Dynamics of the phosphatidylserine-expressing cell surface during endocytosis of Annexin A5

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Contents

Chapter 1

General introduction

This introduction is partly based on:

CPM Reutelingsperger, E Dumont, PW Thimister, H van Genderen, H Kenis, S van de Eijnde, G Heidendal and L Hofstra. Visualization of cell death *in vivo* with the annexin A5 imaging protocol. Journal of Immunological Methods. 265(1-2):123-32 (2002).

H van Genderen, H Kenis, E Dumont, W van Heerde, L Hofstra and CPM Reutelingsperger. Cell proliferation and apoptosis. Chapter 7, Membrane alterations in dying cells. Editors, D Hughes and H Mehmet. Taylor & Francis, (2003).

The annexins

The Annexins are a family of membrane binding proteins that share structural properties and biological activities associated with membrane related processes[1]. Each annexin consists of a core structure of four domains of 70 amino acids with the exception of annexin A6 which consists of two core structures connected via a short peptide linker [2]. Members of the annexin family are able to bind to negatively charged phospholipids in the presence of calcium ions. In solution annexin molecules are present in the form of monomers. After binding to a phospholipid surface some annexin members remain bound as monomers while other annexins form a two-dimensional crystal lattice by protein-protein interactions. Two-dimensional lattice formation has been found to occur with the human annexin A4, annexin A5 and annexin A6 [3-6]. Some annexins can interact with other proteins via their amino terminal tail, which is different in length and amino acid composition for each family member. For instance annexin A1 interacts with S100A11 while annexin A2 interacts with S100A10 [7-10]. The latter can also form a tetramer complex consisting of two annexin A2 and two S100A10 proteins. Annexins are pleiotropic proteins meaning that there function is dependent on tissue location and changes in the surrounding environment. For example, annexin A1 has been proposed to act intracellularly as an inhibitor of phospholipase A2 by competing for phosphatidylserine (PS) binding sites [11-13]. Extracellular annexin A1 binds to neutrophils possibly via the amino terminal tail and accelerates apoptosis via the induction of a calcium influx into the cell [14]. Pleiotropy is further displayed by annexin A2. In the cytosol annexin A2 plays a role in vesicle trafficking during endocytosis [15, 16] but in the extracellular environment annexin A2 acts as a membrane "receptor" for tissue plasminogen activator [17] or as an opsonin for the phagocytosis of apoptotic cells [18]. Knockout mice have been created for annexin A1 and annexin A2 in order to examine the *in vivo* function of these proteins. Analysis of the annexin A1 knockout mouse has demonstrated that annexin A1 plays a role in inflammation [19]. Examination of the annexin A2 knockout mouse showed the involvement of annexin A2 in the coagulation process [20].

Exocytosis of annexins

Annexins are considered cytosolic proteins because they lack a 5'-leader sequence in their messenger RNA. However, annexin A1, annexin A2 and annexin A5 have been found in the circulation indicating that they are released into the extracellular environment [21-24]. Proteins that are released into the extracellular environment contain a leader sequence in their messenger RNA that directs them to the endoplasmatic reticulum (ER). Synthesized protein is then transported via the Golgi apparatus to the plasma membrane and subsequently into the extracellular environment [25]. A possible explanation for the presence of annexins in the circulation might be the release of cytosolic content from necrotic cells into the surrounding environment. There is however

evidence for the existence of protein release pathways independent of the ER and Golgi apparatus. For instance, interleukin-1β and fibroblast growth factor 1 (FGF-1) are proteins that lack a signal peptide and they are present in the circulation. Several non-classical exocytosis pathways have been proposed for the release of interleukin-1β and FGF-1 [26]. The plasma membrane located ABC-A1 transporter has been suggested to play a role in the release of interleukin-1β [27]. While release of FGF-1 is suggested to be mediated by a cytosol located multi-protein complex comprised of Cu^{2+}, S100A13, synaptotagmin-1 p40 and FGF-1 [28].

Similar to interleukin-1β and FGF-1, annexins have been proposed to be released by non-classical exocytosis pathways. Annexin A1, present in pituitary folliculo-stellate cells, has been found to be released into the extracellular environment when these cells are treated with the glucocorticoid dexamethasone. Release of annexin A1 occurs after serine-phosphorylation and has been proposed to be mediated by the ABC-A1 transporter [29-31]. The release mechanism for annexin A2 has not been elucidated but release occurs during a brief period of temperature stress [32]. The circumstances under which annexin A5 is released and the mechanism responsible for this release are at present unknown.

Structure and PS-binding properties of annexin A5
Annexin A5, a member of the annexin family, is a protein that binds calcium-dependently to PS-expressing membranes [33, 34]. The tertiary structure of the soluble form of Annexin A5 has been solved with X-ray crystallography by Huber *et al.* (Fig. 1a) [35]. X-ray crystallography has revealed that the tertiary structure of annexin A5 consists of a core of four domains that are arranged in a cyclic array. This arrangement gives the molecule a slightly curved shape with a convex and a concave face (Fig. 1a). Each domain consists of 5 helices linked to each other via a short turn. The calcium- and PS binding sites are located at the convex, membrane-facing side of the protein [36]. A short amino terminal tail is located at the concave site of the molecule.

The Annexin A5 core contains potential post-translational modification sites that can be O-glycosylated. In addition, the amino terminal tail contains two threonine residues which are potential sites for phosphorylation. However, annexin A5 has not been found to be glycosylated or phosphorylated. The amino terminal tail contains the only post translational modification of annexin A5: an alanine residue located acetylation [37, 38].

Electron crystallography of membrane-bound annexin A5 has revealed that annexin A5 molecules can associate with each other via protein-protein interactions (Fig. 1b) [39]. In solution annexin A5 molecules are monomers, but upon binding to a PS-containing surface they self-assemble into a two-dimensional crystal lattice. The annexin A5 trimer is the basic building block of the two-dimensional crystal lattice (Fig. 1c). The two-dimensional crystal lattice consists of trimers, dimers of trimers and trimers of trimers arranged with p3 or

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p6 symmetry [40]. The type of two-dimensional crystal that is formed depends on the content of PS in the membrane. The two-dimensional lattice with p6 symmetry forms at a PS content that closely matches that found in biological membranes. The crystal with p3 symmetry is formed at a phospholipid membrane containing a higher PS content [40].

Electron crystallography has further revealed that the membrane bound form of the annexin A5 monomer has nearly the same structure as the soluble form [41]. This suggests that the various domains have an unequal distance to the phospholipid membrane surface. Both electron crystallographic data and atomic force microscopy suggest that domain III is the most distant from the membrane surface and domain II the closest [41, 42]. The convex shape of membrane bound annexin A5 potentially influences the shape of the PS-expressing membrane surface. All four domains contain a calcium binding motif making it possible that membrane-bound annexin A5 influences the PS-expressing membrane surface to adopt a shape that is complementary to the convex shape of the annexin A5 molecule [41].

Zinc ions affect the calcium-dependent binding of annexin A5 to PS-expressing membranes [34]. Annexin A5 does not bind to PS-containing membrane surfaces when zinc ions are present and calcium ions are absent. However, the presence of zinc ions in addition to calcium increases the binding affinity of annexin A5 for PS. The zinc-induced increase in binding affinity has been attributed to a cysteine residue in the annexin A5 molecule located at amino acid site 315 (unpublished data, Reutelingsperger *et al.*). A zinc ion possibly binds to the cysteine-315 residue and influences the calcium-dependent binding affinity via an unknown mechanism. Zinc ions are present in the circulation in a concentration of 12-16 µmol L^{-1} [43]. This possibly leads to an increase in binding affinity of annexin A5 for PS-expressing membrane surfaces when annexin A5 is present in the circulation.
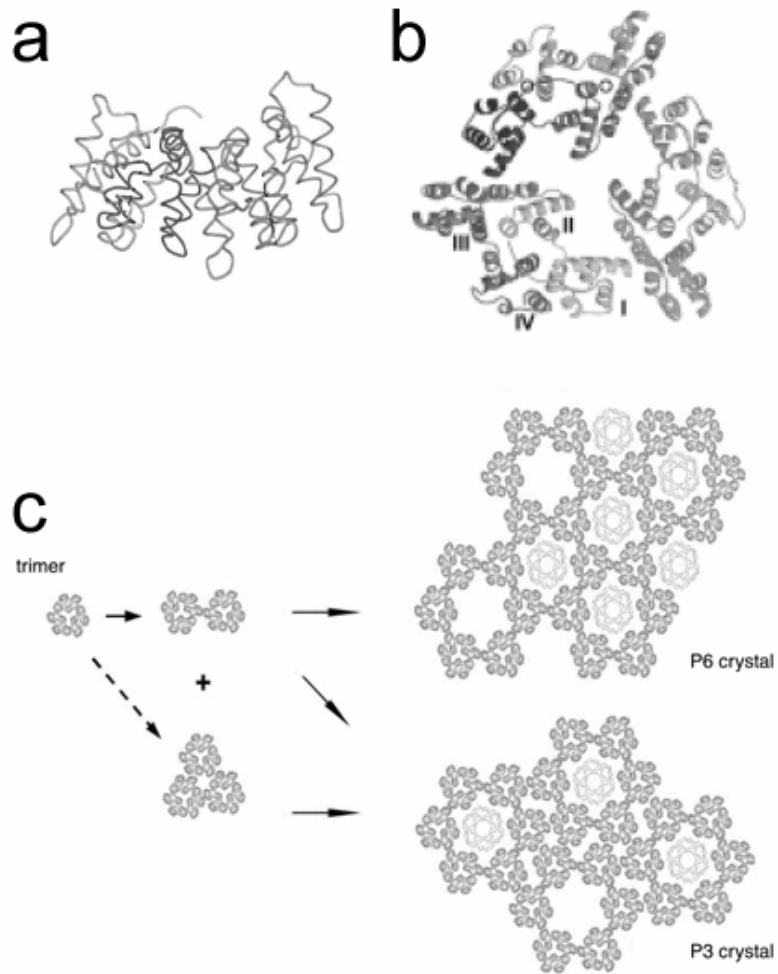
Figure 1, **Molecular structure of annexin A5.** Side view of a model of a monomeric annexin A5 molecule (a), top view of a model of three annexin A5 molecules organized in a membrane bound trimer, each annexin A5 molecule is oriented in such a manner that domain II is localized in the inside and domain IV at the outside of the trimer (b) [Adapted from Sopkova *et al*, 1993; numbering of annexin A5 domains according to Huber *et al*, 1990], (c) top view of a model of an annexin A5 trimer building block organized into a dimer of trimers and a trimer of trimers with p6 and p3 symmetry [Adapted from Oling *et al*, 2001].

Biological functions of annexin A5

Similar to other annexins, annexin A5 has been found to be a pleiotropic protein. For example, membrane interactions of annexin A5 can occur in a calcium-dependent and independent manner. In addition, membrane interactions can occur when annexin A5 is present in the intra- and extracellular environment.

Data obtained from *in vitro* studies have suggested that annexin A5 functions in the intracellular environment as an inhibitor of phospholipase A2. Phospholipase A2 is an enzyme that uses PS as a substrate in the formation of free fatty acid and 2-lysophospholipid [44]. The free fatty acid in the form of arachidonic acid that is released by phospholipase A2 plays a role in signal transduction processes. Studies with artificial membranes or membranes derived from lysed cells show that binding of phospholipase A2 to PS-containing membranes can be inhibited by annexin A5 [45-47]. Inhibition of phospholipase A2 activity by annexin A5 in these model systems occurs by competition for PS binding sites in a calcium-dependent manner. However, inhibition of phospholipase A2 by annexin A5 has not been studied in intact cellular systems. Thus, it is not known whether annexin A5 functions in the intracellular environment as an inhibitor of phospholipase A2.

Annexins have been proposed to function as calcium conducting ion channels [48, 49]. How calcium ions are channeled through the plasma membrane has been an object of debate because annexins are peripheral membrane proteins. In an early model that attempted to explain the calcium conducting behavior of annexin A5 the calcium ions were supposed to flow through the hydrophilic center of the annexin molecule [50-52]. In this model annexin A5 was located peripheral to the plasma membrane. Recent models have incorporated the idea that during calcium conduction the annexin molecule is inserted into the cytosolic site of the plasma membrane. Membrane insertion occurs in an environment with a very low calcium concentration. Moss *et al*. have shown that annexin A5 is able to associate with phospholipid membranes in a calcium-independent manner in the presence of peroxide. They subsequently proposed that calcium is conducted across the membrane after a peroxide-induced insertion of annexin A5 into the plasma membrane at neutral pH [53]. Another model is proposed by Haigler *et al*. In this model, calcium is conducted when human annexin A5 or hydra annexin B12 is inserted into the plasma membrane in a calcium-independent manner at a mildly acidic pH [54]. The annexin B12 molecule is proposed to change conformation so that the protein traverses the entire bilayer in such a manner that ions are able to cross the membrane [55]. There is at present, however, no direct structural evidence from röntgen or electron diffraction analysis studies that support these models.

The capacity of annexin A5 to bind calcium-dependently to PS has lead to the proposal that annexin A5 plays a role in the anti-thrombotic arm of blood coagulation [56]. PS expressed by platelets supports the formation of the prothrombinase complex and the subsequent formation of thrombin [57]. *In vitro*

studies have shown that shielding of PS binding sites by annexin A5 inhibits the formation of thrombin by the prothrombinase complex [58]. Rand and co-workers have suggested that the anti-thrombotic behavior of annexin A5 plays a role in coagulation processes in the placenta of pregnant woman [59]. Thrombosis is prevented in the placenta by shielding PS-expressing membrane surfaces through the formation of the two-dimensional crystal lattice of annexin A5. Disruption of the two-dimensional crystal lattice by anti-phospholipid antibodies is proposed to be responsible for recurrent pregnancy losses in women with the phospholipid antibody syndrome [60, 61].

Brachvogel and co-workers have created an annexin A5 knock-out mouse to examine the *in vivo* function of annexin A5 [62]. Analysis of various physiological parameters of the knock-out mouse showed that the phenotype of the knock out mouse was not different when compared to the wild type mouse. A possible explanation for this result is that other annexins have an identical function as annexin A5. Possible candidates are annexins that have a close evolutionary relationship to annexin A5 such as annexin A4 and annexin A6 [63]. The creation of a knock-out mouse in which more than one annexin gene is deleted may be necessary to determine the function of annexin A5 *in vivo*.

Cell death by apoptosis

This thesis focuses on the cellular processes that are influenced by the calcium-dependent binding of annexin A5 on PS-expressing cell surfaces. Cell surface expression of PS is one of the hallmarks of apoptosis. In several studies described in this thesis we have chosen to examine the interaction of annexin A5 with apoptotic PS-expressing cell surfaces. Therefore we describe in some detail the biochemical and cellular processes which occur during apoptosis in the following sections of the introduction.

Apoptosis

Unwanted cells in developing tissues and damaged or senescent cells are removed by phagocytes after induction of cell death [64-66]. The most common form of cell death is apoptosis. Apoptosis is a cellular process in which the members of a family of proteases, the so-called caspases, selectively cleave target proteins [67]. The cleavage of these proteins results in the breakdown of the cell in an ordered manner. Caspases are cysteine proteases, which reside in the cytosol as inactive zymogens. After cleavage they become active and target aspartate residues on substrate proteins. Caspases are divided into initiator and executioner caspases. Initiator caspases such as caspase 8 and 9 are involved in the first step of activating the caspase cascade [68, 69]. They cleave proteins involved in the apoptotic machinery such as other pro-caspases. Executioner caspases such as caspase 3, 6 and 7 are mainly involved in the cleavage of cellular substrates. Some of the more profound phenotypic changes that occur after caspase activation in apoptotic cells are rounding-up of the cell, cell surface expression of PS [70], the formation of plasma

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membrane blebs, shedding of plasma membrane derived apoptotic bodies and micro-particles [71, 72], chromatin condensation, fragmentation of DNA [73] and in the final phase cellular shrinkage [74]. Caspases are activated either via the extrinsic or the intrinsic pathway.

Extrinsic pathway of caspase activation
Activation via the extrinsic pathway occurs via death receptors present on the plasma membrane such as the CD95/Fas-receptor, TRAIL-receptor and the tumor necrosis factor-α (TNF-α)-receptor [75]. An extensively studied activation pathway is the CD95/Fas-receptor-induced apoptotic pathway. The Fas-receptor is present in the plasma membrane as a monomer when this pathway has not been activated. However, after binding of Fas-ligand, the monomers co-localize and form trimers in a structure called the death inducing signaling complex (DISC) [76, 77]. The cytoplasmic domain of each receptor in the DISC is linked via an adaptor protein (FADD) to a pro-caspase 8 molecule. Receptor trimerization brings each pro-caspase 8 present in the DISC in close proximity to another pro-caspase 8. The presence of two pro-caspase 8 molecules in close proximity to each other results in a catalytic process in which each pro-caspase 8 cleaves its neighbor [78]. Activated caspase 8 cleaves pro-caspase 3 and/or the protein BID into truncated BID. Truncated BID is involved in the release of pro-apoptotic proteins from the inner membrane of the mitochondrion [79]. Activated caspase 3 cleaves the various proteins present in the cell that contain a caspase 3 cleavage site.

Intrinsic pathway of caspase activation
The intrinsic pathway is not activated via ligation of plasma membrane-located death receptors but via activation of intracellular pathways. A diverse array of stimuli like ultraviolet radiation, chemical agents such as etoposide, cisplatin or staurosporin and deprivation of serum factors can cause the activation of these pathways [80-82]. The various pathways converge on the release of proteins present in the inner membrane of the mitochondrion into the cytosol [83]. The proteins which are released include cytochrome C, apoptosis inducing factor (AIF), endonuclease G and Smac/DIABLO. The next step is the formation of a complex known as the apoptosome from released cytochrome C, apoptotic protease activating factor (Apaf-1), pro-caspase 9 and ATP [84, 85]. The apoptosome activates the caspase cascade by converting inactive pro-caspase 9 into active caspase 9. One of the first targets of caspase 9 in the caspase cascade is pro-caspase 3 which is converted into its activated state. The intrinsic and the extrinsic pathway cleave identical substrates after activation of caspase 3.
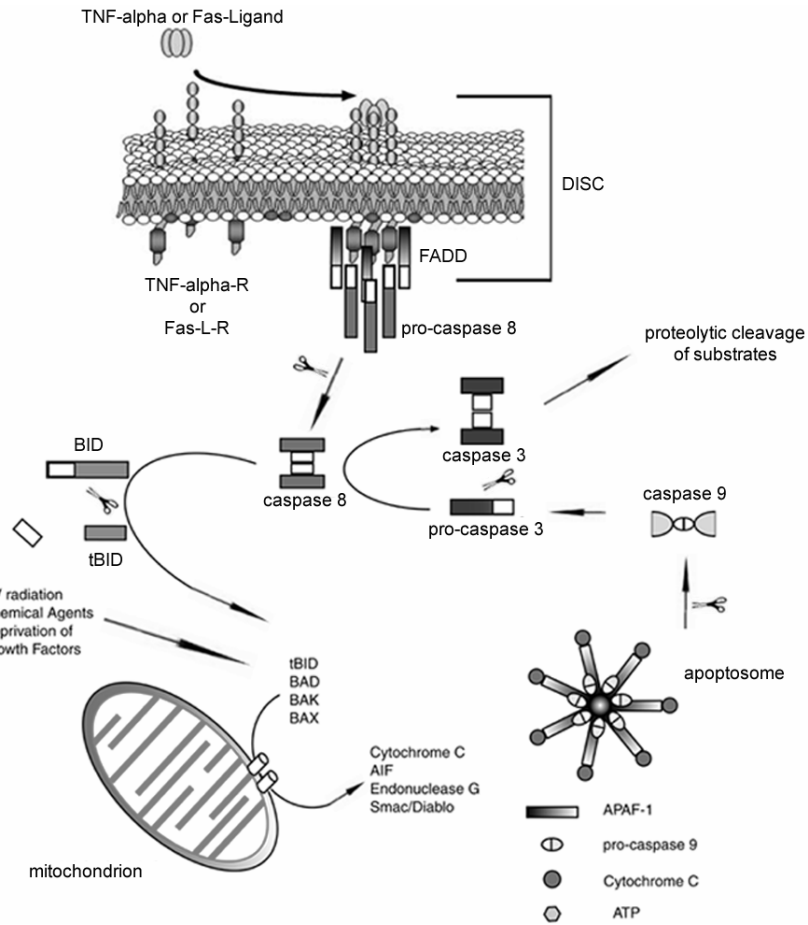
Figure 2, **The caspase activation cascade.** Simplified model depicting the activation of the intrinsic and extrinsic caspase activation pathway. (Further details are given in the text).

Phospholipid translocation across the plasma membrane
The interior of a cell is separated from the extracellular environment via the plasma membrane. The plasma membrane consists of a phospholipid bilayer which is composed of charged aminophospholipids, like phosphatidylserine (PS) and phosphatidylethanolamine (PE) which reside predominantly in the inner leaflet and uncharged phospholipids, such as phosphatidylcholine (PC) and sphingomyelin (SM), which reside in the inner and outer leaflet [86].
In normal healthy cells this asymmetry of the plasma membrane is actively maintained, however asymmetry is lost in activated platelets adhered to

collagen or in dying cells [87, 88]. Loss of membrane asymmetry on the cell surface is however not restricted to dying cells. In recent years several studies have appeared that report on cell surface expression of PS in specific circumstances independent of cell death. PS expression occurs on the cell surface of leukocytes for instance on monocytes that differentiate into macrophages, positively selected B-cells and on a subpopulation of T-lymphocytes. Cell surface expression of PS can also occur after stimulation of monocytes and activated neutrophils with the β-galactoside-binding proteins galectin-1, -2 and -4 [89-93]. Also, myoblasts fusing into myotubes transiently express PS on their cell surface [94].

It is thought that the active maintenance of phospholipid asymmetry is carried out by peripheral membrane proteins or by proteins present in the plasma membrane. Several transporters have been postulated to exist [95]. Inward translocation is mediated by the aminophospholipid translocase. Translocation is ATP-dependent and is specific for PS and with a lesser efficiency also for PE. A rise in intracellular calcium concentration causes inactivation of the inward translocation process. A possible candidate for the aminophospholipid translocase, a P-type ATPase, has been identified by Tang and co-workers [96]. Scramblase is an anti-porter that translocates phospholipids from the outer leaflet to the inner leaflet and vice versa. Translocation occurs in an ATP-independent manner and activation occurs after a sufficient rise in intracellular calcium concentration. Sims and co-workers have proposed that the members of a gene family known as the phospholipid scramblase family are responsible for bi-directional translocation of phospholipids [97, 98]. Recent results have however casted doubt on this claim because a phospholipid scramblase-1 knockout mouse showed no defects in PS-expression and haemostatic processes [99]. Outward translocation is carried out by ABC-C1, a member of the multi drug resistance (MDR) protein family [100, 101].

The intracellular pathways involved in the inactivation of the aminophospholipid translocase and the activation of scramblase and ABC-C1 are mostly unknown. PS expression on the cell surface of dying cells is dependent on active caspase 3 during Fas-ligand-induced apoptotic cell death and active cathepsin B during TNF-α-induced cell death [102, 103]. How caspase 3 and cathepsin B inactivate the aminophospholipid translocase and activate scramblase and ABC-C1 is at present unclear. It is thought that caspase 3 and cathepsin B act on intermediate proteins which are responsible for (in)activation of the phosphoplipid translocation proteins. The pathways responsible for cell surface expression of PS independent of cell death are currently unknown.
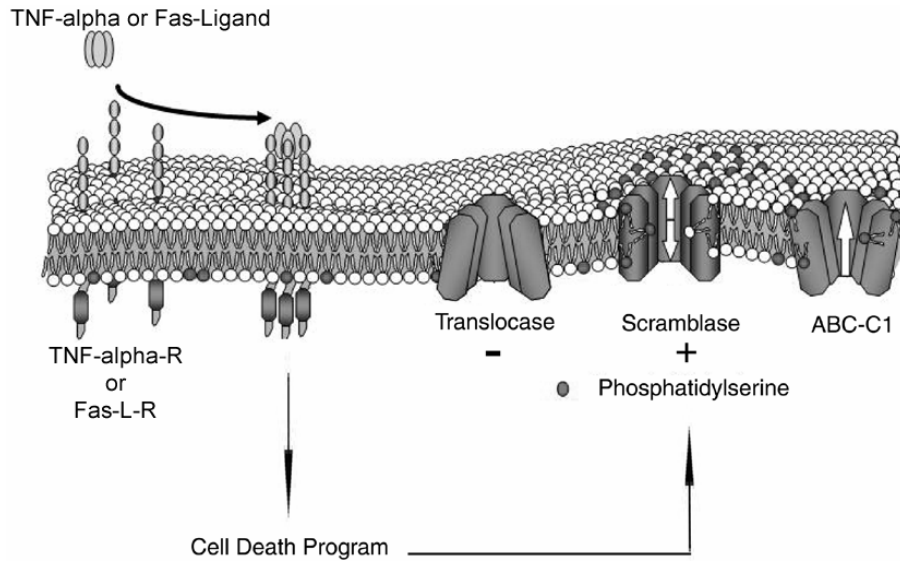
Figure 3, **Translocation of plasma membrane localized phospholipids.** Model depicting the translocation of phospholipids between the plasma membrane leaflets during cell death.

