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Annexin V, the regulator of phosphatidylserine-catalyzed inflammation and coagulation during apoptosis

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Abstract. Annexin V belongs to a family of phospholipid binding proteins, the Annexins. It binds in the presence of Ca^{2+}-ions with high affinity to negatively charged phospholipids like phosphatidylserine (PS). On the basis of its protein structure and biological activity Annexin V is considered as a protein exhibiting its hitherto unknown function within the intracellular environment. One argument comes from the understanding that PS is predominantly located in membrane leaflets, which face the cytosol. However, recent findings show that each cell type has the molecular machinery to expose PS at its cell surface. This machinery is activated during the execution of apoptosis. Once PS is exposed at the cell surface it exhibits procoagulant and proinflammatory activities. Annexin V will bind to the PS-exposing apoptotic cell and can inhibit thereby the procoagulant and pro-inflammatory activities of the dying cell. These findings together with the presence of Annexin V in the extracellular space depict a novel (patho)physiological significance for Annexin V in vivo.
Key words. Annexin V; phosphatidylserine; apoptosis; inflammation; coagulation.

Annexins are a class of proteins that share structural and functional features. To date this family comprises thirteen members whose primary structure has been resolved.

The criteria for being a member of the Annexin family are structural and functional. The Annexin primary structure is characterized by tandem homologous domains, each of which contains the so-called endonexin loop. This conserved amino acid motif harbours the phospholipid binding site, which conveys the functional property of binding to phospholipids. Most Annexins behave as extrinsic membrane proteins which bind reversibly to phospholipid membranes in a manner that depends on Ca^{2+}-ions. Annexins also bind preferentially to the negatively charged phospholipid species.

During the past decade a great deal of information about the structure and biological activity of Annexins has been elaborated without, however, solving the enigma of the (patho)physiological significance of the individual Annexin or the whole family. Most of the biological data on Annexins arise from in vitro studies and point to roles in interfacial processing occurring at or involving phospholipid membrane structures. These processes include membrane trafficking, modulation of membrane architecture, transmembrane transport of compounds, membrane receptor function, generation of membrane-derived second messengers, regulation of membrane-dependent enzymes, and so forth (for recent reviews see refs 1 and 2).

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Since Annexins lack a secretory signal sequence they are supposed to be intracellular proteins and act as such. This concept fits very well with the Annexin structure and ability to bind to phospholipid, because the intracellular cytoplasmic environment is constitutively surrounded by phospholipid membrane leaflets bearing negatively charged phospholipid species. Some Annexins, like Annexin V, have, however, been reported to have extracellular localization. From the point of view of an Annexin the extracellular space is surrounded by membrane leaflets which are devoid of negatively charged phospholipids. What kind of functions can the extracellular Annexin fulfil if binding to negatively charged phospholipids is mandatory? Novel insights into the regulation of cell surface exposure of phosphatidylserine (PS) shed light on this question and stimulate a line of reasoning embedding a (patho)-physiological significance of Annexins in the extracellular space. This essay uses the paradigm of Annexin V to depict a concept and stimulate discussion and investigations into the significance of the extracellular Annexin.

Annexin V binding to phospholipid model membranes

Annexin V is also known by various synonyms, indicating widespread interest and reflecting its abundant presence in the organism as well as its activity in diverse biological systems [3]. Annexin V was isolated from the human umbilical cord artery by virtue of its anticoagulant activity [4], which could be explained by its binding to negatively charged phospholipids [5, 6]. In the presence of Ca^{2+}, Annexin V binds to most phospholipid

