Procoagulant Expression in Platelets and Defects Leading to Clinical Disorders

Nils Olav Solum

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.


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 Vα, 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.3 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,5 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, erythrocytes, and lymphocytes from patients suffering from Scott syndrome,13,14 which indicated that 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.10,11 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.5 The thioesterified fatty acid acyl group(s) in the cytoplasmic region of the scramblase may also be involved in the activation.11 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 scramblase 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 is a prerequisite for microvesiculation, whereas an increased surface expression of aminophospholipids can occur without formation of microvesicles.
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.\(^{18}\) 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.\(^{18}\) 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 showed 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.\(^{18}\) 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.\(^{18}\)

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.\(^{22}\) 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 \(\approx 100\)-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,\(^{18}\) concentrations in the order of \(10^{-5}\) mol/L induced both the scramblase and the calpain activities.\(^{18}\) An important point is that these activations are influenced by the extracellular calcium ion concentration, indicating an influx of such ions during activation.\(^{6,18}\) 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 P2X\(_{1}\) purinoreceptor linked to the influx of calcium ions through a nonspecific cation channel in the platelet cytoplasmic membrane.\(^{23}\) 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.\(^{24}\) Because the aminophospholipid scramblase is inhibited by calcium ions,\(^{8}\) 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.\(^{5}\) 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 proteolyzed in this disorder,\(^{4,25}\) 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.\(^{26}\) Analyses of other members of the same American family,\(^{13}\) as well as a French family described in 1996,\(^{14}\) show that this is a bleeding disorder with well-defined criteria, suggesting a specific syndrome consistent with an autosomal recessive hereditary pattern.\(^{14}\)

Studies involving the originally described patient\(^{16,27,28}\) 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.\(^{26,29}\) 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.\(^{16}\) Calpain is also normally activated.\(^{19}\) 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.\(^{15}\) 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 \(\text{pH} 7.4\) or by acidification.\(^{15}\) However, inside-out vesicles from Scott erythrocytes containing the membrane-associated scramblase still demonstrated the deficiency as observed on treatment with calcium ions.\(^{15}\) 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-
terified fatty acid acyl groups in the scramblase have recently been implicated in the activation process.11

**Other Clinical Conditions With Aberrant Platelet Factor 3 Activity**

Isolated “platelet factor 3” deficiency is very rare. In a survey of 316 patients suspected of having a bleeding tendency, one was characterized as a probable congenital platelet factor 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 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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