Phagocytosis of dying cells

Removal of dying cells occurs mainly by professional phagocytes such as macrophages and dendritic cells [66]. Other cells such as high endothelial venule cells or fibroblasts have also been found to clear dying cells [104, 105]. The difference between professional phagocytes and other cells appears to be the time involved in the clearance process. Professional phagocytes remove dying cells much faster than non-professional phagocytes [106].

Interaction of phagocytes with dying cells occurs in several phases: recognition, tethering, phagocytosis and anti-inflammatory responses. Dying cells can be recognized by various molecular markers present on the plasma membrane. The best-explored molecular marker is PS but other markers such as the protein ICAM-3 present on dying leukocytes and an altered sugar composition on the plasma membrane have also been found [107-111]. Dying cells release a chemotactic lipid compound, lyso-phosphatidylcholine, which attracts monocytic cells and professional phagocytes [112]. Cell surface-expressed PS on dying cells has been proposed to be recognized by a putative PS-receptor [113]. The identity of this PS-receptor has been disputed because a PS-receptor knockout mouse showed no defects *in vivo* or *in vitro* in the clearance of apoptotic cells [114, 115]. In addition, the protein identified as the PS-receptor has been located inside the nucleus of cells suggesting that the proposed PS-receptor is in fact a nuclear protein [116, 117].

Recognition of dying cells is further mediated by cell surface adhered PS binding opsonins such as milk-fat-globule-E8 (MFG-E8), protein S, annexin A1 and annexin A2 [118-120, 18]. Tethering of the phagocyte to the dying cell is followed by phagocytosis via a macropinocytic-like mechanism [121]. Phagocytes that have engulfed dying cells further prevent an immune response by releasing the anti-inflammatory mediators TGF-beta, platelet activating factor (PAF) and prostaglandine E2 (PGE2) [122].

Imaging of cell death *in vivo* with annexin A5

Fadok *et al*. showed in their 1992 paper that dying cells express PS on the plasma membrane [107]. An *in vitro* assay was developed by Reutelingsperger *et al*. in 1994 to measure PS-expression by staining dying cells with annexin A5 [123, 124]. The following years many *in vitro* studies were published that showed the usefulness of this assay. These studies focused on PS expression of dying cells in *in vitro* systems. In 1997 van den Eijnde *et al*. published a study which showed that dying cells could be visualized in the complex environment of developing tissue [125]. They visualized dying cells by injecting a mouse embryo with biotin labeled annexin A5. The mouse was subsequently sacrificed and embedded in paraffin. Peroxidase conjugated streptavidin was then used to show binding of biotin-labeled annexin A5 to PS-expressing cells in coupes of the interdigiting tissue in the mouse paw. Visualization of dying cells in tissue was further demonstrated by Dumont *et al*. in a mouse model of the ischemic heart [126]. The coronary artery of the mouse was ligated for 30 minutes and reperfusion was allowed for 90 minutes. Biotin-labeled annexin A5 was injected into the circulation before sacrifice and paraffin embedding of the heart. Annexin A5 localization was visualized in a similar manner as van den Eijnde *et al*. These studies showed that dying cells could be visualized in tissues during development and during pathological conditions. One drawback of the procedure used in these studies was that changes in the occurrence of dying cells could not be followed in time because the animals were sacrificed for histological analysis. This problem was resolved by using radio nuclide and fluorescence-labeled annexin A5. In two studies performed by Blankenberg *et al*. it was shown that technetium-99m (Tc-99m) labeled annexin A5 could be used in mice to visualize apoptosis *in vivo* without sacrificing the target organ [127, 128]. A similar result was obtained by Dumont *et al.* using fluorescence-labeled annexin A5 [129].

Imaging studies performed by van den Eijnde *et al* in 2001 and by Mari *et al.* in 2004 showed that fluorescence-labeled annexin A5 was not only localized on the plasma membrane of myoblast and apoptotic neural cells but also in the cytosol suggesting that annexin A5 was taken up by these cells [94, 130]. The endocytic pathway responsible for annexin A5 uptake was however not further investigated in these studies.

The annexin A5 methodology was further extended into a clinical setting by using Tc-99m-labeled annexin A5. Tc-99m-labeled annexin A5 has been used

with success to image apoptosis in a patient with a cardiac tumor [131]. Tc-99m-labeled annexin A5 has also been used in patients with cardiac allograft rejection, myocardial infarction and unstable atherosclerotic plaques [132-135]. One of the current drawbacks of the use of annexin A5 in patients is the unfavorable biodistribution of annexin A5 [136]. The clearance process carried out by the liver and the kidneys results in a high accumulation of annexin A5 in these organs independent of the presence of dying cells. Accumulation of conjugated annexin A5 leads consequently to a high back ground signal in these organs.

A short overview of endocytic pathways
Our investigations into the consequences of annexin A5 binding to cell surface expressed PS revealed that annexin A5 is internalized by cells that express PS on the cell surface. Here we describe the endocytic pathways that are used by cells to internalize proteins, present in the extracellular environment, into the cytosol.

The exchange of large molecules between the extracellular environment and the interior of a cell is accomplished via the process of endocytosis. Endocytosis is characterized by uptake of extracellular material in membrane encapsulated vesicles. There are several endocytic pathways present in cells, the clathrin-mediated pathway, the caveolar pathway and the macropinocytic pathway [137]. Internalization via the clathrin-mediated pathway occurs at coated pits; these are specialized sites on the plasma membrane responsible for the internalization of cell surface receptors and ion-channels. Internalization is initiated after crystallization of the clathrin/AP-2 complex at the cytosolic side of the plasma membrane. The plasma membrane is curved inward by the two-dimensional crystal lattice of the clathrin/AP-2 complex. Membrane curvature leads to the formation of a spherical vesicle which is fissured from the plasma membrane in a GTP-dependent manner by dynamin proteins.

Another internalization pathway is mediated by lipid micro-domains on the cell surface known as caveolae [138]. Caveolae are a subset of discrete membrane compartments known as lipid rafts. They consist of plasma membrane invaginations comprised of polymerized caveolin-1 proteins, cholesterol and glycosphingolipids. Fission of vesicles from the plasma membrane is mediated by dynamin proteins. Caveolae have been found to play a role in the internalization of plasma membrane proteins such as tissue factor [139].

Macropinocytosis is involved in the non-specific internalization of solutes [140]. Internalized vesicles, macropinosomes, are formed at sites of membrane ruffling. They contain an actin coat and they are heterogeneous in size. Macropinocytosis occurs constitutively in macrophages, dendritic cells and in some types of tumor cells [141-143].

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Aim of this thesis

The aim of this thesis was to examine the effect of PS binding and two-dimensional crystal formation of annexin A5 on the dynamics of the PS-expressing cell surface. In chapter 2 of this thesis we describe the concept that annexin A5 can be used as a tool to detect cell surface expression of PS. We define the cell physiological processes under which cell surface expression of PS occurs and we describe the experimental conditions necessary for annexin A5-PS binding. In chapter 3 we examined cell surface changes of apoptotic Jurkat cells. We found that annexin A5 is able to inhibit the formation of PS-expressing microparticles generated by apoptotic Jurkat cells. We further found that annexin A5 is internalized by PS-expressing apoptotic Jurkat cells and viable HeLa tumor cells. We found that internalization of annexin A5 on cell surface expressed PS is dependent on trimer formation of annexin A5. In chapter 4 we examined whether annexin A5 induces internalization of cell surface expressed tissue factor. And in chapter 5 we examined the plasma membrane changes that occur during etoposide-induced apoptosis in THP-1 monocytic cells using annexin A5 to detect the onset of apoptosis. We found that annexin A5-binding monocytic cells have lost the capacity to roll and adhere to P-selectin exposing platelets. We discovered that this is the result of plasma membrane changes occurring at the onset of the apoptotic process. This thesis ends with a general discussion in which the relevance of the results presented in this thesis are discussed in relation to current knowledge.

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Chapter 2

Visualizing cell death through the phosphatidylserine binding property of annexin A5

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Summary

One of the hallmarks of cell death is cell surface expression of phosphatidylserine. Expression of phosphatidylserine at the cell surface can be measured *in vitro* with the phosphatidylserine-binding protein annexin A5 conjugated to fluorochromes. This measurement can be made by flow cytometry or by confocal scanning-laser microscopy. The annexin A5 affinity assay comprises the incubation of cells stimulated to execute cell death with fluorescence-labeled annexin A5 and propidium iodide. Living cells are annexin A5 negative and propidium iodide negative, cells in the early phases of cell death are annexin A5 positive and propidium iodide negative, and secondary necrotic cells are annexin A5 positive and propidium iodide positive. The entire procedure takes about 30 minutes for flow cytometry and 45 minutes for confocal scanning-laser microscopy. Various precautions and considerations are discussed further in the protocol described here.

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Introduction

Of the various methods available for detecting dying cells, the measurement of phosphatidylserine (PS) expressed at the cell surface is the most rapid and convenient. PS is an aminophospholipid that resides in the inner leaflet of the plasma membrane of living cells. In dying cells, PS is actively externalized to the plasma membrane's outer leaflet that faces the extracellular environment [1]. PS serves as an 'eat me' signal recognized by phagocytes via a putative PS receptor and via a plethora of proteins that opsonize the PS-expressing membrane surface [2]. PS expressed at the cell surface can be measured with the PS-binding protein annexin A5 conjugated to fluorochromes by flow cytometry and by confocal scanning-laser microscopy (CSLM). In conjunction with the permeability probe propidium iodide, a distinction can be made between dying cells with intact plasma membrane integrity and (secondary) necrotic cells [3, 4].

Cell death

The multicellular organism strives to maintain a balanced number of cells by regulating cell creation versus cell demise. The latter occurs mainly through developmental cell death. Developmental cell death is part of a set of various types of cell death that can become manifest in morphologically and biochemically distinct forms depending on the cell type and the trigger inducing cell death. These types of cell death include apoptosis, anoikis, necrosis, mitotic catastrophe, autophagy, excitotoxicity, wallerian degeneration and cornification[5]. Apoptosis, the most prominent form of cell death, is defined by changes occurring at the morphological level, such as rounding-up of the cell, retraction of pseudopodes, reduction in cellular volume (pyknosis), condensation of chromatin, fragmentation of the nucleus (karyorhexis), little or no ultrastructural modification of cytoplasmic organelles, plasma membrane blebbing and the maintenance of an intact plasma membrane until late phases of the process. Anoikis is defined as apoptosis induced by loss of attachment to the substrate or to other cells. Necrosis is also defined by alterations at the morphological level, such as cytoplasmic swelling, mechanical rupture of the plasma membrane, dilation of cytoplasmic organelles and moderate chromatin condensation. Cell death by mitotic catastrophe occurs during or shortly after a dysregulated or failed mitosis. This form of cell death is characterized by the presence of micronuclei (chromosomes that have not been distributed evenly between two daughter cells) or 'multi-nuclei' (more than one nucleus). Autophagy is defined as a form of cell death without chromatin condensation. Cell death is accompanied by morphological changes in the cytoplasm, such as the appearance of two-membraned autophagic vacuoles that contain degenerating organelles or cytosol. The other forms of cell death, excitotoxicity and wallerian degeneration, which occur in the nervous system, and the cornification of epidermal cells, have been incompletely characterized. The various types of cell death are not strictly defined by biochemical analysis alone.

For example, DNA laddering is a frequent phenomenon during apoptosis but does not occur with all forms of apoptosis. Furthermore, caspase activation is one of the hallmarks of apoptotic cell death, but inhibition of the caspase cascade does not always prevent cell death. However, it has become evident that most forms of cell death share the phenomenon of cell surface expression of PS [6]. Externalization of PS is an early event in the sequence of steps leading to cell death; it starts well before the plasma membrane integrity is compromised [7]. PS is therefore a likely ligand for target-specific measurement of cell death. Green fluorescent annexin A5 is the most widely used target-specific probe for measurement of cell death. The annexin A5 affinity assay discriminates among living cells, cells in the early phase of cell death and (secondary) necrotic cells that have a compromised cell membrane [3, 4].

Cell death–independent PS externalization
The molecular pathways involved in the externalization of PS to the cell surface are mostly unknown. Expression of PS relies on activated caspase 3 in CD95-induced apoptotic cell death, whereas cathepsin B activity is necessary for PS expression in tumor necrosis factor-α–induced caspase-independent cell - death [8, 9]. Several studies have shown that pathways exist that cause cell surface expression of PS by living cells in specific conditions. Cell surface expression of PS has been noted in many types of leukocytes, such as monocytes differentiating into macrophages, a subpopulation of T lymphocytes and positively selected B lymphocytes [10–12]. Furthermore, some cells can be stimulated to express PS independently of cell death, such as stimulation of monocytes and formyl-Met-Leu-Phe–activated neutrophils with the β-galactoside-binding protein galectin-1 [13]. Also, myoblasts fusing into myotubes transiently express PS at their cell surface [14]. Hence, targeting PS to measure cell death would produce false-positive results in those cases.

The annexin A5 affinity assay to measure cell death
Annexin A5 is a member of the annexin protein family that binds in a calcium-dependent way to PS-containing membranes [15]. The tertiary structure of annexin A5 consists of a core of four domains arranged in a cyclic array, giving the molecule a slightly curved shape with a convex face and a concave face. The calcium- and PS binding sites are located at the convex, membrane-facing curve of the protein. Annexin A5 binds extrinsically and reversibly to PS-expressing membranes with a K d in the nanomolar range. The annexin A5 affinity assay is based on the incubation of unfixed cells with annexin A5 conjugated to a detectable flag such as a fluorochrome and with the vital dye propidium iodide. Other vital dyes such as 7-amino-actinomycin D can be used instead of propidium iodide. The annexin A5 affinity assay measures the fraction of cells that are annexin A5 negative and propidium iodide negative; annexin A5 positive and propidium iodide negative; or annexin A5 positive and

propidium iodide positive. The assay requires that the following conditions be met or avoided:

1. The incubation mixture should contain Ca^{2+} ions at a concentration between 0.5 mmol L^{-1} and 3 mmol L^{-1}. The preferred concentration for Ca^{2+} is 1 mmol L^{-1}. The Ca^{2+} concentration should not exceed 5 mmol L^{-1}, because annexin A5 increases affinity toward other phospholipid species such as phosphatidylcholine with increasing Ca^{2+} concentration. Bound annexin A5 dissociates from the PS-containing membrane if the Ca^{2+} ions are removed, such as by chelation with EDTA.

2. The mixture should not be buffered on the basis of phosphates. Recommended buffers are HEPES and Tris-hydroxymethylaminoethane. The assay can also be done in cell culture medium. Measurements in RPMI 1640 medium are not recommended because of the relatively high concentration of phosphate and low concentration of Ca^{2+}. Media that have been found to satisfy the PS-binding conditions of annexin A5 include DMEM and Medium 199.

3. Flow cytometry of adherent cell lines requires the detachment of cells from the culture flask or plate. However, trypsinization of adherent cells has been found to induce binding of annexin A5, leading to false-positive results. False-positive results are prevented when cells are detached by scraping after annexin A5–propidium iodide incubation [16]. Flow cytometry of the resulting cell suspension produces an annexin A5–versus–propidium iodide dot plot with living cells (annexin A5 negative and propidium iodide negative), living cells with a compromised membrane due to scraping (annexin A5 negative and propidium iodide positive), dying cells (annexin A5 positive and propidium iodide negative) and secondary necrotic cells (annexin A5 positive and propidium iodide positive).

4. Cells should not be fixed before incubation with annexin A5. Fixation with paraformaldehyde induces cell surface expression of PS on living cells, resulting in false-positive results. If fixation is required, the cells should be incubated with annexin A5 first, then be washed with Ca^{2+}-containing buffer and subsequently be fixed in a buffer containing 1–3 mmol L^{-1} Ca^{2+} ions.

5. The calcium-dependent PS-binding properties of annexin A5 are lost below a pH of 5.2 and with prolonged incubation above a temperature of 42°C.

6. Fluorescence-labeled annexin A5 should be stored refrigerated at 2–8°C. It should not be stored frozen because it will lose biological activity because of dimerization.

Measurement of cell death: a comparison of various assays
The progress of cell death is often assessed by measurement of caspase activation and with the TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling) method. Caspases are proteolytic enzymes that exist as inactive zymogens in living cells. Induction of apoptosis leads to the activation of caspases by proteolytic cleavage of the inactive zymogen into the active, cleaved caspase. Activated caspases can be detected by Western blot

with antibodies to cleaved caspase fragments and by flow cytometry using fluorescent probes [17, 18]. Caspases are activated early in the apoptotic process before PS expression, so with this method it is possible to detect cells at the onset of apoptosis [8]. Assessment of caspase activity consumes more time than the annexin A5 affinity assay does. Furthermore, caspase activation does not occur in all types of cell death. The TUNEL method consists of the visualization of fragmented DNA in the nucleus [19]. Fragmentation of DNA occurs in the late stages of cell death, after PS expression has started [7]. This method consists of many steps and is consequently time consuming. Furthermore, degradation of DNA does not occur in all types of cell death; thus, assessment of cell death with TUNEL may lead to false-negative results. Annexin A5 can be conjugated with various fluorochromes, such as fluorescein isothiocyanate, FP488, Alexa Fluor dyes, phycoerythrin and carbocyanines. This offers versatility for use with other fluorescence-conjugated probes, such as in multicolor flow cytometry. As annexin A5 detects various forms of cell death, it is recommended that more than one assay be used for studying a particular type of cell death. For example, caspase activity, DNA fragmentation and cell-morphological characteristics can be assessed in addition to the annexin A5 affinity assay.

Materials
Reagents
. Antibody to Fas (the receptor for Fas ligand; clone 7C11; Immunotech) or other cell death–inducing agents
. Binding buffer (see Reagent setup)
. Fixation buffer (see Reagent setup)
. Green fluorescent annexin A5 (Nexins Research BV, Invitrogen, Interchim)
. Propidium iodide (Invitrogen)
. Ionomycin (Sigma)
. Paraformaldehyde (Merck)
. Bovine serum albumin (Sigma)
. Nail polish
. Glycerol

Equipment
. Flow cytometer
. Confocal scanning-laser microscope

Reagent setup
10x concentrated binding buffer:
The 10x concentrated binding buffer is 250 mmol L^{-1} HEPES plus NaOH, pH 7.4, 1.4 mol L^{-1} NaCl and 10 mmol L^{-1} CaCl$_2$. Dilute 10x concentrated binding buffer 1:10 in water for use, to 25 mmol L^{-1} HEPES plus NaOH, pH 7.4, 140 mmol L^{-1} NaCl and 1 mmol L^{-1} CaCl$_2$. Store binding buffer at 2–8°C. The binding buffer will be stable for 6 months if sterilized by filtration through a filter with a pore size of 2 µm.
4% paraformaldehyde fixation buffer:
To prepare 100 mL of 4% (wt/vol) paraformaldehyde fixation buffer, dissolve 4 g paraformaldehyde in 80 mL distilled water. Add some drops of 5 mol L^{-1} NaOH and heat the solution to facilitate dissolution of the paraformaldehyde. When the paraformaldehyde is dissolved, add 10 mL of 10x concentrated binding buffer. Adjust the pH to 7.4 with 1 mol L^{-1} HCl. Add distilled water to 100 mL.
▲ Critical: Paraformaldehyde solution must be freshly prepared on the day of use.

Protocol
As described above, there are two main options used for measuring cell death by annexin A5. To measure PS expression by flow cytometry, follow option A; to visualize cell surface expression of PS by CSLM, follow option B.

(A) **Measurement of PS expression by flow cytometry**
(i) Suspend cells at a density of 1 × 10^6 cells per mL in the appropriate medium at 37°C and induce cell death with a selected agent; for example, with 200 ng mL^{-1} antibody to Fas. As a positive control for cell surface expression of PS, incubate 1 × 10^6 cells per mL with 5 µmol L^{-1} ionomycin in binding buffer for 10 min. Ionomycin is a calcium ionophore that causes an increase in the intracellular calcium concentration and subsequent expression of PS at the cell surface.
(ii) Obtain samples of 5 × 10^4 cells (50 µL) at predetermined time points and dilute each sample with 450 µL binding buffer. Add 250 ng mL^{-1} green fluorescent annexin A5 and 250 ng mL^{-1} propidium iodide and incubate the mixture in the dark for 5–15 min at 20–25°C (ambient temperature) or on ice, depending on the research question.

? Troubleshooting

(iii) Do two-color flow cytometry. Binding of green fluorescent annexin A5 at the cell surface and propidium iodide uptake can be measured without the necessity of including a washing step. If washing steps are required, the wash buffer should contain 1–3 mmol L^{-1} $CaCl_2$. The presence of Ca^{2+} ions ensures that the cell-bound annexin A5 remains bound during the washing procedure. The final washing step should also include 250 ng mL^{-1} propidium iodide.

(iv) Analyze the flow cytometry data 'offline'. Set the quadrants in the green fluorescent annexin A5–versus–propidium iodide dot plot of the untreated control cells. Living cells, annexin A5–binding, and propidium iodide–positive cells are found in the bottom left quadrant, bottom right and top right quadrant, respectively (Fig. 1).
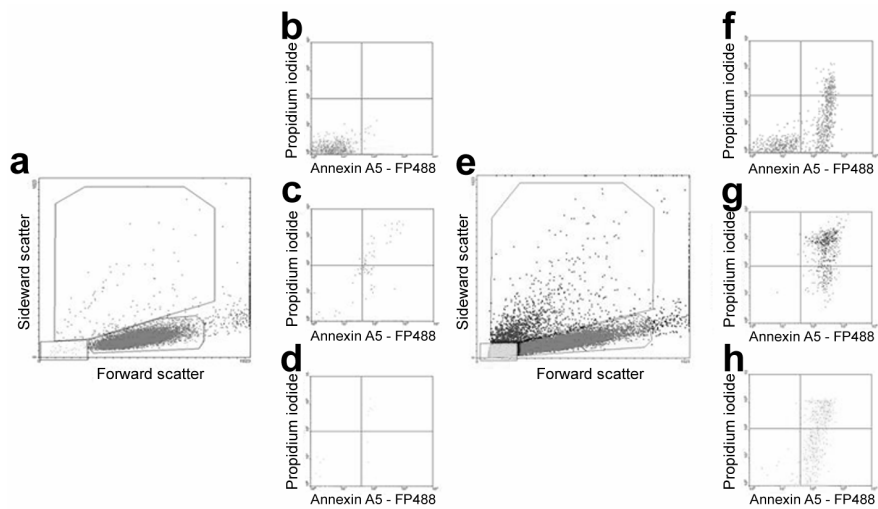
Figure 1, **Flow cytometry of apoptotic Jurkat cells with annexin A5–FP488 and propidium iodide.** Forward- and side-scatter plots of untreated control Jurkat cells (a) and Jurkat cells treated with antibody to Fas (e). (b–d, f–h) Annexin A5–FP488 and propidium iodide dot plots of living and early apoptotic cells (grey; b, f), dot plots of dead cells with compromised integrity of the plasma membrane (black; c, g) and dot plots of apoptotic bodies (light grey; d, h). Bottom left quadrants, living cells; bottom right quadrants, cells in the early phase of cell death; top right quadrants, cells in the phase of cell death with leaky plasma membrane.

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(B) **Visualization of cell surface expression of PS by CSLM**

(i) Suspend cells at a density of 1 x10^6 cells per mL in the appropriate medium at 37°C and induce cell death with a selected agent; for example, with 200 ng mL^{-1} antibody to Fas.

(ii) Add a sample of 5 x10^5 cells per mL to a microcentrifuge tube and spin cells for 2 min at 200g in a microcentrifuge.

(iii) Remove the supernatant and resuspend cells in 500 µL binding buffer. Add 250 ng mL^{-1} of fluorescence labeled annexin A5 and 250 ng mL^{-1} propidium iodide and incubate for 5–15 min at ambient temperature or on ice, depending on the research question.

? Troubleshooting

(iv) Spin cells in a microcentrifuge for 2 min at 200g and resuspend the cells in 500 µL binding buffer.

(v) Spin cells in a microcentrifuge for 2 min at 200g. Remove the supernatant, resuspend cells in 500 µL of 4% paraformaldehyde in binding buffer (the fixation buffer should be freshly prepared) and incubate for 15 min at ambient temperature.

▲ Critical step

Cell samples should not be fixed with paraformaldehyde before incubation with fluorescence-labeled annexin A5 because this produces false-positive results. The paraformaldehyde buffer solution should contain CaCl$_2$ at a concentration ranging from 1 mmol L^{-1} to 3 mmol L^{-1}.

(vi) Spin cells in a microcentrifuge for 2 min at 200g. Remove the supernatant and resuspend cells in 500 µL binding buffer supplemented with 1% (wt/vol) bovine serum albumin.