species in model systems [5–9]. Whereas Annexin V hardly associates with phosphatidylcholine (PC) and sphingomyelin (at <5 mM Ca^{2+}) [6] it binds with high affinity to negatively charged phospholipids like phosphatidylserine (PS), probably because Annexin V bears a binding pocket specific for the phosphoserine headgroup [10]. Binding to a PC model membrane containing PS occurs with a Kd of less than 0.1 nM [5, 6]. Its interaction with the membrane is cooperative and depends on both the phospholipid composition and the Ca^{2+} concentration [11]. The fraction of PS in such membranes, together with the Ca^{2+} level, determines the number of Annexin V binding sites [6, 9]. Bound to the phospholipid surface Annexin V forms two-dimensional lattices, which are stabilized by protein-protein interactions [12, 13]. Probably, the first Annexin V molecule associates with its binding site comprising PS and acts as an initiator from which crystallization on the surface may proceed without the necessity for PS at high Ca^{2+} levels [13]. Binding of Annexin V to phospholipid membrane is reversible and the rates of association and dissociation are very rapid, suggesting that Annexin V does not penetrate the membrane [6, 14]. Taking these data together, the following picture emerges: Annexin V associates rapidly and reversibly with PS-containing membranes under the influence of Ca^{2+}. It then starts to crystallize two-dimensionally and may cover the whole phospholipid surface with triskelions of Annexin molecules. This process depends on phospholipid composition and Ca^{2+} level.

Cells treat PS in a specific manner which has consequences for the perception of the (patho)physiological significance of Annexin V (see below).

Phosphatidylserine localization and (patho)physiological significance

Phosphatidylserine (PS) belongs to the aminophospholipids and carries a phosphoserine headgroup, which gives this phospholipid a negative charge at pH 7.4. As was first shown for erythrocytes and platelets, PS localizes predominantly in those membrane leaflets facing the cytosol. It is now generally accepted that this PS asymmetry is ubiquitous. It is not a fixed situation but requires generation and maintenance. In 1984 Seignereut and Devaux demonstrated that the cell uses energy to generate and maintain the phospholipid asymmetry of its membranes [15]. A model exists in which various membrane-associated proteins are directly responsible for the distribution of the phospholipid species between the two leaflets of the membrane [16]. These proteins, which have not yet been identified, transport PS from the outer to the inner leaflet (aminophospholipid translocase) or vice versa (floppase), or bidirectionally (scramblase) [17]. The aminophospholipid translocase selectively transports aminophospholipids whereas the floppase and scramblase exhibit less specificity and also translocate cholinephospholipids. Under viable and nonperturbing conditions the aminophospholipid translocase activity dominates by creating a situation in which PS is exclusively localized to the inner leaflets. Blood platelets were the first cells for which it was demonstrated that a pathophysiological change in PS asymmetry is invoked by the action of agonists like thrombin and collagen [18, 19]. Stimulation of these cells results in a rise of cytosolic Ca^{2+}, which causes on the one hand inhibition of the aminophospholipid translocase, and on the other hand activation of the scramblase [20]. Within minutes the architecture of the plasma membrane is changed such that the cell exposes significant amounts of PS at its surface. Comparable mechanisms operate in erythrocyte membranes and the plasma membranes of nucleated cells [17]. Erythrocytes have Ca^{2+}-controlled regulation of phospholipid asymmetry and show an age/density-dependent accumulation of PS in the outer leaflet of the plasma membrane [21]. As was demonstrated for the lymphocyte, the nucleated cell type exposes PS at its surface during apoptosis, which is a well-organized process of cell suicide [22]. It was demonstrated that during apoptosis by lymphocytes the aminophospholipid translocase is inhibited while concomitantly a scramblase is activated [23]. Obviously the cell uses energy to maintain its surface devoid of PS and as soon as termination of existence is preluded the cell transports PS to the outer leaflet of the plasma membrane with a speed that is orders of magnitude faster than the rate of passive diffusion of phospholipids between the membrane leaflets. Hence, the PS topology seems to be of major physiological importance under viable as well as dying conditions.

