Cellular Mechanisms Underlying the Formation of Circulating Microparticles

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Abstract—Microparticles (MPs) derived from platelets, monocytes, endothelial cells, red blood cells, and granulocytes may be detected in low concentrations in normal plasma and at increased levels in atherothrombotic cardiovascular diseases. The elucidation of the cellular mechanisms underlying the generation of circulating MPs is crucial for improving our understanding of their pathophysiological role in health and disease. The flopping of phosphatidylserine (PS) to the outer leaflet of the plasma membrane is the key event that will ultimately lead to the shedding of procoagulant MPs from activated or apoptotic cells. Research over the last few years has revealed important roles for calcium-, mitochondrial-, and caspase-dependent mechanisms leading to PS exposure. The study of Scott cells has unraveled different molecular mechanisms that may contribute to fine-tuning of PS exposure and MP release in response to a variety of specific stimuli. The pharmacological modulation of MP release may have a substantial therapeutic impact in the management of atherothrombotic vascular disorders. Because PS exposure is a key feature in pathological processes different from hemostasis and thrombosis, the most important obstacle in the field of MP-modulating drugs seems to be carefully targeting MP release to relevant cell types at an optimal level, so as to achieve a beneficial action and limit possible adverse effects. (Arterioscler Thromb Vasc Biol. 2011;31:15-26.)

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Microparticles (MPs) are a heterogeneous population of small plasma membrane structures that serve as important signaling structures between cells.1,2 MPs are composed of a phospholipid bilayer that exposes transmembrane proteins and receptors and encloses cytosolic components such as enzymes, transcription factors, and mRNA derived from their parent cells.3 Growing evidence suggests that MPs regulate inflammation, stimulate coagulation, affect vascular functions and apoptosis, and can also play a role in cell proliferation or differentiation.1,4 MPs circulate in the bloodstream, can be detected in the peripheral blood, and may originate from different vascular cell types (eg, platelets, monocytes, endothelial cells, red blood cells, and granulocytes). In health, it has been reported that >80% of circulating MPs express membrane antigens that suggest a platelet origin.5 Typically, increased MP levels have been associated with thrombotic disorders and systemic inflammatory conditions, including atherothrombosis.6,7 A prolonged generation of MPs can be deleterious, as it may exert significant procoagulant and inflammatory effects favoring adverse clinical outcomes in a variety of cardiovascular diseases.1,7 Importantly, circulating MPs can mediate the communication between vascular cells in that they allow membrane interactions between cells at distance.8 Therefore, the elucidation of the molecular mechanisms underlying the generation of circulating MPs is crucial for improving our understanding of their role in health and disease.9,10

It is generally assumed that MPs form when the asymmetrical distribution of lipids between the inner and outer leaflets of a plasma membrane is lost.11 Under resting conditions, phosphatidylserine (PS) is located almost exclusively in the inner monolayer.9 When cells undergo activation or apoptosis, PS externalization is one of the earliest observable indicators of the process. Its translocation to the outer leaflet is the initial event that will ultimately lead to the shedding of procoagulant MPs that are therefore regarded as reliable markers of cell stress.11 The dynamic balance of cell stimulation, cell proliferation, and death within the vessels is reflected by the formation and release of MPs that may thus represent a vascular storage pool of bioeffectors.9

MPs shed from activated, necrotic, or apoptotic cells provide a catalytic phospholipid surface for the assembly of blood coagulation factors, thereby promoting the coag-
ulation cascade and thrombin generation. MPs can harbor active tissue factor (TF), the cellular initiator of blood coagulation in vivo. Because PS and TF are known to act synergistically as potent triggers of blood coagulation, it has been suggested that TF-bearing MPs represent the so-called blood-borne TF. These observations suggest that MPs can be viewed as a major therapeutic target, not only in the inhibition of arterial and venous thrombosis but also in the containment of the systemic inflammatory response and atherosclerosis.

In this review, we review the cellular mechanisms governing the formation and the dynamics of circulating MPs. The significance of MPs as potential therapeutic and diagnostic targets for a number of atherothrombotic diseases with unmet medical needs will be discussed.

