Procoagulant Expression in Platelets and Defects Leading to Clinical Disorders

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Abstract—Hemostasis is a result of interactions between fibrillar structures in the damaged vessel wall, soluble components in plasma, and cellular elements in blood represented mainly by platelets and platelet-derived material. During formation of a platelet plug at the damaged vessel wall, factors IXa and VIIIa form the “tenase” complex, leading to activation of factor X on the surface of activated platelets. Subsequently, factors Xa and Va form the “prothrombinase” complex, which catalyzes the formation of thrombin from prothrombin, leading to fibrin formation. An enhanced expression of negatively charged phosphatidylserine in the outer membrane leaflet resulting from a breakdown of the phospholipid asymmetry is essential for the formation of the procoagulant surface. An ATP-driven and inward-acting aminophospholipid “translocase” and a “floppase” counterbalancing this have been postulated to maintain the dynamic state of phospholipid asymmetry. A phospholipid-nonspecific “scramblase,” believed to be responsible for the fast breakdown of the asymmetry during cell activation, has recently been isolated from erythrocytes, cloned, and characterized. An intracellular calcium-binding segment and one or more thioesterified fatty acids are probably of importance for calcium-induced activation of this transporter protein. Cytosolic calcium ions also activate the calcium-dependent protease calpain associated with shedding of microvesicles from the transformed platelet membrane. These are shed with a procoagulant surface and with surface-exposed P-selectin from the α-granules. Theoretically, therefore, microvesicles can be involved in both coagulation and inflammation. Scott syndrome is probably caused by a defect in the activation of an otherwise normal scramblase, resulting in a relatively severe bleeding tendency. In Stormorken syndrome, the patients demonstrate a spontaneous surface expression of aminophospholipids. Activated platelets and the presence of procoagulant microvesicles have been demonstrated in several clinical conditions, such as thrombotic and idiopathic thrombocytopenia, disseminated intravascular coagulation, and HIV-1 infection, and have been found to be associated with fibrin in thrombosis. Procoagulant microvesicles may also be formed from other cells as a result of apoptosis. (Arterioscler Thromb Vasc Biol. 1999;19:2841-2846.)

Key Words: microvesicles ■ platelets ■ coagulation ■ hemostasis

Hemostasis depends on interactions between fibrillar structures in the damaged vessel wall, soluble proteins in plasma, and cellular elements. Even though many reactions are not known in detail and certain concepts may be disputable, hemostasis may briefly be generalized as follows. When the vessel wall is damaged to such a degree that arrest of bleeding is required, platelets gain access to the subendothelium and adhere to specific structures, particularly collagen fibers. The binding is either direct or is brought about by the interaction of adsorbed adhesive proteins acting as ligands for adhesion molecules on the platelet membrane. At the same time, tissue factor in the damaged vessel wall triggers the coagulation system. Receptors on the surface of the adherent platelets are activated and bind other platelets as aggregates through specific ligands, enhancing the platelet adherence. Substances present in the adherent platelets are secreted from internal storage organelles. Some of these contribute to further adhesion and aggregation. As a result, a “platelet plug” is formed at the damaged area as a first defense in hemostasis. Because of processes that will be discussed later in the present review, a reorganization of the platelet membrane transforms this plug into a catalytic surface for reactions among coagulation factors. These reactions lead to deposition of fibrin in and on the platelet plug, enforcing its sealing capacity. Because thrombosis is considered a pathological process in which many of the above-mentioned reactions are involved, a better knowledge of these reactions will also improve the understanding of thrombosis.