The platelet plays an active role in coagulation if its PS asymmetry is perturbed by the action of agonists like thrombin and collagen. The PS, which becomes surface-exposed, catalyzes reactions of the coagulation cascade [24]. The physiological significance of this phenomenon resides in the effects of accelerating and localizing thrombin formation at the site of the activated platelet. Thrombin generated at those sites exerts diverse humoral and cellular responses of the inflammatory and haemostatic system [25]. The physiological significance of adequate catalysis of thrombin formation, e.g. cell surface exposure of PS, is underscored by Scott syndrome, which is characterized by an impaired Ca^{2+}-induced phospholipid scrambling and a moderately severe bleeding disorder [26, 27].

Ageing of erythrocytes is associated with an accumulation of PS at the cell surface [21]. This surface-exposed PS triggers the reticuloendothelial system, which probably carries an as yet unidentified receptor recognizing the PS ligand at the surface of blood cells [28]. This mechanism scavenges aged cells from the circulation

and thereby prevents PS-exposing and hence procoagulant cells continuing to circulate in the blood.

A similar mechanism of removal of unwanted cells operates in the tissues, where tissue macrophages recognize and engulf PS-exposing cells through receptor-mediated processes. Various tumour cells exhibit aberrant phospholipid asymmetry with cell surface-exposed PS, which is recognized by monocyte-derived macrophages [29]. Fadok and coworkers recognized that cell surface exposure of PS is connected with apoptosis, entailing recognition and engulfment of the dying cell by phagocytes [22]. Among other plasma membrane structures, cell surface-exposed PS appears to signal the termination of viability of the cell. This signal is picked up by phagocytes, probably through a receptor-ligand type of mechanism eliciting phagocytosis [30]. The functionality of this recognition-based removal of cells resides in the physiological need to prevent the dying cell from spilling its pro-inflammatory contents into the environment. Studies employing Annexin V as a tool have revealed that a cell in apoptosis exposes PS at its surface well before plasma membrane integrity becomes compromized (see the section below). Hence, cell surface-exposed PS is physiologically employed to entomb the dying cell before it disintegrates and is able to provoke unnecessary inflammatory responses.

Annexin V and cell surface-exposed PS, a revealing *pas de deux* of apoptosis

Over the last five years apoptosis has enjoyed a steep increase of interest, mainly because it represents a new concept of how multicellular organisms from worms to mammals regulate their cell number. Paradoxically, this concept depicts this form of cell death to be crucial for life in many ways. It became clear that apoptosis is a process which is accurately orchestrated and organized inside the cell by gene products. It is now accepted that every cell type carries the machinery to commit suicide by apoptosis. The molecular biology and biochemistry of this death machinery are starting to be unravelled and already show great diversity in the various cell types and the manner in which apoptosis is induced. Beyond this diversity three functionally distinct phases of apoptosis can be distinguished [31, 32]. The initiation phase is the most heterogeneous one in which death-inducing signals like Fas ligand and tumour necrosis factor alpha (TNFα), a lack of growth and survival signals, or DNA damage may induce the cell to prepare for suicide. This preparation results in the activation of a more general effector phase, in which the cell is able to make the decision to die. This phase is characterized by the activation of proteases of the ICE/ICE-like family (the executioner), which form a cascade amplifying the death signals [33]. The death signals probably target the mitochondrion which subsequently releases the apoptosis inducing factor (AIF), a protease which is inhibited by Z-VAD.fmk just like the ICE-like proteases. This event marks the point of no return; the cell has entered the degradation phase in which ''death'' of cytoplasm and nucleus occurs by a thousand cuts, in a way that seems common to all cells [33, 34].

Table 1. Ubiquity of cell surface exposure of PS during apoptosis.