Figure. Key players in membrane remodeling and shedding of procoagulant MPs. Phospholipids in the plasma membranes of resting cells (left) show an asymmetrical distribution. In particular, aminophospholipids (red headgroups), PS (negatively charged) and phosphatidylethanolamine (PE) are located in the inner leaflet. Membrane phospholipid randomization requires cytoskeleton reorganization involving calcium-activated proteases such as caspases and calpains. A sustained increase in intracellular calcium ions is mandatory to PS exposure, eventually associated with changes in mitochondria permeability. The cleavage and reorganization of the cytoskeleton during apoptosis is dependent on the activation of ROCK-I, ultimately resulting in the release of MPs into the extracellular fluid. The exact role of transmembrane ion transport, transient transmembrane pores, and SOCE in PS exposure deserves further investigations to gain insight into a potential pharmacological approach for thrombotic disorders. Recent studies indicate that mitochondrial membrane depolarization is an integral event leading to the outward transport of PS. Membrane budding ultimately leads to the shedding of MPs that expose PS and hijack membrane antigens and cytoplasm components that promote remote cell cross-talk. At the outer leaflet of activated or apoptotic cells and MPs released in the bloodstream, PS enables the assembly of enzymatic complexes involved in the coagulation cascade.

Loss of Membrane Phospholipid Asymmetry and MP Formation

Under resting conditions, phospholipids are asymmetrically distributed in the membrane of eukaryotic cells. The outer leaflet is enriched in phosphatidylcholine and sphingomyelin, whereas the inner leaflet contains the aminophospholipids PS and phosphatidylethanolamine. The distribution of the membrane lipids is the result of an active process under the dependence of complementary phospholipid transporters governing either the inward (flip) or the outward (flop) translocation. The appearance of PS or phosphatidylethanolamine on the outer leaflet results in a back-transportation to the inner leaflet by an aminophospholipid translocase with “flippase” activity that maintains the normal resting phospholipid distribution (Figure). Because uncatalyzed transbilayer...
transport is slow, lipid asymmetry is stable in quiescent cells. It has been suggested that a “floppase” (ie, an ATP-dependent protein that specifically transfers PS from the interior to exterior leaflet) mediates the rapid outward translocation that is directly responsible for the disruption of asymmetry when cells are activated. In addition, a “scramblase” bidirectional nonspecific lipid transporter—which is activated by an influx of calcium ions—enables all phospholipids to flow down their concentration gradients, thereby favoring randomization. Exposure of PS is the consequence of the rapid overwhelming translocation exerted by floppase(s) and the inhibition of flippase activity by calcium influx. The phospholipid transient mass imbalance between the 2 leaflets due to membrane randomization and the proteolysis of the cytoskeleton promoted by calcium-activated calpains promote the shedding of MPs. Importantly, PS exposure appears to be a nearly universal feature of cells undergoing activation or apoptosis and a common underlying feature of MP release by these cells.

Platelets have the highest scrambling rate known, which ensures a rapid and localized blood coagulation at sites of vascular injury. In other vascular cell types, the scrambling is less effective, with the lowest ability being found in red blood cells.

It is important to emphasize that rapid phospholipid membrane remodeling and PS exposure characterize a physiologically relevant procoagulant response. Accordingly, PS borne by MPs provides additional accessible sites for the assembly of the prothrombinase and tenase complexes, leading to the formation of fibrin. Platelets have the highest scrambling rate known, which enables all phospholipids to flow down their concentration gradients, thereby favoring randomization. Exposure of PS is the consequence of the rapid overwhelming translocation exerted by floppase(s) and the inhibition of flippase activity by calcium influx. The phospholipid transient mass imbalance between the 2 leaflets due to membrane randomization and the proteolysis of the cytoskeleton promoted by calcium-activated calpains promote the shedding of MPs. Importantly, PS exposure appears to be a nearly universal feature of cells undergoing activation or apoptosis and a common underlying feature of MP release by these cells.

