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Review

Surface exposure of phosphatidylserine in pathological cells

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Abstract. The asymmetric phospholipid distribution in plasma membranes is normally maintained by energy-dependent lipid transporters that translocate different phospholipids from one monolayer to the other against their respective concentration gradients. When cells are activated, or enter apoptosis, lipid asymmetry can be perturbed by other lipid transporters (scramblases) that shuttle phospholipids non-specifically between the two monolayers. This exposes phosphatidylserine (PS) at the cells' outer surface. Since PS promotes blood coagulation, defective scramblase activity upon platelet stimulation causes a bleeding disorder (Scott syndrome). PS exposure also plays a pivotal role in the recognition and removal of apoptotic cells via a PS-recognizing receptor on phagocytic cells. Furthermore, expression of PS at the cell surface can occur in a wide variety of disorders. This review aims at highlighting how PS expression in different cells may complicate a variety of pathological conditions, including those that promote thromboembolic complications or produce aberrations in apoptotic cell removal.

Key words. Membrane asymmetry; lipid scramblase; aminophospholipid translocase; apoptosis; blood coagulation; erythrocytes; platelets.

Introduction

Phosphatidylserine (PS) is one of the four major phospholipids that predominates in the plasma membranes of mammalian cells, typically comprising 8–15 mole % of the total phospholipid content. In quiescent cells, it is exclusively located at the cytoplasmic side of the membrane bilayer together with most of the phosphatidylethanolamine (PE), whereas the outer monolayer is mainly composed of phosphatidylcholine (PC) and sphingomyelin (Sph) [1, 2]. Like the other phospholipids, PS contributes to the lipid bilayer structure, but its presence in the cytosolic leaflet is also of functional importance. It facilitates binding of membrane proteins at the endofacial surface, and it forms an essential cofactor for a number of membrane-bound enzymes, such as protein kinase C and Na^+/K^+-ATPase. In addition, PS promotes Ca^{2+}-induced membrane fusion events such as occur during exocytosis. Indeed, cells invest energy to generate and maintain asymmetric phospholipid distribution, and in most cases the non-random orientation of phospholipids is preserved during the life span of the cell.

There are, however, physiologically relevant circumstances under which a collapse of membrane phospholipid asymmetry occurs with concomitant exposure of PS at the cells' outer surface. Lipid layers containing PS promote assembly and catalytic activity of various coagulation factors [3]. In normal blood circulation, the coagulation factors in plasma do not encounter PS, since it is absent from the surface of blood cells and endothelial cells that line the vessel wall. Activation of blood platelets resulting from vessel wall injury can lead to surface exposure of PS, bringing this lipid in contact with the proteins of the coagulation system [4–8]. This results in a dramatic increase in the formation rate of thrombin, the enzyme that converts soluble fibrinogen into an insoluble fibrin clot, and also acts as a potent inducer of platelet secretion

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and aggregation. Collapse of membrane lipid asymmetry with appearance of PS in the outer membrane leaflet is also a hallmark of cells undergoing apoptosis [9–11]. This proces tightly tunes the rate of cell death to balance that of cell division, in order to maintain the number of cells in an organism at a fairly constant level. Macrophages contain a PS receptor that recognizes surface-exposed PS on apoptotic cells [12]. This triggers their uptake and removal, prior to becoming necrotic and inflammatory through spilling of their cellular content into surrounding tissues. Thus, control of membrane lipid asymmetry as well as its collapse seems a requisite for the normal functioning of cells. Aberrations in the mechanisms that regulate phospholipid distributions across plasma membranes may cause or complicate a number of pathologic conditions. This will be the main subject of this review with particular focus on the distribution of PS. We will first briefly discuss how phospholipids are directed to either surface of a plasma membrane by specific membrane proteins that catalyze their transmembrane movement.

Control of membrane phospholipid distributions

Membrane lipid sidedness originates from vectorial biosynthesis of lipids in combination with transporter proteins that move lipids from one side of the membrane to the other [13]. The glycerophospholipids PC, PE and PS are synthesized and simultaneously inserted on the cytosolic half of the endoplasmic reticulum. This membrane has been shown to mediate lipid translocation (particularly PC) to its luminal face within minutes [14–16], which is many orders of magnitude faster than spontaneous transbilayer movement in artificial phospholipid vesicles. This has led to the general concept that lipid movement across the endoplasmic reticulum is protein-mediated, and partial purification of such a lipid transporter has been reported [17]. The protein does not seem to require energy and may also act bidirectionally to equilibrate phospholipids over both sides of the bilayer. Newly synthesized lipids move to the plasma membrane by vesicular transport, which implies that PC becomes located mainly on the external surface, whereas the aminophospholipids PS and PE remain primarily on the cytoplasmic side where they were inserted. Sph (as well as glycosphingolipids) are formed from ceramides by enzymes exposed at the luminal surface of the Golgi [13]. Since these lipids are presumably not substrates for intracellular lipid transporters, they remain predominantly on the non-cytosolic half of cell membranes.

The non-random phospholipid distribution thus introduced during plasma membrane growth is normally preserved until the cell enters the apoptotic stage. Some cells, like blood platelets, lose membrane lipid asymmetry when activated, and perturbations in membrane lipid distributions are also not uncommon in pathological cells [18]. Since spontaneous transbilayer movement of phospholipids is a rare event, these phenomona dictate that plasma membranes contain specific lipid transporters that rapidly move phospholipids back and forth between the two membrane leaflets. These membrane proteins catalyze uni- or bidirectional transport of lipids from one membrane leaflet to the other. At least three types of lipid transporters have been distinguished: ‘flippase’, which catalyzes inward transport of lipids; ‘floppase’, which promotes outward migration of lipids; and ‘scramblase’, which mixes the lipids between the two layers (fig. 1). While the first two activities primarily generate and maintain membrane lipid asymmetry, scramblase activity promotes its collapse.

Lipid transporters

Flippase

The virtual complete absence of PS from the outer surface of normal quiescent cells is primarily caused by a ubiquitous flippase that rapidly shuttles PS with half times of 5–10 min, and PE at a slower rate, from the outer to inner leaflet of plasma membranes against their respective con-

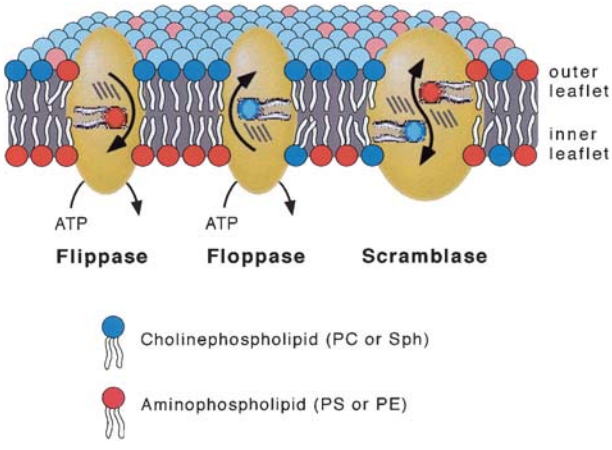

Figure 1. Transporter-controlled exchange of phospholipids between both lipid leaflets of the cell membrane. Unidirectional phospholipid transport by flippase is directed inward, whereas floppase promotes outward directed transport. Both transporters are ATP-dependent and frequently move phospholipids against their respective concentration gradients. For example, aminophospholipid translocase (flippase) rapidly shuttles PS and PE from outer to inner leaflet, while ABCC1 (floppase) moves both choline- and aminophospholipids more slowly towards the outer leaflet (see text). The concerted action of both transporters is thought to create a dynamic asymmetric steady state, in which the outer monolayer is rich in cholinephospholipids, whereas aminophospholipids predominantly occupy the inner leaflet. Bidirectional phospholipid transport is catalyzed by a scramblase, activation of which may occur following Ca^{2+} influx or when cells go into apoptosis. Since scramblase activity moves all major phospholipid classes back and forth between the two leaflets, it promotes collapse of membrane phospholipid asymmetry with appearance of PS at the cells’ outer surface.

centration gradients [1, 2, 19–21]. Since this transporter does not move other phospholipids, it is referred to as aminophospholipid translocase. Transport activity is sensitive to vanadate and to sulfhydryl oxidation. In red cells, one molecule of ATP is hydrolyzed per molecule of lipid transported [22], and the activity is presumably associated with a ~115-kDa P-type Mg^{2+}-ATPase [2, 21, 23, 24], possibly in conjunction with a 31-kDa protein at the endofacial membrane side [1, 20]. It has been argued that aminophospholipid translocase(s) may belong to a subfamily of P-type ATPases, but no definite identification has so far been achieved [23]. Aminophospholipid translocase activity is also present in a variety of intracellular membranes [2, 21] where it contributes to keeping PS located at the cytosolic side.

Floppase

A less specific ATP-requiring floppase promotes transport of both choline- and aminophospholipids from the inner to the outer leaflet of plasma membranes, albeit at a 10 times slower rate than that of the aminophospholipid translocase [25, 26]. This floppase appears to be identical to the ATP-binding cassette (ABC)-transporter encoded by the gene ABCC1, previously known as multidrug resistance protein MRP1 [27, 28]. This protein belongs to a superfamily of ABC transporters known to drive transport of various molecules and hydrophobic drugs from the cytoplasmic leaflet to the outer layer or to an acceptor molecule [29, 30]. ABCC1 is a large integral membrane protein (MW ~ 180 kDa) with 17 putative membrane-spanning domains and two ATP binding sites at its cytoplasmic region. Prolonged inhibition of ABCC1 results in a slow redistribution (halftime ~ 24 h) of endogenous PC to adopt a more random orientation, but does not influence the asymmetric distribution of PE and PS [31]. Both ABCC1 floppase and aminophospholipid translocase seem to be permanently active in normal quiescent cells. Their concerted action is thought to secure a dynamic asymmetric steady state in which all phospholipids are slowly but continuously moved to the outer membrane leaflet, whereas the aminophospholipids are rapidly shuttled back to the inner leaflet [18, 26, 32]. This equips the cell with a flexible machinery able to rapidly correct perturbations in membrane phospholipid asymmetry, such as those arising from membrane fusion events during endo- or exocytosis.

Another ABC transporter that has been suggested to exert lipid floppase activity is ABCA1, expressed in nearly all mammalian cells and one of the largest ABC proteins known (MW ~ 260 kDa) [29]. This transporter has been implicated to promote transient and local surface exposure of PS in apoptotic cells [33], without any effect on the redistribution of other phospholipid classes [34]. However, its precise role in apoptosis remains to be elucidated since apoptosis is usually accompanied by extensive scrambling of all major phospholipid classes rather than by selective egress of PS alone [35–37]. While ABCA1 knockout mice display partially impaired engulfment of apoptotic cells, they are also markedly deficient in promoting efflux of cholesterol and cholinephospholipids from cells to distinct serum apolipoprotein acceptors (e.g. apoA1), resulting in dramatic reduction of HDL cholesterol [33, 38]. This lipidemic profile is characteristic of Tangier disease, known to be caused by defects in the ABCA1 gene [39, 40]. The PS-selective floppase activity of ABCA1 has also been proposed to create a contact site for apoA1 on the cell membrane to allow loading of cholesterol and PC to HDL [34].

Scramblase

Simply inhibiting aminophospholipid translocase and ABCC1 floppase (e.g. by ATP depletion or by sulfhydryl reagents [25, 26]) does not rapidly lead to exposure of PS at the cell surface, due to the sluggish rate of spontaneous transbilayer migration of phospholipids. However, many cells harbor a Ca^{2+}-dependent mechanism (scramblase) that can rapidly move phospholipids back and forth between the two membrane leaflets (flip-flop), promoting within minutes a collapse of membrane phospholipid asymmetry. The archetype with this phenomenon is the blood platelet, the first cell in which this was observed

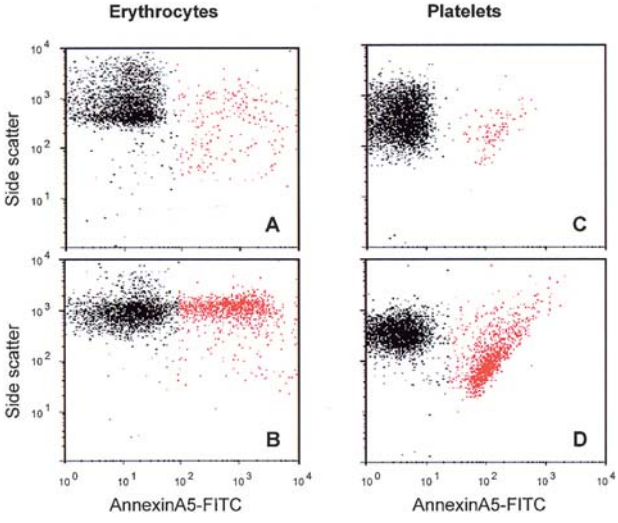

Figure 2. Flow cytometric analysis of the binding of fluorescently (FITC) labelled annexin A5 to Ca^{2+}-loaded red cells and activated platelets. Typical dot plots of fluorescence versus side scatter of (*A*) control erythrocytes, (*B*) erythrocytes plus 3 μM Ca^{2+} ionophore for 10 min, (*C*) control platelets and (*D*) platelets activated by 10 μg/ml collagen plus 1 nM thrombin for 5 min. Annexin A5 binding to PS-exposing cell fraction is represented by the red dots. Scramblase is active in the annexin A5-positive cells (red dots), whereas aminophospholipid translocase is active in the annexin A5-negative cells (black dots).