Cell type	Apoptosis initiating stimulus
Leukocytes	Plasma membrane receptor/ligand
neutrophil	lack of growth factor
T lymphocyte	Fas/Fas ligand interaction
B lymphocyte	TNFR/TNFα interaction
monocyte	Intracellular receptor/ligand
Tissue cells	glucocorticoid
endothelial cell	Intracellular signalling
smooth muscle cell	C2-ceramide
fibroblast	staurosporine
neuron	olomoucine
Tumours	Macromolecular synthesis
leukemic cell	actinomycin D
carcinoma cell	cycloheximide
Mouse tissue	DNA
all embryonic cell types	etoposide
Plant cells	camptothecin
Nicotiana plumbaginifolia	UV irradiation

In 1992 Fadok and coworkers reported that apoptotic leukocytes expose PS at their surface, probably to serve the physiological need to remove the dying cell by phagocytosis [22]. At that time Annexin V was known as a PS-binding protein (see section above) and appreciated for its ability to bind to PS-exposing cells like activated platelets [35] and ovarian tumour cells [36]. The publication of Fadok and coworkers triggered us to investigate the binding of Annexin V to apoptotic cells. Using leukocytes it was demonstrated that Annexin V exhibits low affinity for the cell surface of the leukocyte unless apoptosis is being executed [37–39]. Combination of the vital dye propidium iodide with fluoresceinated Annexin V revealed that the apoptotic cell generates Annexin V binding sites at its surface while maintaining the plasma membrane integrity [37–39]. Competition experiments using phospholipid vesicles demonstrated that the binding site for Annexin V on the apoptotic cell comprises PS [40]. The Annexin V assay to measure cell surface exposure of PS [41] rapidly increased our knowledge of the regulation of PS exposure during apoptosis. Apoptosis-associated cell surface exposure of PS happens during the effector phase, probably downstream of the point where the mitochondrion gets involved [42] and releases AIF into the cytoplasm [43]. Indirect evidence for this notion comes from experiments with Bcl-2, which is an anti-apoptotic protein. Bcl-2 inhibits both the release of AIF from mitochondria [43] and cell surface PS-exposure of cells treated with pro-apoptotic agonists [40, 42, 44]. Studies with Jurkat cells showed that Fas-mediated apoptosis activates the executioner, which subsequently turns on the

machinery for exposing PS at the cell surface [44]. This PS-exposing machinery is probably a scramblase [23], which resides constitutively in the plasma membranes of the viable cell [44] as is the case for platelets and erythrocytes. As with platelets and erythrocytes, activation of the scramblase during apoptosis does not require the involvement of the nucleus [42, 44]. Whether the scramblase of nucleated cells is identical to the scramblase of platelets and erythrocytes remains to be shown. Using Annexin V it turned out that cell surface exposure of PS is a general phenomenon of apoptosis occurring in hemopoietic [40, 41] and tissue-embedded cells [45, 46] regardless of the initiating stimulus (table 1). This ubiquitous phenomenon also appeared to be part of apoptosis in vivo, as was shown by van den Eijnde and coworkers by injecting Annexin V-Biotin into the bloodstream of living mouse embryos [47].

A recent study showed that plant cells expose PS at their cell surface during execution of apoptosis indicating that, just like apoptosis itself, the mechanism of regulating PS asymmetry during life and death is conserved through evolution and is apparently an absolute necessity for multicellular life forms as we know them [48].

Annexin V, a potential regulator of the biological activity of PS on the surface of apoptotic cells

Annexin V has been detected in extracellular space such as blood plasma [49, 50], seminal plasma [51], amniotic fluid [50] and cell conditioned medium [52, 53]. Since the prevailing Ca^{2+} levels are around 1 mM, the presence of Annexin V in the extracellular space implies that plasma membranes containing PS in their outer leaflet are covered by Annexin V if its concentration is in the nM range or higher. As has been shown in vitro for platelets [54], endothelial cells [55], and tumour cells [36, 56] this binding to the PS membrane results in an inhibition of coagulation reactions, which depend on the catalytic activity of PS.