**Platelets**

Platelets are activated upon stimulation by agonists (eg, collagen) that induce the release of procoagulant MPs. These MPs are enriched in P-selectin, CD63, GPIIb-IIIa, or calpains. These observations suggest that an efficient generation of procoagulant MPs is accompanied by
d a major remodeling of the platelet membrane, as well as by platelet granule secretion (see below). Connor et al have reported that up to 80% of platelet-derived CD41-positive MPs circulating in the platelet poor plasma (PPP) of healthy donors cannot be labeled by annexin V. In addition, the proportion of procoagulant MPs generated in the platelet-rich plasma following exposure to collagen was less than 40%. Taken together, these data question our current understanding of the nature and significance of such circulating nonprocoagulant platelet-derived vesicles in plasma. Recently, Flahmenhaft et al have reported that megakaryocytes constitute a physiological source of circulating procoagulant MPs characterized by the exposure of PS, CD42, and CD41, as well as the presence of full-length filamin.

As the methodological approach is crucial for the assessment of MP shedding, it is difficult to reconcile the large amount of data currently available in the literature. For example, it is possible that experimental conditions can impair the use of annexin V as a probe for PS or immunologic labeling, particularly when using blood samples in the absence of washing steps. As recently confirmed by Connor et al using a functional coagulation assay and flow cytometry, there is no doubt that annexin V is appropriate and specific for the detection of procoagulant MPs, at least at tightly regulated calcium concentrations allowing a specific binding to PS. Notably, cell activation and treatment can further complicate the analytic methodology, thus making the preanalytical sample treatment of crucial importance. In this regard, annexin V is known to inhibit platelet MP release, whereas calcium-induced calpain activity is considered mandatory for platelet shedding following exposure to agonists. The potential contribution of the exosomes present in platelet-rich plasma during platelet activation and shedding remains a matter of debate. Indeed, these exosomes expose very low (if any) PS and cannot be detected by flow cytometry because of their small size.

The nature of the annexin V−negative vesicles detected by flow cytometry in platelets or endothelial cells—as well as the mechanisms enabling their release—is poorly understood. One potential explanation is that they can reflect basal tissue homeostasis. Alternatively, they can result from multiple fusion events between cell debris or small endosomal-secreted vesicles with the medium or plasma components, possibly through interactions with lipophilic proteins. Another putative mechanism may rely on the release from the plasma membrane due to a particular cytoskeleton cleavage. This would allow the maintenance of the phospholipid asymmetrical distribution, as observed in megakaryocytes (see below). Nevertheless, the generation of MPs from resting platelets cannot be visualized by real-time imaging.

Although the functional differences between activated cells and cell-derived MPs are not entirely understood in vivo, these findings suggest that cell-derived MPs are not only potent procoagulant effectors in atherothrombosis but can also convey a variety of different molecules, such as adhesion receptors, cytokines, bioactive lipids, mRNA, caspasas, and calpains, to the neighboring cells (see below).
Membrane Remodeling and Microparticle Formation: Insights From Scott Syndrome

The physiological relevance of membrane remodeling and the shedding of MPs in hemostasis is well exemplified by Scott syndrome. This rare bleeding disorder is characterized by provoked hemorrhages caused by the reduced floppase activity of vascular cells, the diminished externalization of negatively charged phospholipids, and reduced MP shedding.38,39 As a consequence, the binding of coagulation factors and their assembly to the platelet surface is decreased and thrombin generation limited.40–42 Low prothrombin consumption in the serum is the sole abnormal hemostasis parameter. Although initial studies could not clearly distinguish between a hereditary and an acquired bleeding disorder, recent evidence unequivocally indicates that Scott syndrome has a genetic etiology.39,43 The hereditary nature of the disorder has been corroborated by the discovery of a single, inbred colony of German shepherd dogs with virtually identical symptoms.43 In both human patients and affected dogs, a defective floppase activity is considered the core pathological defect of Scott syndrome.43,44 Pedigree studies have shown that this defect equally affects males and females, consistent with an autosomal recessive inheritance pattern of the disorder.42