(vii) Spin cells in a microcentrifuge for 2 min at 200g. Resuspend cells in 10 µL binding buffer and 30 µL glycerol.

(viii) Place 15 µL of the cell suspension on a glass slide and place a glass coverslip over the cell suspension. Seal the glass coverslip onto the glass slide with nail polish and analyze the cells by CLSM using wavelength excitation at 488 nm for green fluorescent annexin A5 and 568 nm for propidium iodide.

Troubleshooting

Steps (A) (ii) and (B) (iii)

Annexin A5 is an 'adopted name' according to a consensus taxonomy that accommodates the growing list of members of the annexin family. Its former name was annexin V. That name is still used in many publications and by vendors of fluorescence labeled annexin A5 kits. Some vendors do not describe the contents of fluorescence-labeled annexin A5 in the kit. In those cases, the dilution recommended by the protocol included with the kit should be used or the optimal dilution should be determined by construction of a dose-response relationship.

▋Timing

The total amount of time necessary for completing the flow cytometry procedure is about 30 min. The procedure comprises 10 min for ionomycine treatment, 15 min for the annexin A5–propidium iodide staining and 5 min for flow cytometry measurement.

The total amount of time necessary for completion of the CSLM procedure is about 45 min. The procedure comprises a microcentrifuge step of 2 min, annexin A5–propidium iodide staining for 15 min, a microcentrifuge step of 2 min in which the supernatant with annexin A5–propidium iodide is removed, a microcentrifuge step of 2 min in which the cells are washed, a fixation step of 15 min, a microcentrifuge step of 2 min in which the supernatant with paraformaldehyde solution is removed, a microcentrifuge step of 2 min in which the cells are washed, and a short step in which the cells are suspended in glycerol and binding buffer and subsequently mounted on a glass slide.
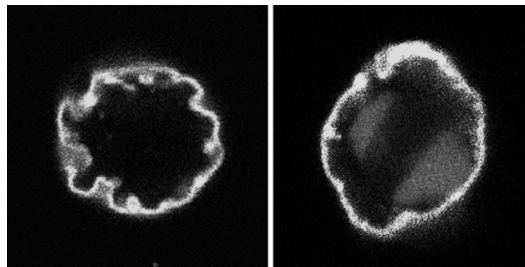

Figure 2, **CSLM analysis of apoptotic Jurkat cells with annexin A5–FP488 and propidium iodide.** Images are of an early apoptotic Jurkat cell (a) and a late apoptotic Jurkat cell with a leaky plasma membrane (b). Original magnification, ×63.

Anticipated results

We measured cell death of the T lymphoma Jurkat cell line using the annexin A5 affinity assay (Fig. 1). We incubated Jurkat cells in the absence (Fig. 1a–d) and presence (Fig. 1e–h) of 200 ng mL^{-1} antibody to Fas at 37ºC, labeled the cells with annexin A5–FP488 and propidium iodide and then analyzed the cells. We obtained forward-scatter and side-scatter plots of untreated control cells (Fig. 1a) and cells treated with antibody to Fas (Fig. 1e). We also obtained annexin A5–FP488–versus–propidium iodide dot plots of living and early apoptotic cells (Fig. 1b,f, red), dot plots of dead cells with compromised integrity of the plasma membrane (Fig. 1c,g, blue) and dot plots of apoptotic bodies (Fig. 1d,h, green). Living cells, cells in the early phase of cell death and cells in the phase of cell death with leaky plasma membrane are in the bottom left, bottom right and top right quadrants, respectively.

We also measured cell death with the annexin A5 affinity assay by CSLM (Fig. 2). We incubated Jurkat cells for 4h with 200 ng mL^{-1} antibody to Fas at 37ºC, labeled the cells with annexin A5–FP488 (green) and propidium iodide (red) and then analyzed them by CSLM. We obtained images of an early apoptotic Jurkat cell (Fig. 2a) and a late apoptotic Jurkat cell with a leaky plasma membrane (Fig. 2b).

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Chapter 3

Trimerization of annexin A5 on plasma membrane expressed phosphatidylserine is a prerequisite for internalization

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Summary

Expression of phosphatidylserine (PS) at the cell surface is part of the membrane dynamics of apoptosis. Expressed phosphatidylserine functions as an "eat me" flag toward phagocytes. Here, we report that expressed PS forms part of a hitherto undescribed endocytic pathway. Annexin A5 is a protein which binds in a calcium dependent manner to PS-expressing membranes. Membrane binding is followed by trimeriztion of annexin A5 molecules and the subsequent formation of a trimer based two-dimensional crystal lattice. PS-binding of annexin A5 at the PS-expressing cell surface prevents apoptotic body formation without interfering with the progression of apoptosis as demonstrated by activation of caspase-3 and DNA fragmentation. Internalization of annexin A5 was observed with apoptotic Jurkat lymphoma cells and viable Hela cells. The two-dimensional crystal lattice bends the membrane patch nanomechanically into the cell and elicits budding and endocytic vesicle formation. Internalization was not observed with a mutant annexin A5 which was able to bind PS but unable to form a membrane bound trimer. In addition annexin A1, which binds to PS without forming a two-dimensional crystal lattice, does not induce the formation of endocytic vesicles. This novel pinocytic pathway differs from macropinocytosis, caveolar or clathrin-mediated endocytosis. In conclusion, this novel endocytic pathway depends on the presence of cell surface expressed PS and on the capacity of annexin A5 to form a trimer based two-dimensional crystal lattice.

Introduction

Cells which are unwanted, damaged or senescent turn into apoptosis, an abundant form of cell death [1]. Apoptotic cells are removed from tissues by phagocytes in a silent and non-provocative manner [2]. Removal of apoptotic cells plays an essential role in development and tissue homeostasis of the multicellular organism. Apoptosis is further found in tissues which are damaged by pathological insults such as heart tissue in ischemia/reperfusion injury caused by myocardial infarction. The phenotypic changes observed in apoptotic cells are caused by proteolytic enzymes known as caspases [3]. Caspases are activated shortly after ligation of death receptors present on the cells surface. Activation of one of the caspases, caspase 3, causes various changes on the cell surface such as the appearance of a variety of "eat me" signals and the generation of microparticles [4-7]. Phosphatidylserine (PS) is the most well-explored "eat me" signal. It is expressed at the cell surface very early after the onset of apoptosis, regardless of the cell type and the apoptosis inducing trigger[8]. Clearance of the apoptotic cell is carried out by phagocytic cells that recognize cell surface expressed PS by a recently described PS-receptor [9].

Annexin A5 is a member of an evolutionary conserved multigene family of calcium and phospholipid binding proteins [10]. It binds calcium-dependently, with high affinity and in a reversible manner to PS-expressing membranes [11]. Membrane binding is followed by the formation of annexin A5 trimers which subsequently associate, via protein-protein interactions, to form larger structures such as dimers of trimers and trimers of trimers [12, 13]. The association of trimers leads to the formation of a two-dimensional crystal lattice which is characterized by P3 or P6 symmetry [14]. This phenomenon was explored using model membranes but is also believed to occur on the apoptotic cell surface. In a recent study Gidon-Jeangirard *et al*. observed that in the presence of annexin A5 the generation of microparticles by apoptotic cells was inhibited [15]. They proposed that the inhibition of microparticle formation was caused by the two-dimensional crystal lattice formed by annexin A5.

Annexin A5 is used to detect cell surface expression of PS on apoptotic cells [16]. In this study we examined the involvement of the formation of annexin A5 trimers and the subsequent two-dimensional crystal lattice on the membrane dynamics of the PS-expressing cell surface. We demonstrate that the inhibition of microparticle formation by annexin A5 is not dependent on the capacity of annexin A5 to form a two-dimensional crystal lattice. We observed that annexin A5 was internalized by cells that expressed PS on the cell surface. Endocytosis of annexin A5 by PS-expressing cells was dependent on the capacity of annexin A5 to form trimers.

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Materials and methods
Chemicals
Human apo-transferrin was purchased from Sigma and Tris-(hydroxymethyl) Aminomethane (Tris) was obtained from Acros Organics.

Cell culture
The T-lymphoma cell line Jurkat (ATCC) was routinely grown in RPMI 1640 medium (Gibco BRL), containing 100 units mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin (Gibco BRL), and 10% Fetal Bovine Serum (PAA Laboratories GmbH). HeLa cells (ATCC), were maintained in Modified Eagle Medium (MEM) with Earl's salts (Gibco BRL) supplemented with 10% Fetal Bovine Serum, 1% non-essential amino acids, 100 units mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin (Gibco BRL). The cells were grown at 37°C in a humidified atmosphere and 5% CO_2.

Molecular modeling and site directed mutagenesis
The Internal Coordinate Mechanics (ICM) software package form Molsoft (San Diego, USA) was used to create a three-dimensional model of a membrane bound annexin A5 trimer. The structure of the annexin A5 trimer was based on data published by Sopkova *et al*. [17]. The model was subsequently used to determine the location of putative amino acids involved in protein-protein interactions responsible for trimer formation. Site-directed mutagenesis was performed on annexin A5 cDNA using the Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions generating the annexin A5 mutant 2D1-6 with amino acid substitutions R63A, K70A, K101A, E138A, D139G and N160A.

Cloning, expression, purification and labeling of annexin A5, 2D1-6, M1234 and annexin A1
cDNA encoding for the annexin A5 mutant M1234 (E72D, D144N, E228A and D303N) having four defective calcium binding sites was kindly provided by Prof. Dr. F. Russo-Marie (BIONEXIS-Pharmaceuticals). M1234 cDNA was recloned to remove the 5' base pairs responsible for the 3 extra N-terminal amino acids after expression [18].

Annexin A5 and M1234 and 2D1-6 cDNA's were cloned into the bacterial expression vector pET-5a (Novagen). Proteins were expressed in Escherichia coli and purified to homogeneity as assessed by silver stained SDS-PAGE and Western blotting. Annexin A5, M1234 and 2D1-6 were labeled with Alexa568-succinimidylester according to the manufacturers protocol (Molecular Probes). The proteins which were labeled with 1:1 stoichiometry were purified from the mixtures by MonoQ chromatography with Äcta Explorer (Amersham-Pharmacia). Annexin A1 was kindly provided by Dr. E. Solito (London, UK). Annexin A1 was labeled with Alexa568-succinimidylester according to the

manufacturers protocol (Molecular Probes). The PS-binding capacity of annexin A1-Alexa568 was verified by ellipsometry.

Plasmid and transfection

The plasmid caveolin-1-GFP was a kind gift of Dr. D. Mundy (University of Texas, Southwestern Medical Center, Dallas) and is described in [19]. The plasmid was transfected in HeLa cells with the transfection agent Fugene 6 (Roche Applied science).

Confocal scanning laser microscopy (CSLM)

Jurkat cells (2×10^6 cells mL^{-1}) were stimulated with anti-Fas (200 ng mL^{-1}) in the presence of annexin A5-oregon green (20 μg mL^{-1}), annexin A1-Alexa568 (20 μg mL^{-1}) and cytoskeletal agents if indicated. After 3h the cells were pelleted (2 min 1000g), washed with EDTA-buffer (140 mmol L^{-1} NaCl, 25 mmol L^{-1} Hepes, 5 mmol L^{-1} EDTA, pH 7.4) and resuspended in Ca^{2+}-buffer (10 mmol L^{-1} Hepes, 150 mmol L^{-1} NaCl, 5 mmol L^{-1} KCl, 1 mmol L^{-1} MgCl$_2$, 2.5 mmol L^{-1} CaCl$_2$, pH 7.4). The cells were stained with annexin A5-alexa568 (250 ng mL^{-1}) for 5 min, washed and resuspended in Ca^{2+}-buffer containing 4% paraformaldehyde. After a fixation period of 10 min, the cells were stained with Phalloidin-TRITC according to the manufacturers' protocol (Invitrogen). HeLa cells were seeded on glass cover slips and left to adhere in culture medium. After 24h one or a combination of the fluorescent labeled proteins was added in a concentration of 100 nmol L^{-1}. HeLa cells were incubated at 37°C and 5% CO$_2$ for 3h. The HeLa cells were washed sequentially with phosphate buffered saline (PBS), EDTA-buffer and PBS. The cells were then fixed for 15 min in Ca^{2+}-buffer containing 4% paraformaldehyde (pH 8.0) and washed three times for 20 min in PBS. The fixed Jurkat or HeLa cells were mounted in 90% glycerol in 0.02 mol L^{-1} Tris-HCl pH 8.0 with addition of 2% 1,4-diazobicyclo-[2,2,2]-octane (Merck). Slides were examined with a confocal scanning laser microscope (Bio-Rad) equipped with a Crypton/Argon mixed gas laser (Ion Laser Technology). Images were recorded and analyzed with NIH image, ImageJ and Adobe Photoshop. Variations on this standard protocol were used to study the internalization of annexin A5-Alexa568 (100 nmol L^{-1}), M1234-Alexa568 (100 nmol L^{-1}), annexin A1-Alexa568 (100 nmol L^{-1}), 2D1-6-Alexa568 (100 nmol L^{-1}), transferrin-Oregon Green488 (100 nmol L^{-1}) and Oregon Green labeled Tris (20 μmol L^{-1}) with the succinimidylester group inactivated.

Two-dimensional crystallization and electron crystallography

The experimental conditions and procedure for growing annexin A5 2D-crystals onto functionalized monolayer are described in details in [20]. In our case 17 μL of 60 μg mL^{-1} annexin A5 and of the 2D1-6 solutions in 10mmol L^{-1} HEPES, pH 7.4, containing 150 mmol L^{-1} NaCl, 2 mmol L^{-1} CaCl$_2$ and 3 mmol L^{-1} NaN$_3$ were deposited in a Teflon well of 4 mm diameter and 1 mm depth. 0.6 μL lipid solution containing 150 mmol L^{-1} DOPS / 450 mmol L^{-1} DOPC in chloroform / n-

hexane (1:1 w/w) was deposited on the top of the solution to form the functionalized lipid monolayer. The 2D protein-lipid domains were formed onto the lipid/buffer interface after overnight incubation at 21^oC in a humid chamber. The two-dimensional protein-lipid domains were transferred onto carbon-coated electron microscopy grids and negatively stained with 1% Uranyl acetate solution as described previously (20, 21). The grids were observed in a JEOL 2010 transmission electron microscope operated at 200kV, equipped with a LaB6 and 2x2k CCD ultra-scan digital camera (Gatan, Inc) with 15 micron / pixel resolution. All data were collected as 2048x2048 digital images at a total magnification of 38700 times and low-electron dose conditions (< 1500 electron / nm^2). The Digital Micrograph Images were converted in MRC format using the EMAN software (http://ncmi.bcm.tmc.edu/~stevel/EMAN/doc/) and further analyzed with the CRISP 2D crystallography software (http://www.calidris-em.com).

Analysis of microparticle formation

Jurkat cells were washed and resuspended at 10^6 cells per mL in DMEM (Gibco BRL). Apoptosis was induced with anti-Fas (200 ng mL^{-1}) in the presence of annexin A5, 2D1-6, M1234, annexin A1 or ROCK-1 inhibitor Y-27632. The course of apoptosis was determined by flow cytometry using the annexin A5-FITC staining protocol (Nexins). Microparticle formation was quantified with flow cytometry and subsequent off-line calculation of the percentage of events with reduced forward and sideward scatter. Off-line analysis of the flow cytometer files was performed with WinMDI 2.8 (free share software designed by Joseph Trotter).

Results

Phosphatidylserine binding of Annexin A5 inhibits microparticle formation

In order to asses the effect of two-dimensional lattice formation of annexin A5 on plasma membrane dynamics we examined the interaction of annexin A5 with the cell surface of apoptotic cells. The progress of the apoptotic program in anti-Fas stimulated Jurkat cells was analyzed in the presence and absence of annexin A5. Exposure of PS on the plasma membrane of apoptotic cells is accompanied with the generation of PS-exposing microparticles. We observed that co-incubation of Jurkat cells with anti-Fas and annexin A5 changed the apoptotic membrane dynamics such that microparticle formation was inhibited (fig. 1a).

We examined whether the capacity of annexin A5 to bind PS was necessary for the inhibition of microparticle formation by incubating anti-Fas treated Jurkat cells with the annexin A5 mutant M1234. M1234 lacks calcium binding sites and is consequently unable to bind to PS. Flow cytometric analysis revealed that M1234 did not inhibit the formation of microparticles indicating that the PS binding property of annexin A5 is necessary in inhibition of microparticle formation (not shown).

Microparticles are formed after phosphorylation of the myosin light chain by caspase activated ROCK-1. In our model system inhibition of ROCK-1 with Y-27632 inhibits microparticle formation indicating that ROCK-1 activation is necessary for the formation of microparticles. We examined the possibility that annexin A5 binding to apoptotic Jurkat cells inhibits the cleavage and the subsequent activation of ROCK-1. Co-incubation of Jurkat cells with anti-Fas and annexin A5 showed that parts of the apoptotic program such as caspase-3 activation (fig. 1b), DNA fragmentation (fig. 1c) and ROCK-1 activation (fig. 1d) were not altered when compared to control cells. This result indicates that annexin A5 does not inhibit the cleavage and activation of ROCK-1.

Annexin A5 forms a two-dimensional lattice on a PS-expressing membrane surface while annexin A1 does not form a two-dimensional lattice. We examined the possibility that two-dimensional lattice formation of annexin A5 is necessary for inhibition of microparticle formation. Co-incubation of Jurkat cells with anti-Fas and annexin A1 showed that annexin A1 inhibits, dose-dependently, the formation of microparticles suggesting that two-dimensional lattice formation of annexin A5 is not necessary for inhibition of microparticle formation (fig 1e). Taken together these results indicate that inhibition of microparticle formation by annexin A5 is dependent on the capacity of annexin A5 to bind calcium-dependently to PS.
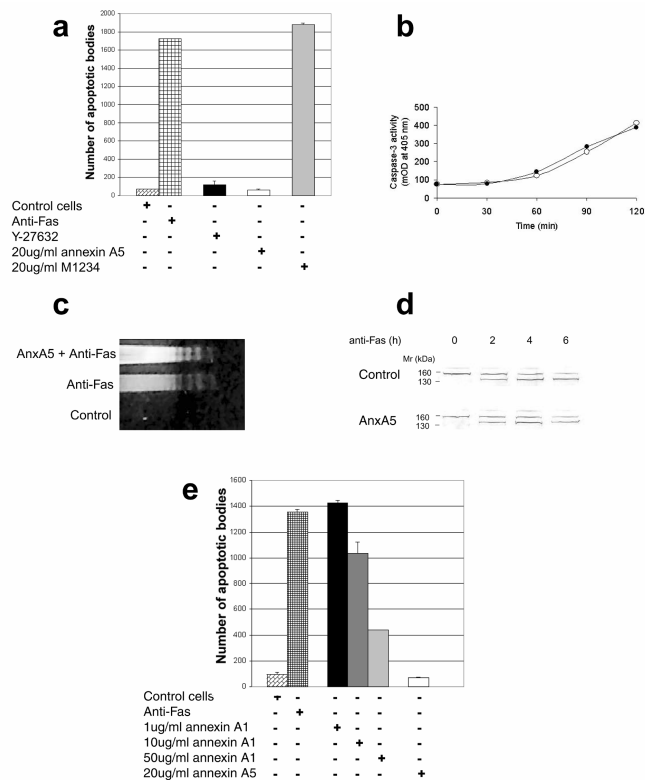
Figure 1, **Annexin A5 inhibits the formation of apoptotic bodies.** Microparticle formation of Jurkat cells after treatment with 200 ng mL^{-1} anti-Fas antibody for 3h in the absence and presence of 10 µg mL^{-1} annexin A5 and M1234 (fig.1a). Time course of caspase-3 activation of anti-Fas-treated Jurkat cells in the absence (open circles) and presence (filled circles) of 10 µg mL^{-1} annexin A5 (fig. 1b). DNA laddering after 3h of anti-Fas treatment in the absence and presence of 10 µg mL^{-1} annexin A5 (fig. 1c). Time course of ROCK-1 cleavage of Jurkat cells treated with anti-Fas antibody in the presence and absence of 10 µg mL^{-1} annexin A5 (fig 1d). Dose-response of microparticle formation of Jurkat cells after treatment with 200 ng mL^{-1} anti-Fas antibody for 3h in the presence of 1, 10 and 50 µg mL^{-1} annexin A1 (fig. 1e).
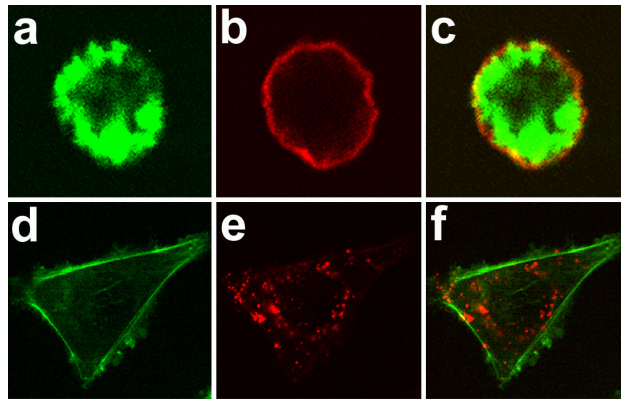
Figure 2, **Annexin A5 is internalized via a PS-dependent pathway.** CSLM of anti-Fas-treated Jurkat cells incubated for 3h with 20 µg mL^{-1} annexin A5-oregon green (fig. 2a) and counter stained for 5 min. with annexin A5-alexa568 (fig 2b). Figure 2c shows the composition of figure 2a and 2b. CSLM of HeLa cells incubated with 100 nmol L^{-1} of annexin A5-alexa568 (Fig. 2e) and counter stained with phalloidin-oregon green (fig. 2d). Fig. 2f shows the composition of figure 2d and 2e.
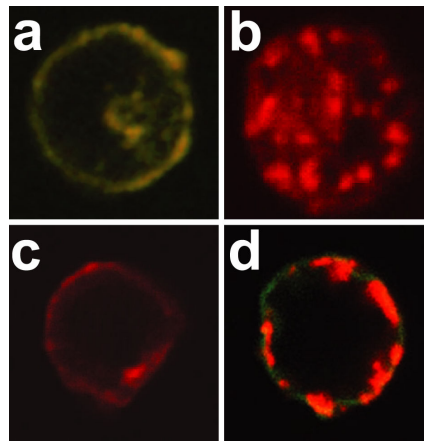
Figure 3, **Annexin A5-containing vesicles in apoptotic Jurkat cells are coated with actin.** CSLM of anti-Fas-treated Jurkat cells in the presence annexin A5-Oregon Green and stained for F-actin with TRITC-labeled phalloidin (fig. 3a). CSLM of anti-Fas-treated Jurkat cells in the presence (fig. 3b) and absence (fig. 3c) of cold annexin A5. CSLM of Jurkat cell treated with 0.5 µmol L^{-1} of latrunculin B (fig. 3d).

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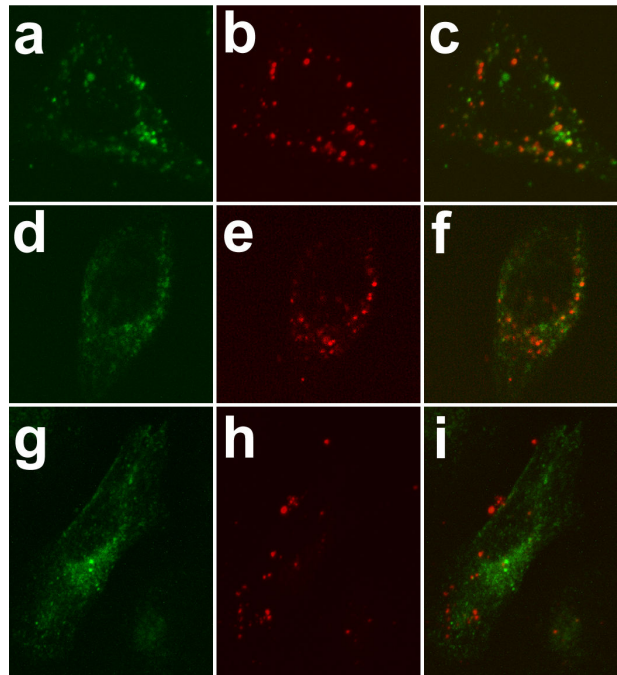
Figure 4, **Annexin A5 is not internalized by HeLa cells via a known endocytic pathway.** CSLM of HeLa cell which has internalized the macropinocytic probe Oregon Green (4a; green) and annexin A5-Alexa568 (4b; red). Fig. 4c was obtained after merging of 4a and 4b. HeLa cells were transfected with caveolin 1-enhanced green fluorescent protein and 24h later incubated with 100 nmol L^{-1} annexin A5-Alexa568. HeLa cell containing caveolin 1-enhanced green fluorescent protein (4d; green) and annexin A5-alexa568 (4e; red). Fig. 4f was obtained after merging of 4d and 4e. CSLM of HeLa cells that have internalized transferrin-Oregon Green488 (4g; green) and annexin A5 alexa568 (4h; red). Fig. 4i shows the composition of figure 4g and 4h.