In vivo the platelet is the classic provider of these catalytic sites for the coagulation system. The novel insights into the regulation of PS asymmetry by nucleated cells open up the possibility that cells in apoptosis express this platelet type of function in the coagulation cascade. Apoptotic cells indeed catalyze coagulation [22, 57]. Moreover this procoagulant activity of apoptotic cells is inhibited by Annexin V [57], indicating that, in vivo, extracellular Annexin V can regulate the catalytic activity of both providers and, consequently, regulates the participation of these cells in coagulation and inflammation [25]. Under physiological conditions apoptotic cells hardly play a role in those systems because of their efficient removal by phagocytes. Pathological conditions with a high incidence of apoptotic cells have been described [58]. In this case, the phenotype of apoptosis, e.g. cell surface-exposed PS, may contribute to coagulation and inflammation and extracellular Annexin V may regulate this contribution (summarized in fig. 1). The Annexin V shielding of PS on the surface of apoptotic cells could produce an adverse effect since PS functions as a recognition signal for phagocytes. Phagocytes have developed redundancy in

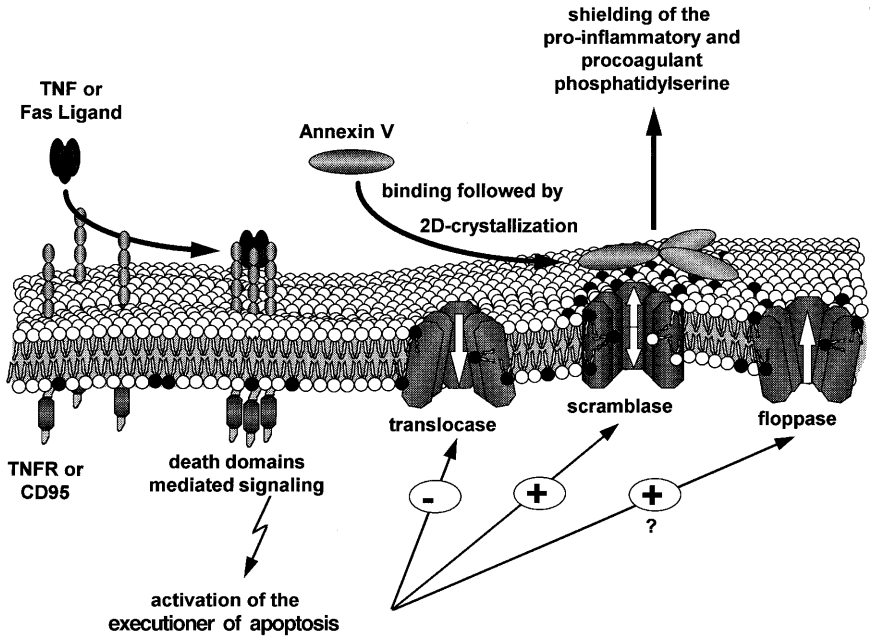

Figure 1. Schematic presentation of the regulation of PS asymmetry of the plasma membrane during life and death, and the role of Annexin V if PS (closed symbol) is exposed at the cell surface. For a detailed explanation see text.

the mechanisms by which they recognize dying cells [30]. Recently it was shown that Annexin V could not completely block phagocytosis of apoptotic bodies [46], indicating selective anticoagulant and anti-inflammatory potential of Annexin V in the interplay between PS-exposing cells and the extracellular environment.