[4, 5]. Flow cytometry studies employing fluorescent labeled annexin A5 to detect PS-exposing platelets have revealed that platelet activation by collagen and/or thrombin causes a distinct part of the total platelet population to react with annexin A5 (fig. 2), even though secretion (release reaction) occurs in all platelets except those activated by collagen under non-stirring conditions [7]. Only Ca ionophore is able to produce PS exposure in the total platelet population. Recent experiments in our laboratory have shown that the annexin A5-positive populations clearly display scramblase activity, whereas aminophospholipid translocase activity is abrogated; the annexin A5-negative populations still retain an active aminophospholipid translocase without activation of scramblase.

Lipid scrambling is bidirectional and involves all major phospholipid classes, although glycerophospholipids move somewhat faster than Sph [41–44]. Scrambling requires the continuous presence of cytoplasmic calcium [45]; extrusion of calcium can restore membrane lipid asymmetry provided that aminophospholipid translocase has not been irreversibly degraded by intracellular calpain [46]. Unlike the energy-dependent aminophospholipid translocase and the ABCC1 floppase, scramblase activity does not require hydrolyzable ATP. However, its activity slowly deteriorates upon ATP depletion but can be restored by ATP repletion [41], suggesting that the lipid transporter may be constitutively phosphorylated to be eligible for stimulation by calcium.

Ca^{2+}-dependent scramblase activity has been reconstituted in artificial lipid vesicles recombined with proteins fractionated from blood platelets [47] or erythrocytes [48]. The reconstituted activities appeared to be pronase- and heat-sensitive. Although these observations confirm the notion that one or more proteins are responsible for scramblase activity, its identity has so far remained elusive. A 37-kDa membrane protein was initially proposed as the Ca^{2+}-dependent phospholipid scramblase [8, 49], but subsequent experiments have ruled out this protein as a probable candidate [8, 50, 51]. However, this protein (often referred to as phospholipid scramblase 1) may play an important role in exposing PS at the surface of apoptotic cells. Following expression of phospholipid scramblase 1 in Chinese hamster ovary cells, which are normally devoid of this protein, ultraviolet (UV) irradiation of these cells showed earlier and enhanced PS exposure, increased caspase activation and nuclear changes, all indicative for apoptosis [52].

Progressive dissipation of membrane phospholipid asymmetry is usually accompanied by outward blebbing of the cell membrane followed by shedding of microvesicles in which the phospholipids are randomized over the vesicular membrane [46, 53–56]. In addition to lipid scrambling, this process of microvesiculation is also dependent on Ca^{2+}-induced activation of intracellular calpain [55, 56], a protease which degrades cytoskeletal membrane proteins facilitating their detachment from the plasma membrane. It has been proposed that surface exposure of PS during apoptosis also results from activation of Ca^{2+}-dependent phospholipid scramblase with simultaneous inhibition of aminophospholipid translocase [57, 58]. There are, however, notable differences between PS exposure in activated cells and in apoptotic cells. Although both transport processes are bidirectional and non-specific regarding lipid polar headgroup, generation of apoptosis-induced PS exposure occurs over a much longer time scale (hours) than the Ca^{2+}-induced scrambling process (minutes) [23, 37]. It has become widely appreciated that the so-called intrinsic apoptotic signalling pathway converges at the mitochondria [59], resulting in decrease or even collapse of the mitochondrial membrane potential [60], and peroxidation of cardiolipin enabling release of cytochrome c [61] that assists in the activation of effector caspases [62]. How these phenomena are linked to PS exposure during apoptosis is not clear. It has been suggested that cytochrome c release from mitochondria may promote PS oxidation at the cytoplasmic leaflet of the cell membrane, followed by migration of oxidized PS to the cells' outer surface [63, 64]. Another possibility concerns cytosolic acidification resulting from the collapse of mitochondrial membrane potential [65], since phospholipid scrambling can be stimulated by $pH < 6$ [66]. Whatever these mechanisms, Ca^{2+} influx into erythrocytes, which have no mitochondria, readily produces loss of membrane asymmetry [41–43, 46]. Moreover, whereas PS exposure in activated blood platelets requires Ca^{2+} influx from the extracellular medium [45], treatment of blood platelets with local anesthetics in the absence of extracellular Ca^{2+} leads to typical apoptotic events with surface exposure of PS, collapse of mitochondrial membrane potential and release of cytochrome c into the cytoplasm [67]. Remarkably, these events could not be prevented when the cells were loaded with an intracellular Ca^{2+} chelator [67], confirming similar observations with apoptotic promonocytes and Jurkat cells [68]. Finally, Ca^{2+}-induced scramblase activity is clearly deficient in blood cells from patients with Scott syndrome (vide infra), but PS exposure during apoptosis is not aberrant in these cells [37]. Blood cells from Scott syndrome have a normal expression of phospholipid scramblase 1 [50], the 37-kDa membrane protein that has been proposed to catalyze egress of PS during apoptosis [52].

PS exposure and cell pathology

Although membrane phospholipid asymmetry is the rule for normal viable cells, changes in lipid distributions that lead to appearance of PS at the cell surface may accompany a variety of diseases. The properties of PS-expressing cells to provide a procoagulant membrane surface or to be

marked for phagocytosis can form a substantial complication of the disorder or be a matter of secondary importance. At present, no defects or deficiencies are known regarding aminophospholipid translocase or ABCC1 floppase activities, but a defective Ca^{2+}-induced scramblase activity has been shown to be the underlying cause of a bleeding disorder.

Scott syndrome

The physiological importance of scramblase-induced surface exposure of PS in activated blood platelets is clearly illustrated in Scott syndrome, a rare, moderately severe, bleeding disorder, characterized by an impaired ability of the patients' platelets to promote blood coagulation in response to agonists [69, 70]. While stimulation of these platelets results in normal secretion and aggregation, they are markedly deficient in Ca^{2+}-induced scramblase activity, resulting in a strongly reduced transport of PS to the cells' outer surface [71]. The cells also display an impaired capacity to shed membrane-derived, PS-expressing microvesicles, even though calpain activation is normal [53, 72]. In healthy platelets, PS exposure generates a catalytic membrane surface that promotes assembly and catalysis of two consecutive coagulation factor complexes, i.e. the tenase complex that converts the zymogen factor X into factor Xa, and this protease subsequently associates in the prothrombinase complex that rapidly converts prothrombin into thrombin [3, 73]. Indeed, Scott platelets are equally impaired in their ability to promote both tenase- and prothrombinase activity [71], though coagulation factor levels are normal [69]. Family studies [74], and studies on dogs with Scott syndrome [75], have indicated that this bleeding disorder is transmitted as an autosomal recessive trait (fig. 3).

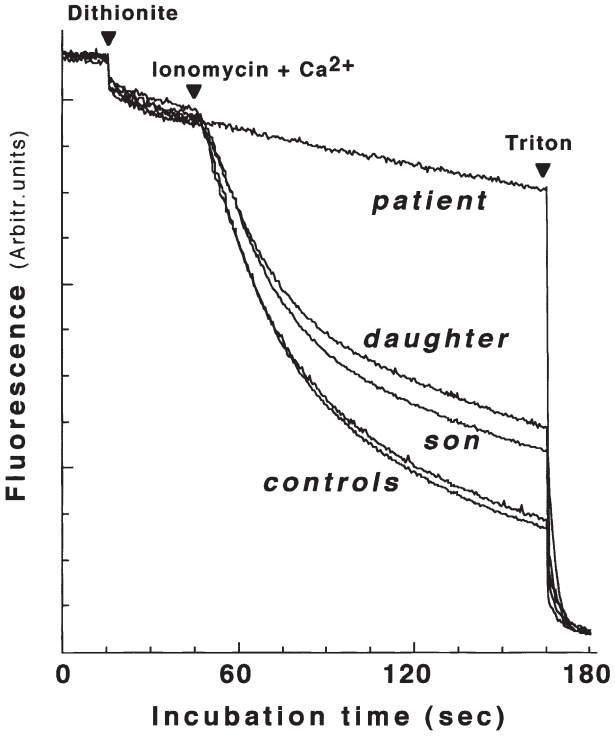

Figure 3. Ca^{2+}-induced scramblase deficiency in Scott syndrome. Platelets are first loaded with the fluorescent lipid probe NBD-phosphatidylserine (NBD-PS), which is translocated to the membranes' inner leaflet by aminophospholipid translocase. Addition of non-permeable dithionite to the NBD-PS-loaded platelets immediately quenches all fluorescence present at the cells' outer surface. Activation of control platelets by Ca^{2+} and ionomycin produces rapid scrambling of phospholipids and egress of NBD-PS, detected as a fluorescence decay by dithionite from outside the cell. Decay of NBD-PS fluorescence is negligible following addition of Ca ionophore to platelets from a patient with Scott syndrome, and intermediate with platelets from two of the patients' children. Addition of Triton X-100 at the end of the experiment rapidly quenches all remaining NBD-PS (adapted from [70]).

The defect in Ca^{2+}-induced lipid scrambling is not restricted to platelets but can also be demonstrated in erythrocytes and erythrocyte ghosts [76], and in other peripheral blood cells, including Epstein-Barr virus-transformed B lymphocytes [8, 37, 74]. Also, the morphology of Scott red blood cells after Ca^{2+} influx is in marked contrast to that of normal Ca^{2+} loaded erythrocytes. While Ca^{2+} entry promotes efflux of potassium and water leading to cell shrinkage, membrane blebbing and microvesicle shedding [77], Scott red cells do not shrink or shed vesicles, and even retain the biconcave shape [76]. Whether or not Scott syndrome is related to defects in Ca^{2+}-activated K^+ channels has, however, not been explored.

Phosphorylation studies on Scott platelets and erythrocytes have revealed a reduced extent of tyrosine phosphorylation following Ca influx [72, 78]. However, studies employing scramblase- and kinase inhibitors have argued that this is a consequence rather than a cause of impaired lipid scrambling [78], although the possibility of a defect in constitutive phosphorylation of lipid scramblase has not been ruled out. While the molecular basis of Scott syndrome is still unresolved, most of the studies are consistent with a deletion or mutation in multiple hematological lineages that either affects lipid scramblase directly or alters its Ca^{2+}-induced activation pathway.

Studies on Scott syndrome have clearly illustrated that there are differences between Ca^{2+}-induced lipid scrambling and the egress of PS that occurs when cells are steered into apoptosis. Whereas B lymphocytes from Scott syndrome do not expose PS following Ca^{2+} influx, PS expression is normal in apoptotic lymphocytes from these patients [37, 79]. Moreover, surface exposure of PS in Scott platelets is normal when the cells are treated with amphiphilic membrane drugs such as tetracaine and propranolol [72], which have been shown to produce mitochondria-related apoptotic events [67]. Once the lipid scrambling process is activated in apoptotic Scott lymphocytes, no increase in rate of scrambling is seen when these cells are challenged with Ca^{2+} [37]. These observa-

tions are not inconsistent with the view that a single scramblase can be activated via a Ca^{2+}-dependent and a Ca^{2+}-independent pathway, and that only the first route would be defective in Scott syndrome. However, the observation that Scott cells normally express phospholipid scramblase 1 [50], which has been implicated in PS exposure during apoptosis [52], strongly suggests that the Ca^{2+}-dependent scramblase is different from the scramblase that is activated during apoptosis. This also corroborates the finding that mice deficient in phospholipid scramblase 1 display no hemostatic abnormalities that are characteristic for Scott syndrome [51]

Antiphospholipid syndrome

The presence of circulating 'antiphospholipid' antibodies in association with arterial and venous thrombosis, recurrent abortions and thrombocytopenia are the characteristic features of the antiphospholipid syndrome [80]. A salient example of this syndrome is systemic lupus erythematosis, but these antibodies are also observed in diseases that are accompanied by increased cell surface exposure of PS, such as sickle cell anemia, thalassemia, malaria, uremia, diabetes, pre-eclampsia, cancer and conditions associated with elevated levels of circulating microvesicles (vide infra).

Although these antibodies were first believed to recognize anionic phospholipids directly, it is now generally appreciated that the antibodies are addressed towards plasma proteins when they interact with anionic phospholipids, particularly PS and cardiolipin [81–84]. The most frequently occurring lipid-bound antigens are β_2-glycoprotein I [81–82] and prothrombin [83], although several other plasma proteins have been implicated as well. Both proteins interact with PS-expressing membranes, such as those of activated platelets, apoptotic cells, cell-derived microvesicles or artificial PS-containing lipid vesicles, although the binding is rather weak with Kd's in the micromolar range [84]. The immunoglobulin G (IgG) antibodies potentiate this binding by at least two orders of magnitude, likely resulting from bivalent, trimolecular immune complexes of the IgG molecules with the lipid-bound proteins [85–87]. While this would explain that binding of the antibodies to β_2-glycoprotein I and prothrombin in solution is negligible, it remains possible that the antibodies recognize conformation-induced neo-epitopes [83], as β_2-glycoprotein I and prothrombin undergo shape changes upon binding to an anionic phospholipid surface [3, 88–90].