Internalization of annexin A5 occurs via a PS-dependent endocytic pathway

Data from *in vivo* imaging of apoptotic cells with annexin A5 have suggested that annexin A5 not only binds to the PS exposing cell surface but also that annexin A5 internalizes into the apoptotic cell (Ph. D. thesis, H. Kenis). This observation prompted us to investigate this phenomenon closer in an *in vitro* system. Jurkat cells were induced to undergo apoptosis with anti-Fas treatment and incubated with annexin A5-oregon green. CSLM analysis revealed the presence of intracellular vesicles containing annexin A5-oregon green (Fig. 2 a). We demonstrated that these vesicular structures are endocytic vesicles and not part of an open cannicular system by i) washing the cells with EDTA and ii) incubating them shortly with annexin A5-alexa568 prior to CSLM analysis (fig. 2b). This procedure neither removed annexin A5-oregon green from the vesicular structure nor resulted in co-localization of annexin A5-oregon green and annexin A5-alexa568. The latter bound to the plasma membrane only. Internalization of annexin A5 was not observed in healthy Jurkat cells nor did internalization occur in apoptotic Jurkat cells with mutant M1234-alexa568. Execution of apoptosis results in the surface expression of PS, which is a requirement for internalization of annexin A5 in apoptotic Jurkat T-lymphocytes. In order to determine whether apoptosis is a necessity for internalization we incubated HeLa cells that were not submitted to apoptotic stimuli with annexin A5-Alexa568. HeLa cells were incubated for 3 hours with annexin A5-alexa568. Fig. 2d shows a CSLM image of a HeLa cell stained with the actin dye phalloidin-oregon green. CSLM analysis further demonstrated the presence of intracellular vesicles of various sizes containing annexin A5 (Fig. 2e). Annexin A5 inside the vesicles could not be removed by treatment with EDTA-buffer. The internalization was not coupled to the execution of the apoptotic program because i) zVAD-fmk, a caspase inhibitor, did not inhibit annexin A5 internalization and ii) the cells with internalized annexin A5 were viable (not shown). Internalization of M1234-alexa568 in HeLa cells was not observed. We conclude from these results that annexin A5 is taken up in endocytic vesicles in apoptotic Jurkat and HeLa tumor cells via a PS-dependent mechanism.

Annexin A5 is internalized via a novel endocytic pathway

Cells can internalize proteins from the extracellular environment via several endocytic pathways. We examined the possibility that annexin A5 is internalized via one of these pathways. The size distribution of the annexin A5 endocytic vesicles suggests that the uptake is a macropinocytic process. This process is dependent on actin dynamics which is involved in ruffling of the cell surface [22]. Jurkat cells were co-incubated for three hours with anti-Fas and annexin A5-Oregon Green. Staining of F-actin with Phalloidin-Texas Red revealed the presence of an actin coat around the annexin A5-containing vesicles, suggesting the involvement of the actin cytoskeleton (Fig. 3a). When anti-Fas treated Jurkat cells were incubated with annexin A5 we observed intracellular

vesicles coated with actin (Fig 3b). These vesicles were not observed in the absence of annexin A5 (fig. 3c). Treatment of Jurkat cells with latrunculin B, an actin-depolimerizing agent, did not prevent the uptake of annexin A5 in endocytic vesicles indicating that annexin A5 is not internalized via macropinocytosis (Fig. 3d). The macropinocytic pathway is further characterized by internalization of solutes in a non-specific manner. HeLa cells were co-incubated with annexin A5-alexa568 and the macropinocytic probe oregon-green. CSLM analysis showed the presence of two distinct populations of vesicles, one containing annexin A5-alexa568 (fig. 4a) and the second Oregon Green (fig. 4b). These two populations of intracellular vesicles did not co-localize with each other (Fig. 4c). Co-incubation of HeLa cells for 3 hours with M1234-alexa568 and Oregon Green showed that only Oregon Green was internalized but not M1234 demonstrating that annexin A5 is internalized via a PS-dependent pathway distinct from macropinocytosis (not shown).

We investigated whether annexin A5 is internalized via the clathrin-mediated endocytic pathway. The endocytic vesicles generated by the clathrin-mediated pathway have a uniform size. The reported diameter of clathrin coated vesicles is 100 nm [23]. The annexin A5-containing vesicles are heterogeneous is size. In both apoptotic Jurkat and living HeLa cells the annexin A5-containing vesicles have a diameter ranging from 0.2 to 1.0 µm. We co-incubated HeLa cells with transferrin-oregon green, a protein internalized via the clathrin mediated pathway, and annexin A5-alexa568. Fig. 4d shows a CSLM image of a HeLa cell which has internalized transferrin-oregon green and annexin A5-alexa568 (fig. 4e). Fig. 4f demonstrates the difference in diameter between vesicles containing transferrin and annexin A5. CSLM analysis shows that transferrin-containing vesicles do not co-localize with annexin A5-containing vesicles.

We next examined a possible involvement of the caveolar endocytic pathway in the internalization of annexin A5. Lymphocytes do not express the membrane protein caveolin-1 which is part of the molecular machinery of the caveolar endocytic pathway indicating that annexin A5 is not internalized in Jurkat lymphoma cells via caveolae. We examined the possibility that annexin A5 is internalized by HeLa cells via the caveolar endocytic pathway. HeLa cells were transfected with EGFP-caveolin-1-expressing plasmid and incubated 24h later with annexin A5-alexa568. Fig. 4g shows a CSLM image of a HeLa cell expressing EGFP-caveolin-1 which has internalized annexin A5-alexa568 (fig. 4h). CSLM analysis showed that EGFP-caveolin-1 did not co-localize with annexin A5-alexa568-containing intracellular vesicles (fig. 4i) demonstrating that annexin A5 is not internalized via caveolae.

Taken together our data show that annexin A5 is internalized through a process that differs mechanistically from macropinocytosis, clathrin-mediated and caveolar endocytosis.

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Trimer formation of annexin A5 is essential for endocytosis
The tertiary structure of annexin A5 shows that its phospholipid binding side has a convex shape. When bound to the membrane annexin A5 forms trimers of which each monomer retains the convex shape at its phospholipid binding side. We hypothesized that the trimers bend the membrane and provide the driving force for membrane movement. In order to test this hypothesis we created the annexin A5 mutant 2D1-6 which was able to bind PS but unable to form a trimer. Several criteria were used to establish that 2D1-6 had lost its ability to form trimers but not its capacity to bind calcium-dependently to PS. We examined the possibility that the introduction of mutations in the annexin A5 protein results in a change in structure. Analysis of the secondary structure of 2D1-6 by circular dichroism showed however no significant change when compared to annexin A5 (not shown). We next examined the ability of 2D1-6 to bind calcium-dependently to PS. Elipsometric measurements demonstrated that the calcium-dependent binding properties of 2D1-6 were virtually the same as annexin A5 (not shown). This was further corroborated by CSLM analysis which showed that 2D1-6-alexa568 (fig. 5a) was able to bind to the surface of ionomycin and calcium-treated HeLa cells. Fig. 5b shows binding of annexin-alexa568 to ionomycin and calcium-treated HeLa cells. We finally examined the ability of 2D1-6 to form a membrane-bound two-dimensional crystal lattice. Fourier analysis of the electron diffraction pattern generated by electron-irradiation of membrane bound annexin A5 showed a characteristic hexagonal pattern, previously reported for two-dimensional crystals of annexin A5 obtained in similar conditions (Fig. 5c) [12, 13]. Further analysis showed the typical trimeric organization of annexin A5. Fourier analysis did not reveal a hexagonal pattern and trimeric organization for membrane-bound 2D1-6. This result indicates that according to Fourier analysis 2D1-6 does not form an observable trimer-based two-dimensional crystal lattice.

Our results so far suggested that the two-dimensional crystal lattice is not responsible for the inhibition of microparticle formation. We confirmed this by demonstrating that 2D1-6 can inhibit the formation of apoptotic Jurkat cell derived microparticles (not shown). To investigate the involvement of the two-dimensional crystal lattice in the endocytosis of annexin A5, we incubated HeLa cells for 3h with 2D1-6-alexa568. Fig. 5d shows HeLa cells stained with the actin dye phalloidin-oregon green. CSLM analysis of these HeLa cells revealed that endocytic vesicles containing 2D1-6-alexa568 were absent (Fig. 5e). We obtained a similar result for apoptotic Jurkat cells incubated with 2D1-6-alexa568 (not shown). These experiments indicate that trimerization of annexin A5 and the subsequent two-dimensional crystal formation is necessary for the endocytosis of annexin A5 by cells that express PS on the cell surface. This conclusion was supported by experiments in which apoptotic Jurkat and viable HeLa cells were incubated with annexin A1-alexa568. Fig. 6a shows a CSLM image of apoptotic Jurkat cells with intracellular vesicles containing annexin A5-alexa568. Annexin A1-alexa568 was able to bind the PS-expressing cell surface

of the apoptotic Jurkat cell however intracellular vesicles containing annexin A1-alexa 568 were absent (fig. 6b). HeLa cells contained intracellular vesicles containing annexin A5-alexa568 (fig. 6d) but intracellular vesicles containing annexin A1-alexa568 were not observed (fig. 6e). Interestingly, when apoptotic Jurkat cells were co-incubated with annexin A5-oregon green and annexin A1-alexa568 we observed intracellular vesicles containing both annexin A5 and annexin A1 suggesting that annexin A5 is able to internalize PS-binding proteins (fig 6c). We observed a less pronounced co-localization of annexin A5 and annexin A1 when HeLa cells were co-incubated with annexin A5-oregon green and annexin A1-alexa668 (fig 6f). In some vesicles we did not observe co-localization of annexin A5 and annexin A1 while in other vesicles annexin A1 was marginally present.
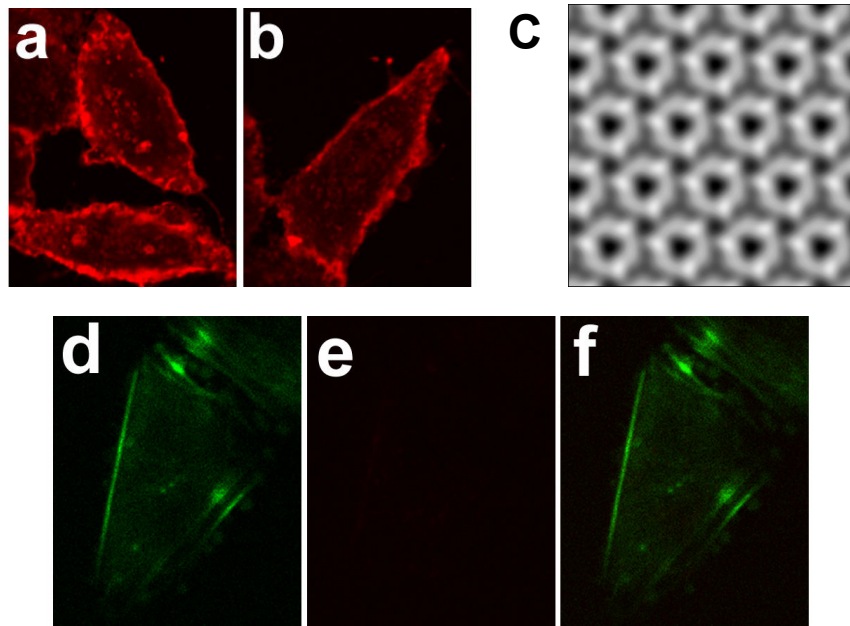
Figure 5, **Endocytosis of annexin A5 is dependent on annexin A5 trimer formation.** HeLa cells treated with ionomycin and stained with annexin A5-alexa568 (fig 5a; red) and 2D1-6-alexa568 (fig 5b; red). Two-dimensional density projection map of annexin A5 two-dimensional crystals at 2.0 nm resolution (fig. 5c). The protein density is in white, P3 symmetry has been imposed. The unite cell dimensions (from hole to hole) are a = b = 11nm, γ = 120°, phase residual 11.6°. HeLa cell stained with phalloidin-oregon green (fig 5d; green) and incubated for 3h with 100 nmol L^{-1} of 2D1-6-alexa568 (fig 5e; red). Fig. 5f shows the composition of figure 5d and 5e.

Discussion

In this study we examined the effect of the formation of trimers and two-dimensional crystallization of annexin A5 on the membrane dynamics of the PS-expressing cell surface. Apoptotic cell death causes various changes on the cell surface such as the externalization of PS to the outer membrane leaflet and the shedding of receptor bearing microparticles [4-7]. Recently, it was reported that annexin A5 inhibits microparticle formation by the application of a physical constraint, i.e. a two-dimensional crystal lattice, on the plasma membrane [15]. We confirmed that annexin A5 inhibits, dose-dependently, the formation of microparticles. Inhibition of microparticle formation was however not caused by the two-dimensional crystal lattice because 2D-1-6 and annexin A1 (which are able to bind PS but which are unable to form a two-dimensional crystal lattice) were able to inhibit microparticle formation.

During our study into the inhibition of microparticle formation by annexin A5 we observed that annexin A5 was internalized by apoptotic Jurkat cells and viable HeLa cells. Our investigations into this phenomenon revealed the existence of an endocytic pathway that involves annexin A5 and cell surface-expressed PS. We found that annexin A5 was not internalized via one of the known endocytic pathways. The endocytic vesicles containing annexin A5 were heterogeneous in size which suggested that annexin A5 was internalized via macropinocytosis. The formation of macropinosomes is dependent on the activity of actin polymerisation process. However, the experiments with the actin disruption agent latrunculatin B showed that annexin A5 is internalized by apoptotic Jurkat cells when the actin polymerisation process is inhibited. Furthermore, in HeLa cells annexin A5 did not co-localize with endocytic vesicles containing a macropinocytosis marker. We further demonstrated that annexin A5 was neither internalized by the caveolar nor by the clathrin-mediated endocytic pathway.

In order to obtain more insight into the mechanism that is responsible for endocytosis of annexin A5 we focused our investigation on the two-dimensional crystallization property of annexin A5. Oling *et al*. have demonstrated that annexin A5 molecules do not significantly change conformation after binding to a PS-expressing membrane surface [13]. This means that annexin A5 molecules in solution and the molecules bound to PS have a convex shape at the phospholipid binding side. This further indicates that the various domains have an unequal distance to the phospholipid membrane surface [13]. Domain III is the most distant from the membrane surface and domain II the closest. All four domains contain a calcium-binding motif making it possible that membrane-bound annexin A5 influences the PS-expressing membrane surface to adopt a shape that is complementary to the convex shape of the annexin A5 molecule.

We hypothesized that the convex shape of annexin A5 monomers, that is retained when annexin A5 is assembled into a two-dimensional crystal lattice, imposes a curvature to the PS-expressing plasma membrane on which annexin A5 is bound. Membrane curvature is subsequently followed by the formation of a spherical endocytic vesicle. To test this hypothesis we generated an annexin

A5 mutant that was able to bind to PS but unable to form a trimer. Sopkova *et al*. have published data on the crystal form-1 from which it is possible to deduce the presence of two different trimer conformations (Fig. 7a) [17]. In the first trimer conformation the annexin A5 molecules are oriented in such a manner that domain two of each individual annexin A5 molecule is present inside and domain three on the outside of the trimer (Fig 7b). In the second trimer conformation the annexin A5 molecules are oriented in a reverse manner. Each annexin A5 molecule in this trimer is oriented with domain three present in the inside and domain two on the outside (Fig. 7c). Both trimers are not formed with equal likelihood because the formation of the first trimer (Fig. 7b) is energetically more favourable than the second trimer (Fig. 7c).

The annexin A5 mutant, 2D1-6, which was unable to form trimers was created by introduction of mutations at the putative amino acid locations that were predicted to be involved in the formation of the first trimer structure (Fig. 7b). These amino acids were identified after modelling the trimer structure using protein-protein docking protocols in the Molsoft software package. To test whether the 2D1-6 mutant had lost its capacity to induce internalization, we incubated apoptotic Jurkat lymphoma and viable HeLa tumor cells with fluorescently labeled 2D1-6. We demonstrated with CSLM analysis that there were no endocytic vesicles containing 2D1-6 present inside these cells. We concluded from this result that trimerization and the formation of a two-dimensional crystal lattice were necessary for endocytosis of annexin A5. This conclusion was supported by experiments which showed that fluorescently labeled annexin A1 was not internalized by apoptotic Jurkat and viable HeLa cells.

Studies with artificial membranes have revealed that annexin A5 trimers can be packed in such a way that four two-dimensional crystal types are formed. These types include the crystal form-1, the crystal form-2 and the crystals characterized by P6 (fig. 7d) and P3 (fig. 7e) symmetries [14]. The crystal characterized by P6 symmetry is commonly found on artificial PS-expressing membranes while the other three types are rarely found. The crystal lattice characterized by P6 symmetry is found on membrane surfaces which consists of 25 mol% PS and 75 mol% PC [14]. The crystal characterized by P3 symmetry is found on membranes with a higher PS content. It is not known whether or not the P6 or P3 crystal lattices are formed on biological membranes. This might depend on the manner in which PS is distributed on the plasma membrane. PS might be localized in micro-domains or it is homogeneously distributed. It is possible that both P6 and P3 crystal types are formed on the plasma membrane when there are micro-domains present which have a high PS content.
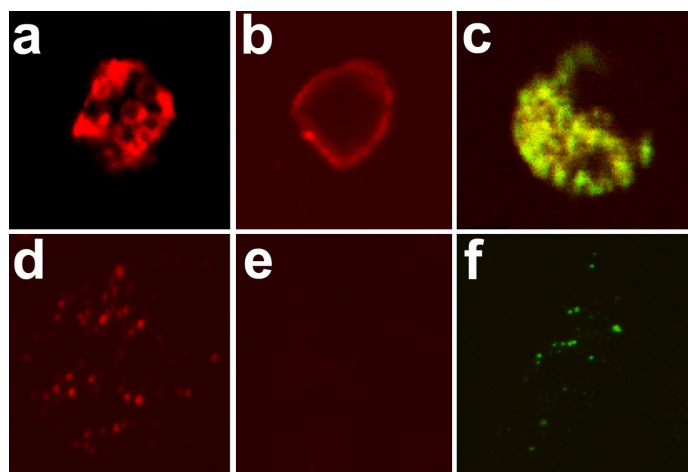
Figure 6, **Annexin A5 and not annexin A1 opens a novel portal of cell entry.** a–c, CSLM analyses of Jurkat cells that were co-incubated with anti-Fas and annexin A5-Alexa568 (fig. 6a), annexin A1-Alexa568 (fig. 6b), or the combination of annexin A5-Oregon Green and annexin A1-Alexa568 (fig. 6c). CSLM analyses of living HeLa cells that were incubated with annexin A5-Alexa568 (fig. 6d), annexin A1-Alexa568 (fig. 6e) or the combination of annexin A5-Oregon Green and annexin A1-Alexa568 (fig. 6f).

We hypothesize that the two-dimensional crystal lattice causes bending of the membrane which is followed by the formation of an endocytic vesicle via an unknown mechanism. The vesicles that are present in the cytosol of both apoptotic Jurkat and HeLa tumor cells are heterogeneous in size. The following two hypothetical mechanisms may account for the heterogeneous vesicle size: (i) PS-containing micro-domains on the cell surface can have various diameters. Large and small endocytic vesicles are then created as annexin A5 crystallizes on PS-containing micro-domains that have respectively large and small diameters. (ii) Annexin A5 trimers can associate to form P6 or P3 crystals on the cell surface, however the extent as to which these crystal types induce membrane curvature is different. The difference in membrane curvature may subsequently lead to large and small endocytic vesicles.

The relevance of the annexin A5 endocytic pathway during apoptosis is debatable. Why should a cell determined to die be capable of activating endocytic pathways? Recent articles have reported on cell surface expression of PS on viable cells independent of cell death. PS expression has been found to occur on living leukocytes for example on positively selected B-cells, a subpopulation of T-lymphocytes and on monocytes that differentiate into macrophages [24-26]. Furthermore monocytes and activated neutrophils express PS independent of cell death after stimulation with dimeric galectin-1 [27]. These findings suggest that it is possible that the annexin A5-PS endocytic pathway is

not only active on dying cells but also on living cells. In this paper we demonstrated the likelihood of this possibility by showing that the annexin A5 endocytic pathway exists in living HeLa cells.
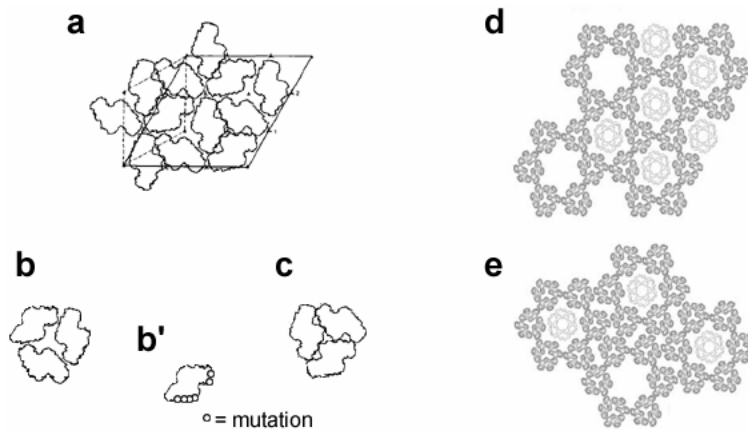
Figure 7, **Trimer structure and two-dimensional lattice formation of annexin A5.** Figure 7a shows the crystal form-1 of annexin A5. This crystal lattice consists of two types of trimer structures shown in fig. 7b and 7c. Figure 7b' shows the location of the mutations that were introduced into annexin A5 to obtain 2D1-6. Trimers can associate into a crystal lattice characterized by P6 symmetry (fig. 7d) or P3 symmetry (fig. 7e). Figures 7a-c are adapted from Sopkova *et al*. (J Mol Biol., 1993) and figures 7d-e are adapted from Oling *et al*. (J Struct Biol., 2001).

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Chapter 4

Annexin A5 induces endocytosis of the complex of tissue factor with factor VIIa

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Summary

Annexin A5 is a protein that binds calcium-dependently to phosphatidylserine (PS)-expressing membrane surfaces. Cell surface binding is followed by an endocytosis process that is dependent on the capacity of annexin A5 to bind PS and to form a two-dimensional crystal lattice. In this study we have investigated annexin A5-induced endocytosis of tissue factor (TF) on viable HeLa cells. Endocytosis of TF was studied with fluorescently labeled inactivated factor VIIa (FVIIai-F) on HeLa cells transfected with TF cDNA. Confocal scanning laser microscopy (CSLM) revealed that the TF:FVIIai-F complex was predominantly localized on the cell surface at sites of cell-cell contact. Localization at sites of cell-cell contact was independent on the cytoplasmic domain of TF. CSLM analysis further demonstrated that annexin A5 induces the endocytosis of TF:FVIIai-F complex in viable HeLa cells. Annexin A5-induced endocytosis of TF:FVIIai-F complex is dependent on the capacity of annexin A5 to bind PS and to form a two-dimensional crystal lattice. Endocytosis of TF did however not result in a decrease of TF activity suggesting that annexin A5-induced endocytosis of TF on viable HeLa cells does not play a role in down regulation of coagulation processes initiated by TF-bearing cell surfaces.

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Introduction

The annexins are a family of membrane binding proteins that share structural properties and biological activities associated with membrane-related processes[1]. One of the members of this family, annexin A5, binds reversibly to phosphatidylserine (PS)-expressing membranes with high affinity in a calcium-dependent manner[2]. Binding to PS is accompanied by the formation of a two-dimensional crystal lattice of annexin A5 molecules through protein-protein interactions[3]. Recently, we have reported that apoptotic cells internalize annexin A5 via a novel route of internalization[4]. This endocytic pathway is characterized by the generation of endocytic vesicles that are heterogeneous in size ranging from 0.2 to 1.0 μm. Endocytosis of annexin A5 is dependent on its properties to bind PS and to form a two-dimensional crystal lattice. In addition internalization of annexin A5 does not occur via the known endocytic pathways such as macropinocytosis and caveolar or clathrin-mediated endocytosis. It is hypothesized that the first steps of endocytosis comprise an annexin A5-induced invagination by annexin A5 as a result of nanomechanical bending of the PS-expressing membrane patch[4]. Viable cells restrict PS to inner leaflets of the plasma membrane by employing aminophospholipid translocases (APLT). The APLT P-type ATPase rapidly transports aminophospholipids inwards and in an ATP-dependent manner[5]. PS is also transported to the exofacial leaflet of the plasma membrane via ATP-binding-cassette transporter C1, albeit at low rates[6]. During apoptosis net aminophosholipid transport shifts towards externalization leading to prolonged surface exposure of PS. The shift results from the inhibition of APLTs and concomitant activation of scramblases[7]. Cell surface expressed PS acts as an "eat me" signal towards phagocytes[8]. If apoptosis occurs in the presence of annexin A5 the PS-expressing membrane patches are internalized resulting in inhibition of phagocytosis[9] and down-regulation of receptors embedded in PS-containing membrane patches[10]. The latter was shown for tissue factor (TF) and apoptotic THP-1 cells. The relevance of this mechanism operating during apoptosis, however, is not well understood.

