V, it could be demonstrated that the apoptotic lymphocytes expose PS at their outer membrane early after onset of the execution phase of apoptosis. PS appears at the outer leaflet of the plasma membrane, the integrity of which has not been compromised at this stage. PS exposure seems to last from the early execution phase of apoptosis until the final stage, at which the cell has broken up into apoptotic bodies.

**Fig. 1.** Schematic representation of the loss of membrane lipid asymmetry during apoptosis. Vital cells maintain a strictly asymmetric lipid bilayer composition, with PS residues (red circles) facing the cytosol. During apoptosis these PS molecules become exposed at the outer membrane leaflet. Hapten-labeled annexin V can bind with high affinity to the exposed PS in the presence of millimolar Ca\(^{2+}\)-concentration.

**Fig. 2.** MR65 cells, grown on glass slides are cultured in the presence of 200 µM olomoucine. After 4.5 h, the vital cultures were incubated with annexin V-FITC, PI, and Hoechst 33342. The cell cultures were subsequently analyzed using the BioRad MRC600 confocal scanning laser microscope. A: Merged image of annexin V fluorescence and differential interference contrast. Cells showing the characteristic bud formation are clearly annexin V positive, while vital adherant cells, including the metaphase figure in the centre of the image, are negative. Note the presence of small annexin V-positive vesicles. B,C: Merged images of annexin V (green), PI (red), and Hoechst 33342 (blue) fluorescence of a single apoptotic (B) and a single dead (C) MR65 cell. Note that some buds are devoid of nuclear remnants.
Based on the phenomenon that PS is exposed during apoptosis and on the ability of annexin V to bind to PS with high affinity, Koopman et al. (28) were the first to describe a method using extrinsically applied hapten (i.e., FITC or biotin) labeled annexin V to detect apoptosis. As schematically represented in Figure 1, hapten-labeled annexin V binds in the presence of millimolar Ca\(^{2+}\) to PS residues that are exposed at the outer leaflet of the plasma membrane of apoptotic cells. Annexin V is not able to bind to normal vital cells since the molecule is not able to penetrate the phospholipid bilayer. In dead cells, however, the inner leaflet of the membrane is available for binding of extrinsically applied annexin V, since the integrity of the plasma membrane is lost. To discriminate between dead and apoptotic cells, a membrane impermeable DNA stain, such as propidium iodide (PI) can be added simultaneously to the cell suspension. In this way vital, apoptotic and dead cells can be discriminated on basis of a double-labeling for annexin V and PI, and analyzed either by flow cytometry or fluorescence microscopy.

DETECTION OF APOPTOSIS USING THE ANNEXIN V-ASSAY FOR IMMUNOCYTOCHEMISTRY

When examined by light microscopy, the cells showing morphological aspects characteristic for apoptotic cells (e.g., bud formation) also show affinity for annexin V (Fig. 2a). Most of these cells are devoid of PI staining indicating the intactness of the membrane. Figure 2b shows an example of an annexin V-positive/PI-negative cell. When the membrane looses its integrity, the cell becomes both annexin V and PI positive, indicating the necrotic stage of the cell (Fig. 2c). In annexin V-positive/PI-negative cells, addition of the supravital DNA dye Hoechst 33342 allows the study of nuclear morphology. In Figure 2b,c, annexin V-positive buds can be observed, some showing nuclear remnants, some devoid of nuclear material.

QUANTIFICATION OF APOPTOTIC CELLS USING THE ANNEXIN V-ASSAY FOR FLOW CYTOMETRY

For the quantification of annexin V-positive apoptotic cells, flow cytometry can best be applied using a single cell suspension prepared from the cells or tissue under examination. Figure 3 shows an example of a flow cytometric analysis of the annexin V labeling assay to detect apoptosis in a dexamethasone treated rat thymocyte culture. For this purpose, rat thymocyte cultures were exposed to dexamethasone for various periods of time. The cytogram in Figure 3A shows the bivariate PI/annexin V analysis of the unexposed cell suspension. Vital cells are negative for both PI and annexin V (R1), apoptotic cells are PI negative and annexin V positive (R2), while dead cells are positive for both PI and annexin V (R3). After 4.5-h exposure to dexamethasone, the number of apoptotic cells increased from 4.1% in the control culture to 43.6%, while the number of dead cells remained constant (Fig. 3B).
Recently, van Engeland et al. (57) showed that it is feasible to use the bivariate PI/annexin V analysis also for adhering cells in cultures. The method does not work after routinely applied cell harvesting techniques, such as trypsinization. Using this technique, membrane changes are induced which result in PS exposure in the outer plasma membrane, probably due to bleb formation during harvesting (1).

The method described by us (57) was designed to overcome these pitfalls. It makes use of adhering cells works with adhering cell cultures which were labeled with annexin V prior to quantitative harvesting with a rubber policeman. The cell suspension thus obtained is then counterstained with PI. Figure 4 shows an example of the annexin V assay performed on adherent cells, harvested by scraping. The different labeling patterns in this assay identify the different cell populations, i.e., region R1: vital cells (PI-negative/annexin V-negative), region R2: apoptotic cells (PI-negative/annexin V-positive), region R3: dead cells (PI-positive/annexin V-positive) and region R4: damaged cells (PI-positive/annexin V-negative).