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1 Raynal P. and Pollard H. B. (1994) Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. Biochim. Biophys. Acta **1197:** 63–93
2 Seaton B. A. (ed.) (1996) Annexins: molecular structure to cellular function. Book series: Molecular Biology Intelligence Unit, R. G. Landes Company, Austin, USA
3 Van Heerde W. L., Degroot P. G. and Reutelingsperger C. P. M. (1995) The complexity of the phospholipid binding protein annexin V. Thromb. Haemost. **73:** 172–179
4 Reutelingsperger C. P. M., Hornstra G. and Hemker H. C. (1985) Isolation and partial purification of a novel anticoagulant from arteries of human umbilical cord. Eur. J. Biochem. **151:** 625–629
5 Tait J. F., Gibson D. and Fujikawa K. (1989) Phospholipid binding properties of human placental anticoagulant protein-I, a member of the lipocortin family. J. Biol. Chem. **264:** 7944–7949
6 Andree H. A., Reutelingsperger C. P. M., Hauptmann R., Hemker H. C., Hermens W. T. and Willems G. M. (1990) Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. J. Biol. Chem. **265:** 4923–4928
7 Blackwood R. A. and Ernst J. D. (1990) Characterization of Ca2(+)-dependent phospholipid binding, vesicle aggregation and membrane fusion by annexins. Biochem. J. **266:** 195–200
8 Meers P., Daleke D., Hong K. and Papahadjopoulos D. (1991) Interactions of annexins with membrane phospholipids. Biochemistry **30:** 2903–2908
9 Tait J. F. and Gibson D. (1992) Phospholipid binding of annexin V: effects of calcium and membrane phosphatidylserine content. Arch. Biochem. Biophys. **298:** 187–191
10 Swairjo M. A., Concha N. O., Kaetzel M. A., Dedman J. R. and Seaton B. A. (1995) Ca^{2+}-bridging mechanism and phospholipid head group recognition in the membrane-binding protein annexin V. Nature Structural Biology **2:** 968–974
11 Schlaepfer D. D. and Haigler H. T. (1987) Characterization of Ca^{2+}-dependent phospholipid binding and phosphorylation of lipocortin I. J. Biol. Chem. **262:** 6931–6937
12 Mosser G., Ravanat C., Freyssinet J. M. and Brisson A. (1991) Sub-domain structure of lipid-bound annexin-V resolved by electron image analysis. J. Molec. Biol. **217:** 241–245
13 Andree H. A., Stuart M. C., Hermens W. T., Reutelingsperger C. P. M., Hemker H. C., Frederik P. M. et al. (1992) Clustering of lipid-bound annexin V may explain its anticoagulant effect. J. Biol. Chem. **267:** 17907–17912
14 Meers P. and Mealy T. (1993) Calcium-dependent annexin V binding to phospholipids: stoichiometry, specificity, and the role of negative charge. Biochemistry **32:** 11711–11721
15 Seigneuret M. and Devaux P. F. (1984) ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape change. Proc. Natl Acad. Sci. USA **81:** 3751–3755
16 Diaz C. and Schroit A. J. (1996) Role of translocases in the generation of phosphatidylserine asymmetry. J. Membr. Biol. **151:** 1–9
17 Zwaal R. F. A. and Schroit A. J. (1997) Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood **89:** 1121–1132
18 Bevers E. M., Comfurius P., van Rijn J. L. M. L., Hemker H. C. and Zwaal R. F. A. (1982) Generation of prothrombin converting activity and the exposure of phosphatidylserine at the outer surface of platelets. Eur. J. Biochem. **122:** 429–436
19 Bevers E. M., Comfurius P. and Zwaal R. F. A. (1983) Changes in membrane phospholipid distribution during platelet activation. Biochim. Biophys. Acta **736:** 57–66