Recent studies have shown that viable cells can express PS on the cell surface under certain circumstances such as positively selected B-cells, a subset of T-lymphocytes and neutrophils stimulated with galectin-1[11-13]. Living HeLa cells in culture express PS and internalize annexin A5 with a mechanism similar to annexin A5 internalization by apoptotic cells[4]. These findings prompted us to examine whether annexin A5 regulates expression of plasma membrane receptors of living HeLa cells. Hereto, we have chosen to investigate TF internalization by HeLa cells. TF, a 45-kDa type II cytokine receptor that possesses one transmembrane domain and a short intracellular COOH-terminal tail, initiates the blood coagulation cascade upon complexation with blood coagulation factor VIIa in an environment with PS[14, 15]. Since, HeLa cells have a low expression of TF on the cell surface, functional TF expression was provoked by transfection with TF cDNA. TF expression and cell surface location was examined with a TF activity assay and by confocal scanning laser

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microscopy (CSLM). The role of annexin A5 in the internalization of TF was examined by using fluorescently-labeled inactivated factor FVIIa.
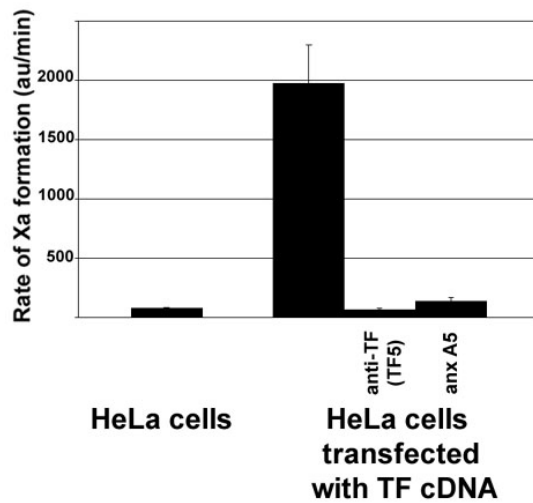
Figure 1, **Activity of cell surface expressed tissue factor.** The ability of HeLa cells and TF-expressing HeLa cells to convert factor X in a factor VIIa-dependent manner was measured using a TF activity assay. The TF neutralizing antibody TF5 (10 μg mL^{-1}) and annexin A5 (10 μg mL^{-1}) inhibited the ability of TF-expressing HeLa cells to convert FX into FXa.
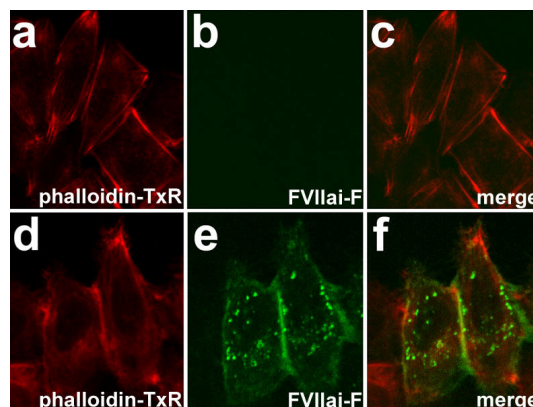
Figure 2, **FVIIai-F localizes in HeLa cells predominantly at sites of cell-cell contact.** CSLM images of HeLa cells incubated with 10 nmol L^{-1} FVIIai-F (green; Fig. 2b) and counterstained with phalloidin-texas red (red; Fig. 2a). CSLM images of HeLa cells transfected with TF cDNA and subsequently incubated for 1.5h with 10 nmol L^{-1} FVIIai-F (green; Fig. 2e) and counter stained with phalloidin-texas red (red; Fig. 2d). Figure 2c and 2f show the composition of 2a-b and 2d-e respectively.

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Materials and methods
Cell culture conditions
HeLa cells, obtained from ATCC, were maintained in modified eagle medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS, PAA Laboratories), 1% non-essential amino acids, 100 units mL^{-1} penicillin and 100 μg/mL streptomycin (Gibco BRL). The cells were grown at 37°C in a humidified atmosphere and 5% CO_2.

Cloning, expression, purification and labeling of proteins
cDNA encoding for the annexin A5 mutant M1234 (E72D, D144N, E228A and D303N) having four defective calcium binding sites was kindly provided by Prof. Dr. F. Russo-Marie (BIONEXIS-Pharmaceuticals) [16]. Annexin A5 and M1234 cDNAs were cloned into the bacterial expression vector pET-5a (Novagen). Proteins were expressed in Escherichia coli and purified to homogeneity as assessed by silver-stained SDS-PAGE and Western blotting. Annexin A5, M1234 and annexin A1 were labeled with Alexa568-succinimidylester according to the manufacturer's protocol (Invitrogen). The labeled proteins with 1:1 stoichiometry were purified from the mixtures by MonoQ chromatography with Äcta Explorer (Amersham Biosciences). Annexin A1 was kindly provided by Dr. E. Solito (Imperial College London, London, England). FITC-labeled inactivated factor VIIa (FVIIai-F) was prepared as described earlier [10]. Briefly, N-α-[(acetylthio)acetyl]-D-Phe-Pro-Arg-CH$_2$Cl was linked to the active site of factor VIIa (Novoseven, NovoNordisk). Then, 1 mg mL^{-1} acetylthioacetate-Phe-Pro-Arg-FVIIai was mixed with 0.5 mg mL^{-1} iodoacetamide-fluorescein, to which 50 μL of 2 mol L^{-1} NH$_2$OH was added. This mixture was incubated for 1h, protected from light. The fluorescein isothiocyanate-labeled FVIIai was separated from free dye on a PD-10 column equilibrated in phosphate buffered saline (PBS).

Plasmids and site directed mutagenesis
Human Tissue Factor cDNA was obtained from RZPD Deutsches Ressourcenzentrum fur Genomforschung (Berlin, Germany). Tissue Factor cDNA was amplified by polymerase chain reaction (PCR) from RZPD clone IRAUp969D0152D6 from library IRAU MGC Human verified full length cam cDNA. TF cDNA containing the full-length coding sequence was amplified by PCR using the sense primer 5'-GGTACCATGGAGACCCCTGCCT -3' and the anti-sense primer 5'-GGATCCTTATGAAACATTCAGTGG -3'. The sense primer contained a Kpn1 restriction site and the anti sense primer contained a BamH1 restriction site. The PCR fragment was ligated into pGem-t-easy (Promega). Tissue factor cDNA was subcloned into pcDNA3.1 (Invitrogen) via restriction sites Kpn1 and BamH1 obtaining the plasmid pcDNA3.1-tissue factor. A Site Directed Mutagenesis kit (Stratagene) was used to introduce a stop codon in the cDNA of TF at amino-acid site 246 by conversion of AGA to TGA obtaining the plasmid pcDNA3.1-TF-ΔCT. Plasmid DNA sequence was analyzed with BigDey Terminator v3.1 cycle sequencing kit from Applied Biosystems.

Confocal scanning laser microscopy (CSLM)

HeLa cells were seeded on glass coverslips and 24 hours later transfected with plasmid. The cells were used for experiments 36 hours after transfection. HeLa cells were incubated at 37°C and 5% CO_2 for 1.5 h with 100 nmol L^{-1} annexin A5-allexa568, M1234-alexa568, annexin A1-alexa568 and/or 10 nmol L^{-1} FITC-labeled inactivated factor VIIa in $CaCl_2$-containing DMEM (Gibco) supplemented with 10% FBS. The cells were washed twice with PBS. The cells were then fixed for 15 min in 4% paraformaldehyde (pH 8.0) and washed twice with PBS. The microtubule disruption agent colchicine (Sigma) was added 1h before starting an experiment using a concentration of 10 µg mL^{-1}. Actin was visualized by staining fixed cells with Texas-red or Oregon-green labeled phalloidin (Invitrogen) according to the manufacturer's instructions. After phalloidin staining, cells were washed twice with PBS. The coverslips with HeLa cells were examined with a confocal scanning laser microscope (Bio-Rad) equipped with a Crypton/Argon mixed gas laser (Ion Laser Technology) using excitation wavelengths of 488nm for green fluoresce and 568nm red fluorescence. Images were recorded and analyzed with Adobe Photoshop 5.0 and Image J. The fluorescence intensity of membrane bound FVIIai-F was measured as the integrated density (ID) using Image J. The ID was defined as the sum of grey values present within a region of interest. The average ID was measured of eight positions which were randomly selected on the plasma membrane of a TF-expressing cell.

Transfection and measurement of tissue factor activity

HeLa cells were seeded in the wells of a 96 wells plate. Transfection with pcDNA3.1-TF and pcDNA3.1-TF-ΔCT was carried out 24h after the seeding procedure. TF activity was determined 36h after transfection by incubating transfected cells with a reaction mixture containing: 100 nmol L^{-1} factor X, 100 pmol L^{-1} factor VIIa, 3 mmol L^{-1} $CaCl_2$, and 200 µmol L^{-1} Pentafluor FXa substrate (Pentapharm) as previously described [17]. Fluorescence tracings were recorded in a plate spectrofluorometer (Spectramax Gemini XS, Molecular Devices, Sunnyvale, CA, USA) at 37°C with an excitation and emission wavelength of 350 and 450 nm, respectively.

Statistical analysis

To determine the statistical significance of means, probability values were obtained with a Student T-test. Probability values of < 0.05 were considered significant. Error bars indicate standard deviation.
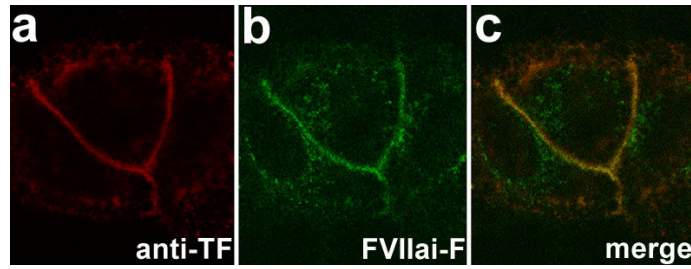
Figure 3, **FVIIai-F binds specifically to cell surface expressed tissue factor.** TF-expressing HeLa cells were incubated with 10 nmol L^{-1} FVIIai-F (fig 3b) and subsequently stained with primary polyclonal anti-TF antibody (American diagnostica) and secondary alexa568-labeled goat-anti-rabit-antibody (fig. 3a). Fig. 3c shows the composition of fig. 3a and 3b.
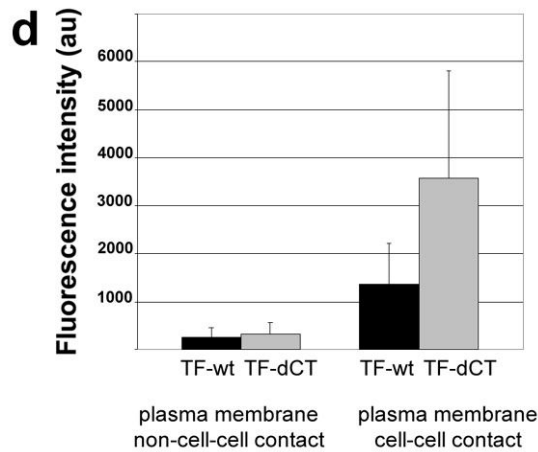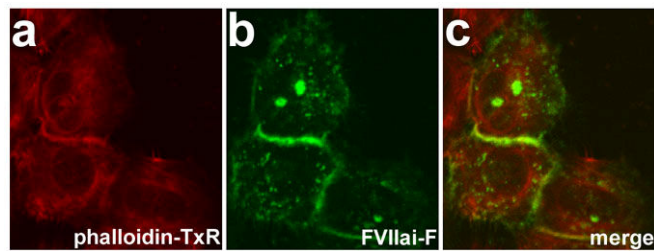
Figure 4, **TF is not associated to sites of cell-cell contact via the cytoplasmic domain.** Hela cells transfected with TF-ΔCT (fig. 4a-c) were incubated for 1.5h with 10 nmol L^{-1} FVIIai-F and stained with phalloidin-texas red. Fig. 4a shows a CSLM image of cells stained with phalloidin-texas red. Fig. 4b shows cells incubated with FVIIai-F. Fig. 4c was obtained after merging fig. 4a and 4b. Fig. 4d shows the fluorescence intensity of membrane bound FVIIai-F expressed as the average integrated density.

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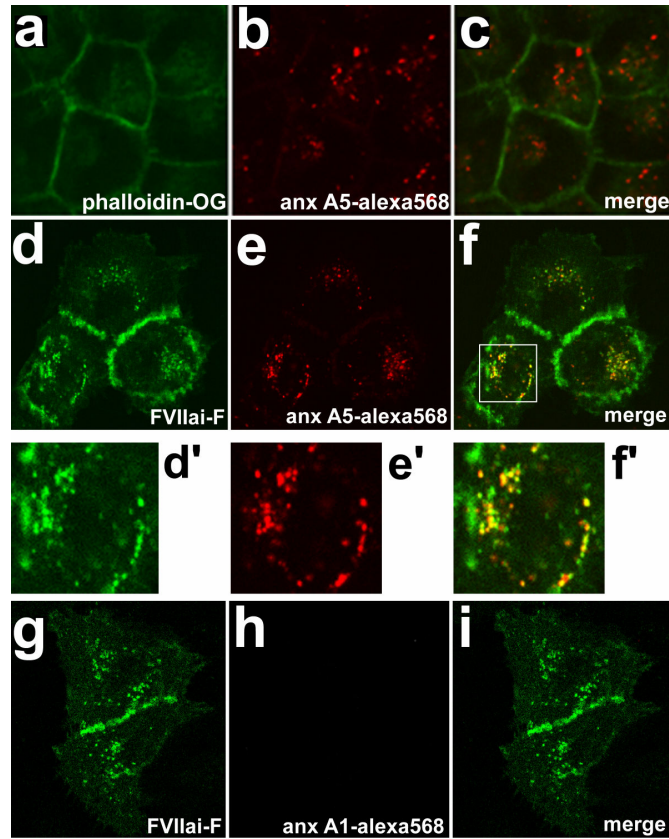
Figure 5, **Annexin A5 induces endocytosis of tissue factor:factor VIIia-F complex in HeLa cells.** CSLM image of HeLa cell stained with phalloidin-oregon green (green; Fig. 5a) after incubated for 1.5h with 100 nmol L^{-1} annexin A5-alexa568 (red; Fig. 5b). Fig. 5c was obtained after merging of image 5a and 5b. CSLM image of HeLa cells transfected with TF cDNA and incubated for 1.5h with 10 nmol L^{-1} FVIIai-F (green; Fig. 5d) and 100 nmol L^{-1} annexin A5-alexa568 (red; Fig. 5e). Fig. 5f was obtained after merging of image 5d and 5e. Fig. 5d', 5e' and 5f' are magnifications of fig. 5d, 5e and 5f respectively. CSLM images of HeLa cells transfected with TF cDNA were incubated with 10 nmol L^{-1} FVIIia-F (green; Fig. 5g) and 100 nmol L^{-1} annexin A1-alexa568 (red; Fig. 5h). Fig. 5i was obtained after merging fig. 5g and 5h.

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Results

Localization and activity of cell surface expressed TF

To examine annexin A5-induced endocytosis of cell surface expressed TF we used HeLa cells transiently transfected with TF cDNA as a model system. Expression and functionality of cell surface expressed TF was assessed by a TF activity assay and confocal scanning laser microscopy (CSLM), respectively. Fig. 1 shows that TF cDNA transfected HeLa cells have a 25-fold higher TF activity than non-transfected HeLa cells. Factor Xa generation by transfected and non-transfected cells could be fully blocked by anti-TF antibody TF5 and by annexin A5, indicating that activation of factor X at the cell surface is dependent on the presence of both TF and PS.

We also examined cell surface expression of TF with CSLM. HeLa cells transfected with TF cDNA stained on the plasma membrane with primary antibody TF5 and secondary phycoerythrin-labeled goat-anti-mouse antibody (Dako) indicating the presence of cell surface-located TF and non-transfected HeLa cells did not stain for TF (data not shown). Fluorescein-labeled inactivated factor VIIa (FVIIai-F) binds stoichiometrically with high affinity to TF. To examine cell surface expression of TF in more detail non-transfected HeLa cells were incubated for 1.5h with FVIIai-F in a calcium-containing medium. Fig. 2a shows a CSLM image of HeLa cells stained with the actin dye phalloidin-texas red. Binding of FVIIai-F to non-transfected cells was not observed (Fig. 2b). We next studied binding of FVIIai-F to the cell surface of TF cDNA transfected HeLa cells. Hereto, transfected cells were incubated with FVIIai-F for 1.5h and then analyzed by CSLM. For reference, cells were also stained for actin (Fig. 2d). As shown in Fig. 2e, FVIIai-F is localized at a high density in membrane regions where cell-cell contact occurred. Non-contact regions showed a much lower density. Fig. 2e also shows an intracellular location for FVIIai-F, suggesting that the complex of TF:FVIIai-F was partly internalized during the 1.5 hour incubation period. The absence of intracellular FVIIai-F in non-transfected cells strongly supports the notion that FVIIai-F only internalizes in complex with TF (Fig.2b).

The question of whether internalization of TF:FVIIai-F caused a reduced TF activity on the cell surface was addressed by measuring TF-dependent FX activation. After 1.5h of treatment with FVIIa, TF activity was not significantly decreased (P value > 0.05) when compared to non-treated cells. This finding seemingly contrasts the notion that TF is internalized together with FVIIai-F (Fig. 2e). A plausible explanation could be a very rapid recycling of internalized TF from cytosol to cell surface.

FVIIa has the capacity to bind via its gamma-carboxyglutamic acid (GLA) domain to a PS-containing membrane surface [15]. To confirm that FVIIai-F binds to cell surface expressed TF and not to PS, we incubated transfected HeLa cells for 1.5h with FVIIai-F and subsequently stained these cells with primary polyclonal anti-TF antibody (American Diagnostica) and secondary alexa568-labeled goat-anti-rabbit antibody (Invitrogen). Fig. 3c shows that staining of TF

with primary anti-TF antibody and secondary alexa568-labeled antibody (Fig. 3a) co-localizes with cell surface bound FVIIai-F (Fig. 3b) indicating that FVIIai-F specifically binds to TF.

## Role of the TF cytoplasmic domain in localization of TF at sites of cell-cell contact

We next, examined the role of the TF cytoplasmic domain on the preference of TF to associate with sites of cell-cell contact. Staining of HeLa cells with the actin dye phalloidin-alexa568 showed that actin is present in high concentrations at sites of cell-cell contact (Fig 2d). Because the cytoplasmic domain of TF has been reported to interact with the actin cytoskeleton via the adaptor protein filamin A [18], we chose to examine whether interaction of the TF cytoplasmic domain with the cytoskeleton is involved in the localization of TF at sites of cell-cell contact. To this end we transfected HeLa cells with the TF deletion mutant TF-ΔCT which lacks the cytoplasmic domain. HeLa cells transfected with TF-ΔCT cDNA were incubated for 1.5h with FVIIai-F. Actin stained cells are shown in Fig. 4b as a reference. It is apparent from the CSLM recordings that the cellular localization of TF-ΔCT is not different from that of TF-wild type (cf. Fig. 2e and Fig. 4b).

The localization of membrane bound FVIIai-F was further analyzed by measuring the fluorescent intensity of FVIIai-F as recorded by CSLM. The fluorescent intensity was expressed as the average integrated density (ID) of the grey values of FVIIai-F. Measurement showed that the ID of FVIIai-F present on HeLa cells expressing TF was significantly higher (P value < 0.01) on the plasma membrane at sites of cell-cell contact compared to sites that were not in contact with other cells (fig. 4d). A similar result was found for HeLa cells expressing TF-ΔCT. These findings suggest that TF association to sites of cell-cell contact is not mediated via the intracellular domain but likely by its extracellular domain. In addition, the very same pattern of intracellular distribution of FVIIai-F was observed for cells transfected with TF-wild type and TF-ΔCT, suggesting that endocytosis of TF:FVIIai-F complex is not dependent on the cytoplasmic domain of TF.

Annexin A5 induces in viable HeLa cells internalization of TF:FVIIai-F

Non-transfected viable HeLa cells were incubated for 1.5h with annexin A5-Alexa568. Fig. 5a shows a CSLM image of a HeLa cell stained with the actin dye phalloidin-oregon green. CSLM analysis of this HeLa cell further revealed the presence of intracellular vesicles containing annexin A5-alexa568 (fig. 5b). When non-transfected HeLa cells were co-incubated with annexin A5-alexa568 and FVIIai-F we observed intracellular vesicles containing annexin A5-alexa568 only and no vesicles containing FVIIai-F (not shown). This result indicates that annexin A5 does not induce the internalization of FVIIai-F by non-transfected HeLa cells.
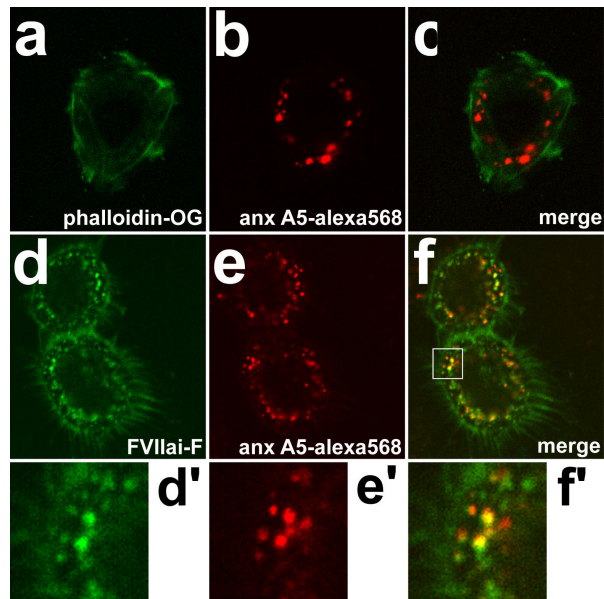
Figure 6, **Transport of endocytic vesicles containing both annexin A5 and TF:factor VIIai-F complex is microtubule-dependent.**
HeLa cells were pretreated with 10 µmol L^{-1} colchicine and incubated for 1.5h with 100 nmol L^{-1} annexin A5-alexa568. CSLM image of HeLa cell stained with phalloidin-oregon green (green; Fig. 6a) and incubated with annexin A5-alexa568 (red; Fig. 6b). Fig. 6c was obtained after merging of image 6a and 6b. CSLM image of HeLa cells transfected with TF cDNA, pretreated with colchicine and incubated for 1.5h with 10 nmol L^{-1} FVIIai-F (green; Fig. 6d) and 100 nmol L^{-1} annexin A5-alexa568 (red; Fig. 6e). Fig. 6f was obtained after merging of image 6d and 6e. Fig. 6d', 6e' and 6f' are magnifications of fig. 6d, 6e and 6f respectively.

We next determined the effect of extracellular annexin A5 on the internalization process of TF bound FVIIai-F in TF cDNA transfected viable HeLa cells. After a co-incubation of FVIIai-F and annexin A5-alexa568 for 1.5h, we observed two enclosing populations of endocytic vesicles. The first containing only FVIIai-F and the second containing both FVIIai-F and annexin A5-Alexa568 (Fig. 5d-f). Endocytic vesicles containing only annexin A5-Alexa568 were rarely observed in TF-transfected cells. This result prompted us to hypothesize that the concomitant endocytosis of annexin A5 together with TF:FVIIai-F complex is induced by membrane binding, two-dimensional crystallization of annexin A5 and by the subsequent endocytic vesicle formation.
To confirm that internalization of annexin A5 together with TF:FVIIai-F complex is dependent on the capacity of annexin A5 to bind PS, we performed the same experiment with the non-lipid binding annexin A5 mutant M1234 [19]. HeLa cells transfected with TF cDNA that were incubated with M1234-alexa568 and FVIIai-

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F only comprised vesicles that contained FVIIai-F indicating that the PS binding property of annexin A5 is critical for its endocytosis by HeLa cells (not shown). Confirming an annexin A5-independent pathway of TF:FVIIai endocytosis. Endocytosis of annexin A5 was earlier shown to be dependent on the ability of annexin A5 to form a two-dimensional crystal lattice [4]. We examined whether annexin A5-induced endocytosis of TF is also dependent on the ability of annexin A5 to form a membrane bound crystal lattice. Another member of the annexin family, annexin A1, binds to PS but does not form a membrane bound two-dimensional crystal lattice [18]. Transfected HeLa cells expressing TF were co-incubated with annexin A1-alexa568 and FVIIai-F. CSLM analysis showed cell surface binding of FVIIai-F and intracellular vesicles containing FVIIai-F (fig. 5g). Fig. 5h shows that intracellular vesicles containing annexin A1-alexa568 are absent from these cells. This result indicates that not only PS binding but also two-dimensional crystal formation of annexin A5 is necessary for annexin A5 to internalize together with TF:FVIIai-F complex.