To date, only 3 documented human cases of Scott syndrome have been reported.42 The paucity of human cases has hindered genetic analyses of the trait. As the underlying defect seems to be the deficiency of phospholipid floppase, the initial candidate gene for Scott syndrome was the canonical phospholipid scramblase (PLSCR1).45 PLSCR1 mediates accelerated transblayer unspecific migration of phospholipids, facilitating rapid translocation of PS to the cell surface on elevation of intracellular calcium concentrations. Although expression levels of PLSCR1 mRNA have a good degree of correlation with scrambling efficiency in different cell types,45 PLSCR1-null mice show normal hemostasis and PS externalization.46 In addition, the product of PLSCR1 gene is normally expressed in Scott cells.47,48 The fact that occasional and specific PS translocators probably exist in the membrane represent the main obstacle in the identification of the PS-floppase necessary for the hemostatic response. Like most occasional PS translocators, multidrug resistance protein-1 (MDR1) or ABCA1 are members of the ATP-binding cassette protein family49,50 that have been investigated in Scott cells.51 In humans, the loss of function of ATP-binding cassette transporter ABCA1 leads to the dystrophic phenotype of Tangier disease, an autosomal recessive disorder of lipid metabolism characterized, at the cellular level, by the defective translocation of membrane lipids to specific apolipoprotein acceptors. In mice, ABCA1 promotes Ca2+-induced exposure of PS at the outer leaflet of the plasma membrane of Cu2+-ionophore-stimulated red blood cells, as determined by a prothrombinase assay, membrane shedding, and measurement of transblayer redistribution of spin-labeled phospholipids. In addition, the rate of PS translocation has been found sensitive to the altered expression of ABCA1 in knock-out mice. In a patient with Scott syndrome, Albrecht et al49 identified a heterozygous missense mutation (Arg1925Gln) in the ATP-binding cassette transporter ABCA1 gene, which was not found in unaffected family members or controls. However, the authors found no causative mutation for this phenomenon in the ABCA1 gene or its proximal promoter and suggested that a putative second mutation in a transacting regulatory gene might be involved.49

In the canine hereditary bleeding disorder, ABCA1 has been firmly excluded as the causative genetic defect.32 Recently, a genome-wide linkage scan in German shepherd dogs has localized canine platelet procoagulant deficiency (Scott syndrome) to canine chromosome 27.53 Of note, this region does not contain the canine homologues of human PLSCR1 or ABCA1 genes.53 Another rare bleeding disorder reported by Castaman and coworkers shares clinical and laboratory findings with Scott syndrome.42 This defect was found to be associated with an inability to form platelet-derived MPs on cell activation, despite normal PS expression on the platelet surface.52 Although the reason for this defective shedding remains elusive, both disorders highlight the importance of platelet-derived MPs for efficient hemostasis.

Previous studies have shown that the abnormal membrane remodeling in Scott syndrome can also affect red blood cells and lymphocytic cells, indicating that it occurs in multiple hematopoietic lineages.41,42 Importantly, Scott lymphoblasts show normal exposure of PS and MPs shedding when subjected to apoptotic stress.54 It is clear that the elucidation of molecular pathways active in Scott syndrome—different from those operating in the process of membrane remodeling typical of hemostatic responses—holds great promise for our understanding of the mechanisms governing MPs formation in health and disease.