20 Williamson P., Bevers E. M., Smeets E. F., Comfurius P., Schlegel R. A. and Zwaal R. F. A. (1995) Continuous analysis of the mechanism of activated transbilayer lipid movement in platelets. Biochemistry **34:** 10448–10455
21 Connor J., Pak C. C. and Schroit A. J. (1994) Exposure of phosphatidylserine in the outer leaflet of human red cells. Relationship to cell density, cell age and clearance by mononuclear cells. J. Biol. Chem. **269:** 2399–2404
22 Fadok V. A., Voelker D. R., Campbell P. A., Cohen J. J., Bratton D. L. and Henson P. M. (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J. Immunol. **148:** 2207–2216
23 Verhoven B., Schlegel R. A. and Williamson P. (1995) Mechanism of phosphatidylserine exposure, a phagocyte recognition signal on apoptotic T lymphocytes. J. Expl Med. **182:** 1597–1601
24 Rosing J., Van Rijn J. L. M. L., Bever E. M., Van Dieijen G., Comfurius P. and Zwaal R. F. A. (1985) The role of activated human platelets in prothrombin and factor X activation. Blood **65:** 319–332
25 Esmon C. T. (1993) Cell mediated events that control blood coagulation and vascular injury. Ann. Rev. Cell Biol. **9:** 1–26
26 Rosing J., Bevers E. M., Comfurius P., Hemker H. C., Van Dieijen G., Weiss H. J. et al. (1985) Impaired factor X and prothrombin activation associated with a decreased phospholipid exposure in platelets from a patient with a bleeding disorder. Blood **65:** 1557–1561
27 Weiss H. J. (1994) Scott syndrome: a disorder of platelet coagulant activity. Semin. Hematol **31:** 312–319
28 Schroit A. J., Madsen, J. W. and Tanaka Y. (1985) In vivo recognition and clearance of red blood cells containing phosphatidylserine in their plasma membranes. J. Biol. Chem. **260:** 5131–5138
29 Connor J., Bucana C., Fidler I. J. and Schroit A. J. (1989) Differentiation dependent expression of phosphatidylserine in mammalian plasma membranes: quantitative assessment of outer leaflet lipid by prothrombinase complex formation. Proc. Natl Acad. Sci. USA **86:** 3184–3188
30 Savill J. (1995) The innate immune system: recognition of apoptotic cells. In: Apoptosis and the Immune Response, pp. 341–369, Gregory C. D. (ed.), Wiley-Liss Inc, New York
31 Thompson C. B. (1995) Apoptosis in the pathogenesis and treatment of disease. Science **267:** 1465–1462
32 Kroemer G., Petit P. X., Zamzami N., Vayssiere J.-L. and Mignotte B. (1995) The biochemistry of apoptosis. FASEB J. **9:** 1277–1287
33 Martin S. J. and Green D. R. (1995) Protease activation during apoptosis: death by a thousand cuts. Cell **82:** 349–352
34 Susin S. A., Zamzami N. and Kroemer G. (1996) The cell biology of apoptosis: evidence for the implication of mitochondria. Apoptosis **1:** 231–242
35 Thiagarajan P. and Tait J. F. (1990) Binding of annexin V/placental anticoagulant protein I to platelets. Evidence for phosphatidylserine exposure in the procoagulant response of activated platelets. J. Biol. Chem. **265:** 17420–17423
36 Rao L. V., Tait J. F. and Hoang A. D. (1992) Binding of annexin V to a human ovarian carcinoma cell line (OC-2008). Contrasting effects on cell surface factor VIIa/tissue factor activity and prothrombinase activity. Thromb. Res. **67:** 517–531
37 Koopman G., Reutelingsperger C. P. M., Kuijten G. A. M., Keehnen R. M. J., Pals S. T. and Vanoers M. H. J. (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood **84:** 1415–1420
38 Homburg C. H. E., Dehaas M., Vondemborne A. E. G. K., Verhoeven A. J., Reutelingsperger C. P. M. and Roos D. (1995) Human neutrophils lose their surface Fc gamma RIII and acquire annexin V binding sites durig apoptosis in vitro. Blood **85:** 532–540