Tissue factor is a protein complexed with phospholipid (a cell membrane or a membrane fragment). It triggers the coagulation cascade by activating factor VII and forms a complex with the activated coagulation factor. (By convention, the activated form of a coagulation factor is written with lowercase a.) This complex directly activates some factor X. It also activates factor IX, which then binds, together with factor VIIIa, to negatively charged phospholipid surfaces. (Some factor VIII is believed to be activated by the first thrombin molecules formed.) These 2 activated coagulation
factors form the so-called intrinsic “tenase” complex on the surface of activated platelets. Here factor IXa is acting as an enzyme with factor VIIIa as a cofactor. This combination leads to the proteolytic activation of factor X. As symbolized in the Figure, factor Xa supported by factor Va, which is bound to phospholipid, acts as the proteolytic enzyme of the “prothrombinase” complex on the platelet surface. This converts prothrombin into thrombin, the enzyme that subsequently transforms fibrinogen into fibrin monomers. These polymerize into a clot that is “stabilized” by a transamidase reaction catalyzed by factor XIIIa, resulting in covalent linkages between adjacent fibrin fibrils. The factor VIIa–tissue factor–phospholipid complex probably is most important as a trigger of coagulation, in view of the fact that it is soon inactivated by the “tissue factor pathway inhibitor” in complex with factor Xa. It has been suggested that specific receptors contribute to the binding of the activated coagulation factors to the activated platelet.3 Thus, the effector cell protease receptor-1 is expressed on the surface of the activated platelet and is supposed to act as a receptor for factor Xa. This is in conjunction with factor Va and phospholipid and in the presence of calcium ions. Traditionally, the contribution of platelets to the coagulation process has been described as “platelet factor 3 activity.”

Coagulation Processes on the Platelet Surface

Whereas the tissue factor complex can provide the necessary phospholipid surface for triggering the coagulation process as depicted above, the surface of the activated platelets in the platelet plug is considered to be of major importance for coagulation in hemostasis. This directs the fibrin formation to the site of damage and enforces the sealing effect of the platelet plug. Factor V is derived from either plasma or platelets. Platelet factor V is found in the α-granules and is released or translocated to the membrane during secretion as a partly proteolyzed active molecule. It has been calculated that the platelets contribute approximately one fifth of the total factor V of whole blood (see Reference 4). Except for tiny amounts of factor Xa from intracellular platelet stores, factor X and factors VIII and IX are exclusively found in plasma. The catalytic subunit of factor XIII, the α chain, is present in significant amounts in platelets, surprisingly not in the α-granules but in the cytosol. Factors IXa and Xa, which bind calcium ions with high affinity because of their content of γ-carboxylglutamic acid, demonstrate a strongly enhanced binding on platelet activation. This is directly related to the breakdown of the phospholipid asymmetry of the platelet surface membrane. The inner leaflet of the nonactivated platelet membrane contains most of the aminophospholipids and almost all of the phosphatidylserine, but a considerable amount of this is found in the outer monolayer after platelet activation.6 By use of isolated platelets and different types of platelet activators in the presence of defined concentrations of the other reactants, a direct quantitative relation comparing the platelet prothrombinase activity and the percentage of the total amount of phosphatidylserine exposed on the platelet surface has been shown.7 The negative charge of the phosphatidylserine, hydrophobic interactions, and the calcium-binding properties of the vitamin K–dependent coagulation factors are essential for the catalytic activity at the activated platelet surface. In the presence of calcium ions, an anticoagulant protein from placenta, annexin V, binds to the platelet surface with a markedly increased affinity after platelet activation. Because it is available as a recombinant protein, the fluorescence-labeled protein is much used as a probe in flow cytometry to demonstrate the presence of exposed phosphatidylserine on the surface of activated platelets.