Recently, van Engeland et al. (57) showed that it is feasible to use the bivariate PI/annexin V analysis also for adhering cells in cultures. The method does not work after routinely applied cell harvesting techniques, such as trypsinization. Using this technique, membrane changes are induced which result in PS exposure in the outer plasma membrane, probably due to bleb formation during harvesting (1).

The method described by us (57) was designed to overcome these pitfalls. It makes use of adhering cells works with adhering cell cultures which were labeled with annexin V prior to quantitative harvesting with a rubber policeman. The cell suspension thus obtained is then counterstained with PI. Figure 4 shows an example of the annexin V assay performed on adherent cells, harvested by scraping. The different labeling patterns in this assay identify the different cell fractions, i.e. PI-negative/annexin V-negative vital cells (R1), PI-negative/annexin V-positive apoptotic cells (R2), PI-positive/annexin V-positive dead cells (R3), and PI-positive/annexin V-negative cells, damaged during the isolation procedure (R4).

The annexin V-labeled cells can be fixed in cold methanol, which then enables detection and quantification of other intracellular antigens in the different cell populations. For this purpose, biotin conjugated annexin V is used for labeling of the cells (57). After fixation annexin V binding is visualized with FITC-labeled Streptavidin.

For example, Figure 5 shows an example of lamB expression during induction of apoptosis in a lung cancer cell line. As shown in Figure 5A, most of the annexin V-positive apoptotic cells are devoid of lamB staining. A small proportion of the annexin V-positive cells is, however, positive for lamB (R3), while low numbers of lamB-negative, annexin V-negative cells are observed (R1), representing mitotic cells (Fig. 5B). This trivariate annexin V/lamB/DNA analysis also shows that DNA loss, resulting in the characteristic “sub-G1 peak” in the DNA histogram (Fig. 5C), is detected mainly in the apoptotic, annexin V-positive/lamB-negative cells (Fig. 5E), while the annexin V-positive/lamB-positive cells show a normal cell cycle distribution (Fig. 5D).

**HISTOCHEMICAL DETECTION OF APOPTOTIC CELLS USING ANNEXIN V**

In order to use the annexin V-assay for the detection of apoptotic cells in situ, biotin-labeled annexin V was injected into mice. In vivo labeling with biotinylated annexin V during 30 minutes was followed by dissection, routine formalin fixation and paraffin embedding of the tissues. After dewaxing and rehydration the tissue sections were incubated with peroxidase conjugated streptavidin and visualized by enzyme histochemistry. In this way apoptosis could be detected in developing mouse embryos (18). Figure 6 shows an example of an in situ-labeled section mouse thymus, dissected approximately 30 min after injection with biotin-labeled annexin V. Control sections, which were incubated with annexin V after fixation and embedding of the tissue and stained using peroxidase conjugated streptavidin, show integral membrane staining in all cells as a result of detectability of the PS in the inner leaflet of the plasma membrane. As shown at low magnification, thymocytes, dying by the process of apoptosis, are not randomly scattered throughout the tissue, but die in small groups. At higher magnification, the specific membrane staining of the individual cells can clearly be seen.

**PS EXPOSURE: A UNIVERSAL MECHANISM DURING APOPTOSIS?**

PS exposure during apoptosis seems to be a universal phenomenon during apoptosis, not limited to mammalian cells, but also occurring in insect and plant cells (41).

Hence, PS exposure appears as ubiquitous as apoptosis itself and likely constitutes an integral part of this process of cell suicide. Martin et al. (35) recently showed that PS exposure during apoptosis occurs in most if not all cells under the action of most if not all triggers of apoptosis. The authors measured PS exposure in several murine and human cell lines, exposed to a wide variety of initiating stimuli, including ligation of FAS or CD3/T cell receptor complex, treatment with synthetic ceramides, glucocorticoids, a variety of cytotoxic drugs and UV irradiation. All...
treatments resulted in PS externalization and binding of FITC-labeled Annexin V. This Annexin V-binding could specifically be inhibited by exogenous phosphatidylserine and not by other phospholipids. Furthermore, the authors provided evidence that PS exposure is indeed intimately coupled to the process of programmed cell death, since overexpression of known repressors of apoptosis, such as Abl and Bcl2, inhibit PS externalization.