39 Vermes I., Haanen C., Steffens-Nakken H. and Reutelingsperger C. P. M. (1995) A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J. Immunol. Methods **184:** 39–52
40 Martin S. J., Reutelingsperger C. P. M., McGahon A. J., Rader J., van Schie C. A. A., LaFace D. M. et al. (1995) 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. Expl Med. **182:** 1545–1557
41 Martin S. J., Reutelingsperger C. P. M. and Green D. R. Annexin V, a specific probe for apoptotic cells. In: Techniques in Apoptosis. A User's Guide, pp. 107–120, Cotter T. G. and Martin S. J. (eds), Portland Press Ltd, London
42 Castedo M., Hirsch T., Susin S. A., Zamzami N., Marchetti P., Macho A. et al. (1996) Sequential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. J. Immunol. **157:** 512–521
43 Susin S. A., Zamzami N., Castedo M., Hirsch T., Marchetti P., Macho A. et al. (1996) Bcl-2 inhibits the mitochondrial release of an apoptogenic protein. J. Expl Med. **184:** 1331–1341
44 Martin S. J., Finucane D. M., Amarantemendes G. P., Obrien G. A. and Green D. R. (1996) Phosphatidylserine externalization during CD95-induced apoptosis of cells and cytoplasts requires ICE/CED-3 protease activity. J. Biol. Chem. **271:** 28753–28756
45 Van Engeland M., Ramaekers F. C. S., Schutte B. and Reutelingsperger C. P. M. (1996) A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry **24:** 131–139
46 Bennett M. R., Gibson D. F., Schwartz S. M. and Tait J. F. (1995) Binding and phagocytosis of apoptotic vascular smooth muscle cells is mediated in part by exposure of phosphatidylserine. Circulation Res. **77:** 1136–1142
47 van den Eijnde S. M., Boshart L., Reutelingsperger C. P. M., De Zeeuw C. I. and Vermeij-Keers C. (1997) Phosphatidylserine plasma membrane asymmetry in vivo: a pancellular phenomenon which alters during apoptosis. Cell Death Diff. In press
48 O'Brien I., Reutelingsperger C. P. M. and Holdaway K. (1997) The use of Annexin V and TUNEL to monitor the progression of apoptosis in plants. Cytometry. In press
49 Flaherty M. J., West S., Heimark R. L., Fujikawa K. and Tait J. F. (1990) Placental anticoagulant protein-I: measurement in extracellular fluids and cells of the hemostatic system. J. Lab. Clin. Med. **115:** 174–181
50 Romisch J., Schuler E., Bastian B., Burger T., Dunkel F. G., Schwinn A. et al. (1992) Annexins I to VI: quantitative determination in different human cell types and in plasma after myocardial infarction. Blood Coag. Fibrinol. **3:** 11–17
51 Christmas P., Callaway J., Fallon J., Jones J. and Haigler H. T. (1991) Selective secretion of annexin 1, a protein without a signal sequence, by the human prostate gland. J. Biol. Chem. **266:** 2499–2507
52 Koster J. J., Boustead C. M., Middleton C. A. and Walker J. H. (1993) The sub-cellular localization of annexin V in cultured chick-embryo fibroblasts. Biochem. J. **291:** 595–600
53 Solito E., Raugei G., Melli M. and Parente L. (1991) Dexamethasone induces the expression of the mRNA of lipocortin 1 and 2 and the release of lipocortin 1 and 5 in differentiated, but not undifferentiated U-937 cells. FEBS Lett. **291:** 238–244
54 Thiagarajan P. and Tait J. F. (1991) Collagen-induced exposure of anionic phospholipid in platelets and platelet-derived microparticles. J. Biol. Chem. **266:** 24302–24307
55 Vanheerde W. L., Poort S., Vantveer C., Reutelingsperger C. P. M. and Degroot P. G. (1994) Binding of recombinant annexin V to endothelial cells: Effect of annexin V binding on endothelial-cell-mediated thrombin formation. Biochem. J. **302:** 305–312
56 Sugimura M., Donato R., Kakkar V. V. and Scully M. F. (1994) Annexin-V as a probe of the contribution of anionic phospholipids to the procoagulant activity of tumour cell surfaces. Blood Coag. Fibrinol. **5:** 365–373
57 Casciola-Rosen L., Rosen A., Petri M. and Schlissel M. (1996) Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: Implications for coagulation events and antigenic spread in systemic lupus erythematosus. Proc. Natl Acad. Sci. USA **93:** 1624–1629
58 Holmgren L., O'Reilly M. S. and Folkman J. (1995) Dormancy of micrometastasis: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nature Med. **1:** 149–153

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