Two phospholipid transport–mediating proteins, or systems, are postulated to maintain the steady-state phospholipid asymmetry of the cell membrane, whereas a third one has been implicated in the fast breakdown of the phospholipid asymmetry (scrambling) observed on platelet activation. The first one, usually called the aminophospholipid translocase and first described in erythrocytes,7 is specific for aminophospholipids (phosphatidylserine and phosphatidylethanolamine). This protein is considered responsible for the transport of aminophospholipids against a gradient from the outer to the inner membrane leaflet in a process that is dependent on ATP and is inhibited by calcium ions and sulfhydryl-blocking agents.8 A slow, outwardly acting phospholipid-nonspecific “floppase,” supposed to counterbalance the aminophospholipid translocase, has also been postulated.8 A bidirectional phospholipid-nonspecific “scramblase” has been isolated from erythrocytes, cloned, and sequenced.9 This protein is believed to be identical or similar to the one responsible for the fast breakdown of the phospholipid asymmetry during platelet activation.10 Human erythrocyte scramblase is a proline-rich type II membrane protein with a short C-terminal external sequence, a single transmembrane segment, and a long cytoplasmic extension.9 The cytoplasmic region contains a calcium-binding segment as well as several cysteinyl residues with at least one thioesterified fatty acid acyl group.11,12 The biological activator is believed to be calcium ions, the cytosolic level of which is significantly increased on platelet activation (see Figure). Knowledge of the scramblase activity was to a large extent obtained by use of platelets, erythrocytes, and lymphocytes from patients suffering from Scott syndrome,13,14 which indicated that the phospholipid transporters act similarly in these cells. The syndrome is characterized first of all by a reduced ability to expose aminophospholipids on the surface of the cells in response to an increased cytosolic calcium ion concentration. (Clinical aspects are discussed below.) Furthermore, scramblase activity has been studied by use of “inside-out” vesicles from erythrocyte membranes containing endogenous scramblase and also by use of liposomes with detergent-extracted scramblase incorporated.10,13 The picture that has developed from these studies is that of a phospholipid transporter protein that is activated by calcium ions with the activation related to a conformational alteration of the molecule. Under experimental conditions, activation can be obtained by lowering the pH to <6.0, even in the absence of calcium ions.15 The thioesterified fatty acid acyl group(s) in the cytoplasmic region of the scramblase may also be involved in the activation.15 It has been suggested that these thioesterified fatty acid acyl groups may function by placing the calcium-binding segment in the right position relative to the membrane.12 In Scott syndrome, the aminophospholipid-specific translocase appears to function normally.16 The term scramblase has been used in the present review even though the idea of scrambling in the sense of a total randomization of the
phospholipid distribution during platelet activation has been challenged. 17

**Microvesiculation**

During regular platelet activation, 3 phenomena are observed, namely, secretion, surface exposure of aminophospholipids, and microvesiculation, which means the formation of small membrane vesicles containing cytoplasmic material. These are also called microparticles. In the vesiculation process, small areas of the surface membrane are shed in a true budding process. However, even if the microvesicles are formed in an outside-out configuration, they possess a procoagulant surface that can bind annexin V,18,19 the anticoagulant protein from placenta. P-selectin, which in the nonactivated platelet is present in the α-granule membrane, is also present on the microvesicular surface.18,20 This is explained by the hypothesis that both the phospholipid scrambling and the secretion from the α-granules occur before the shedding of the microvesicles. In line with this, it was observed that annexin V that is present extracellularly during platelet activation allowed the aminophospholipid surface to be formed but prevented the shedding of microvesicles.18 This observation also has a bearing on whether there is a direct causal relation between the phospholipid scrambling and the formation of microvesicles. It suggests that the surface expression of aminophospholipids in itself is not sufficient for vesiculation even though it is a prerequisite for the process. Whereas an increased surface expression of aminophospholipids can occur without formation of microvesicles, microvesiculation has never been reported to happen without an increased surface expression of aminophospholipids. Protein tyrosine phosphatases are clearly involved in microvesiculation,21 but the exact function of these enzymes is unknown at present. Physiologically, the surface expression of phosphatidylserine is probably the most important feature of the microvesicles, because this means that they are shed