These results indicate that PS exposition on the plasma membrane of HeLa cells is necessary for the internalization of annexin A5. When HeLa cells were incubated with annexin A5-alexa 568 we did not detect binding of annexin A5 on the plasma membrane with CSLM (not shown). This result suggests that plasma membrane binding of annexin A5-alexa568 is below the detection limit of CSLM measurement. However, annexin A5 does inhibit TF activity of HeLa cells, indicating that PS is present on the plasma membrane.

In order to determine whether transfection of HeLa cells with TF cDNA induces exposure of PS on the plasma membrane we incubated transfected cells for 10 minutes with annexin A5-alexa 568. We did not detect membrane binding of annexin A5-alexa568 with CSLM analysis indicating that the transfection procedure did not induce upregulation of PS exposition on the cell surface of viable HeLa cells (not shown).

Finally, we studied the effect of annexin A5 on TF internalization by measuring the FVIIa-dependent factor X converting activity of TF. TF-expressing HeLa cells were incubated for 1.5h with FVIIa in the presence and absence of annexin A5. The cells were washed with EDTA-containing buffer before measuring TF activity to remove annexin A5 from the cell surface. We did not find a significant difference (P value > 0.05) in the factor X-converting activity of TF between cells treated with annexin A5 and cells that were not treated with annexin A5 (not shown) suggesting that annexin A5-mediated internalized TF, like annexin A5-independent internalized TF, is rapidly recycled back to the cell surface.

Transport of annexin A5-containing vesicles is mediated by microtubuli

CSLM analysis shows that intracellular vesicles containing both annexin A5 and TF:FVIIai-F complex are localized in the perinuclear region (fig. 5f) suggesting that these vesicles are subjected to an intracellular transport process. Microtubuli have been reported to play a role in the transport of endocytic vesicles into the cytosol [20]. We, therefore, examined the effect of colchicine, a microtubule disruption agent, on the transport process of annexin A5-containing vesicles. HeLa cells were pre-treated for 1h with colchicine and subsequently incubated for 1.5h with annexin A5-alexa568. Fig. 6a shows a CSLM image of a HeLa cell stained with the actin dye phalloidin-oregon green. Colchicine treatment caused loss of cellular shape resulting in rounding-up of the cell. CSLM analysis of this HeLa cell shows that in colchicine-treated cells, annexin A5-alexa568-containing vesicles remain localized nearby the plasma membrane indicating that colchicine treatment inhibits the transport of annexin A5-containing vesicles (fig 6b). We next examined the effect of colchicine treatment on the transport of vesicles containing both annexin A5 and FVIIai-F. Transfected HeLa cells expressing TF were pre-treated with colchicine and subsequently incubated with annexin A5-alexa568 and FVIIai-F. Fig. 6d shows that FVIIai-F binds to the cell surface and that FVIIai-F is present in vesicles in the cytosol. Fig. 6e further shows that annexin A5-alexa568-containing vesicles are present close to the plasma membrane. Vesicles of various sizes containing both annexin A5 and TF:FVIIai-F are present in the cytosol near the cell surface indicating that the transport process of these vesicles is also inhibited by colchicine (fig. 6f). These results demonstrate that vesicles containing annexin A5 and vesicles containing both annexin A5 and TF:FVIIai-F complex are transported into the cytosol via a microtubule-dependent process.

Discussion

In a previous study we found indications for an annexin A5-mediated internalization of TF expressed on apoptotic THP-1 macrophages [10]. The relevance of the annexin A5-mediated endocytic pathway of TF during apoptosis, however, is debatable. Why should a cell determined to die be capable of activating endocytic pathways? It should however be emphasized that the annexin A5 endocytic pathway in apoptotic cells could be a remnant of the pinocytic capabilities of the living cell. In addition, internalization of TF by apoptotic cells in contact with the blood circulation could be an important mechanism in the down regulation of thrombus formation. To find further support for our notions on the role of annexin A5 and PS in endocytosis of transmembrane proteins, and in particular that of TF, we performed a study on the internalization of TF using living HeLa cells.

TF-dependent FX activation measurements and CSLM visualization with active site labelled FVIIa (FVIIai-F), however, revealed that HeLa cells express only small amounts of TF, if any, on their surfaces. Moreover, upon incubation of

HeLa cells with FVIIai-F, no internalization could be detected. We therefore used in further experiments HeLa cells that were transfected with human TF. Transfected HeLa cells showed a 25-fold higher TF activity than non-transfected cells. Exposure of PS in the outer leaflet of the plasma membrane was not affected by the transfection procedure. The FXa-forming activity could be completely neutralized by annexin A5 and an anti-TF antibody, indicating the critical dependence of both TF and PS.

We found that the average fluorescent intensity of TF:FVIIai-F on the plasma membrane at sites of cell-cell contacts was significantly higher when compared with plasma membrane that was not in contact with other cells. Localization of TF at cell-cell contacts has been earlier observed by Luther *et al.* in intercalated discs between cardiomyocytes [21]. These authors suggested that TF interacts via its cytoplasmic domain with the adaptor protein filamin A, which in turn binds to actin. However, we found that the mutant TF lacking the intracellular domain (TF-ΔCT), like wild type TF, was predominantly localized at sites of cell-cell contact, suggesting that interaction with filamin A is not involved in the cellular distribution of TF. Taken together, these findings suggest that clustering of TF at sites of cell-cell contact probably arises from the interaction of the extracellular domains of TF.

Using FVIIai-F indicated that TF-expressing HeLa cells internalize TF:FVIIai-F complex into the cytosol. We did not observe internalization of FVIIai-F in wild type HeLa cells indicating that TF-FVIIai-F complex formation is essential for FVIIai-F endocytosis. Our findings on the internalization of TF are in agreement with those reported by Hanssen *et al.* [22]: endocytosis of TF:FVIIai-F complex is not dependent on the cytoplasmic domain of TF. This conclusion is based on the observation that HeLa cells that express the TF deletion mutant TF-ΔCT on the cell surface enclose endocytic vesicles containing FVIIai-F. This observation indicates that the complex of TF-ΔCT and FVIIai-F is internalized by HeLa cells.

In an earlier study we established that internalization of annexin A5 by HeLa cells is dependent on the capacity of annexin A5 to bind PS and to form a membrane bound two-dimensional crystal lattice [4]. This proposition is supported by the following observations: 1) annexin A5 mutant M1234, which is unable to bind to PS, is not internalized by HeLa cells, demonstrating that PS binding of annexin A5 is necessary for the endocytosis process, 2) annexin A1, which binds PS but does not form a two-dimensional crystal lattice, is not internalized by HeLa cells, demonstrating that two-dimensional crystallization is essential for the internalization process, and 3) HeLa cells co-incubated with annexin A1 and annexin A5 enclosed endocytic vesicles containing both annexin A1 and annexin A5, suggesting that annexin A1, bound to the PS-expressing membrane close to annexin A5 is internalized by HeLa cells. We further demonstrated that internalization of annexin A5 by HeLa cells did not occur via macropinocytosis, caveolar or clathrin mediated endocytic pathways.

In the present study we investigated the possibility that internalization of annexin A5 by HeLa cells is accompanied by the concomitant internalization of

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membrane receptors by examining the internalization process of TF in the presence of annexin A5. We found that annexin A5, exogenously added to TF-expressing HeLa cells, internalizes together with TF:FVIIai-F inside the same endocytic vesicles indicating that both annexin A5 and TF:FVIIai-F are internalized via the same endocytic pathway. To determine whether HeLa cells internalize TF:FVIIai-F after annexin A5 induction as previously described for apoptotic THP-1 cells [10], we investigated whether PS binding and two-dimensional crystallization of annexin A5 were necessary for the internalization process of TF. The experiments with M1234 and annexin A1 showed that both proteins were not internalized together with TF:FVIIai-F by HeLa cells, indicating that indeed annexin A5-induced internalization of TF:FVIIai-F might be responsible for the internalization process. As earlier shown in the study by Kenis *et al.* [4] for the simultaneous internalization of annexin A5 and annexin A1 these results suggest that the close presence of TF:FVIIai-F to membrane bound annexin A5 results in the concomitant internalization of both annexin A5 and TF:FVIIai-F. We further observed that TF-expressing HeLa cells enclosed two populations of endocytic vesicles: vesicles containing only FVIIai-F and vesicles containing both FVIIai-F and annexin A5. This finding suggests that two pathways are present for the internalization of TF: 1) a FVIIai-F-induced pathway and 2) an annexin A5-induced pathway. This conclusion is based on the observation that FVIIai-F is internalized independently of annexin A5. In addition annexin A5 is not present in all vesicles that contain FVIIai-F indicating that annexin A5 is not internalized in these vesicles and thus not via this pathway. Ravassa *et al.* [10] observed that annexin A5-induced internalization of TF:FVIIai-F in apoptotic THP-1 cell results in a significant decrease in FVIIa-dependent TF activity. We found however that in HeLa cells both FVIIa-induced and annexin A5-induced internalization of TF does not result in a decrease of FVIIa-dependent TF activity. A possible explanation for this result is that after internalization TF is rapidly recycled from the cytosol back to the cell surface. Indeed, Iakhiaev *et al.* showed for fibroblast cells that FVIIa-induced internalization of TF is followed by recycling of TF back to the cell surface [23].

In conclusion our findings indicate that annexin A5 induces internalization of TF:FVIIai-F in viable HeLa cells. However, internalization of TF:FVIIai-F by viable HeLa cells does not result in a decrease in TF activity, suggesting that annexin A5 does not play a significant role in down regulation of the coagulation processes initiated by TF-bearing cells.

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Chapter 5

Rolling and adhesion of apoptotic monocytes is impaired by loss of functional cell surface expressed P-selectin glycoprotein ligand-1

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Summary

Background: Apoptosis induces cellular membrane changes that are thought to be linked to thrombotic processes, e.g. surface exposure of procoagulant phosphatidylserine (PS), up regulation of tissue factor (TF) and microvesicle formation. The latter, though, could down-regulate this cellular response by shedding prothrombotic membrane elements, e.g. integrins and TF. To test this hypothesis, etoposide treated, apoptotic, monocytic cells (THP-1) were examined for rolling and adhesion on adherent platelets and for TF expression. Methods and results: Etoposide treatment did not result in a significant change in TF antigen expression. However, TF activity, measured in a continuous factor Xa generation assay, was increased 5-fold concomitantly with increased exposure of PS. Laminar flow adhesion assays specific for interaction between P-selectin and P-selectin glycoprotein ligand 1 (PSGL-1) revealed that in contrast to non-treated cells, apoptotic cells did not roll or firmly attach on adherent platelets. Lack of apoptotic THP-1 platelet interaction could be attributed to both a loss of cell surface expressed PSGL-1 and loss of functional PSGL-1 as a result of disruption of the binding of PSGL-1 with the cytoskeleton. Conclusion: Etoposide-induced apoptosis in THP-1 cells evokes a procoagulant response by increasing TF activity associated with an increased PS exposure. However, in contrast to TF, PSGL-1 shedding and loss of function, makes that apoptotic monocytes are unlikely involved in a thrombotic action because of their inability to adhere to an injured vessel wall or developing thrombus.

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Introduction

Apoptosis is a genetically conserved process intended to remove unwanted, damaged or senescent cells. Proteolytic enzymes known as caspases are activated after ligation of death receptors or after the activation of the intrinsic pathway with for example etoposide [1]. Caspase activation leads to the initial stages of leukocyte apoptosis such as changes on the plasma membrane. These changes include plasma membrane exposition of phosphatidylserine (PS), membrane bleb formation, and generation of microparticles with concomitant loss of membrane receptors [2-4]. Recently it was hypothesized that apoptosis of monocytes contributes to the thrombogenicity of atherosclerotic plaques by upregulation of tissue factor (TF) antigen expression [5]. In addition, cell surface expression of PS catalyzes procoagulant reactions culminating in the formation of thrombin [6]. An important prerequisite for its thrombogenic nature is that the monocyte adheres to a lesion of the vessel wall. Monocytes interact via the constitutively expressed membrane protein P-selectin glycoprotein ligand-1 (PSGL-1) with perturbed endothelial cells and platelets that expose P-selectin [7]. P-selectin, stored in Weibel-Palade bodies of endothelial cells or alpha-granula of platelets, is expressed on the cell surface during vascular injury or inflammatory conditions [8]. The interaction of monocyte PSGL-1 and P-selectin results in rolling and eventually in stable adhesion of these cells [9]. A recent study showed that the attachment of PSGL-1 to the actin cytoskeleton is essential for the rolling capacity of the cell [10]. Rolling of cells expressing PSGL-1 was diminished by disruption of the actin - PSGL-1 interaction via the ezrin-radixin-moesin complex. Apoptosis of mouse L929 cells causes the translocation of moesin from the plasma membrane to the cytosol [11]. Thus, apoptosis may cause impaired tethering and rolling of monocytes through inducing the translocation of moesin with a concomitant loss of PSGL-1 function.

To gain more insight into the effects of apoptosis on the prothrombotic role of monocytes we investigated the change in procoagulant and adhesive properties of the monocytic THP-1 cell line upon treatment with etoposide. Changes in the procoagulant response were evaluated with a continuous factor Xa generation assay that is dependent both on TF and PS exposure. Expression of TF antigen and PS were measured by flow cytometry. A laminar flow adhesion assay was used to evaluate the PSGL-1 dependent interaction of apoptotic THP-1 cells with a monolayer of surface-adherent platelets. Apoptotic THP-1 cells were distinguished from non-apoptotic cells by the use of fluorescently labeled annexin A5. Annexin A5 is a protein that binds calcium-dependently with high affinity to plasma membranes that expose PS, and is therefore extensively used as a tool to detect apoptosis [12, 13]. We investigated the effect of apoptosis on the interaction of PSGL-1 with the cytoskeleton by flow cytometry and confocal scanning laser microscopy (CSLM) using Moesin-EGFP. The results obtained show that etoposide-induced apoptosis increases monocytic TF activity as a result of increased PS expression. However, the interaction of apoptotic

monocytes with P-selectin-expressing cell surfaces was greatly impaired because of loss of functional PSGL-1.

Materials and methods
Reagents
Etoposide and bovine serum albumin (BSA) were purchased from Sigma. FITC-labeled anti-TF antibody was obtained from Diagnostica Stago. TF neutralizing antibody (TF-5) was purchased from Sanquin. Apoptame-Q was a product from MP Biomedicals and Phyco Erythrin (PE)-annexin A5 and propidium iodide were purchased from Invitrogen. Annexin A5, alexa 568-annexin A5, Oregon Green-annexin A5 and FITC-annexin A5 were from Nexins. Hepes buffer (pH 7.45) contains 10 mmol L^{-1} Hepes, 136 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 2 mmol L^{-1} MgCl$_2$, 5 mmol L^{-1} glucose and 0.1 % BSA.

Cell culture conditions
The human monocytic THP-1 cell line was obtained from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 with glutamax-I and Phenol Red (Gibco), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; PAA Laboratories), 25 mmol L^{-1} Hepes, 100 U mL^{-1} penicillin and 100 µg/mL streptomycin. Mouse fibroblast 3T3 cells, obtained from ATCC were maintained in DMEM with glutamax-I supplemented with 10% heat-inactivated FBS (v/v), 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin. Cells were maintained at 37ºC in a humidified atmosphere with 5% CO$_2$ and resuspended in Hepes buffer containing 3 mmol L^{-1} CaCl$_2$ immediately before use.

Blood cell preparation
Washed platelets were prepared as described previously [14]. Briefly, blood was drawn from healthy volunteers by puncture of antecubital vein and freely drained on 0.17 vol. ACD (80 mmol L^{-1} trisodium citrate, 52 mmol L^{-1} citric acid and 180 mmol L^{-1} glucose). Platelet rich plasma was obtained by centrifugation at 190g for 15 min and diluted with 0.1 vol. of ACD and centrifuged at 650g for 15 min. The resulting pellet was resuspended in Hepes buffer (pH 6.6) and 0.1 vol. ACD and centrifuged at 650g for 15 min. The resulting pellet was resuspended in Hepes buffer (pH 7.4) at 2x10^8 platelets mL^{-1}, stored at room temperature and used within 3 hours.
Monocytes were isolated from blood donated from healthy volunteers as described earlier (15). Briefly, Human monocytes were isolated from leukocyte-rich plasma by hyperosmotic NycoPrep 1.068 density gradient centrifugation (Nycomed, Norway) and platelets were removed by 300g washes. Monocytes were suspended in RPMI containing 10% FBS and used within 4 hours.

Etoposide-induced apoptosis
THP-1 cells (2x10^6/mL) in RPMI 1640 supplemented with 10% FBS were incubated with etoposide (100 µmol L^{-1}) during 4h at 37°C. Apoptosis was

established by binding of FITC-labeled annexin A5 and propidium iodide. Analyses were performed with an EPICS XL flow cytometer (Coulter) and data were analyzed with WinMDI 2.8. (http://facs.scripps.edu/software.html).

Laminar flow adhesion assay

Washed platelets (2×10^8/mL) were allowed to adhere and spread for up to 30 min at room temperature in rectangular glass micro-capillaries (0.2x2.0 mm and length 50 mm; Composite Metal Services). Non-adherent platelets were removed by perfusion (wall shear rate 100 s^{-1}) with Hepes buffer (pH 7.4). The glass capillaries were mounted on an inverted microscope and a suspension of THP-1 cells (2×10^6/mL) in Hepes buffer containing 2 mmol L^{-1} Ca^{2+} was perfused into the capillary with a shear rate of 100 s^{-1}. Flow was then stopped to allow the cells to settle onto the substrate for 45 s at stasis. Flow was initiated (shear rate of 100 s^{-1}) and continued for an additional 4 min. All flow experiments were conducted at ambient temperature (21±1°C). Images (225 µm^2) were visualized with a Diaphot 200 inverted microscope (Nikon) by using 40x oil immersion objective lens. Oregon Green (OG) fluorescence imaging was performed with a 485 nm excitation filter, a 505 nm dichroic long-pass filter, and a 530 nm emission filter. Data were captured at 25 frames per second with high resolution charge-coupled device cameras and recorded on a SVHS recorder [16]. Cells were considered as stationary when they moved less than 1 cell radius within 10 s. Rolling cells were defined as cells moving more than their own diameter during 10 s before arresting or leaving the platelet monolayer. Rolling velocities were determined using Image J software (National Institutes of Health).

Flow cytometry

Non-treated THP-1 cells and etoposide-treated THP-1 cells were incubated for 30 min on ice in Hepes buffer containing 2 mmol L^{-1} Ca^{2+} with FITC-labeled anti-TF (Diagnostica Stago) and annexin A5-PE. The same procedure was used with FITC-labeled anti-PSGL-1 antibody PL2 (Santa Cruz) and annexin A5-PE. The cells were washed ones in Hepes buffer containing 2 mmol L^{-1} Ca^{2+} before flow cytometry analysis.

Tissue factor activity assay

TF activity expressed by THP-1 cells was determined as previously described[17]. Briefly, a cell suspension was incubated with a reaction mixture containing: 100 nmol L^{-1} factor X, 0.1 nmol L^{-1} factor VIIa, 3 mmol L^{-1} CaCl$_2$, and 200 µmol L^{-1} Pentafluor FXa substrate (Pentapharm). Fluorescence tracings were recorded in a 48-well plate spectrofluorometer (Spectramax Gemini XS, Molecular Devices) at 37°C with an excitation and emission wavelength of 350 and 450 nm, respectively. Rate of factor Xa generation is expressed as percentage of control (non-treated) cells.

Plasmid transfection

The plasmid pMoesin-Enhanced Green Fluorescent Protein (EGFP) containing the fusion gene of cDNA of murine moesin and EGFP attached to the carboxy amino terminus of moesin was a kind gift of Dr. D. Crouch (University of Dundee, Biomedical Research Centre, Dundee UK) [18]. 3T3 cells were seeded in wells containing a poly-l-lysine coated glass bottom (MatTek Corporation). Plasmid pMoesin-EGFP was transfected in 3T3 cells at 60% confluency with the transfection agent Fugene 6 (Roche Applied Science). THP-1 cells were electroporated with 10 μg mL^{-1} plasmid pMoesin-EGFP. THP-1 cells were suspended in Optimem (Gibco, Life Sciences) at a density of 4×10^7/mL and electroporation was carried out at 250 V and 900 μF with Bio-Rad Gene Pulser-II.

Confocal Scanning Laser Microscopy (CSLM)

THP-1 cells were incubated with 100 μmol L^{-1} etoposide at 37°C and 5% CO_2 for 4h. The THP-1 cells were washed with phosphate buffered saline (PBS). The cells were then incubated on ice with alexa 568-annexin A5 and anti-PSGL-1 antibody KPL1 for 30 min in Hepes buffer containing 2 mmol L^{-1} Ca^{2+}. In the latter case a PE-labeled goat-anti-mouse secondary antibody was used to detect bound KPL1.

The cells were washed once in Hepes buffer containing 2 mmol L^{-1} Ca^{2+}. The cells were then fixed for 15 min in Hepes buffer containing 2 mmol L^{-1} Ca^{2+} and 4% paraformaldehyde (pH 8.0) and washed once in Hepes buffer containing 2 mmol L^{-1} Ca^{2+}. The fixed THP-1 cells were mounted in 90% glycerol in 2 mmol L^{-1} Tris-HCl pH = 8.0 with addition of 2% 1,4-diazobicyclo-[2,2,2]-octane (Merck). Slides were examined with a confocal scanning laser microscope (Bio-Rad) equipped with a Crypton/Argon mixed gas laser (Ion Laser Technology). Images were recorded and analyzed with NIH Image (National Institutes of Health).

Time-lapse fluorescence microscopy

Imaging was started 24h after transfection of 3T3 cells with pMoesin-EGFP using an inverted fluorescence microscope (Zeiss) with appropriate excitation and emission filters (Leica Microsystems). Image acquisition was carried out in intervals of 15 min using Openlab (Improvision) software. Deconvolution software was applied to remove out-of-focus information from selected images using the Openlab package.

Statistical analysis

To determine the statistical significance of means, P values were obtained with a Student T-test. Error bars indicate standard deviation.

Results
Apoptotic THP-1 monocytes have an increased tissue factor activity
In order to asses the effect of apoptosis on the procoagulant activity of THP-1 cells, we analyzed the plasma membrane expression of TF and PS on non-treated and etoposide-treated (100 μmol L^{-1}) THP-1 cells. Flow cytometry of THP-1 cells using a FITC-labeled anti-TF antibody showed that virtually all THP-1 cells expressed TF and only a small subpopulation (5%) of THP-1 cells bound annexin A5 (Fig. 1A). Following treatment with etoposide a marked increase in annexin A5 binding cells (44% of the total population) was observed (Fig. 1B). However, TF expression of apoptotic, annexin A5 binding, cells was not diminished when compared with non-treated cells.

We next determined the effect of etoposide treatment on TF activity, measured as the rate of factor VIIa-dependent factor X activation. Fig. 1C shows that etoposide-treated cells have a 5-fold higher TF activity than non-treated cells. In both cases, factor Xa generation could be fully blocked by a TF neutralizing antibody (TF-5) and by annexin A5. No factor Xa generation was detected in the absence of THP-1 cells. These findings indicate that THP-1 associated factor Xa generation depends on the presence of both TF and PS expressed at the THP-1 surface.

Figure 1,

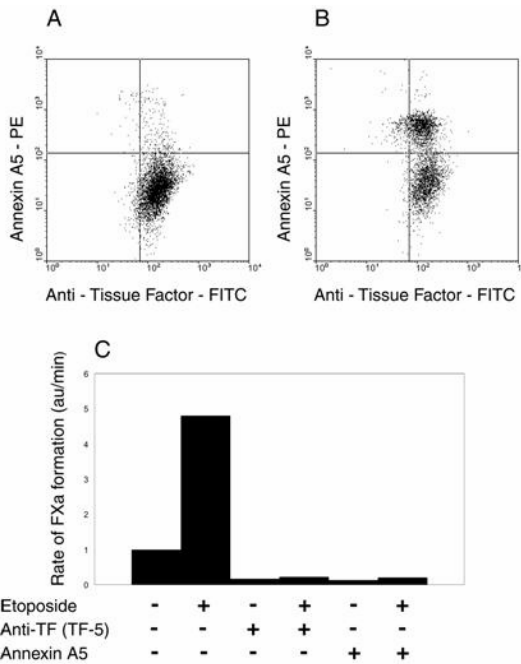**TF antigen expression and TF activity of etoposide-treated THP-1 cells.**

THP-1 cells were incubated (4 hours) in the absence (A) and presence (B) of 100 μmol L^{-1} etoposide at 37°C, labeled with FITC-anti-TF and PE-annexin A5 and analyzed by flow cytometry. C, factor VIIa-dependent factor Xa generation in the absence and presence of 10 μg mL^{-1} annexin A5 and 10 μg mL^{-1} anti-TF antibody (TF-5).