It may be expected that any protein or protein complex with a high affinity for PS-expressing membrane surfaces would inhibit blood clotting by competing with the binding of coagulation factors to these membranes, and interfere with the scavenging of apoptotic cells via the PS receptor on phagocytes. Indeed, the anticoagulant PS binding protein annexin A5 prevents assembly and catalytic activity of the tenase- and prothrombinase complex [91], and inhibits the sequestration of apoptotic cells by macrophages in vitro [92]. However, these notions do not accurately apply to the antiphospholipid syndrome. Although IgG-β_2-glycoprotein I- and IgG-prothrombin complexes restrict assembly of the prothrombinase complex on PS-expressing membranes [86, 87, 93], patients with antiphospholipid syndrome have no bleeding tendency but have an increased risk for thrombosis [94]. It has been suggested that the circulating antibodies would reflect a normal immune response towards persistent thrombogenic PS-expressing cells rather than an aberrant auto-immune response [83, 95, 96]. Prolonged surface exposure of PS in these patients could be due to a disturbed balance between aminophospholipid translocase and scramblase activity, an increased rate of apoptosis [97], or may be caused by a defective scavenging mechanism by which procoagulant cells, cell-derived microvesicles and apoptotic cells are cleared from the circulation [98]. This would reflect a thrombotic condition, while binding of prothrombin and β_2-glycoprotein I to these cells serves as a target for the production of antibodies [99, 100]. It has also been suggested that these antibodies may enhance blood coagulation by stimulating monocytes to increase the expression of tissue factor, a transmembrane protein that initiates blood coagulation [101].

Other explanations for the thrombotic complications in patients with antiphospholipid syndrome have been forwarded, the most noteworthy of which concerns interference with the protein C anticoagulant system. Protein C (after being activated by thrombin) is a serine-protease that cleaves the non-enzymatic coagulation cofactors Va and VIIIa of the prothombinase- and tenase complex, respectively, but this only occurs effectively when both complexes are assembled on a PS-expressing membrane surface [3, 102, 103]. Degradation of the two cofactors decomposes and inactivates both coagulation complexes, thus providing feedback inhibition of thrombin formation. This PS-dependent reaction is of cardinal importance to limit the rate and extent of thrombin generation, illustrated by the fact that patients with heterozygous protein C deficiency suffer from severe venous thrombosis [103]. In this regard, it has been suggested that the tight binding of IgG-β_2-glycoprotein I- and IgG-prothrombin complexes to PS-expressing cells hampers the anticoagulant action of protein C, thus leading to thrombotic complications [104, 105].

Sickle cell anemia

Sickle cell disease, which results from a point mutation in the β-chain of hemoglobin (HbS), is characterized by hemoglobin polymerization and sickling of erythrocytes under deoxygenated conditions being responsible for the

hemolytic anemia and vaso-occlusive episodes. A prominent feature of this disorder is a partial collapse of membrane phospholipid asymmetry with exposure of PS on the surface of a subpopulation of sickled cells [106–109], and on sickle cell-derived microvesicles, which are produced during repeated cycles of oxygenation and deoxygenation [110]. Flow-cytometric studies using fluorescent labelled annexin A5 have indicated that between 0.5 and 10% of the red blood cells of sickle cell patients expose substantial amounts of PS on their surface, whereas normal donors have very few annexin A5-positive cells (less than 0.3%) [106, 107]. While the small fraction of PS-exposing normal red cells presumably reflects the dense senescent cells known to expose PS [111], the annexin A5-positive subpopulation of sickle cells contains both the densest and the very light cells [108]. Apart from containing reticulocytes, the light fraction harbors those mature cells that have ion transport abnormalities that lead to high Na^+ and low K^+ content. PS-exposing sickle cells have no apparent aminophospholipid translocase activity, possibly due to increased formation of reactive oxygen species that distrain the supply of ATP [112]. Sickling of erythrocytes is accompanied by transient periods of increased cytosolic Ca^{2+} particularly in cells with ion transport abnormalities [113], which may lead to temporary activation of the scramblase. Since the sickled cells do not show a sustained high cytosolic Ca^{2+} level [108, 112], PS exposure may be corrected in those cells in which the aminophospholipid translocase has not been irreversibly damaged.

PS-expressing sickle cells can contribute to microvascular occlusion during sickle cell crisis in several ways. Although the size of the PS-exposing subpopulation may look rather small (average: 4%), numerically this equals to more than half the number of blood platelets in the circulation. Exposure of PS promotes blood coagulation, which may contribute to the thrombotic episodes during sickle cell crisis [114]. Moreover, thrombosis in sickle cell patients can be aggravated by circulating 'antiphospholipid' antibodies, which may have been generated in response to cells with a sustained PS exposure [115]. Apart from thrombosis, microvascular occlusion may be promoted by the propensity of PS-exposing red cells to adhere to vascular endothelium and to the endothelial matrix protein thrombospondin [116–118]. In addition to contributing to sickle cell crisis, the exposure of PS on sickle cells could be partially responsible for the strongly decreased red blood cell survival as observed in transgenic murine models of sickle cell anemia [119, 120], since these cells would be prone to clearance by macrophages [9, 11, 12].

Improvement of anemia and other clinical symptoms of sickle cell patients has been achieved following administration of hydroxyurea. Significantly, red cells from patients receiving treatment with hydroxyurea have been reported to display a more than 50% reduction in annexin A5-positive cells relative to non-treated patients [121]. A similar reduction in PS-expressing platelets was observed in sickle cell patients receiving hydroxyurea. However, these observations are difficult to reconcile with a study reporting that hydroxyurea induces apoptosis and surface expression of PS in a lymphoblastoid cell line [122].

Thalassemia

Thalassemia is a congenital hemolytic anemia caused by a partial or complete absence of the alpha- or beta-chain of hemoglobin. Homozygous carriers suffer from severe anemia and other serious complications from early childhood. Similar to sickle cells, subpopulations of thalassemic erythrocytes are present which expose PS on their external surface [123–127]. The number of PS-expressing cells can vary considerably between different patients, from as low as those found in normal cells (less than 0.3%) to as high as 20% [127]. While the mechanism by which thalassemic cells express PS is unknown, it has been suggested that it may involve cells with oxidized membranes resulting from the frequently observed iron overload in these patients [127–128], which for example could inactivate the oxidation-sensitive aminophospholipid translocase [18, 20]. Alternatively, oxidative stress, energy depletion or hyperosmotic shrinkage of erythrocytes is known to activate a non-selective cation channel (Gardos channels) promoting influx of Ca^{2+} and effux of K^+ via a Ca^{2+}-sensitive K^+ channel [77]. In thalassemic red cells, these manoeuvers induce a significantly higher Ca^{2+}-induced K^+ efflux, accompanied by increased numbers of PS-expressing cells as compared to erythrocytes from healthy controls [129]. Very similar phenomena are observed with sickle cells and glucose-6-phosphate-dehydrogenase deficient red cells (favism). This enhanced vulnerability has been proposed to contribute to the shortened life span of the affected red cells [130].

Due to their procoagulant nature, PS-exposing red cells are also thought to contribute to the profound thromboembolic complications in thalassemia, among which cerebral thrombosis, deep venous thrombosis and pulmonary embolism are the most common [125, 128, 131]. Moreover, these cells may be more rapidly removed from the circulation by phagocytes that recognize PS, contributing to the anemia. Particularly, splenectomized patients lacking a significant reservoir of macrophages have the highest number of PS-expressing cells and an increased thrombotic tendency, although a direct correlation between the severity of the anemia and the proportion of these cells could not be identified [127, 131]. It is evident that apart from early clearance from the circulation, ineffective erythropoiesis of thalassemic red cells also aggravates the anemia.

Stomatocytosis

While a wide variety of hemolytic anemias are associated with often life-threatening, thrombotic complications [132], most of the literature on a possible link with red cell surface exposure of PS is restricted to sickle cell anemia and thalassemia. In a recent study on two patients with hereditary hydrocytosis, a rare variant of hereditary stomatocytosis with an increased thrombotic risk, a moderate rise in PS-expressing red cells and an increased propensity to adhere to endothelial monolayers was observed [133]. While the molecular basis of hydrocytosis is unknown, these cells display a marked increase in cation permeability, forming swollen overhydrated mouth-shaped erythrocytes, but it remains unclear if or how this relates to surface exposure of PS [134]. Moreover, the very small sample size of two patients precludes any definite conclusions about the mechanisms of thrombosis in this disorder.

Uremia

Uremia, a toxic condition associated with accumulation of by-products of protein metabolism (e.g. urea) in the blood, is a frequent aspect of chronic renal failure. A subpopulation of about 3% of uremic erythrocytes has been found to expose PS at their outer surface [135], and to be preferentially removed from the circulation by phagocytosis [136]. Phagocytosis of uremic erythrocytes is strongly inhibited in vitro when macrophages are pretreated with glycerophosphorylserine, a structural derivative of PS, whereas no inhibition is observed with the equivalent derivative of PE, glycerophosphorylethanolamine. Also, annexin A5 strongly hampers macrophage recognition of uremic erythrocytes. PS externalization promotes increased adhesion of uremic erythrocytes to endothelium, possibly via interaction with matrix thrombospondin [137]. Apart from suppressed erythropoiesis, a shortened red blood cell life span has been found to contribute to anemia, a common feature in chronic renal failure. The toxic origin of shortened red cell survival in chronic renal failure is well known: red blood cells from uremic patients have a normal life span in healthy subjects, whereas red cells from healthy individuals have a reduced life span in uremic patients. Interestingly, in vitro incubation of uremic plasma with normal red blood cells promotes both PS exposure and erythrophagocytosis, the latter being independent of interaction between plasma and macrophages [136]. Conversely, a marked decrease in PS-expressing uremic erythrocytes occurs following incubation in normal plasma, with a concurrent decrease in their propensity to be recognized by phagocytes. Although the molecular mechanism of this toxicity is unknown, a putative uremic compound has been found that produces PS exposure in red cells [136]. This uremic factor, having an apparent molecular weight about 10 kDa, seems to be highly lipophilic and is presumably associated to plasma proteins [138].

The form of dialytic treatment of uremic patients is of influence on the abnormal surface exposure of PS. Patients on continuous ambulatory peritoneal dialysis have a lower percentage of PS-expressing red cells than patients on hemodialysis, which also correlates to the lower degree of anemia in the group of patients on peritoneal dialysis [135, 139]. The type of dialysis membrane employed also seems to be important. It seems that the better its capacity to retain the putative 10-kDa uremic compound, the higher the potential benefit for uremic patients [138].

Surface exposure of PS in uremia is not restricted to red cells. Flow-cytometric studies on uremic platelets showed a 2–3 times increased number of annexin A5-positive cells with a concurrent capacity of the platelets to promote prothrombinase activity [140]. Circulating platelet-derived microvesicles with procoagulant activity, originating from PS-exposing platelets are also found in these patients [141]. Activation of uremic platelets by collagen and thrombin produces a 2 times higher procoagulant response than observed in activated control platelets. Increased PS externalization in uremic platelets is partly linked to enhanced activity of effector caspases, suggesting that apoptotic-like events are involved [140]. These findings may help to explain the thrombotic accidents in these patients. Indeed, platelet-derived microvesicles were found to be significantly higher in uremic patients with thrombotic events than in those without [141]. However, impaired platelet function and regular bleeding accidents are also well-recognized complications of chronic renal failure. A number of studies have suggested that the bleeding tendency in uremia is associated with decreased expression and/or dysfunction of platelet membrane glycoproteins [142–144].

Kidney stone disease

Abnormal distribution of PS in renal epithelial cell membranes has been suggested to play a role in kidney stone disease [145–147]. Exposure of cultured renal epithelial cells to oxalate produces apoptotic-like events [148] with surface exposure of PS, which in turn promotes binding of calcium oxalate crystals to the cell surface [145]. This process may foster crystal retention and stone formation within the kidney. While the mechanism of oxalate-induced PS exposure is unclear, it may involve a direct physical interaction of oxalate with membrane lipids, rather than interfering with lipid transporters. This possibility is supported by the observation that calcium oxalate causes a redistribution of PS in artificial phospholipid vesicles that lack the biochemical machinery to maintain phospholipid asymmetry [146].

Diabetes and hyperglycemia

Erythrocytes and platelets from patients with diabetes mellitus have been found to exhibit a partial collapse of membrane phospholipid asymmetry with increased surface exposure of PS [149–151]. In a study of 25 diabetic patients, 12–18% of PS in the patients' erythrocytes was found to be hydrolyzed by exogenous phospholipase A2, or labelled by the non-permeant agent trinitrobenzene sulfonic acid, compared to none in normal red cells [149]. These results suggest a partial loss in lipid asymmetry, but may also reflect a destabilization of erythrocyte membranes in diabetic patients, causing increased accessibility to phospholipase A2 degradation. In blood platelets from diabetic patients, a 3–7 times enhanced capacity to catalyze prothrombinase activity has been been observed [152], which is most likely due to increased surface exposure of PS. Alterations in membrane surface characteristics may contribute to an increase in spontaneous aggregation of diabetic erythrocytes and platelets, as well as promoting microvascular occlusion by abnormal adherence of blood cells to vascular endothelium. Indeed, adhesion of red cells to human umbilical vein endothelial cells under flow conditions can be inhibited by PS liposomes and by annexin A5, clearly indicating the PS dependence of these interactions [116–117]. In addition, diabetes is associated with several defects of coagulation and fibrinolysis, which together with PS-expressing blood cells and increased platelet-derived procoagulant microvesicles [153] predispose to a thrombogenic tendency. A slightly increased number of PS-expressing red cells is also found in obese mice that lack a functional receptor for leptin, the major regulator for fat storage in mammals [154]. These animals have an uncontrolled rise in blood sugar and display many of the characteristics of non-insulin-dependent diabetes, including a reduced life span of erythrocytes.