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![Diagram of reactions leading to procoagulant expression in platelets based on current knowledge and theories, with focus on the role of Ca^{2+} ions in these processes. In the experimental studies discussed in the review, the cytosolic Ca^{2+} level has been increased by ionophore-mediated transport of extracellular Ca^{2+} through the membrane, by diffusion of Ca^{2+} through pores formed by the Csb-9 complement complex, by inhibition of Ca^{2+}-ATPases, or by a combination of the physiological stimulators thrombin and collagen. Thrombin activates protease-activated receptor-1 (PAR-1), which interacts with G proteins and stimulates phospholipase Cβ (PLCβ) to split phosphatidylinositol-4,5-bisphosphate (PIP2) into 1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). Collagen binds to the receptor integrin α2β1, also called glycoprotein (GP) Ia/IIa, and activates platelets through tyrosine phosphorylation of the Fc receptor γ-chain (FcγR-γ-chain), which is associated with the membrane glycoprotein GPVI. This leads to tyrosine phosphorylation and activation of PLCγ2, which like PLCβ, splits PIP2 and produces IP3. The IP3 opens Ca^{2+} channels in the intracellular membrane system called the dense tubular system, which serves as an intracellular Ca^{2+} store. ATP-driven Ca^{2+} pumps both in these membranes and in the plasma membrane carry Ca^{2+} out of cytosol by active transport. This can be inhibited by specific Ca^{2+}-ATPase inhibitors. Further platelet activation via DAG and protein kinase C and activation of phospholipase A2 with formation of thromboxane A2 are not shown. As discussed in detail in the text, activation of the phospholipid scramblase by Ca^{2+} ions and a concomitant Ca^{2+}-mediated inhibition of the ATP-dependent aminophospholipid translocase that serves to maintain the normal phospholipid asymmetry lead to translocation of negatively charged phosphatidylserine to the outer membrane bilayer. This is a prerequisite for formation of the tenase and prothrombinase complexes of coagulation factors on the platelet surface. A Ca^{2+}-induced activation of the proteolytic enzyme calpain is important for the formation of microvesicles. Protein tyrosine phosphatase activities are associated with calpain activation and microvesiculation in an as-yet-unknown manner. The microvesicles express a procoagulant surface. Substances that activate or inhibit the various processes are labeled with + or −, respectively. FVa and FXa indicate factors Va and Xa, respectively. See References 1 and 2, along with other references mentioned in the text.**
with a procoagulant surface. In addition, the exposure of adhesion molecules like P-selectin may be important in leukocyte interactions and inflammatory reactions.

**Calcium Ions in Aminophospholipid Scramblase Activity and Microvesiculation**

The relation between the formation of a procoagulant platelet surface and the microvesicle formation, particularly an involvement of the Ca$^{2+}$-dependent protease calpain in these events, has been a matter of debate (see discussion in Reference 22). Activation of calpain also means degradation of the cytoskeletal proteins filamin, talin, and myosin, resulting in a reorganization of the cytoskeleton. It seems generally accepted today that the scramblase-mediated transmembrane transport of phospholipids does not require the activation of calpain. Experiments using inhibitors of intracellular Ca$^{2+}$-ATPases of varying specificity and efficiency strongly suggest that it is the level of cytosolic calcium ions that determines whether only the phospholipid scramblase activity or both the scramblase and the calpain activities will be triggered. They further suggest that an activated calpain molecule is a necessary, but not sufficient, condition for the shedding of microvesicles. The additional requirement for the shedding is not known, but the experiment with the annexin V that is present extracellularly during platelet activation indicates that conditions favoring membrane fusion may be important.

Whereas activation of calpain is not required for the expression of a procoagulant surface, such an activation may have an additive effect, in view of the fact that it has been shown that membrane-penetrating inhibitors of calpain can reduce the production of the procoagulant activities to some degree. However, whether the additive effect is related to shedding of procoagulant microvesicles is not known.

The free calcium ion concentration in the cytosol of resting platelets is generally reported to be $\approx 10^{-7}$ mol/L, but strong activators like the calcium ionophore A23187 can increase this $>100$-fold in the presence of extracellular calcium ions. Whereas cytosolic concentrations of $\approx 3$ times the basal level were found to activate the phospholipid scramblase but not calpain, concentrations in the order of $10^{-5}$ mol/L induced both the scramblase and the calpain activities. An important point is that these activations are influenced by the extracellular calcium ion concentration, indicating an influx of such ions during activation. In experimental studies, calcium ionophores and formation of the terminal complement complex C5b-9 allow such influx because of hydrophobic permeability or passage through membrane pores. Physiological agonists require other mechanisms (see Figure). It is interesting that in addition to mobilization of calcium ions from intracellular stores, ADP (and ATP) can activate a $P_{2X1}$ purinoreceptor linked to the influx of calcium ions through a nonselective cation channel in the platelet cytoplasmic membrane. Because these 2 substances are released from platelet "dense bodies," such an influx may take place secondary to secretion. This may be why normal platelets increase their cytosolic calcium ion concentration during the first 10 minutes after an initial rise induced by a combination of collagen and thrombin, whereas platelets from patients with dense granule defects were not able to sustain the increased cytosolic calcium ion concentration. Because the aminophospholipid translocase is inhibited by calcium ions, a sustained calcium concentration is probably necessary to maintain the scrambled surface and the procoagulant activity (see Figure).