Apoptotic THP-1 cells have a diminished interaction with adherent platelets

The results so far established that etoposide treatment of monocytic THP-1 cells causes an increase in TF-mediated procoagulant activity. We next wished to establish whether or not these apoptotic cells can adhere to a surface of adherent platelets. Therefore, non-treated and etoposide-treated THP-1 cells were perfused into a capillary with a monolayer of adherent platelets, the flow was stopped for 45 s and then continued for 4 min. In case of non-treated cells a large fraction (90%) of the settled cells rolled over the surface while a few remained firmly attached (Fig 2A). Typical tracings of translocating THP-1 cells are shown in Fig. 2C. The variance in flow velocities is apparent: cells that role continuously, cells that role for a short time and become firmly bound and cells that become firmly attached during the stasis phase.

Flow cytometry analysis of THP-1 suspensions treated with etoposide revealed that $33\pm4\%$ (mean $\pm$ SD, n=5) of the total cell population bound annexin A5. These cells were considered to be apoptotic. To distinguish apoptotic cells from living cells in the flow adhesion assay, cells were incubated for 1 min with OG-annexin A5 and visualized by fluorescence microscopy. Fig. 2B demonstrates that apoptotic THP-1 cells do not role, nor stably adhere when flow is initiated after a 45 s period of stasis. Simultaneous real-time phase contrast and fluorescence microscopy revealed that OG-annexin A5 negative cells (living cells) present in the cell suspension that was treated with etoposide rolled and firmly adhered as observed in cell suspensions not treated with etoposide shown in Fig. 2A (data not shown).

To assess more precisely the ability of non-treated cells and apoptotic cells to interact with platelets, the number of stationary and rolling cells was determined 4 min after the restart of the perfusion from 10-15 randomly chosen microscopic fields. Fig. 2D shows that for non-treated cells the number of firmly attached cells was 8.1 ± 1.1 (mean $\pm$ SD) per microscopic field. Occasionally a rolling cell was observed (0.2 ± 0.2 cells per microscopic field). The number of stationary cells drastically decreased when monocytic cells were treated with etoposide (attached THP-1 cells versus attached etoposide treated THP-1 cells, P value < 0.025). The number of adherent apoptotic cells was 1.3 ± 0.9 (mean $\pm$ SD) per microscopic field. Interestingly, treatment of THP-1 cells with the PSGL-1 antibody KPL1 (10 µg mL^{-1}) [19] resulted in the same drastic reduction in the number of adherent cells (Fig. 2D). To confirm that monocytes isolated from human blood are equally affected by etoposide as demonstrated for THP-1 cells, flow adhesion assays were performed with non-treated and etoposide-treated monocytes. After 4 min perfusion of non-treated cells (1.5×10^6 cells/mL) through capillaries with adherent platelets, the number of rolling cells was 13.4 ± 3.2 (mean $\pm$ SD) per microscopic field. Only a few stationary cells were observed. Etoposide treatment greatly reduced the number of rolling cells (1.4 ± 1.4 per microscopic field). No stationary cells were observed.

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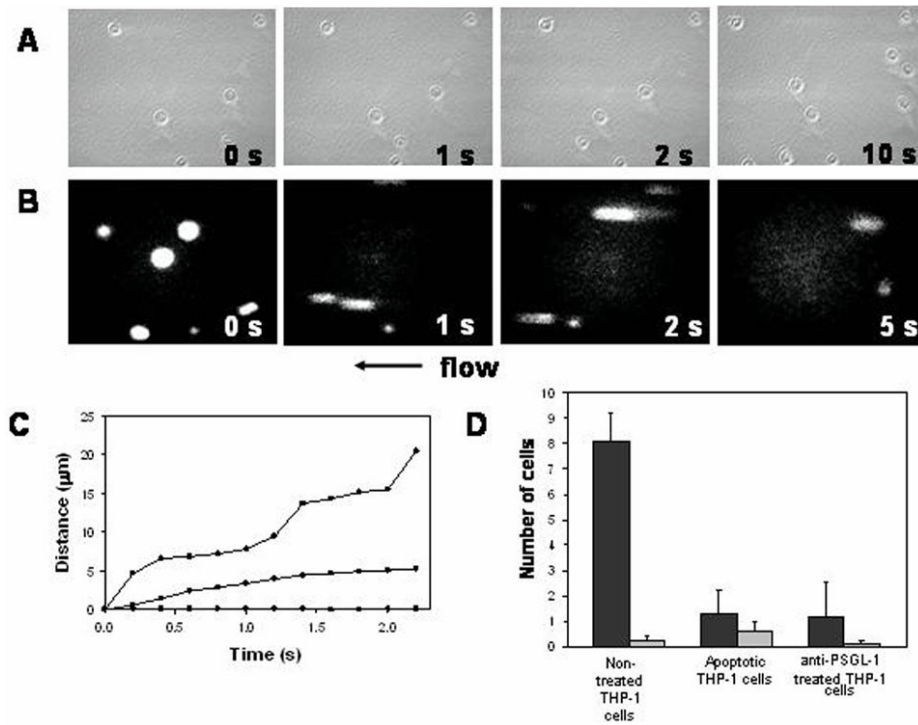
Figure 2, **Interaction of THP-1 cells with a monolayer of adherent platelets.**
A: a suspension of THP-1 cells (2×10^6/mL) was infused in a glass capillary coated with a monolayer of platelets. After 45 s of stasis the flow was started (t=0 s) and phase contrast images were grabbed at the times indicated. B: a similar experiment as described in panel A but with etoposide-treated THP-1 cells (2×10^6/mL) incubated with OG-annexin A5 and visualized by fluorescence microscopy. C: representative translocations of THP-1 cells in the direction of the flow. D: the number of firmly adherent (black bar) and rolling cells (gray bar) as determined 4 min after the restart of perfusion with non-treated cells, apoptotic cells and cells that were treated with 10 µg mL^{-1} of the anti-PSGL-1 antibody KPL-1 from 10-15 different microscopic fields in 3 independent perfusion experiments.
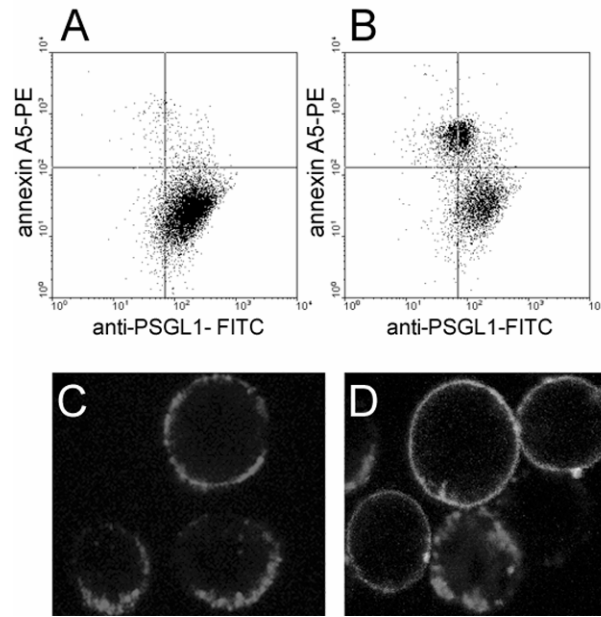
Figure 3, **Effect of etoposide on PSGL-1 exposure.** THP-1 cells were incubated for 4 hours in the absence (A) and presence (B) of 100 µmol L^{-1} etoposide at 37°C, labeled with FITC-anti-PSGL-1 and PE-annexin A5 and then analyzed by flow cytometry. Non-treated cells (C) and etoposide-treated cells (D) were stained with anti-PSGL-1 antibody (KPL1) followed by a secondary PE-goat-anti-mouse antibody (red) and FITC-annexin A5 (green) and analyzed by CSLM.

Apoptosis induces loss of functional PSGL-1

We examined two possibilities which could be responsible for the altered rolling and adherence behavior of apoptotic THP-1 cells. Firstly, apoptosis could induce the shedding of PSGL-1 from the THP-1 cell surface and secondly, apoptosis could cause an impaired attachment of PSGL-1 to the actin cytoskeleton resulting in loss of function. We first compared the surface density of PSGL-1 before and after treatment with etoposide. THP-1 cells expressed PSGL-1 on the cells surface as shown with flow cytometry using a FITC-labeled anti-PSGL-1 antibody (Fig. 3A). Flow cytometry demonstrated that apoptotic, annexin A5 binding, THP-1 cells have a reduced surface density of PSGL-1 (Fig. 3B). The mean fluorescence intensity (MFI) for FITC-labeled anti-PSGL-1 antibody on annexin A5 binding cells was 28% compared to non annexin A5 binding cells. CSLM of non-treated THP-1 cells that were stained with the primary antibody KPL1 and a secondary PE labeled goat-anti-mouse antibody showed the presence of PSGL-1 in high density at discrete sites on the plasma membrane (Fig. 3C). However, PSGL-1 could not be detected on the plasma

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membrane of etoposide treated THP-1 cells that bind FITC-annexin A5 (Fig. 3D). Pre-incubation of THP-1 cells with 10 µmol L^{-1} pan caspase inhibitor apoptame-Q prevents etoposide-induced loss of PSGL-1 (data not shown), confirming that the apoptotic process reduces the amount of surface-expressed PSGL-1.

An alternative or additional explanation for the aforementioned impaired adhesion properties of apoptotic THP-1 cells is loss of the moesin-controlled association of PSGL-1 to actin in microvilli. It has been demonstrated that the association of PSGL-1 with the actin cytoskeleton is essential for the rolling ability of leukocytes over P-selectin exposing cells [10]. This notion prompted us to examine the cellular location of moesin during etoposide treatment. Moesin location was examined with a plasmid containing a cDNA for green fluorescent protein tagged to murine moesin. We first examined the location of moesin-EGFP during etoposide treatment in murine 3T3 cells to study the behavior of moesin in a homologous cellular system. The location of moesin-EGFP in transfected 3T3 cells was followed during apoptosis by time lapse fluorescence microscopy taking images with intervals of 15 min. Transfected 3T3 cell showed a patchy-like localization of moesin-EGFP at the plasma membrane. Etoposide treatment induced a dissociation of moesin-EGFP from the plasma membrane into the cytosol concomitant with alexa 568-labeled annexin A5 binding to the plasma membrane. Apoptosis-induced translocation of moesin-EGFP could be inhibited by incubation with apoptame-Q (data not shown). We next turned to THP-1 cells to examine moesin-EGFP location after etoposide treatment. The location of moesin in THP-1 cells was visualized by CSLM after electroporation of THP-1 cells with plasmid containing the moesin-EGFP gene. CSLM demonstrated that moesin-EGFP is located at the plasma membrane and in the cytosol of THP-1 cells (Fig. 4A). In etoposide treated THP-1 cells moesin-EGFP was only seen in the cytosol and not at the plasma membrane of annexin A5 binding THP-1 cells (Fig. 4D, 4E). These findings indicate translocation of moesin-EGFP from the plasma membrane to the cytosol before or during the exposition of PS on the plasma membrane both in murine 3T3 cells and human THP-1 cells.
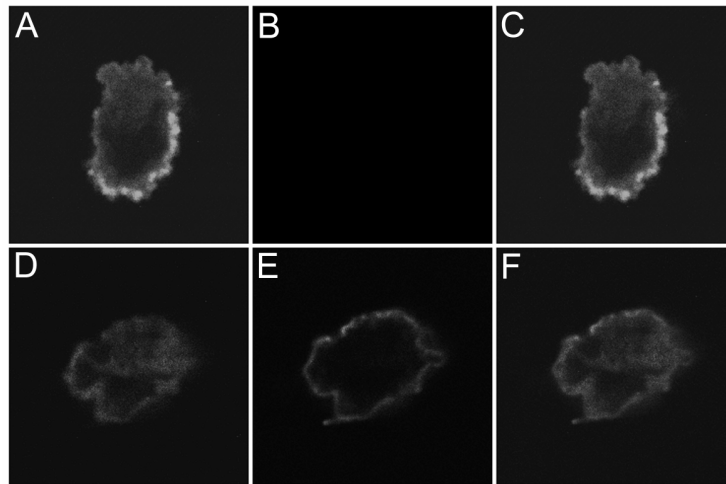
Figure 4, **Etoposide-induced translocation of moesin-EGFP in THP-1 cells.**
THP-1 cells transiently expressing moesin-EGFP were incubated for 4 hours in the absence (A, B, C) and presence (D, E, F) of 100 µmol L^{-1} etoposide at 37°C. Alexa 568-annexin A5 is absent on THP-1 cells (B), Moesin-EGFP is present in both the membrane bound and in the free cytosolic form in THP-1 cells (C). Moesin-EGFP is present in cytosolic but not in membrane bound form in etoposide treated THP-1 cells (D), THP-1 cell with plasma membrane bound Alexa 568-annexin A5 (E). F depicts the merged images of D and E.

Discussion

The processes involved in the interaction between leukocyte PSGL-1 and cells exposing P-selectin are currently under intense investigation. PSGL-1 is a leukocyte membrane protein involved in the initial rolling and attachment events of leukocytes on platelets and endothelial cells exposing P-selectin and/or L-selectin. Recent studies demonstrate that the interaction of leukocyte expressed PSGL-1 with membrane exposed P-selectin not only plays a role in inflammatory processes but also in haemostasis [20, 21].

In this study we examined apoptosis-induced changes in PS exposure, TF and PSGL-1 expression on the plasma membrane of monocytic THP-1 cells. We established with flow cytometry and a factor X activation assay that TF is present on THP-1 monocytes. During etoposide-induced apoptosis TF expression was not changed. However the activity of TF was increased. The apoptosis-induced increase in TF activity is associated with the externalization of PS. We conclude that apoptosis-induced membrane exposure of PS transforms cryptic TF into active TF by supplying anionic phospholipids that support the assembly of the extrinsic tenase complex. Etoposide-induced up-regulation of TF activity by circulating monocytes poses a risk of thrombosis on patients treated with this cytostatic agent, provided that the etoposide-treated monocytes retain the ability to adhere to vascular sites with activated platelets and endothelial cells. In this paper we demonstrate for THP-1 cells that etoposide induces apoptosis and reduces concomitantly the adhesive properties of THP-1 cells by downregulation of surface expression of functional PSGL-1. Since differences between THP-1 cells and human blood monocytes have been reported we compared both cell types on their adhesive and rolling properties [22]. Similar to THP-1 cells, the human monocytes lose their ability to adhere and roll on a layer of activated platelets if treated with etoposide, indicating that THP-1 cells are a representative model for monocyte interaction with platelets.

Flow cytometry and CSLM indicate that annexin A5 binding cells have a reduced surface density of PSGL-1. We hypothesize that apoptosis-induced shedding of PSGL-1 bearing microparticles or protease-catalyzed cleavage of the extracellular domain of PSGL-1 causes loss of cell surface-located PSGL-1 during externalization of PS. Alternatively or additionally, the inability of apoptotic THP-1 cells to form firm interactions with adherent platelets could also be caused by an impaired moesin-mediated interaction between PSGL-1 and the actin cytoskeleton [10]. Similar to observations made by Kondo *et al* [11] in mouse L929 cells, we have shown that moesin is not present in the membrane bound form during apoptosis of mouse 3T3 cells and human THP-1 cells. Snapp *et al* have shown that the association of PSGL-1 with the actin cytoskeleton is essential for the rolling ability of leukocytes over P-selectin exposing cells [10]. We propose that translocation of moesin during apoptosis and the concomitant dissociation of PSGL-1 from the actin cytoskeleton decreases the interaction of PSGL-1 with P-selectin. Collectively, our data suggest that

apoptosis induces two processes that impair the interaction of THP-1 cells with P-selectin-bearing cells: a) loss of membrane associated PSGL-1 and b) loss of functionality of membrane-associated PSGL-1.

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Chapter 6

Summary and general discussion

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Introduction

Annexin A5 is a protein of 36 kDalton that binds in a calcium-dependent manner to phosphatidylserine (PS)-containing membrane surfaces. Membrane binding is followed by the formation of annexin A5 trimers which subsequently associate, via protein-protein interactions, to form larger structures such as dimers of trimers and trimers of trimers [1, 2]. The association of trimers leads to the formation of a two-dimensional crystal lattice which is characterized by P3 or P6 symmetry. In this thesis we have investigated whether the PS binding property of annexin A5 and the subsequent trimer formation can influence the membrane dynamics of the PS-expressing plasma membrane. In the studies presented here we have chosen to examine PS binding of annexin A5 with the PS-containing outer membrane surface of cells because: (i) The calcium concentration present in the circulation is 1000 µmol L^{-1} and in the cytosol, at resting level, is approximately 0.1 µmol L^{-1} [3-5]. (ii) The half maximal adsorption of annexin A5 to a phospholipid layer consisting of a ratio of 20/80 mol% and 5/95 mol% of phosphatidylserine to phosphatidylcholine occurs at a calcium concentration of 220 and 1500 µmol L^{-1} respectively [6]. From both conditions we can conclude that annexin A5 binding to a PS-containing membrane surface does much more readily occur when annexin A5 is present in the circulation than in the cytosol.

Annexin A5 has been suggested to function as an anti-coagulant protein by competing for PS binding sites in a calcium-dependent manner with prothrombin on a PS-expressing membrane surface [7]. Apart from playing a role in the anticoagulant arm of the thrombotic system annexin A5 has also been suggested to play a role in other processes occurring on a PS-expressing cell surface. For instance, binding of annexin A5 to the plasma membrane of apoptotic cells has been reported to influence the progress of the apoptotic program [8]. Both examples give reason to expect that annexin A5 might play a role on other cell types during various circumstances. In the studies presented in this thesis we have investigated the effect of annexin A5 binding to PS on various cell types. In chapter 3 we examined PS binding of annexin A5 on apoptotic Jurkat cells and viable HeLa tumor cells. It was shown that PS binding of annexin A5 influences membrane dynamics of the PS-expressing cell surface by inhibiting apoptotic body formation and by inducing a novel endocytic pathway that depends on PS-expression. Here we discuss the implication of the annexin A5-mediated inhibition of apoptotic body formation in relation to physiological processes in which apoptotic bodies play a role. In chapter 3 and 4 we examined endocytosis of annexin A5 and the annexin A5-induced endocytosis of the membrane protein tissue factor in viable HeLa cells. We discuss the physiological relevance of this endocytic pathway. And finally in chapter 5 we examined plasma membrane changes occurring during apoptotic cell death of monocytes.

Function of the annexin A5 two-dimensional crystal lattice

In chapter 3 is investigated whether plasma membrane dynamics of PS-expressing cells is influenced by the trimer based two-dimensional crystal lattice formed by annexin A5. The two-dimensional crystal lattice has been suggested to function in several membrane-related processes. The coagulation proteins factor Xa, factor Va and prothrombin associate on a PS-expressing membrane to form the prothrombinase complex. Andree *et al*. proposed that annexin A5 not only inhibits the formation of the prothrombinase complex by competing for PS binding sites but also by the formation of the two-dimensional crystal lattice[9]. They suggested that the membrane bound crystal lattice of annexin A5 inhibits the formation of the prothrombinase complex by preventing the lateral movement of the coagulation factors on the PS-expressing membrane surface. Another function of the two-dimensional crystal lattice was suggested by Gidon-Jeangirard *et al*. [8]. Cells induced to undergo apoptosis are characterized by membrane changes such as externalization of PS to the outer membrane leaflet and shedding of microparticles. Gidon-Jeangirard *et al*. observed that cell surface binding of annexin A5 resulted in the inhibition of microparticle generation by cells which were undergoing apoptotic cell death [8]. They proposed that inhibition of microparticle generation by annexin A5 was the result of a physical constraint on the cell surface carried out by the crystal lattice formed by annexin A5 on the membrane.

Inhibition of microparticle generation by annexin A5

We studied the ability of annexin A5 to inhibit microparticle generation with a model system comprised of T-lymphoma Jurkat cells induced to execute apoptosis with an anti-body directed against the Fas-receptor. Annexin A5 was able to inhibit, dose-dependently, the generation of Jurkat cell derived microparticles. In order to determine the role of the two-dimensional crystal lattice formed by annexin A5 in inhibiting the generation of microparticles we used the annexin A5 mutant 2D1-6 which binds PS but is unable to form a two-dimensional crystal lattice. We found that 2D1-6 was able to inhibit microparticle generation indicating that two-dimensional crystal formation is not involved in inhibiting the generation of microparticles. This was further supported by experiments with annexin A1 (a member of the annexin family which binds PS but is unable to form a two-dimensional crystal lattice) which showed that annexin A1 was also able to inhibit microparticle generation. These experiments demonstrate that PS binding of annexin A5 and not two-dimensional crystal formation causes the inhibition of microparticle generation. How annexin A5 inhibits microparticle generation is at present unclear.

In recent years microparticles, containing (intrinsic membrane) proteins, have been suggested to function in intercellular communication processes and in the transfer of plasma membrane receptors between cells [10-16]. Monocytes for instance, generate microparticles carrying the membrane proteins tissue factor and P-selectin Glycoprotein Ligand-1 (PSGL-1). These microparticles have

been reported to accumulate on P-selectin-expressing platelets located at a developing thrombus during vascular injury [14, 15]. Annexin A5 might regulate these microparticle-mediated processes if the following conditions are fulfilled (i) the microparticles express PS on the membrane surface and (ii) annexin A5 is present in the circulation in a concentration that results in inhibition of microparticle generation.

The annexin A5-PS endocytic pathway
Our investigations into the inhibition of microparticle generation by annexin A5 revealed that annexin A5 but not annexin A5 mutant M1234 (which is unable to bind calcium and consequently does not bind PS) was internalized by apoptotic Jurkat cells and HeLa tumor cells. Internalization of annexin A5 was shown to be mechanistically distinct from macropinocytosis or from clathrin or caveolar-mediated endocytosis. These results indicate that annexin A5 is internalized via a novel endocytosis pathway that requires the cell surface expression of PS.

Annexin A5 has been reported to function as a plasma membrane localized calcium conducting ion-channel [17, 18]. Calcium signaling plays a role in the initiation of various biochemical processes. It might be possible that the formation of annexin A5 calcium channels is involved in the initiation of annexin A5 endocytosis. However, channel formation of annexin A5 through the plasma membrane occurs, according to current models, in an environment with a low calcium concentration. Internalization of annexin A5 is consequently not initiated by channel formation because of the high calcium concentration that was used in our experiments.

Having ruled out the involvement of annexin A5-mediated calcium signaling in the endocytosis process we examined the involvement of the two-dimensional crystal formed by annexin A5. Apoptotic Jurkat cells and HeLa tumor cells were incubated with fluorescently labelled 2D1-6. CSLM analysis did not show the presence of endocytic vesicles containing 2D1-6 indicating that two-dimensional crystal formation of annexin A5 is involved in the endocytosis process. This conclusion was further corroborated by experiments that showed that annexin A1 also failed to internalize in apoptotic Jurkat cells and HeLa tumor cells.

A possible mechanism for the endocytosis of annexin A5 might be the annexin A5-induced curvature of the membrane which leads to the formation of an endocytic vesicle. The tertiary structure of annexin A5 shows that its phospholipid binding side has a convex shape [19]. When bound to the membrane annexin A5 forms trimers of which each monomer retains the convex shape at its phospholipid binding side [20, 21]. Domain III is the most distant from the membrane surface and domain II the closest [20]. All four domains contain a calcium-binding motif making it possible that membrane-bound annexin A5 influences the PS-expressing membrane surface to adopt a shape that is complementary to the convex shape of the annexin A5 molecule. We propose that the trimers bend the membrane and provide the driving force for the internalization of membrane-bound annexin A5.

Annexin A5-induced endocytosis of tissue factor

Annexin A5 has been proposed the play a role in anti-coagulant processes by binding to PS and by the formation of a two-dimensional crystal lattice. PS binding of annexin A5 prevents binding of factor Va, Xa and prothrombin to the PS-expressing cell surface [7]. And the two-dimensional crystal lattice of annexin A5 molecules prevents the lateral movement of PS bound Va, Xa and prothrombin to combine into the pro-thrombinase complex [9]. In a recent study by Ravassa *et al.* a third anti-coagulant mechanism in which annexin A5 plays a role was proposed [22]. Tissue factor (TF) was shown to be internalized concomitantly with annexin A5 by apoptotic PS-expressing THP-1 macrophages. TF is the cell surface receptor of the serine protease factor VIIa (FVIIa) [23]. The complex of TF with FVIIa converts the inactive factor X (FX) into the active factor X (FXa). The formation of the complex of TF, FVIIa and FX and the subsequent conversion of FX into FXa requires the presence of PS on the cell surface. Ravassa *et al.* showed that annexin A5-induced internalization of TF on apoptotic THP-1 cells resulted in a significant decrease in TF activity [22]. This result indicates that annexin A5 plays a role in anti-coagulant processes initiated by TF-expressing apoptotic cells. In this thesis we have investigated whether annexin A5 also plays a role in down regulating TF activity on living HeLa cells. The physiological relevance of annexin A5-induced TF internalization during apoptosis is not well understood and can be debatable. Apoptotic cells that express PS on the plasma membrane are rapidly removed from tissue by phagocytes [24]. If the annexin A5-PS endocytic pathway plays a role of significance it has to be active before the apoptotic cell is cleared by phagocytosis. To investigate the possibility that annexin A5-induced endocytosis of TF occurs in living cells we studied TF internalization in a model system comprised of living HeLa cells expressing TF cDNA. Using this model system we demonstrated annexin A5-induced endocytosis of TF by showing that fluorescently labeled FVIIa was internalized by annexin A5 in HeLa cells transfected with TF cDNA. This indicates that the annexin A5 endocytic pathway can internalize TF in living cells. Annexin A5-induced internalization of TF did however not cause a significant decrease in TF activity on living HeLa cells. These results indicate that annexin A5 plays a role in down regulating TF activity on apoptotic cells but not on living cells. A possible explanation for this result is that living HeLa cells recycle TF back to the cell surface while recycling of TF does not occur in apoptotic THP-1 cells. Apoptotic cells contain active proteolytic enzymes known as caspases [25]. The intracellular proteins responsible for recycling receptors back to the cell surface might be the target of proteolytic cleavage by caspases with the result that TF is retained in the cytosol of apoptotic THP-1 macrophages.