It has been claimed that the alterations in membrane phospholipid asymmetry observed in diabetic red cells can be brought about by in vitro incubations of normal red cells in hyperglycemic buffers [155, 156]. For example, erythrocytes incubated for 18 h with 20 mM glucose exhibited a strongly increased accessibility of PS and PE, and a decrease in the accessibility of Sph and PC, to exogenous phospholipases. However, these red cells had a normal aminophospholipid translocase activity, were only marginally able to stimulate prothrombinase and did not exhibit significant annexin A5 binding [155]. These inconsistencies suggest that extensive lipid reorientation may have occured during phospholipase treatment and not during prolonged incubation in hyperglycemic media. It has been shown that incubation of red cells in high glucose buffers causes depletion of vitamin E and accumulation of vitamin E-quinone and malondialdehyde, an end product of lipid peroxidation [156]. This is accompanied by a slight increase in red cell procoagulant activity, which can be prevented by preincubation with reducing agents like N-acetylcysteine. Indeed, if oxidation of PS occurs in hyperglycemic media, it may migrate to the membrane outer surface [63, 64]. On the other hand, malondialdehyde rapidly interacts with PE to form an anionic lipid adduct that can be mistakenly identified as PS since it readily binds annexin A5 and is moderately procoagulant [157].

Viral and bacterial infections

It has become widely appreciated that many viruses modulate apoptotic cell death [158]. As a rule of thumb, viruses forestall apoptosis by various mechanisms and provide for a living host cell to enhance virus replication in an advantageous intracellular environment. Conversely, the host response is directed towards triggering the apoptotic mechanisms in infected cells, limiting microbial propagation [159]. When apoptosis gets the upper hand, infected cells will express PS at their outer surface, marking these cells as a pathological target for elimination by phagocytes. This corroborates the finding that various virus-infected cells exhibit a procoagulant phenotype [160], as first shown for cytomegalovirus-infected endothelial cells [161]. One of the best-documented examples concerns influenza-infected HeLa cells [162, 163]. While there is no evidence that influenza virus has direct access to apoptosis execution factors, caspase-dependent exposure of PS at the cell surface occurs several hours post-infection at about the time that phagocytosis by peritoneal macrophages becomes detectable. Uptake is largely inhibited by PS-containing liposomes, suggesting a role for a PS-receptor in the removal of virus-infected cells [164].

Several obligate and facultative intracellular bacteria are implicated in promoting procoagulant activity in vascular cells. Moreover, infection may be associated with atherosclerosis and has been considered a risk factor for myocardial infarction [160]. A bacterial agent that has attracted wide attention is *Chlamydia pneumoniae*, frequently found in atherosclerotic lesions [160, 165]. Infection by this agent causes rapid (5 min post-infection) externalization of PS in a wide variety of host cells [166]. Egress of PS was found to be Ca^{2+}-dependent and could not be inhibited by a broad spectrum caspase inhibitor. PS exposure depends on the continuous presence of *Chlamydia* since removal of inoculum leads to disappearance of PS from the surface. Also, *Chlamydia*-infected cells accelerate plasma clotting and are susceptible to PS-dependent uptake by phagocytes.

Malaria

Protozoan parasites such as malaria, which have elaborate life cycles within human erythrocytes and liver cells,

have been suspected to perturb membrane phospholipid asymmetry. Flow-cytometric studies with human erythrocytes infected with *Plasmodium falciparum* – the most severe of the malaria-causing parasites – have shown that these cells bind annexin A5, provided that the extent of parasitemia is in excess of 25% [167], with most of the parasites being in the trophozoite and/or schizont stages [168]. Previous reports disagree as to whether or not malaria parasites promote a collapse of lipid asymmetry [169, 170], but this may depend on the different parasitic forms, the extent of parasitemia and the duration of infection employed in these studies. It should also be noted that malaria infection is accompanied by a progressive oxidative assault on the red cells coupled with an enhanced flux of Ca^{2+} through the non-selective Ca^{2+} channel [171, 172] that may activate the Ca^{2+}-dependent lipid scramblase. However, the extent of oxidation may well depend on the number of infected red cells, which determines the concentration of reactive oxygen species. Considering the much lower extent of parasitemia in malaria patients compared to that used in the laboratory studies, it remains doubtful if PS exposure contributes to any of the clinical manifestations of *Plasmodium falciparum* infection. It should be mentioned, however, that the majority of falciparum malaria patients are positive for anti-phospholipid antibodies [173], which may reflect a response against PS-expressing cells (vide supra) and may underlie the frequently observed thrombocytopenia.

Pre-eclampsia

Pre-eclampsia is a serious multisystem disorder characterized by hypertension, proteinuria and a hypercoagulable state during the second half of pregnancy [174]. Red cells from patients with pre-eclampsia have been shown to display a twofold increase, relative to erythrocytes from normotensive pregnant women, in their ability to promote prothrombinase activity when used as a source of phospholipid [175]. Whilst this may reflect a slightly increased surface exposure of PS, its significance should not be underrated considering the abundance of circulating red cells, relative to other peripheral blood cells. A procoagulant contribution from activated blood platelets may be less important, considering that a significantly lower number of PS-expressing, platelet-derived microvesicles are found in pre-eclampsia, relative to that found during normal pregnancy [176]. In some but not all cases, patients with pre-eclampsia are positive for antibodies directed against lipid-bound β_2-glycoprotein I [177–179]. Both conditions have been proposed to contribute to thrombosis in the intervillous spaces on the maternal side of the placenta, impeding placental perfusion.

Hyperbilirubinemia

Hyperbilirubinemia is a frequently observed complication in neonates during the first week of life, resulting from increased bilirubin production and decreased elimination. Unconjugated bilirubin binds to erythrocytes, particularly when the molar ratio of bilirubin-to-albumin exceeds unity. This leads to toxic manifestations [180], such as crenation of red cells, hemolysis, anemia, and release of phospholipids and cholesterol from the erythrocyte membrane [181]. Release from the cell of PC, PE and Sph starts at bilirubin-to-albumin molar ratios of ~ 0.5, whereas release of PS occurs when this ratio becomes greater than unity. Incubation of red cells at a bilurubin-to-albumin ratio of 3 results in about 8% of the red cells becoming positive for annexin A5, suggesting transbilayer movement of PS from the cells' inner to outer membrane leaflet [181]. In samples pretreated with N-ethylmaleimide to inhibit inward movement of PS by aminophospholipid translocase, nearly 20% of the bilirubin-treated cells express PS at the outer surface, indicating that bilirubin does not inhibit translocase activity per se. Bilirubin-induced egress of PS requires the presence of Ca^{2+}, which raises the possibility that bilirubin provokes Ca^{2+} influx by opening non-selective Ca^{2+} channels leading to scramblase activation. Irrespective of the molecular mechanism, lipid scrambling and release of lipids from the red cell may facilitate hemolysis and promote erythrophagocytosis, contributing to anemia during severe neonatal jaundice where bilirubin-to-albumin ratios higher than 1 are not uncommon.

Neoplasia

Despite the biological heterogeneity of tumor cells, cancer is presently understood as an improper control of the cell cycle associated with a loss of the cells' ability to steer into apoptosis [182]. Notwithstanding, many tumor cells have been shown to exhibit elevated expression of PS in the outer leaflet of the cell membrane [183–188]. This is particularly evident for undifferentiated, tumorigenic cells, which may express about 5 times as much PS at their cell surface as their differentiated, non-tumorigenic counterparts [184]. Tumor cells also release PS-expressing microvesicles, similar to microvesicle release in other cells during lipid scrambling [18]. Increased surface expression of PS in tumor cells and in their shed microvesicles may promote blood coagulation, and could be responsible for fibrin deposits often seen in solid tumors [185, 186].

In vitro, surface exposure of PS in tumorigenic cells directly correlates with their ability to be recognized and bound by macrophages [183, 184]. This may seem rather enigmatic considering that tumor cells often have faulty apoptotic pathways to escape from programmed cell death and subsequent elimination by phagocytes. Indeed,

chemotherapy often activates the apoptotic program to dictate tumorigenic cells to commit suicide [189–192]. It has long been known, however, that tumor-associated mononuclear phagocytes accumulate in neoplastic tissues near the tumor-host tissue interface, and this may even amount to half of the tumor's mass [193, 194]. The question as to whether this inflammatory infiltrate helps or hinders tumor growth is still open to debate. Increased consensus exists, however, that phagocytes form part and parcel of the inflammatory responses that promote tumor growth and progression, rather than mounting an effective antitumor response directed at elimination of neoplastic cells [195]. Conceivably, surface exposure of PS by oncogenic cells facilitates recruitment of inflammatory phagocytes and cytokines to the benefit of tumor growth and progression.

Cystic fibrosis and bronchiectasis

Cystic fibrosis is a common autosomal recessive trait, characterized by a mutation of an ATP-dependent plasma membrane protein which functions as part of a cyclic AMP-regulated chloride channel in epithelial cells. The slightly misfolded mutant protein, which would function normally if it would reach the plasma membrane, is retained in the endoplasmic reticulum and discarded before being transported to the cell membrane. This aberration produces a generalized dysfunction of the exocrine glands with formation of viscid mucus, which progressively plugs their ducts. Particularly, obstruction of the bronchi promotes persitant pulmonary infections, e.g. by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which are also characteristic for bronchiectasis in which the lung periphery fails to develop, resulting in focal bronchial dilation accompanied by inflammatory destruction of bronchial walls. These chronic bacterial infections evoke a sustained influx of polymorphonuclear neutrophils (PMNs) into the airways, where they die and release intracellular proteases that overwhelm antiprotease defenses. This produces a protease/antiprotease imbalance, leaving proteases unimpeded to injure airways and impair host defence [196, 197].

Resolution of inflammation is normally accomplished by phagocytosis of dying apoptotic inflammatory cells before disruption of the plasma membrane and leakage of potentially harmful intracellular components. However, airway fluid from patients with cystic fibrosis and bronchiectasis contains an abundance of apoptotic and necrotic cells, much more than seen e.g. in patients with chronic bronchitis or with acute respiratory distress syndrome [196–198]. Contrary to the latter disorders, there is a protease/antiprotease imbalance in cystic fibrosis and bronchiectasis airway fluid, with PMN elastase in excess of its major inhibitors. In spite of prominent surface exposure of PS on the apoptotic PMNs, cystic fibrosis and bronchiectasis airway fluid inhibits removal of these cells by alveolar macrophages in a PMN-elastase-dependent manner [198]. PMN elastase cleaves the PS receptor on phagocytes in vitro, implying a potential mechanism for defective apoptotic cell removal in vivo. Thus, while collapse of membrane phospholipid asymmetry occurs normally in apoptotic inflammatory neutrophils in the lungs of these patients, PS exposure is no longer recognized as a signal for cell removal due to elastase-catalyzed clipping of the PS receptor on macrophages.

PS receptor deficiency

A possible relationship between a defective PS receptor on macrophages and pulmonary dysfunction is also suggested by studies of knockout mice lacking the PS receptor [199]. These mice suffer from abnormal lung and brain development leading to perinatal lethality. No phagocytosis of apoptotic cells was observed in PS receptor-deficient lungs, and necrotic cells were readily detectable. Remaining apoptotic cells may undergo secondary necrosis that is associated with respiratory distress and abnormal lung development in these mice.

Besides pulmonary dysfunction, PS receptor-deficient mouse embryos also display severe anemia and developmental defects of erythroid and T lymphoid cells [200]. Particularly, red cell differentiation was found to be arrested at an early erythroblast stage. During normal erythroblast proliferation, the cell extrudes its nucleus to become a reticulocyte that leaves the bone marrow and passes into the bloodstream. Erythrocyte clones develop in the bone marrow on the surface of a macrophage, which starts to engulf the membrane lobe surrounding the nucleus even before the segregation of the two bodies is complete [201]. Moreover, the portion of the plasma membrane which surrounds the lobe of the cell containing the extruding nucleus, stains with the dye merocyanin 540, which reflects a looser packing of the lipids frequently associated with lipid scrambling [202]. The membrane surrounding the reticulocyte lobe of the erythroblast does not stain with merocyanin and is not recognized by phagocytes. Assuming that phagocytosis of the nucleus-containing membrane lobe is PS-dependent, this may provide a link to anemia in PS receptor deficient mice.

It should, however, be mentioned that a recent study on PS receptor knockout mice showed a completely normal phagocytosis of apoptotic cells, though confirming that this protein is essential for the development and differentiation of multiple organs during embryogenesis [203]. This casts some doubt as to whether this protein really plays a crucial role in the recognition and uptake of PS-expressing cells. This notion is fortified by observations which suggest that this protein is localized in the cell nucleus, rather than in the plasma membrane [204].