**Clinical Aspects**

As mentioned before, factor V present in platelets accounts for $\approx 20\%$ of the total amount of factor V in blood. Patients severely deficient in plasma factor V are generally deficient in platelet factor V as well. In a rare variant of the defect (factor V Quebec), the platelet factor V activity was reported as $<6\%$ of normal, whereas the plasma level was nearly normal. This finding was associated with an abnormal bleeding tendency, pointing to a role for platelet factor V in hemostasis. However, recent studies have shown that other $\alpha$-granular proteins are also proteolized in this disorder, and the reason for the bleeding tendency is unclear.

The clinical condition associated to a defect in platelet procoagulant activity that is most studied and best understood is Scott syndrome.

**Scott Syndrome**

Studies involving a patient with a relatively severe bleeding disorder, from whom the name Scott syndrome was derived, were first reported in 1979. Analyses of other members of the same American family, as well as a French family described in 1996, show that this is a bleeding disorder with well-defined criteria, suggesting a specific syndrome consistent with an autosomal recessive hereditary pattern.

Studies involving the originally described patient showed that the immediate defect is a reduced ability to promote factor X and prothrombin activation that is due to a diminished surface exposure of phosphatidylserine and that this is associated with a reduced shedding of microvesicles. The defect is not associated with defects in aggregation, secretion, or granule storage function. The patient’s erythrocytes and lymphocytes also demonstrate the same basic defect. This is believed to reflect a deficiency in the function of the phospholipid scramblase described above, whereas the ATP- and sulfhydryl-dependent aminophospholipid translocase apparently functions normally. Calpain is also normally activated. A series of experiments indicate, however, that scramblase, as such, is a normal molecule in Scott syndrome and that the deficiency is related to a defect in the activation mechanism. Thus, the phospholipid scramblase activity of Scott erythrocyte membranes could be induced by lowering the pH to <6.0. This was also true after incorporation of the scramblase isolated from a detergent extract of Scott syndrome erythrocyte ghosts into liposomes. Furthermore, in this system, the phospholipid scramblase activity could be induced with the patient’s protein and with the protein obtained from normal erythrocytes whether activation was induced by addition of calcium ions at pH 7.4 or by acidification. However, inside-out vesicles from Scott erythrocytes containing the membrane-associated scramblase still demonstrated the deficiency as observed on treatment with calcium ions. This indicates that some additional component had been removed or modified in the detergent-extracted scramblase compared with that in the intact membrane. As already mentioned, one or more thioes-
One may speculate that the multifaceted aspects of the syndrome rates of 650 s\(^{-1}\) a clearly reduced thrombus volume was observed at shear 3–deficient patient. 30

Recent studies have described a life-long bleeding disorder in 4 patients from 3 apparently unrelated families with a deficient platelet microvesiculation but a normal prothrombinase activity. 31 Analyses of phospholipid distribution were not presented.

Stormorken syndrome, first described in 1985, 32 has certain features that may justify its characterization as an “inverse Scott syndrome membrane anomaly.” However, whereas the abnormality in Scott syndrome apparently is related to one basic anomaly, Stormorken syndrome is a multifaceted one in which the underlying cause of the abnormality is unknown. It refers to a family in which a grandmother, mother, and one son have been hospitalized several times for various ailments, including a bleeding tendency. Mother and son show essentially the same platelet deficiencies, the most prominent of which is a nearly full procoagulant activity even in the absence of stimulating agents. 33 This phenomenon also corresponds to an increased binding of annexin V to their nonactivated platelets, as determined by flow cytometry. In accordance with this binding, microvesicles are present in nonactivated samples of the patients’ platelet-rich plasma at a higher level than that usually observed in patients with activated platelets.