In recent years cell surface expression of PS has been found to occur on several cell types under specific conditions and independent of cell death. For instance, monocytes that differentiate into macrophages or monocytes stimulated with the dimeric protein galectin-1 expose PS [26-28]. The results

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presented in chapter 4 raise the possibility that the annexin A5-PS endocytic pathway might by involved in the internalization of TF in these cell types. The data presented in chapter 4 suggest that the annexin A5-PS endocytic pathway might also be involved in the internalization and regulation of plasma membrane-localized receptors besides TF. Other potential membrane receptors that could be internalized by the annexin A5-PS endocytic pathway might be receptors homologous to TF such as the interferon-gamma (IFN-γ) receptor and other receptors of the class II cytokine receptor family [29].

Functionality of cell surface expressed PSGL-1 during apoptosis
In chapter 5 we studied the effect of plasma membrane changes occurring during apoptosis on monocyte expressed TF and PSGL-1. Monocytes generate microparticles that express the membrane proteins TF and PSGL-1 [14, 15] indicating that TF and PSGL-1 are closely localized to each other on the cell surface. Because TF was shown to be internalized by annexin A5 we investigated the possibility that the annexin A5-PS endocytic pathway affects the functionality of PSGL-1 by internalizing this protein. Our investigations into the functionality of PSGL-1 on the apoptotic plasma membrane showed however that this protein had lost its functionality independent from the annexin A5-PS endocytic pathway. Monocytes are able to roll and adhere to P-selectin exposing adhered platelets via cell surface expressed PSGL-1 [30]. We demonstrated that THP-1 monocytes had lost this ability during the apoptotic process because of loss of function of cell surface expressed PSGL-1 and because of disruption of the binding of PSGL-1 to the cytoskeleton.

Future perspectives of annexin A5 in anti-tumor treatment
Apoptotic cells are frequently found in pathologically insulted tissues. Solid tumors, for example, often contain apoptotic PS-expressing cells because of the insufficient supply of oxygen and nutrients into the tumor tissue. In addition, the number of apoptotic cells present in the tumor increases when cancer patients are treated with cytostatic apoptosis-inducing agents. PS-expressing endothelial cells are further found in angiogenic blood vessels present in solid tumors [31]. The presence of PS-expressing cells in tumors offers an opportunity to devise strategies to treat tumors using annexin A5 as a targeting agent.
An example of such a strategy is described in a study recently published by Bondanza and co-workers [32]. Dendritic cells (DC) are able to phagocytose apoptotic tumor cells and to present apoptotic tumor cell-derived antigens via the major histocompatibility complex I (MHC-I) on the cell surface [33]. MHC-I presented tumor antigens then become available for recognition by cytotoxic T-lymphocytes. The strategy Bondanza and co-workers used was based on a difference in phagocytic abilities displayed by macrophages and DC's. Macrophages recognize and phagocytose PS-expressing apoptotic cells via a putative PS-receptor [24]. DC's, however, are less dependent on PS recognition during phagocytosis of apoptotic cells because DC's actively internalize cells

and substrates via macropinocytosis in a constitutive manner [32, 34, 35]. Bondanza and co-workers introduced apoptotic lymphoma cells concomitantly with annexin A5 into the circulation of mice. They reasoned that disrupting the PS-dependent clearance of apoptotic cells by macrophages with annexin A5 would result in unscavenged apoptotic tumor cells, available for uptake and processing by DC's. The DC's would subsequently present tumor derived antigens via MHC molecules on the cell surface and activate a cytotoxic T-lymphocyte response. Bondanza and co-workers observed a cyotoxic T-lymphocyte response against lymphoma tumor cells when the same mice were later challenged with viable lymphoma cells. The cytotoxic T- lymphocyte response in these mice resulted in a significant decrease in tumor development. Two other strategies which are currently under development are possible: (i), using the PS binding property of annexin A5 it might be possible to target toxic agents to PS-expressing cells present on or inside the tumor. This strategy has been used earlier for targeting the bacterial pseudomonas exotoxin to malignant glioma tumor cells. Glioma tumor cells are a type of neural tumor cells which overexpress the interleukin-4 receptor on the cell surface. Puri and co-workers created a chimera protein that consisted of the exotoxin protein tagged to interleukin 4 [36]. The interleukin 4 part of the chimera protein was used in this strategy as a tumor-targeting agent. Once introduced close to the glioma tumor this chimera protein would accumulate at the tumor by binding to the interleukin 4 receptor. The accumulated chimera protein would then exert its toxic effect on the tumor cells. A similar strategy is now pursued by Reutelingsperger and co-workers. The PS binding property of annexin A5 is used to target pseudomonas exotoxin tagged to annexin A5 to PS-expressing cells in the tumor. This strategy aims to accumulate a high concentration of pseudomonas exotoxin inside the tumor which would subsequently decrease in size because of the toxic effects of the exotoxin.

 (ii), The PS binding property of annexin A5 could also be used to specifically target apoptosis-inducing cytostatic agents to the site in the body where the solid tumor is located. It is desirable to administrate cytostatic agents only to the site where the solid tumor is located. Cytostatic agents are introduced into the circulation which leads to a systemic distribution of the agent. The systemic distribution of the cytostatic agent leads to the destruction of the tumor but also to damage of organs that do not contain tumor cells. A strategy which aims to solve this problem is currently under development by Reutelingsperger and co-workers. Reutelingsperger and co-workers use annexin A5 to target liposomes that contain a cytostatic agent to PS-expressing tumor cells. Annexin A5 tagged liposomes containing cytostatic agents are introduced into the circulation. These liposomes subsequently accumulate on PS-expressing cells present on the solid tumor. Annexin A5 targeting of liposome-containing cytostatic agents leads to destruction of the tumor while non-specific distribution to other parts in the body is prevented.

In this thesis we have described the existence of an endocytic pathway present on PS-expressing cells that internalize annexin A5. Other strategies in anti-tumor treatment can be envisioned that use the capacity of annexin A5 to internalize into PS-expressing cells. One such strategy might be the introduction of apoptosis-inducing agents in PS-expressing endothelial cells lining the inner surface of angiogenic blood vessels present inside solid tumors.

Conclusions

Previously annexin A5 was known to bind to PS-expressing cell surfaces in a calcium-dependent manner. In this thesis it was shown that annexin A5 not only binds to the PS-expressing cell surface but that PS binding and the formation of a two-dimensional crystal lattice also influences the membrane dynamics of the PS-expressing cell surface. It was shown that annexin A5 binding to PS inhibits microparticle formation by PS-expressing apoptotic Jurkat lymphoma cells. It was further shown that annexin A5 induces internalization of PS microdomains by inducing curvature of the cell surface. In addition, annexin A5 induces internalization of the intrinsic membrane protein TF present in the PS microdomain. The annexin A5-PS endocytic pathway also makes it possible to devise novel strategies in anti-tumor treatment.

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Samenvatting

Inleiding

De annexine familie bestaat uit eiwitten die gemeenschappelijke structurele en functionele eigenschappen hebben. Annexine A5, een lid van deze familie, is in staat om in de aanwezigheid van calcium ionen aan een membraan te binden die negatief geladen fosfatidylserine (PS) bevat. De tertiaire (drie-dimensionale) structuur van annexine A5 bestaat uit vier domeinen, met elk 70 aminozuren, die cyclisch georganiseerd zijn. Annexine A5 moleculen gaan in een oplossing, in afwezigheid van calcium of een PS bevattende membraan, geen interacties met elkaar aan. Echter membraan gebonden annexine A5 moleculen (monomeren) associëren met elkaar, via eiwit-eiwit interacties, tot structuren (trimeren) die elk opgebouwd zijn uit drie annexine A5 moleculen. Trimeren kunnen vervolgens een twee-dimensionaal membraan gebonden kristal rooster vormen die beschreven kan worden door middel van P3 of P6 rotatie symmetrie.

In dit proefschift hebben we onderzocht of binding van annexine A5 aan PS en de daaropvolgende trimer vorming invloed heeft op de dynamica van de PS bevattende plasma membraan. We hebben gekozen om de interactie van annexin A5 met PS te bestuderen op PS bevattende cel oppervlakken die bloot gesteld zijn aan de omgeving buiten de cel om de volgende redenen. (1) Annexin A5 moleculen komen voor in het binnenste van een cel en in bloed. (2) De calcium concentratie binnen in een cel is ongeveer 0,1 micro-molair en de calcium concentratie van bloed is 1000 micro-molair. (3) Annexine A5 kan het oppervlak van een PS bevattende membraan die bestaat uit 20 mol% en 5 mol% PS voor de helft bezetten bij een calcium concentratie die gelijk is aan respectievelijk 1500 en 220 micro-molair. Uit het voorgaande kan afgeleid worden dat annexine A5 veel makkelijker bindt aan een PS bevattende membraan als dit eiwit zich in het bloed bevindt.

Er zijn verschillende processen waarbij een PS bevattende membraan een rol speelt. Bloed plaatjes die aan collageen vast gehecht zitten brengen PS tot expressie op het membraan oppervlak. De bloed stollings factoren Va, Xa en prothrombine kunnen op dit PS bevattende membraan binden en het prothrombinase eiwit complex vormen. Het prothrombinase complex is verantwoordelijk voor de omzetting van het inactieve eiwit prothrombine naar het actieve thrombine. Thrombine is daaropvolgend betrokken bij het omzetten van het inactieve en oplosbare eiwit fibrinogeen naar fibrine. Fibrine moleculen kunnen onderling associaties aangaan die leiden tot de formatie van lange polymeren (kettingen) die bestaan uit fibrine moleculen. De fibrine polymeren worden gevormd op plaatsen waar een beschadiging van het bloedvat heeft plaats gevonden. Het netwerk van bloed plaatjes en fibrine polymeren is verantwoordelijk voor het voorkomen van het lekken van bloed uit het beschadigde bloedvat.

Er is voorgesteld dat annexine A5 moleculen een rol spelen in het remmen van het bloed stollings proces. Annexin A5 moleculen kunnen een competitie aangaan met de stollings factoren prothrombine, factor Va en factor Xa door het

binden aan een PS bevattende membraan. In dit competitie proces kunnen annexine A5 moleculen de vorming van het prothrombinase complex voorkomen en de daaropvolgende activering van het eiwit thrombine. Een ander proces waarbij een PS bevattende membraan een rol speelt is het cel dood proces. De cel inhoud wordt van de omgeving afgeschermd door middel van een plasma membraan. De zijde van de plasma membraan die bloot gesteld is aan de omgeving bevat normaal geen negatief geladen PS moleculen. In geval van een beschadiging van de cel, cellen die een hoge ouderdom hebben of cellen die ongewild zijn kan de plasma membraan PS bevatten. Het presenteren van PS op de plasma membraan aan de omgeving van de cel is één van de vele eigenschappen die karakteristiek zijn voor een cel die het cel dood proces ondergaat. Er is voorgesteld dat binding van annexine A5 op de PS bevattende plasma membraan het verloop van het cel dood proces kan vertragen. De voorgaande voorbeelden geven aanleiding tot de gedachte dat annexin A5 invloed heeft op biochemische processen die zich afspelen op de PS bevattende plasma membraan van cellen. In dit proefschrift hebben we de invloed van annexine A5 op verschillende processen die zich afspelen op de PS bevattende plasma membraan onderzocht. Tijdens het cel dood proces wordt er niet alleen PS op de plasma membraan gepresenteerd maar er worden ook bolvormige membraan omsloten fragmenten (micro-partikels) afgescheiden. In hoofdstuk 3 staat beschreven dat annexine A5 de vorming van micro-partikels kan remmen. Er staat ook beschreven dat annexine A5, na binding aan de PS bevattende plasma membraan, opgenomen wordt door de cel (endocytose) via een nog niet eerder beschreven endocytose proces. In hoofdstuk 4 staat beschreven dat annexine A5 verantwoordelijk is voor de endocytose van het membraan eiwit tissue factor. En in het laatste hoofdstuk staan de veranderingen beschreven die optreden op de plasma membraan van monocyten die het cel dood proces ondergaan.

Welke rol speelt het twee-dimensionale kristal van annexine A5 op de plasma membraan?

In hoofdstuk 3 hebben we onderzocht wat de rol is van het twee-dimensionale kristal van annexine A5 op de PS bevattende plasma membraan. Er zijn verschillende voorstellen gedaan voor de rol van het twee-dimensionale kristal van annexine A5 op de plasma membraan. De bloed stollings factoren Va, Xa en prothrombine kunnen het prothrombinase complex vormen op een PS bevattende membraan. Harry Andree en collega's hebben voorgesteld dat annexine A5 de vorming van het prothrombinase complex niet alleen kan remmen via een competitie proces voor PS binding maar ook via de vorming van het twee-dimensionale kristal rooster. De bloed stollings factoren Va, Xa en prothrombine binden en bewegen eerst op het oppervlak van de PS bevattende membraan alvorens het prothrombinase complex te vormen. Harry Andree en collega's hebben voorgesteld dat het twee-dimensionale kristal rooster van annexine A5 de beweging voorkomt van de stollings factoren die al aan PS

gebonden zijn zodat er geen prothrombinase complex gevormd kan worden. Een andere rol van het twee-dimensionale kristal rooster is voorgesteld door Gidon-Jeangirard en collega's. Tijdens het cel dood proces worden bolvormige en membraan omsloten fragmenten (micro-partikels) van de PS bevattende plasma membraan afgescheiden. Gidon-Jeangirard en collega's namen waar dat annexine A5 de vorming van micro-partikels kon remmen. Zij stelden voor dat de aanwezigheid van een mechanische beperking op de plasma membraan in de vorm van het twee-dimensionale kristal rooster van annexine A5 verantwoordelijk was voor remming van micro-partikel vorming.

Remming van micro-partikels door annexine A5

Micro-partikel vorming werd bestudeerd door middel van een model systeem bestaande uit Jurkat lymfoma cellen die gestimuleerd werden om het cel dood proces te ondergaan met behulp van een anti-lichaam gericht tegen de Fas-receptor. We namen waar dat annexine A5, dosis afhankelijk, in staat was de vorming van micro-partikels te remmen. We gebruikten de annexine A5 mutant 2D1-6 om te testen of het twee-dimensionale kristal rooster van annexine A5 verantwoordelijk was voor de remming van micro-partikels. De annexine A5 mutant 2D1-6 kan aan PS binden maar door de aanwezigheid van zes mutaties kan deze mutant geen twee-dimensionale kristal rooster meer vormen. Experimenten uitgevoerd met 2D1-6 lieten zien dat de annexine A5 mutant in staat was micro-partikel vorming te remmen. We concludeerden hieruit dat remming van micro-partikel vorming door annexine A5 niet veroorzaakt werd door het twee-dimensionale kristal rooster. Deze conclusie werd ondersteund door de resultaten van experimenten die uitgevoerd werden met annexine A1, een lid van de annexine familie die aan PS kan binden maar die geen twee-dimensionaal kristal rooster kan vormen. We namen waar dat ook annexine A1, dosis afhankelijk, de vorming van micro-partikels kon remmen.

Hoe annexine A5 de vorming van micro-partikels remt is op dit moment niet duidelijk. In de afgelopen jaren zijn er artikelen verschenen waarin gemeld werd dat, membraan eiwit bevattende, micro-partikels een rol spelen in de communicatie tussen cellen en in de overdracht van membraan eiwitten tussen verschillende soorten cellen. Een voorbeeld hiervan is de door monocyten (een type witte bloed) afgescheiden micropartikels die de membraan eiwitten tissue factor (TF) en P-selectine glycoprotein ligand-1 (PSGL-1) bevatten. Er is gerapporteerd dat deze micropartikels zich ophopen op bloed-plaatjes die aanwezig zijn op plaatsen waar vaatwand beschadiging heeft plaats gevonden. Annexine A5 zou een rol kunnen spelen in deze micropartikel gerelateerde processen als er aan de volgende voorwaarden voldaan wordt: (1), de micropartikels bevatten PS op het membraan oppervlak en (2), de concentratie van annexine A5 in het bloed is hoog genoeg om remming van micropartikel vorming te veroorzaken.

Endocytose van annexine A5

Gedurende het onderzoek naar de door annexine A5 veroorzaakte remming van micropartikel vorming namen we waar dat annexine A5 werd opgenomen (endocytose) door cellen met PS op het cel oppervlak. Endocytose was afhankelijk van de aanwezigheid van PS op het cel oppervlak omdat annexine A5 wel maar de annexine A5 mutant M1234 (welke niet in staat is om PS te binden) niet werd opgenomen. Het mechanisme dat verantwoordelijk was voor endocytose van annexine A5 was niet gelijk aan macropinocytose of de caveolaire en clathrine afhankelijke endocyte. Deze resultaten laten zien dat annexine A5 wordt opgenomen via een nieuw endocytose proces.

Er is gesuggereerd dat annexine A5 in staat is om calcium-ion geleidende kanalen in de plasma membraan te vormen. Calcium speelt een rol in signaal processen in de cel, het is dus mogelijk dat kanaal vorming van annexine A5 een rol speelt bij endocytose van annexine A5. Kanaal vorming van annexine A5 treed echter alleen op als de calcium concentratie laag is zoals binnen in een cel. Buiten de cel, in bloed, treed geen kanaal vorming op zodat het annexine A5 geinduceerde endocytose proces niet wordt veroorzaakt door kanaal vorming van annexine A5.

Nadat we hadden uitgesloten dat kanaalvorming van annexin A5 een rol speelt in het endocytose process besloten we de rol van het twee dimensionaal kristal, gevormd door annexine A5, in het opname proces te bestuderen. We testten de betrokkenheid van het twee-dimensionale kristal in het endocytose proces door apoptotische Jurkat lymfoma cellen en HeLa tumor cellen te incuberen met fluorescente 2D1-6. Analyse van de cellen met behulp van confocale scan laser microscopie (CSLM) toonde aan dat de cellen geen fluorescente 2D1-6 hadden opgenomen. Uit de resultaten van dit experiment trokken we de conclusie dat de vorming van het twee-dimensional kristal noodzakelijk is voor de opname van annexine A5 door beide cel typen. Deze conclusie werd ondersteund door de resultaten van een experiment met fluorescente annexine A1. We vonden dat deze annexine niet werd opgenomen door de apoptotische Jurkat lymfoma en HeLa tumor cellen.

Waarom speelt het door annexine A5 gevormde twee-dimensionale kristal rooster een rol in het endocytose proces? De zijde van het annexine A5 molecuul dat aan negatief geladen PS bind heeft een bolle (convexe) vorm. Annexine A5 behoud zijn convexe vorm als het annexine A5 molecuul aan een PS bevattende membraan bind en een trimer vormt. We stellen voor dat de annexine A5 trimeren, waaruit het twee-dimensionale kristal is opgebouwd, de membraan vervormen en ombuigen zodat er endocytische bolvormige structuren onstaan binnen in de cel. Annexine A5 veroorzaakt in dit hypothetische proces zijn eigen opname door de cel.

Annexine A5 veroorzaakt endocytose van tissue factor

Er is voorgesteld dat annexine A5 een rol speelt in de remming van bloed stollings processen. (1) Annexine A5 kan bloed stolling remmen door aan PS te binden. (2) Annexine A5 kan bloed stolling ook remmen door de vorming van een twee-dimensionaal kristal op de PS bevattende membraan. Ravassa en collega's hebben voorgesteld dat er nog een derde manier bestaat waarmee annexine A5 de bloed stolling kan remmen. Annexine A5 kan de bloed stolling ook remmen door internalisatie van tissue factor te veroorzaken op dode cellen. Tissue factor (TF) is een eiwit die aanwezig is in de plasma membraan van bepaalde cel typen. Dit eiwit is in staat de bloedstollings factor VIIa te binden. Het eiwit complex TF en factor VIIa katalyseerd de vorming van actieve factor X uit inactieve factor X. De vorming van het complex bestaande uit TF, factor VIIa en factor X en de daaropvolgende vorming van factor Xa uit factor X vinden plaats als PS aanwezig is in de membraan.

Er is echter reden tot twijfel dat het proces voorgesteld door Ravassa en collega's fysiologisch van belang is. Cellen die PS op de plasma membraan presenteren worden zeer snel verwijderd door macrofagen. Als het annexine A5 endocytose proces een rol van belang speelt dan moet het endocytose proces plaats vinden voordat de apoptotische cel is verwijderd door macrofagen. We onderzochten of endocytose van TF door annexine A5 plaats vindt in levende cellen in een model systeem bestaande uit HeLa cellen getransfecteerd met TF cDNA. Met dit model systeem lieten we zien dat TF endocytose onder invloed van annexine A5 plaats vind door te laten zien dat annexine A5 endocytose van fluorescent factor VIIa veroorzaakt in TF cDNA getransfecteerde cellen. Dit resultaat laat zien dat annexine A5 niet alleen endocytose veroorzaakt van TF in dode cellen maar ook in levende cellen. Annexine A5 veroorzaakte internalisatie van TF door dode cellen veroorzaakte een afname in de activiteit van TF. We namen echter geen afname van TF activiteit waar in levende HeLa cellen. Dit wordt mogelijk veroorzaakt door recycling van TF na internalisatie terug naar het cel oppervlak door levende cellen. In dode cellen treedt geen recycling van TF op waarschijnlijk door een defect recycling mechanisme.

Er zijn de laatste jaren enkele artikelen verschenen waarin gerapporteerd wordt dat levende cellen PS op de plasma mebraan presenteren. Twee voorbeelden zijn monocyten die differentieren tot macrofagen en monocyten gestimuleerd met het eiwit galectine-1. De resultaten die beschreven zijn in hoofdstuk 4 geven aanleiding tot de gedachte dat annexine A5 bij deze cellen de endocytose van TF kan veroorzaken.

De concentratie van receptoren op de plasma membraan kan gereguleerd worden door de caveolaire en clathrine gemedieerde endocytose processen. De resultaten die beschreven zijn in hoofdstuk 4 geven aanleiding tot de gedachte dat annexine A5 ook betrokken zou kunnen zijn bij de regulatie van plasma membraan receptoren. Receptoren die door annexine A5 geëndocyteerd zouden kunnen worden zijn receptoren die homoloog zijn aan

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TF zoals de interferon gamma (IFN-γ) receptor of andere receptoren die lid zijn van de klasse II cytokine receptor familie.

De functionaliteit van PSGL-1 op apoptotische THP-1 cellen
In hoofdstuk 5 onderzochten we het effect van veranderingen, die optreden op de plasma membraan van apoptotische THP-1 monocyte cellen, op de membraan eiwitten TF en PSGL-1. Monocyten kunnen micropartikels afscheiden die de membraan eiwitten TF en PSGL-1 bevatten. Dit is alleen mogelijk als de eiwitten TF en PSGL-1 zich dicht bij elkaar op de plasma membraan bevinden. Omdat annexine A5 de endocytose van TF veroorzaakt besloten we te onderzoeken of annexine A5 de functionaliteit van PSGL-1, door middel van endocytose, kan beinvloeden. Het onderzoek naar de functionaliteit van PSGL-1 tijdens het apoptose proces liet echter zien dat de functionaliteit van PSGL-1 verandert zonder dat annexine A5 veroorzaakte endocytose betrokken is. Monocyten zijn via PSGL-1 in staat te rollen en te hechten aan vast gehechte bloed plaatjes die P-selectine presenteren aan de omgeving. Wij toonden aan dat apoptotische THP-1 monocyten niet meer in staat waren te rollen en te hechten door dat deze cellen PSGL-1 afscheiden en door dat PSGL-1 niet meer vast gehecht was aan het cytoskelet.

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 22 februari 1974 te Uithoorn, Noord Holland. De middelbare school (HAVO en Atheneum) werd doorlopen bij de Stedelijke Scholen Gemeeschap te Leeuwarden, Friesland. In 1993 werd aangevangen met de studie Biologie aan de Rijks Universiteit Groningen. In 2000 werd de studie succesvol afgesloten met als studie richtingen Klinische Immunologie en Medische Fysiologie. In 2001 werd hij aangesteld als onderzoeker in opleiding (OIO) bij de capaciteits groep Biochemie aan de Universiteit Maastricht. In de daaropvolgende jaren werd onderzoek verricht aan het eiwit annexine A5 onder begeleiding van Dr. Chris Reutelingsperger. Het onderzoek gedurende deze jaren heeft geleid tot dit proefschrift.

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