Nevertheless, the issue remains controversial, the more so as knocking down the PS receptor in zebrafish demonstrated both impairment of embryonic cell development and accumulation of dead apoptotic cells [205].

Clinical aspects of microvesicles

As mentioned above, loss of membrane lipid asymmetry is usually accompanied by shedding of lipid-symmetric microvesicles (also called microparticles) from the cell surface. Circulating microvesicles are mostly derived from activated platelets but sometimes also from other blood cells, including endothelial cells [54, 206–209]. Due to surface-exposed PS, microparticles are procoagulant and in some cases also contain tissue factor, the initiator of blood coagulation. They also expose a variety of membrane proteins, the composition of which depends on their cellular origin. Apart from their distinctive procoagulant activity, they have also been suggested to induce inflammatory reponses in other cells via cell adhesion molecules [209, 210].

The clinical relevance of circulating microvesicles may be well illustrated in immune thrombocytopenic purpura (ITP). In this bleeding disorder, interaction of autoimmune antiplatelet antibodies with platelets strongly reduces platelet counts while provoking microvesicle formation, the extent of which varies among patients. Interestingly, ITP patients with high levels of platelet-derived microvesicles do not bleed despite severe thrombocytopenia, while others with higher platelet counts but lower levels of microvesicles bleed extensively [211]. Those with the highest levels of microvesicles often suffer from small vessel transient ischemic attacks.

Elevated, mainly platelet-derived, microvesicles in the circulation have been observed in many disorders associated with increased thromboembolic risk and vascular damage. Apart from above-mentioned disorders, such as thrombocytopenia, diabetes, uremia, cancer or antiphospholipid syndrome, it occurs in acute coronary syndromes, small vessel strokes and during cardiopulmonary bypass surgery [207–209].

While a correlation between thromboembolic complications and elevated numbers of microparticles has often been demonstrated, circulating microvesicles are not always associated with thrombotic tendencies. For example, in Stormorken syndrome (also referred to as inverse Scott syndrome) PS-expressing platelets are found without deliberate stimulation, and this condition is characterized by considerable spontaneous microvesiculation in the patients' blood [212]. This would be expected to result in a thrombotic disposition, but these patients have a bleeding tendency. Although the reason for this is unclear, it should be noted that PS-exposing microvesicles also promote the anticoagulant protein C pathway [213] that inhibits thrombin formation through inactivation of cofactors Va and VIIIa [102, 103]. The balance between the pro- and anticoagulant effect of a lipid surface may depend on the presence of other lipids such as PE [3]. The presence of PE in PS-containing vesicles enhances their capacity to stimulate both protein C and prothombinase activity, but this effect is much larger for protein C than for prothrombinase. On the other hand, a clear correlation between a bleeding tendency and the inability of activated platelets to release microvesicles upon stimulation, despite normal PS expression on the platelet surface, has been observed in another inherited bleeding disorder known as Castaman's defect [214].

The question remains to what extent microparticles are causative agents in pathology or merely an epiphenomenon. Microvesicles from different cells also circulate in healthy subjects, which may reflect the balance between resting, activated and apoptotic cells. Whatever their clinical relevance with respect to hemostatic disorders, platelet-derived microvesicles may be regarded as a clinical marker of platelet activation.

Concluding remarks

Membrane phospholipid asymmetry was first discovered in human erythrocyte membranes in the early 1970s [215, 216], and many studies since have led to the concept that it is a ubiquitous phenomenon of most if not all mammalian cells. Because quiescent cells invest energy to catalyze transbilayer lipid movement in order to generate and maintain a specific transmembrane phospholipid distribution, it is considered to be of major physiological importance. This is most evident for PS, which is normally restricted to the cytosolic leaflet of cell membranes. The transporter-controlled emergence of PS at the cell's outer leaflet results in the expression of altered surface properties with its consequent impact on the cell's interaction with its environment. Controlled surface emergence of PS during platelet activation clearly plays a pivotal role in promoting blood coagulation, but its overexpression may generate potentially dangerous thromboembolic complications. It is therefore crucial that distinct mechanisms exist for the recognition and ingestion of PS-expressing cells. Sequestration of these cells via PS-receptor(s) on phagocytes is equally important for the orderly removal of apoptotic cells to maintain the balance between cell division and cell death. Defects in membrane transport activities that regulate transbilayer lipid distributions will almost inevitably result in undesired surface expression of PS, which may cause or compromise a wide variety of disorders. It remains to be established, however, to what extent surface exposed PS is a marker of the pathological state. Also, whether or not PS exposure reflects cell activation or apoptosis is often a matter of semantics, even though the mechanisms of both

processes differ as illustrated by lymphocytes from Scott syndrome. Apoptosis is often defined by DNA breakdown and collapse of the mitochondrial membrane potential, but PS exposure under pathological conditions frequently occurs in red cells that lack mitochondria and DNA. Sometimes, PS exposure in red cells is referred to as 'apoptotic-like' even though it is related to Ca influx, typical of cellular activation. Finally, in nearly all cases, a detailed insight in the lipid-transport mechanisms that are dysregulated is lacking, which also holds for the relation between the transport defects and the molecular mechanisms responsible for the various disorders. Thus, further understanding of the mechanisms that generate and regulate lipid sidedness and those that promote its collapse, is crucial to assess the role of lipid transporters in disease.

1 Schroit A. J. and Zwaal R. F. A. (1991) Transbilayer movement of phospholipids in red cell and platelet membranes. Biochim. Biophys. Acta **1071:** 313–329
2 Devaux P. F. and Zachowski A. (1994) Maintenance and consequences of membrane phospholipid asymmetry. Chem. Phys. Lipids **73:** 107–120
3 Zwaal R. F. A., Comfurius P. and Bevers E. M. (1998) Lipid-protein interactions in blood coagulation. Biochim. Biophys. Acta **1376:** 433–453
4 Bevers E. M., Comfurius P., van Rijn J. L., Hemker H. C. and Zwaal R. F. A. (1982) Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. Eur. J. Biochem. **122:** 429–436
5 Bevers E. M., Comfurius P. and Zwaal R. F. A. (1983) Changes in membrane phospholipid distribution during platelet activation. Biochim. Biophys. Acta **736:** 57–66
6 Thiagarajan P. and Tait J. F. (1991) Collagen-induced exposure of anionic phospholipid in platelets and platelet-derived microparticles. J. Biol. Chem. **266:** 24302–24307
7 Dachary-Prigent J., Freyssinet J.-M., Pasquet J.-M., Carron J.-C. and Nurden A. T. (1993) Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: a flow cytometric study showing a role for free sulfhydryl groups. Blood **81:** 2554–2565
8 Sims P. J. and Wiedmer T. (2001) Unravelling the mysteries of phospholipid scrambling. Thromb. Haemost. **86:** 266–275
9 Fadok V. A., Voelker D., Campbell P. A., Cohen J. J., Bratton D. L. and Henson P.M. (1992) Exposure of phosphatidylserine on the surface of apoptic lymphocytes triggers specific recognition and removal by macrophages. J. Immunol. **148:** 2207–2216
10 Martin S. J., Reutelingsperger C. P. M., McGahon A. J., Rader J. A., van Schie R. C. A. A., LaFace D. M. et al. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and AbI. J. Exp. Med. **182:** 1545–1556
11 Fadok V. A., de Cathelineau A., Daleke D. L., Henson P. M. and Bratton D. L. (2001) Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. J. Biol. Chem. **276:** 1071–1077
12 Fadok V. A., Bratton D. L., Rose D. M., Pearson A., Ezekewitz R. A. B. and Henson P. M. (2000) A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature **405:** 85–90
13 Sprong H., van der Sluijs P. and van Meer G. (2001) How proteins move lipids and lipids move proteins. Nat. Rev. Mol. Cell Biol. **2:** 504–513
14 Bishop W. R. and Bell R. M. (1985) Assembly of the endoplasmic reticulum phospholipid bilayer: the phosphatidylcholine transporter. Cell **42:** 51–60
15 Zilversmit D. B. and Hughes M. E. (1977) Extensive exchange of rat liver microsomal phospholipids. Biochim. Biophys. Acta **469:** 99–110
16 Chang Q. L., Gummadi S. N. and Menon A. K. (2004) Chemical modification identifies two populations of glycerophospholipid flippase in rat liver ER. Biochemistry **43:** 10710–10718
17 Menon A. K., Watkins W. E. I. and Hrafnsdottir S. (2000) Specific proteins are required to translocate phosphatidylcholine bidirectionally across the endoplasmatic reticulum. Curr. Biol. **10:** 241–252
18 Zwaal R. F. A. and Schroit A. J. (1997) Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood **89:** 1121–1132
19 Seigneuret M. and Devaux P. F. (1984) ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: Relation to shape changes. Proc. Natl. Acad. Sci. USA **81:** 3751–3755
20 Connor J. and Schroit A. J. (1990) Aminophospholipid translocation in erythrocytes: evidence for the involvement of a specific transporter and an endofacial protein. Biochemistry **29:** 37–43
21 Daleke D. L. and Lyles J. V. (2000) Identification and purification of aminophospholipid flippases. Biochim. Biophys. Acta **1486:** 108–127
22 Beleznay Z., Zachowski A., Devaux P. F., Navazo M. P. and Ott P. (1993) ATP-dependent aminophospholipid translocation in erythrocyte vesicles – stoichiometry of transport. Biochemistry **32:** 3146–3152
23 Williamson P. and Schlegel R. A. (2002) Transbilayer phospholipid movement and the clearance of apoptotic cells. Biochim. Biophys. Acta **1585:** 53–63
24 Auland M. E., Roufogalis B. D., Devaux P. F. and Zachowski A. (1994) Reconstitution of ATP-dependent aminophospholipid translocation in proteoliposomes. Proc. Natl. Acad. Sci. USA **91:** 10938–10942
25 Bitbol M. and Devaux P. F. (1988) Measurement of outward translocation of phospholipids across human erythrocyte membrane. Proc. Natl. Acad. Sci. USA **85:** 6783–6787
26 Connor J., Pak C. H., Zwaal R. F. A. and Schroit A. J. (1992) Bidirectional transbilayer movement of phospholipid analogs in human red blood cells. J. Biol. Chem. **267:** 19412–19417
27 Dekkers D. W. C., Comfurius P., Schroit A. J., Bevers E. M. and Zwaal R. F. A. (1998) Transbilayer movement of NBD-labeled phospholipids in red blood cell membranes; outward-directed transport by the multidrug resistance protein 1 (MRP1). Biochemistry **37:** 14833–14837
28 Kamp D. and Haest C. W. M. (1998) Evidence for a role of multidrug resistance protein (MRP) in outward translocation of NBD-phospholipids in the erythrocyte membrane. Biochim. Biophys. Acta **1372:** 91–101
29 Dean M., Hamon Y. and Chimini G. (2001) The human ATP-binding cassette (ABC) transporter superfamily. J. Lipid Res. **42:** 1007–1017
30 Borst P., Zelcer N. and van Helvoort A. (2000) ABC transporters in lipid transport. Biochim. Biophys. Acta **1486:** 128–144
31 Dekkers D. W. C., Comfurius P., van Gool R. J. G., Bevers E. M. and Zwaal R. F. A. (2000) Multidrug resistance protein1 (MRP1) regulates lipid asymmetry in red blood cell membranes. Biochem. J. **350:** 531–535
32 Diaz C. and Schroit A. J. (1996) Role of translocases in the generation of phosphatidylserine asymmetry. J. Membrane Biol. **151:** 1–9
33 Hamon Y., Broccardo C., Chambenoit O., Luciani M. F., Toti F., Chaslin S. et al. (2000) ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. Nat. Cell Biol. **2:** 399–406

34 Hamon Y., Chambenoit O. and Chimini G. (2002) ABCA1 and the engulfment of apoptotic cells. Biochim. Biophys. Acta **1585:** 64–71

35 Emoto K., Toyama-Sorimachi N., Karasuyama H., Inoue K. and Umeda M. (1997) Exposure of phosphatidylethanolamine on the surface of apoptotic cells. Exp. Cell Res. **232:** 430–434

36 Maulik N., Kagan V., Tyurin V. and Das D. K. (1998) Redistribution of phosphatidylethanolamine and phosphatidylserine precedes reperfusion-induced apoptosis. Am. J. Physiol. **274:** H242–H248

37 Williamson P., Christie A., Kohlin T., Schlegel R. A., Comfurius P., Harmsma M. et al. (2001) Phospholipid scramblase activation pathways in lymphocytes. Biochemistry **40:** 8065–8072

38 Christiansen-Weber T. A., Voland J. R., Wu Y., Ngo K., Roland B. L., Nguyen S. et al. (2000) Functional loss of ABCA1 in mice causes severe placental malformation, aberrant lipid distribution and kidney glomerulonephritis as well as high-density lipoprotein cholesterol deficiency. Am. J. Pathol. **157:** 1017–1029

39 Brooks-Wilson A., Marcil M., Clee S. M., Zhang L. H., Roomp K., van Dam M. et al. (1999) Mutations in ABCA1 in Tangier disease and familial high density lipoprotein deficiency. Nat. Genet. **22:** 336–345

40 Bodzioch M., Orso E., Klucken J., Langmann T., Böttcher A., Diederich W. et al. (1999) The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. Nat. Genet. **22:** 347–351