In addition to the presence of platelets with a spontaneously expressed procoagulant surface and microvesicles, clot retraction is clearly reduced in these patients. 33 All other coagulation and fibrinolytic activities tested were normal. 33 Platelet aggregation with citrated platelet-rich plasma was in the lower normal range with all the usual agonists except collagen. This agonist gave a reduced platelet aggregation and secretion of ATP. 33 However, these studies were complicated by a tendency to spontaneous platelet aggregation in whole blood, 33 which may result in a loss of platelets and a selected platelet population after centrifugation. Normal amounts of glycoprotein IIb/IIIa complexes on the platelet surface but a reduced binding of the activation-dependent antibody PAC-1 to glycoprotein IIb/IIIa after platelet activation indicate that some of these aggregation receptors might be in a refractory state. Paradoxically, whereas the existence of the platelets in a “procoagulant” state would predict an increased thrombotic predisposition, the clinical picture of the syndrome is that of a moderate bleeding tendency. The clinical bleeding corresponds, however, to the situation observed under shear in a flow chamber. By use of purified human collagen type III as a trigger for thrombus formation, a clearly reduced thrombus volume was observed at shear rates of 650 s\(^{-1}\) and 2600 s\(^{-1}\), whereas platelet adhesion to the collagen surface was higher than normal. 33 Finally, it should be added that the multifaceted aspects of the syndrome include asplenia, reduced platelet survival time, miosis, dyslexia, muscle fatigue, and ichthyosis. 32 One may speculate whether the high number of microvesicles in plasma reflects the congenital lack of spleen. Surface exposure of phosphatidylserine is considered a signal for clearance for many cells. However, the platelet survival time is clearly reduced in these patients. 33

Another clinical condition associated with bleeding in spite of an increased procoagulant platelet surface is Bernard Soulier syndrome. 34 However, the giant size of the platelets and low platelet count complicate the interpretation of this observation. It is generally believed that the hemostatic defect in this syndrome is related to absence of the adhesion receptor complex glycoprotein Ib/V/IX.

Activated Platelets and Microvesicles in Various Clinical Conditions

The presence of activated platelets and platelet-derived microvesicles in patients has attracted much interest in recent years. Thus, microvesicles have been found in a series of conditions including thrombotic as well as idiopathic thrombocytopenic purpura (ITP), 35 disseminated intravascular coagulation, 36 and HIV-1 infection. 37 Some of these conditions are associated with thrombotic processes, whereas others are not. A study of ITP patients suggested that a high level of microvesicles could exert a protective effect concerning hemostatic defects. 38 However, it was also found that a group of ITP patients demonstrating neurological complications, probably related to thrombosis, had a higher level of microvesicles in their plasma than did patients without such complications. 35 However, there are 2 basic concerns with such observations: (1) whether activated platelets observed in vitro reflect the presence of activated platelets in the circulation and (2) whether platelet activation and microvesicle shedding only are consequences of the disease and do not contribute causally to the disease. These questions have not yet been answered. It should also be noted that in clinical conditions in which activated platelets are observed at a normal or near normal platelet count, these are only a few percent (often ≈5%) of the platelets (an exception is Stormorken syndrome, in which the percentage of activated platelets is significantly higher). Even so, it should be noted that microvesicles are able to bind soluble fibrinogen, bind to immobilized fibrinogen, and coaggregate with platelets. 38

Microvesicles in plasma are normally observed and quantified by use of flow cytometry with the instrument gated with a platelet-specific probe so that only platelet-derived microvesicles are detected. One should keep in mind that apoptosis in other cells also leads to breakdown of the phospholipid asymmetry, the formation of procoagulant surface blebs, 39 and shedding of such membrane fragments, all of which go undetected when this approach is used. The extent and significance of nonplatelet microvesicles in the circulation in disease are not known at present.

It has recently been reported that platelets contain CD40L and express this on the surface after activation. 40 This protein acts as a ligand for CD40 on B cells, monocytes, macrophages, and endothelial cells, triggering inflammatory reactions. We have confirmed the existence of this protein in platelets and found that CD40L from activated platelets is also present in a biologically active soluble form after secretion. In general, activation of endothelial cells and...
monocytes is known to induce synthesis and exposure of tissue factor. Because there seems to be some link between inflammation and coagulation, it should also be mentioned that platelets contain and release platelet-derived growth factor as well as chemokines, such as platelet factor 4, platelet basic protein/NAP-2, and RANTES.37

References
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