Fig. 5. Trivariate (46) annexin V/Lamin B and DNA content analysis of the lung cancer cell line MR65. Cells were cultured in the presence of 200 μM olomoucine for 4.5 h, prior to labeling of the cells with biotin conjugated annexin V. Cells were harvested and the cell suspension was subsequently fixed in ice-cold methanol. Lamin B was detected by routine immunocytochemical protocols. Annexin V binding was visualized using FITC-labeled Streptavidin, Lamin B staining using phycoerythrin conjugated secondary antibodies, and DNA content using PI. Most of the annexin V-positive apoptotic cells are devoid of Lamin B staining (A, R4). A small proportion of the annexin V-positive cells are also positive for lamin B (R3), while a small number of Lamin B-negative, annexin V-negative cells are observed (R1). These cells, which appear as Lamin B-negative in the cytogram of Lamin B versus DNA content (B), represent the mitotic cells. The DNA histograms of the total cell population (C), of the region R4 (E), and of region R3 (D) show that no signs of DNA loss are present in D, the annexin V-positive/Lamin B-positive cells (R3), in contrast to the E, annexin V-positive/Lamin B-negative cells (R4), which accumulate at the sub G1 region of the histogram (for details, see ref. 57).
In the same article evidence was provided that loss of membrane asymmetry is a rather early phenomenon in the apoptotic process, since PS exposure preceded both the characteristic morphological changes as well as nuclear condensation.

**PS EXPOSURE: AN EARLY OR LATE PHENOMENON IN THE APOPTOTIC PROCESS?**

Although the molecular mechanisms of apoptosis are not well understood, we know that the family of caspases, seem to play a pivotal role in initiating this process (37,67). Recent reports in the literature suggests that PS externalization is prevented by inhibitors of the caspase family (32,38). These findings are in line with the observations made by Castedo et al. (7), showing that early during apoptosis, cells undergo a disruption of the mitochondrial transmembrane potential ($\Delta v_{mt}$), prior to exposing PS at the outer membrane leaflet. This $\Delta v_{mt}$ disruption is mediated by the opening of so-called mitochondrial permeability transition pores or megachannels. Susin et al. (51) showed that $\Delta v_{mt}$ disruption leads to the release of an apoptosis inducing factor, which in a cell free in vitro system causes isolated nuclei to undergo apoptotic changes. This apoptosis inducing factor is blocked by the caspase inhibitor Z-VAD.

Taken together, these results would indicate that PS externalization is a downstream event of early caspase activation and possibly an early phenomenon of the so-called execution phase. Evidence for this is provided by the kinetics of PS exposure and breakdown of many putative targets of the caspases. For instance, we provided evidence that PS exposure occurs prior to the loss of nuclear lamins. Loss of nuclear lamina architecture during apoptosis was shown to be the result of proteolysis of this nuclear matrix protein, in contrast to the solubilization during mitosis due to hyperphosphorylation by the mitotic cdk/cyclin kinase complex (40,48). Recently the protease responsible for lamin breakdown was isolated and shown to be a member of the family of caspases (29). Neamati et al. (39) demonstrated that lamin degradation is an early event of apoptosis in thymocytes and suggested that chromatin condensation and breakdown of the nuclear envelope occurs as a result of disruption of the nuclear lamina architecture.

Recently, Tinnemans et al. (56) observed changes in another member of the intermediate filament family, i.e. the cytokeratins, which were degraded early during apoptosis. Double labeling experiments using annexin V and anti-cytokeratin antibodies (57) showed that loss of membrane asymmetry preceeded the degradation of these cytoskeletal elements.

In double labeling experiments, using annexin V-biotin and counterstaining with the supravital DNA dye Hoechst 33342, Koopman et al. (28) showed that all cells showing chromatin condensation were annexin V positive, suggesting that PS exposition in apoptotic cells is at least as early as chromatin condensation. This finding was further substantiated by Stuart et al. (50), who showed annexin V labeling of cells with condensed chromatin using electron microscopy. However, Vanags et al. (59) found that the kinetics of $\alpha$-fodrin degradation and plasma membrane blebbing were similar and difficult to distinguish in time from the nuclear apoptotic changes.

Direct comparison of the TUNEL assay and the annexin V affinity assay, indicates that PS externalizations can be measured prior to the detection of DNA strand breaks (41). All experimental evidence obtained thus far indicates that loss of membrane asymmetry is a very early phenomenon during apoptosis, initiated at a time following the caspase proteolytic cascade but possibly preceeding nuclear condensation and breakdown of intracellular cytoskeletal and nuclear matrix constituents.

Fig. 6. Exogenously applied annexin V localization in formalin fixed, paraffin-embedded tissue sections of a mouse thymus. The mouse was in situ-labeled by injection of annexin V-biotin. The thymus was removed after 30 min and routinely processed for paraffin embedding. Sections cleared from paraffin were incubated with peroxidase-labeled Streptavidin to visualize annexin V-biotin, bound to apoptotic cells. The low-power ($\times 20$) (A) field shows that apoptosis occurs in discrete groups of cells and not randomly dispersed throughout the tissue. At higher magnification ($\times 40$), (B) a clear membranous staining in the positive cells can be observed (for details, see ref. 18).
CONCLUSION

PS exposure at the outer membrane surface of a cell is a universal process occurring during early apoptosis, independent of the species, the cell type or the apoptosis induction system used. Using the annexin V-affinity assay, the number of apoptotic cells in suspension can be determined in a fast, simple, and sensitive way. In vivo or in situ detection of apoptotic cells has become feasible by injecting biotin-labeled annexin V in the bloodstream of animals to be examined. Furthermore, this technique can easily be adapted to incorporate detection of other cellular features, such as chromatin condensation, as well as the presence or absence of specific cellular constituents.

LITERATURE CITED