41 Bevers E. M., Comfurius P., Dekkers D. W. C. and Zwaal R. F. A. (1999) Lipid translocation across the plasma membrane of mammalian cells. Biochim. Biophys. Acta **1439:** 317–330

42 Williamson P., Kulick A., Zachowski A., Schlegel R. A. and Devaux P. F. (1992) Ca^{2+} induces transbilayer redistribution of all major phospholipids in human erythrocytes. Biochemistry **31:** 6355–6360

43 Smeets E. F., Comfurius P., Bevers E. M. and Zwaal R. F. A. (1994) Calcium induced transbilayer scrambling of fluorescent phospholipid analogs in platelets and erythrocytes. Biochim. Biophys. Acta **1195:** 281–286

44 Dekkers D. W. C., Comfurius P., Bevers E. M. and Zwaal R. F. A. (2002) Comparison between Ca^{2+}-induced scrambling of various fluorescently labelled lipid analogues in red blood cells. Biochem. J. **362:** 741–747

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

46 Comfurius P., Senden J. M., Tilly R. H., Schroit A. J., Bevers E. M. and Zwaal R. F. A. (1990) Loss of membrane phospholipid asymmetry in platelets and red cells may be associated with calcium-induced shedding of plasma membrane and inhibition of aminophospholipid translocase. Biochim. Biophys. Acta **1026:** 153–158

47 Comfurius P., Williamson P., Smeets E. F., Schlegel R. A., Bevers E. M. and Zwaal R. F. A. (1996) Reconstitution of phospholipid scramblase activity from human blood platelets. Biochemistry **35:** 7631–7634

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

49 Zhou Q., Zhao J., Stout J. G., Luhm R. A., Wiedmer T. and Sims P. J. (1997) Molecular cloning of human plasma membrane phospholipid scramblase – a protein mediating transbilayer movement of plasma membrane phospholipids. J. Biol. Chem. **272:** 18240–18244

50 Zhou Q., Sims P. J. and Wiedmer T. (1998) Expression of proteins controlling transbilayer movement of plasma membrane phospholipids in the B lymphocytes from a patient with Scott syndrome. Blood **92:** 1707–1712

51 Zhou Q., Zhao J., Wiedmer T. and Sims P. J. (2002) Normal hemostasis but defective hematopoietic response to growth factors in mice deficient in phospholipid scramblase 1. Blood **99:** 4030–4038

52 Yu A., McMaster C. R., Byers D. M., Ridgway N. D. and Cook H. W. (2003) Stimulation of phosphatidylserine biosynthesis and facilitation of UV-induced apoptosis in Chinese hamster ovary cells overexpressing phospholipid scramblase 1. J. Biol. Chem. **278:** 9706–9714

53 Sims P. J., Wiedmer T., Esmon C. T., Weiss H. J. and Shattil S. J. (1989) Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant avtivity. J. Biol. Chem. **264:** 17049–17057

54 Zwaal R. F. A., Comfurius P. and Bevers E. M. (1992) Platelet procoagulant activity and microvesicle formation. Its putative role in hemostasis and thrombosis. Biochim. Biophys. Acta **1180:** 1–8

55 Dachary-Prigent J., Pasquet J. M., Freyssinet J. M. and Nurden A. T. (1995) Calcium involvement in aminophospholipid exposure and microparticle formation during platelet activation: a study using Ca^{2+}-ATPase inhibitors. Biochemistry **34:** 11625–11634

56 Pasquet J. M., Dachary-Prigent J. and Nurden A. T. (1996) Calcium influx is a determining factor of calpain activation and microparticle formation in platelets. Eur. J. Biochem. **239:** 647–654

57 Bratton D. L., Fadok V. A., Richter D. A., Kailey J. M., Guthrie L. A. and Henson P. M. (1997) Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. J. Biol. Chem. **272:** 26159–26165

58 Henson P. M., Bratton D. L. and Fadok V. A. (2001) Apoptotic cell removal. Curr. Biol. **11:** R795–R805

59 Green D. R. and Reed J. C. (1998) Mitochondria and apoptosis. Science **281:** 1309–1312

60 Shidoji Y., Nakamura N., Moriwaki H. and Muto Y. (1997) Rapid loss in the mitochondrial membrane potential during geranygeranoic acid-induced apoptosis. Biochem. Biophys. Res. Commun. **230:** 58–63

61 Shidoji Y., Hayashi K., Komura S., Ohishi N. and Yagi K. (1999) Loss of molecular interaction between cytochrome c and cardiolipin due to lipid peroxidation. Biochem. Biophys. Res. Commun. **264:** 343–347

62 Earnshaw W. C., Martins L. M. and Kaufmann S. H. (1999) Mammalian caspases: structure activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. **68:** 383–424

63 Tyurina Y. Y., Serinkan F. B., Tyurin V. A., Kini V., Yalowich J. C., Schroit A. J. et al. (2004) Lipid antioxydant, etoposide, inhibits phosphatidylserine externalization and macrophage clearance of apoptotic cells by preventing phosphatidylserine oxydation. J. Biol. Chem. **279:** 6056–6064

64 Tyurina Y. Y., Tyrin V. A., Zhao Q., Djukic M., Quinn P. J., Pitt B. R. et al. (2004) Oxidation of phosphatidylserine: a mechanism for plasma membrane phospholpid scrambling during apoptosis? Biochem Biophys. Res. Commun. **324:** 1059–1064

65 Meisenholder G. W., Martin S. J., Green D. R., Nordberg J., Babior B. M. and Gottlieb R. A. (1996) Events in apoptosis. Acidification is downstream of protease activation and BCL-2 protection. J. Biol. Chem. **271:** 16260–16262

66 Stout J. G., Bassé F., Luhm R. A., Weiss H. J. Wiedmer T. and Sims P. J. (1997) Scott syndrome erythrocyte contain a membrane protein capable of mediating Ca2+-dependent transbilayer migration of membrane phospholipids. J. Clin. Invest. **99:** 2232–2238

67 Augereau O., Rossignol R., DiGiorgi F., Mazat J.-P., Letellier T. and Dachary-Prigent J. (2004) Apoptotic-like mitochondrial events associated to phosphatidylserine exposure in blood platelets induced by local anaestetics. Thromb. Haemost **92:** 104–113

68 Hampton M. B., Vanags D. M., Pomares M. I. and Orrenius S. (1996) Involvement of extracellular calcium in phosphatidylserine exposure during apoptosis. FEBS Lett. **399:** 277–282

69 Weiss H. J. (1994) Scott syndrome: a disorder of platelet procoagulant activity. Semin. Hematol. **31:** 312–319

70 Zwaal R. F. A., Comfurius P. and Bevers E. M. (2004) Scott syndrome, a bleeding disorder caused by defective scrambling of membrane phospholipids. Biochim. Biophys. Acta **1636:** 119–128

71 Rosing J., Bevers E. M., Comfurius P., Hemker H. C., van Dieijen G., Weiss H. J. et al. (1985) Impaired factor X and prothrombin activation associated with decreased phospholipid exposure in platelets from a patient with a bleeding disorder. Blood **65:** 1557–1561

72 Dachary-Prigent J., Pasquet J.-M., Fressinaud E., Toti F., Freyssinet J.-M. and Nurden A. T. (1997) Aminophospholipid exposure, microvesiculation and abnormal protein tyrosine phosphorylation in the platelets of a patient with Scott syndrome: a study using physiological agonists and local anaesthetics. Brit. J. Haematol. **99:** 959–967

73 Rosing J., van Rijn J. L. M. L., Bevers E. M., van Dieijen G., Comfurius P. and Zwaal R. F. A. (1985) The role of activated human platelets in prothrombin and factor X activation. Blood **65:** 319–332

74 Toti F., Satta N., Fressinaud E., Meyer D. and Freyssinet J.-M. (1996) Scott syndrome, characterized by impaired transmembrane migration of procoagulant phosphatidylserine and hemorrhagic complications, is an inherited disorder. Blood **87:** 1409–1415

75 Brooks M. B., Catalfamo J. L., Brown H. A., Ivanova P. and Lovaglio J. (2002) A hereditary bleeding disorder of dogs caused by a lack of platelet procoagulant activity. Blood **99:** 2434–2441

76 Bevers E. M., Wiedmer T., Comfurius P., Shattil S. J., Weiss H. J., Zwaal R. F. A. et al. (1992) Defective Ca^{2+}-induced microvesiculation and deficient expression of procoagulant activity in erythrocytes from a patient with a bleeding disorder: a study of the red blood cells of Scott syndrome. Blood **79:** 380–388

77 Lang P. A., Kaiser S., Myssina S., Wieder T., Lang F. and Huber S. M. (2003) Role of Ca^{2+}-activated K+ channels in human erythrocyte apoptosis. Am. J. Physiol. Cell Physiol. **285:** C1553–C1560

78 Dekkers D. W. C., Comfurius P., Vuist W. M. J., Billheimer I., Weiss H. J., Zwaal R. F. A. et al. (1998) Impaired Ca^{2+}-induced tyrosine phosphorylation and defective lipid scrambling in erythrocytes from a patient with Scott syndrome: a study using an inhibitor for scramblase that mimics the defect in Scott syndrome. Blood **91:** 2133–2138

79 Martinez M. C. and Freyssinet J.-M. (2001) Deciphering the plasma membrane hallmarks of apoptosic cells: phosphatidylserine transverse redistribution and calcium entry. BMC Cell Biol. **2:** 20

80 Hughes G.R.V. (1993) The antiphospholipid syndrome: ten years on. Lancet **342:** 341–344

81 Galli M., Comfurius P., Maassen C., Hemker H. C., de Baets M. H., van Breda-Vriesman P. J. et al. (1990) Anticardiolipin antibodies (ACA) are directed not to cardiolipin but to a plasma protein cofactor. Lancet **335:** 1544–1547

82 McNeil H. P., Simpson R. J., Chesterman C. N. and Krilis S. A. (1990) Antiphospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H). Proc. Natl. Acad. Sci. USA **87:** 4120–4124

83 Bevers E. M., Galli M., Barbui T., Comfurius P. and Zwaal R. F. A. (1991) Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. Thromb. Haemost. **66:** 629–632

84 Bevers E. M., Zwaal R. F. A. and Willems G. M. (2004) The effect of phospholipids on the formation of immune complexes between autoantibodies and beta2-glycoprotein I or prothrombin. Clin. Immunol. **112:** 150–160

85 Roubey R. A., Eisenberg R. A., Harper M. F. and Winfield J. B. (1995) 'Anticardiolipin' autoantibodies recognize beta 2-glycoprotein I in the absence of phospholipid. Importance of Ag density and bivalent binding. J. Immunol. **154:** 954–60

86 Willems G. M., Janssen M. P., Pelsers M. M. A., Comfurius P., Galli M., Zwaal R. F. A. et al. (1996) Role of divalency in the high-affinity binding of anticardiolipin antibody-β_2-glycoprotein I complexes to lipid membranes. Biochemistry **35:** 13833–13842

87 Willems G. M., Janssen M. P., Comfurius P., Galli M., Zwaal R. F. A. and Bevers E. M. (2002) Kinetics of prothrombin-mediated binding of lupus anticoagulant antibodies to phosphatidylserine-containing phospholipid membranes: an ellipsometric study. Biochemistry **41:** 14357–14363

88 Borchman D., Harris E. N., Pierangeli S. S. and Lamba O. P. (1995) Interactions and molecular structure of cardiolipin and beta 2-glycoprotein 1 (beta 2-GP1). Clin. Exp. Immunol. **102:** 373–378

89 Chamley L. W., Duncalf A. M., Konarkowska B., Mitchell M. D. and Johnson P. M. (1999) Conformationally altered beta 2-glycoprotein I is the antigen for anti-cardiolipin autoantibodies. Clin. Exp. Immunol. **115:** 571–576

90 Wu J. R. and Lentz B. R. (1994) Phospholipid-specific conformational changes in human prothrombin upon binding to procoagulant acidic lipid membranes. Thromb. Haemost. **71:** 596–604

91 Reutelingsperger C. P. (2001) Annexins: key regulators of haemostasis, thrombosis and apoptosis. Thromb. Haemost. **86:** 413–419

92 Krahling S., Callahan M. K., Williamson P. and Schlegel R. A. (1999) Exposure of phosphatidylserine is a general feature in the phagocytosis of apoptotic lymphocytes by macrophages. Cell Death Differ. **6:** 183–189

93 Simmelink M. J., Horbach D. A. Derksen R. H., Meijers J. C., Bevers E. M., Willems G. M. et al. (2001) Complexes of anti-prothrombin antibodies and prothrombin cause lupus anticoagulant activity by competing with the binding of clotting factors for catalytic phospholipid surfaces. Br. J. Haematol. **113:** 621–629

94 Arnout J. and Vermylen J. (2003) Current status and implications of autoimmune antiphospholipid antibodies in relation to thrombotic disease. J. Thromb. Haemost. **1:** 931–942

95 Bevers E. M., Smeets E. F., Comfurius P. and Zwaal R. F. A. (1994) Physiology of membrane lipid asymmetry. Lupus **3:** 235–240

96 Casciola Rosen L., Rosen A., Petri M. and Schlissel M. (1996) Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosis. Proc. Natl. Acad. Sci. USA **93:** 1624–1629

97 Emlen W., Niebur J. and Kadera R. (1994) Accelerated in vitro apoptosis of lymphocytes from patients with systemic lupus erythematosis. J. Immunol. **152:** 3685–3692

98 Rauch J., Subang R., D'Agnillo P., Koh J. S. and Levine J. S. (2000) Apoptosis and the antiphospholipid syndrome. J. Autoimmunity **15:** 231–235

99 D'Agnillo P., Levine J. S., Subang R. and Rauch J. (2003) Prothrombin binds to the surface of apoptotic, but not viable, cells and serves as a target of lupus anticoagulant antobodies. J. Immunol. **15:** 3408–3422

100 Asano K., Miwa M., Miwa K., Hanayama R., Nagase H., Nagata S. et al. (2004) Masking of phosphatidylserine inhibits apoptotic cell engulfment and induces autoantibody production in mice. J. Exp. Med. **200**: 459–67

101 Zhou H., Wolberg A. S. and Roubey R. A. S. (2004) Characterization of monocyte tissue factor activity induced by IgG antiphospholipid antibodies and inhibition by dilazep. Blood **104:** 2353–2358

102 Esmon C. T., Ding W., Yasuhiro K., Gu J. M., Ferrell G., Regan L. M. et al. (1997) The protein C pathway: new insights. Thromb. Haemost. **78:** 70–74

103 Dahlbäck B. (1995) The protein C anticoagulant system: inherited defects as basis for venous thrombosis. Thromb. Res. **77:** 1–43

104 Oosting J. D., Derksen R. H., Bobbink I. W., Hackeng T. M., Bouma B. N. and de Groot P. G. (1993) Antiphospholipid antibod-

ies directed against a combination of phospholipids with prothrombin, protein C, or protein S: an explanation for their pathogenic mechanism? Blood **81:** 2618–2625
105 Galli M., Ruggeri L. and Barbui T. (1998) Differential effects of anti-beta2-glycoprotein I and antiprothrombin antibodies on the anticoagulant activity of activated protein C. Blood **91:** 1999–2004
106 Kuypers F. A., Lewis R. A., Hua M., Schott M. A., Discher D., Ernst J. D. et al. (1996) Detection of altered membrane phospholipid asymmetry in subpopulations of human red blood cells using fluorescently labeled annexin V. Blood **87:** 1179–1187
107 Wood B. L., Gobson D. F. and Tait J. F. (1996) Increased erythrocyte phosphatidylserine exposure in sickle cell disease: flow-cytometric measurement and clinical associations. Blood **88:** 1873–1880
108 De Jong K., Larkin S. K., Styles L. A., Bookchin R. M. and Kuypers F. A. (2001) Characterization of the phosphatidylserine-exposing subpopulation of sickle cells. Blood **98:** 860–867
109 Yasin Z., Witting S., Palascak M. B., Joiner C. H., Rucknagel D. L. and Franco R. S. (2003) Phosphatidylserine externalization in sickle red blood cells: associations with cell age, density and hemoglobin F. Blood **102:** 365–370
110 Middelkoop E., Lubin B. H., Bevers E. M., OpdenKamp J. A. F., Comfurius P., Chiu D. T. et al. (1988) Studies on sickled erythrocytes provide evidence that the asymmetric distribution of phosphatidylserine in the red cell membrane is maintained by both ATP-dependent translocation and interaction with membrane skeletal proteins. Biochim. Biophys. Acta **937:** 281–288
111 Connor J., Pak C. C. and Schroit A. J. (1994) Exposure of phosphatidylserine in the outer leaflet of human red blood cells: relationship to cell density, cell age and clearance by mononuclear cells. J. Biol. Chem. **269:** 2399–2404
112 Banerjee T. and Kuypers F. A. (2004) Reactive oxygen species and phosphatidylserine externalization in murine sickle red cells. Br. J. Haematol. **124:** 391–402
113 Lew V. L., Ortiz O. E. and Bookchin R. M. (1997) Stochastic nature and red cell population distribution of the sickling-induced Ca^{2+} permeability. J. Clin. Invest. **99:** 2727–2735
114 Setty B. N. Y., Rao A. K. and Stuart M. J. (2002) Thrombophilia in sickle cell disease: the red cell connection. Blood **98:** 3228–3233
115 Westerman M. P., Green D., Gilman-Sachs A., Beaman K., Freels S., Boggio L. et al. (1999) Antiphospholipid antibodies, proteins C and S, and coagulation changes in sickle cell disease. J. Lab. Clin. Med. **134:** 352–362
116 Closse C., Dachary-Prigent J. and Boisseau M. R. (1999) Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. Br. J. Haematol. **107:** 300–302
117 Manadori A. B., Barabino G. A., Lubin B. H. and Kuypers F. A. (1999) Adherence of phosphatidylserine-exposing erythrocytes to endothelial matrix thrombospondin. Blood **95:** 1293–1300
118 Setty B. N. Y., Kulkami S. and Stuart M. J. (2002) Role of erythrocyte phosphatidylserine in sickle red cell-endothelial adhesion. Blood **99:** 1564–1571
119 De Jong K., Emerson R. K., Butler J., Bastacky J., Mohandas N. and Kuypers F. A. (2001) Short survival of phosphatidylserine-exposing red blood cells in murine sickle cell anemia. Blood **98:** 1577–1584
120 Kean L. S., Brown L. E., Nichols J. W., Mohandas N., Archer D. R. and Hsu L. L. (2002) Comparison pf mechanisms of anemia in mice with sickle cell disease and beta-thalassemia: peripheral destruction, ineffective erythropoiesis and phospholipid scramblase-mediated phosphatidylserine exposure. Exp. Hematol. **30:** 394–402
121 Covas D. T., de Lucena Angelo I., Vianna Bonini Palma P. and Zago M. A. (2004) Effects of hydroxyurea on the membrane of erythrocytes and platelets in sickle cell anemia. Haematologica **89:** 273–280
122 Huyghe P., Dassonneville L., Fenaux P. and Bailly C. (2003) Hydroxyurea-induced apoptosis in an EBV-immortalized lymphoblastoid cell line. Oncol. Res. **14:** 235–245
123 Borenstain-Ben Yashar V., Barenholz Y., Hy-Am E., Rachmilewitz E. A. and Eldor A. (1993) Phosphatidylserine in the outer leaflet of red blood cells from beta-thalassemia patients may explain the chronic hypercoagulable state and thrombotic episodes. Am. J. Hematol. **44:** 63–65
124 Srinivasan P. T. and Basu J. (1996) Altered membrane phospholpid organisation and erythrophagocytosis in E beta-thalassemia. Biochim. Biophys. Acta **1285:** 65–70
125 Helley D., Eldor A., Girot R., Ducrocq R., Guillin M. C. and Bezeaud A. (1996) Increased procoagulant activity of red blood cells from patients with homozygous sickle cell disease and beta-thalassemia. Thromb Haemost. **76:** 322–327
126 Ruf A., Pick M., Deutsch V., Patscheke H., Goldfarb A., Rachmilewitz E. A. et al. (1997) In-vivo platelet activation correlates with red cell anionic phospholipid exposure in patients with beta-thalassaemia major. Br. J. Haematol. **98:** 51–56
127 Kuypers F. A., Yuan J., Lewis R. A., Snyder L. M., Kiefer C. R., Bunyaratvej A. et al. (1998) Membrane phospholipid asymmetry in human thalassemia. Blood **91:** 3044–3051
128 Eldor A. and Rachmilewitz E. A. (2002) The hypercoaguable state in thalassemia. Blood **99:** 36–43
129 Lang K. S., Roll B., Myssina S., Schittenhelm M., Scheel-Walter H. G., Kanz L. et al. (2002) Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency. Cell. Physiol. Biochem.**12:** 365–372
130 Lang F., Lang K. S., Wieder T., Myssina S., Birka C., Lang P. A. et al. (2003) Cation channels, cell volume and the death of an erythrocyte. Pflugers Arch. **447:** 121–125
131 Atichartakarn V., Angchaisuksiri P., Aryurachai K., Onpun S., Chuncharunee S., Thakkinstian A. et al. (2002) Relationship between hypercoagulable state and erythrocyte phosphatidylserine exposure in splenectomized haemoglobin E/beta-thalassaemic patients. Br. J. Haematol. **118:** 893–898
132 Barker J. E. and Wandersee N. J. (1999) Thrombosis in heritable hemolytic disorders. Curr. Opin. Hematol. **6:** 71–75
133 Gallagher P. G., Chang S. H., Rettig M. P., Neely J. E., Hillery C. A., Smithe B. D. et al. (2003) Altered erythrocyte endoyhelial adherence and membrane phospholpid asymmetry in hereditary hydrocytosis. Blood **101:** 4625–4627
134 Delauney J., Stewart G. and Iolascon A. (1999) Hereditary dehydrated and overhydrated stomatocytosis: recent advances. Curr. Opin. Hematol, **6:** 110–114
135 Bonomini M., Sirolli V., Settefrati N., Dottori S., Di Liberato L. and Arduini A. (1999) Increased erythrocyte phosphatidylserine exposure in chronic renal failure. J. Am. Soc. Nephrol. **10:** 1982–1990
136 Bonomini M., Sirolli V., Reale M. and Arduini A. (2001) Involvement of phosphatidylserine exposure in the recognition and phagocytosis of uremic erythrocytes. Am. J. Kidney Dis**. 37:** 807–814
137 Bonomini M., Sirolli V., Gizzi F., Di Stante S., Grilli A. and Felaco M. (2002) Enhanced adherence of human uremic erythrocytes to vascular endothelium: role of phosphatidylserine exposure. Kidney Int. **62:** 1358–1563
138 Bonomini M., Ballone E., Di Stante S., Bucciarelli T., Dottori S., Arduini A. et al. (2004) Removal of uraemic plasma factor(s) using different dialysis modalities reduces phosphatidylserine exposure in red blood cells. Nephrol. Dial. Transplant. **19:** 68–74
139 Kong Q. Y., Wu X., Li J., Peng W. X., Ye R., Lindholm B. et al. (2001) Loss of phospholipid asymmetry in red blood cells contributes to anemia in uremic patients. Adv. Perit. Dial. **17:** 58–60
140 Bonomini, M., Dottori S., Amoroso L., Arduini A. and Sirolli V. (2004) Increased platelet phosphatidylserine exposure and caspase activation in chronic uremia. J. Thromb. Haemost. **2:** 1275–1281
141 Ando M., Iwata A., Ozeki Y., Tsuchiya K., Akiba T. and Nihei H. (2002) Circulating platelet-derived microparticles with procoagulant activity may be a potential cause of thrombosis in uremic patients. Kidney Int. **62:** 1757–1763

142 Escolar G., Cases A., Bastida E., Garrido M., Lopez J., Revert L. et. al. (1990) Uremic platelets have a functional defect affecting the interaction of von Willebrand factor with glycoprotein IIb-IIIa. Blood **76:** 1336–1340

143 Sloand E. M., Sloand J. A., Prodouz K., Klein H. G., Yu M. W., Harvath L. et al. (1991) Reduction of platelet glycoprotein Ib in uraemia. Br. J. Haematol. **77:** 375–381

144 Moal V., Brunet P., Dou L., Morange S., Sampol J. and Berland Y. (2003) Impaired expression of glycoproteins on resting and stimulated platelets in uraemic patients. Nephrol. Dial. Transplant. **18:** 1834–1841

145 Wiessner J. H., Hasegawa A. T., Hung L. Y. and Mandel N. S. (1999) Oxalate-induced exposure of phosphatidylserine on the surface of renal epithelial cells in culture. J. Am. Soc. Nephrol. **14:** S441–S445

146 Cao L. C., Jonassen J., Honeyman T. W. and Scheid C. (2001) Oxalate-induced redistribution of phosphatidylserine in renal epithelial cell: implications for kidney stone disease. Am. J. Nephrol. **21:** 69–77

147 Wiessner J. H., Hasegawa A. T., Hung L. Y., Mandel G. S. and Mandel N. S. (2001) Kidney Int. **59:** 637–644

148 Khan S. R., Byer K. J., Thamilselvan S., Hackett R. L., McCormack W. T., Benson N. A. et al. (1999) Crystal-cell interaction and apoptosis in oxalate-associated injury of renal epithelial cells. J. Am. Soc. Nephrol. **14:** S457–S463

149 Wali R. K., Jaffe S., Kumar D. and Kalra V. K. (1988) Alterations in organization of phospholipids in erythrocytes as factor in adherence to endothelial cells in diabetes mellitus. Diabetes **37:** 104–111

150 Lupu F., Calb M. and Fixman A. (1988) Alterations of phospholipid asymmetry in the membrane of spontaneously aggregated platelets in diabetes. Thromb. Res. **50:** 605–616

151 Wahid S. T., Marshall S. M. and Thomas T. H. (2001) Increased platelet and erythrocyte external cell membrane phosphatidylserine in type 1 diabetes and microalbuminuria. Diabetes Care **24:** 2001–2003

152 Lupu C., Calb M., Ionescu M. and Lupu F. (1993) Enhanced prothrombin and intrinsic factor X activation on blood platelets from diabetic patients. Thromb. Haemost. **70:** 579–583

153 Cohen Z., Gonzales R. F., Davis-Gorman G. F., Copeland J. G. and McDonagh P. F. (2002) Thrombin activity and platelet microparticle formation are increased in type 2 diabetic platelets: a potential correlation with caspase activation. Thromb. Res. **107:** 217–221

154 Manadori A. B. and Kuypers F. A. (2002) Altered red cell turnover in diabetic mice. J. Lab. Clin. Med. **140:** 161–165

155 Wilson M. J., Richter-Lowney K. and Daleke D. L. (1993) Hyperglycemia induces a loss of phospholipid asymmetry in human erythrocytes. Biochemistry **32:** 11302–11310

156 Jain S. K., Palmer M. and Chen Y. (1999) Effect of vitamin E and N-acetylcysteine on phosphatidylserine externalization and induction of coagulation by high-glucose-treated human erythrocytes. Metabolism **48:** 957–959

157 Balasubramanian K., Bevers E. M., Willems G. M. and Schroit A. J. (2001) Binding of annexin V to membrane products of lipid peroxidation. Biochemistry **40:** 8672–8676

158 Granville D. J., Carthy C. M., Yang D., Hunt D. W. and McManus B. M. (1998) Interaction of viral proteins with host cell death machinery. Cell Death Differ. **5:** 653–659

159 Dockrell D. H. (2003) The multiple roles of Fas ligand in the pathogenesis of infectious diseases. Clin. Microbiol. Infect. **9:** 766–779

160 Vercellotti G. M. (2001) Overview of infections and cardiovascular diseases. J. Allergy Clin. Immunol. **108:** S117–S120

161 Van Dam-Mieras M. C., Bruggeman C. A., Muller A. D., Debie W. H. and Zwaal R. F. A. (1987) Induction of endothelial cell procoagulant activity by cytomegalovirus infection. Thromb. Res. **47:** 69–75

162 Shiratsuchi A., Kaido M., Takizawa T. and Nakanishi Y. (2000) Phosphatidylserine-mediated phagocytosis of influenza A virus-infected cells by mouse peritoneal macrophages. J. Virol. **74:** 9240–9244

163 Lowy R. J. (2003) Influenza virus induction of apoptosis by intrinsic and extrinsic mechanisms. Int. Rev. Immunol. **22:** 425–429

164 Watanabe Y., Shiratsuchi A., Shimizu K., Takizawa T. and Nakanishi Y. (2002) Role of phosphatidylserine exposure and sugar chain desialylation at the surface of influenza virus-infected cells in efficient phagocytosis by macrophages. J. Biol. Chem. **277:** 18222–18228

165 Kuo C. C., Shor A., Campbell L. A., Fukushi H., Patton D. L. and Grayston J. T. (1993) Demonstration of *Chlamydia pneumoniae* in atherosclerotic lesions of coronary arteries. J. Infect. Dis. **167:** 841–849

166 Goth S. R. and Stephens R. S. (2001) Rapid, transient phosphatidylserine externalization induced in host cells by infection with *Chlamydia* spp. Infect. Immun. **69:** 1109–1119

167 Sherman I. W., Prudhomme J. and Tait J. F. (1997) Altered membrane phospholipid asymmetry in plasmodium falciparum-infected erythrocytes. Parasitol. Today **13:** 242–243

168 Eda S. and Sherman I. W. (2002) Cytoadherence of malaria-infected red blood cells involves exposure of phosphatidylserine. Cell. Physiol. Biochem. **12:** 373–384

169 Schwartz R. S., Olson J. A., Raventos-Suarez C., Yee M., Heath R. H., Lubin B. et al. (1987) Altered plasma membrane phospholipid organization in *Plasmodium falciparum*-infected human erythrocytes. Blood **69:** 401–407

170 Moll G. N., Vial H. J., Bevers E. M., Ancelin M. L., Roelofsen B., Comfurius P. et al. (1990) Phospholipid asymmetry in the plasma membrane of malaria infected erythrocytes. Biochem. Cell. Biol. **68:** 579–585

171 Lang F., Lang P. A., Lang K. S., Brand V., Tanneur V., Duranton C, et al. (2004) Channel-induced apoptosis of infected host cells-the case of malaria. Pflugers Arch. **448:** 319–324

172 Sherman I. W., Eda S. and Winograd E. (2004) Erythrocyte aging and malaria. Cell. Mol. Biol. **50:** 159–169

173 Facer C. A. and Agiostratidou G. (1994) High levels of anti-phospholipid antibodies in uncomplicated and severe *Plasmodium falciparum* and in *P. vivax* malaria. Clin. Exp. Immunol. **95:** 304–309

174 Walker I. D. (2002) Prothrombotic genotypes and pre-eclampsia. Thromb. Haemost. **87:** 777–778

175 Grisaru D., Zwang E., Peyser M. R., Lessing J. B. and Eldor A. (1997) The procoagulant activity of red blood cells from patients with severe preeclampsia. Am. J. Obstet. Gynecol. **177:** 1513–1516

176 Bretelle F., Sabatier F., Desprez D., Camoin L., Grunebaum L., Combes V. et al. (2003) Circulating microparticles: a marker of procoagulant state in normal pregnancy and pregnancy complicated by preeclampsia or intrauterine growth restriction. Thromb. Haemost. **89:** 486–492

177 Yamamoto T., Takahashi Y., Geshi Y., Sasamori Y. and Mori H. (1996) Anti-phospholipid antibodies in preeclampsia and their binding ability for placental villous lipid fractions. J. Obstet. Gynaecol. Res. **22:** 275–83

178 Allen J. Y., Tapia-Santiago C. and Kutteh W. H. (1996) Antiphospholipid antibodies in patients with preeclampsia. Am. J. Reprod. Immunol. **36:** 81–85

179 Fialova L., Mikulikova L., Matous-Malbohan I., Benesova O. and Zwinger A. (2000) Prevalence of various antiphospholipid antibodies in pregnant women. Physiol. Res. **49:** 299–305

180 Brito M. A., Silva R., Tiribelli C. and Brites D. (2000) Assessment of bilirubin toxicity to erythrocytes. Implication in neonatal jaundice management. Eur. J. Clin. Invest. **30:** 239–247

181 Brito M. A, Silva R. F. and Brites D. (2002) Bilirubin induces loss of membrane lipids and exposure of phosphatidylserine in human erythrocytes. Cell. Biol. Toxicol. **18:** 181–192

182 Igney F. H. and Krammer P. H. (2002) Death and anti-death: tumor resistance to apoptosis. Nat. Rev. Cancer **2:** 277–288

183 Connor J., Bucana C. D., Fidler I. J. and Schroit A. J. (1989) Differentiation-dependent expression of phosphatidylserine in mam-

malian plasma membranes: quantitative assessment of outer-leaflet lipid by prothrombinase complex formation. Proc. Natl. Acad. Sci. USA **86:** 3183–3188

184 Utsugi T., Schroit A. J., Connor J., Bucana C. D. and Fidler I. J. (1991) Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. Cancer Res. **15:** 3062–3066

185 Rao L. V., Tait J. F. and Hoang A. D. (1992) Binding of annexin V to a human ovarian carcinoma cell line (OC-2008). Contrasting effects on cell surface factor VIIa/tissue factor activity and prothrombinase activity. Thromb. Res. **67:** 517–531

186 Sigimura M., Donato R., Kakkar V. V. and Scully M. F. (1994) Annexin V as a probe of the contribution of anionic phospholipids to the procoagulant activity of tumour cell surfaces. Blood Coagul. Fibrinolysis **5:** 365–373

187 Ran S., Downes A. and Thorpe P. E. (2002) Increased exposure of anionic phospholipids on the surface of tumor blood vessels. Cancer Res. **62:** 6132–6140

188 Woehlecke H., Pohl A., Alder-Baerens N., Lage H. and Herrmann A. (2003) Enhanced exposure of phosphatidylserine in human gastric carcinoma cells over expressing the half-size ABC transporter BCRP (ABCG2). Biochem. J. **376:** 489–495

189 Kabore A. F., Johnston J. B. and Gibson S. B. (2004) Changes in the apoptotic and survival signaling in cancer cells and their potential therapeutic implications. Curr. Cancer Drug Targets. **4:** 147–63

190 Debatin K. M. and Krammer P. H. Death receptors in chemotherapy and cancer. Oncogene **23:** 2950–2966

191 Sun S. Y., Hail N. Jr and Lotan R. (2004) Apoptosis as a novel target for cancer chemoprevention. J. Natl. Cancer Inst. **96:** 662–672

192 Malaguarnera L. (2004) Implications of apoptosis regulators in tumorigenesis. Cancer Metastasis Rev. **23:** 367–387

193 Sica A., Saccani A. and Mantovani A. (2002) Tumor-associated macrophages: a molecular perspective. Int. Immunopharmacol. **2:** 1045–1054

194 Brigati C., Noonan D. M., Albini A. and Benelli R. (2002) Tumors and inflammatory infiltrates: friends or foes? Clin. Exp. Metastasis **19:** 247–258

195 Balkwill F. and Mantovani A. (2001) Inflammation and cancer: back to Virchow? Lancet **357:** 539–545

196 Birrer P., McElvaney N. G., Rudeberg A., Sommer C. W., Liechti-Gallati S., Kramer R. et al. (1994) Protease anti-protease imbalance in the lungs of children with cystic fibrosis. Am. J. Respir. Crit. Care Med. **150:** 207–213

197 Tsang K. W., Chan K., Ho P., Zheng L. Ooi G. C., Ho J. C. et al. (2000) Sputum elastase in steady state bronchiectasis. Chest **117:** 420–426

198 Vandivier R. W., Fadok V. A., Hoffmann P. R., Bratton D. L., Penvari C., Brown K. K. et al. (2002) Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. J. Clin. Invest. **109:** 661–670

199 Li M. O., Sarkisian M. R., Mehal W. Z., Rakic P. and Flavell R. A. (2003) Phosphatidylserine receptor is required for clearance of apoptotic cells. Science **302:** 1560–1563

200 Kunisaki Y., Masuko S., Noda M., Inayoshi A., Sanui T., Harada M. et al. (2004) Defective fetal liver erythropoiesis and T lymphopoiesis in mice lacking the phosphatidylserine receptor. Blood **103:** 3362–3364

201 Skutelski E. and Danon D. (1967) An electron microscopic study of nuclear elimination from the late erythroblast. J. Cell Biol. **33:** 625–635

202 Schlegel R. A., Phelps B. M., Waggoner A., Terada L. and Williamson P. (1980) Binding of merocyanine 540 to normal and leukemic erythroid cells. Cell **20:** 321–328

203 Böse J., Gruber A. D., Helming L., Schiebe S., Wegener I., Hafner M. et al. (2004) The phosphatidylserine receptor has essential functions during embryogenesis but not in apoptotic cell removal. J. Biol. **3:** 15.1–15.18

204 Cui P., Qin B., Liu N., Pan G. and Pei D. (2004) Nuclear localization of the phosphatidylserine receptor via multiple nuclear localization sig nals. Exp. Cell Res. **293:** 154–163

205 Hong J.-R., Lin G.-H., Lin C. J., Wang W.-P., Lee C.-C., Lin T.-L. et al. (2004) Phosphatidylserine receptor is required for the engulfment of dead apoptotic cells and for normal embryonic development in zebrafish. Development **131:** 5417–5427

206 Solum N. O. (1999) Procoagulant expression in platelets and defects leading to clinical disorders. Arterioscl. Thromb. Vasc. Biol. **19:** 2841–2846

207 Horstman L. L. and Ahn Y. S. (1999) Platelet microparticles; a wide-angle perpective. Crit. Rev. Oncol. Hematol. **30:** 111–142

208 Nomura S. (2001) Function and clinical significance of platelet-derived microparticles. Int. J. Hematol. **74:** 397–404

209 Diamant M., Tushuizen M. E., Sturk A. and Nieuwland R. (2004) Cellular microparticles: new players in the field of vascular disease? Eur. J. Clin. Invest. **34:** 392–401

210 Mesri M. and Altieri D. C. (1999) Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in JNK 1 signalling pathway. J. Biol. Chem. **274:** 23111–23118

211 Jy W., Horstmann L. L. Arce M. and Ahn Y. S. (1992) Clinical significance of platelet microparticles in autoimmune thrombocytopenias. J. Lab. Clin. Med. **119:** 334–345

212 Stormorken H., Holmsen H., Sund R., Sakariassen K. S., Hovig T. and Jellum E. (1995) Studies on the haemostatic defect in a complicated syndrome: an inverse Scott syndrome platelet membrane abnormality? Thromb. Haemost. **74:** 1244–1251

213 Tans G., Rosing J., Thomassen M. C. L., Heeb M. J. Zwaal R. F. A. and Griffin J. H. (1991) Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microvesicles. Blood **77:** 26-41–2648

214 Castaman G., Yu-Feng L., Battostin E. and Rodeghiero (1997) Characterization of a novel bleeding disorder with isolated prolonged bleeding time and deficiency of platelet microvesicle generation. Br. J. Haematol. **96:** 458–463

215 Bretscher M. S. (1972) Asymetrical lipid bilayer structure for biological membranes. Nat. New Biol. **236:** 11–12

216 Verkleij A. J., Zwaal R. F. A., Roelofsen B., Comfurius P., Kastelijn D. and van Deenen L. L. M. (1973) The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze–etch electron microscopy. Biochim. Biophys. Acta **323:** 178–193