Calcium Channels, Ions, and Microparticle Formation

The exposure of procoagulant phospholipids and the shedding of MPs are cellular responses that depend on activating conditions. Notably, membrane remodeling and PS externalization is dependent on an increase in cytosolic calcium.55 Activation of human platelets by a Ca2+-ionophore results in the surface exposure of PS. Conversely, the inhibition of Ca2+ influx abolishes agonist-induced PS externalization and the procoagulant response in activated platelets.56 For more than 20 years, it has been known that calcium ion influx and activation of the Ca2+-dependent protease calpain stimulate platelets to shed MPs. In a landmark study, Chang et al57 have reported that MP formation and the surface exposure of PS show a similar requirement for Ca2+. The Ca2+-driven mechanisms underlying rapid phospholipid redistribution include a number of different events, including the activation of transmembrane outward PS transporters, the inhibition of the inward aminophospholipid translocase, the transmembrane redistribution of PS, and even cell shrinkage with a decrease in lipid packing of both leaflets. As small increases in Ca2+ lead to platelet activation, the maintenance of a stable Ca2+ is essential to keep platelets in a resting state. The mechanisms limiting platelet activation by counteracting Ca2+ leakage from the intracellular stores rely on sarcoplasmic/endoplasmic Ca2+ ATPases that pump the calcium ions back to the stores (targeted by the inhibitor thapsigargin) and on a plasma membrane Ca2+-ATPase that pumps Ca2+ out of the cell.
Calcium entry in the platelet occurs via at least 2 different mechanisms. The first consists in the agonist-induced release of Ca\(^{2+}\) from cytosolic stores, followed by an influx of calcium ions through the plasma membrane. This process is commonly referred to as store-operated calcium entry (SOCE). The second mechanism depends on the purinergic calcium channel P2X1. SOCE may act as a modulator of aminophospholipid exposure (Figure). In platelets, the release of Ca\(^{2+}\) from the endoplasmic reticulum induces SOCE and a rapid PS exposure without the need for ligand-receptor interactions. Importantly, this mechanism of refilling empty internal stores has been found to be reduced in Scott B-lymphoblasts of one Scott patient, although it was normal in others. This clearly suggests that defective SOCE cannot be considered a typical feature of Scott syndrome. Nonetheless, this apparent discrepancy might be explained by the heterogeneity of transformation by Epstein-Barr virus.

In human erythroleukemic cell line (HEL) cells, SOCE and the exposure of PS are regulated through the reorganization of actin cytoskeleton by Rho A (a small GTPase), and not by ROCK, highlight the presence of intricate control pathways (Figure and section below). More recently, STIM1 (stromal-interacting molecule 1), a transmembrane protein located in the tubular dense system, has emerged as the key regulatory protein of SOCE in platelet with Orai1 as principal Ca\(^{2+}\) entry channel after depletion of the Ca\(^{2+}\) stores. The interaction between oligomerized Orai1 and STIM1 leads to channel opening. Research has suggested that mice expressing a mutated form of Orai1 (Arg93Try) show a marked reduction of PS externalization and thrombus formation in one Scott patient, although it was normal in others. This clearly suggests that defective SOCE may act as a coagonist.

Interestingly, platelets from STIM1- and Orai1-null mice correctly exert most of their physiological functions despite a lack of SOCE-mediated calcium entry. These results suggest that Ca\(^{2+}\) store release is sufficient to trigger important basic platelet functions such as shape change, integrin activation, and granule release in a SOCE-independent fashion. It is thus feasible that SOCE may be of special relevance in the processes following initial platelet adhesion and primary activation (PS exposure, coagulant activity, and thrombus stabilization).

It should be kept in mind that Ca\(^{2+}\) signaling pathways may be cell type specific. For example, raft integrity, SOCE involving transient receptor potential channel 1, and cGMP have been shown to play an important role in the completion of PS transmembrane redistribution in megakaryocytic HEL cells.

However, the actual role of SOCE in cells activated by nonphysiological agonists is still a matter of debate. Munnix et al have shown that elevated SOCE-driven Ca\(^{2+}\) elevation is not sufficient to trigger PS exposure. In addition, Ca\(^{2+}\) ionophores may trigger membrane remodeling without a need for store-operated Ca\(^{2+}\) entry. Similarly, the increase in cytosolic Ca\(^{2+}\) elicited by the reticular Ca\(^{2+}\)-ATPase inhibitor thapsigargin in lymphocyte cells is not sufficient to induce scrambling. Importantly, evidence suggests that modulation of intracellular free calcium in human platelets affects the formation of MPs in clinical settings. Labiós et al demonstrated that treatment of hypertensive patients with doxazosin leads to normalization of the kinetics of cytoplasmic microparticle-free calcium, leading to a reduced release of MPs. Further study is needed to confirm whether the pharmacological modulation of procoagulant MP release may improve the clinical outcomes of patients with atherothrombotic disorders.

Although PS externalization is largely believed to be calcium ion–dependent, regardless of the cell lineage, this pathway alone may not be sufficient to promote the release of MPs. Indeed, evidence suggests that flux of ions other than calcium may play a role in the exposure of anionic phospholipids at the cell surface (Figure). Following activation, shrinking is the result of cell dehydration due to a Ca\(^{2+}\)-activated efflux of potassium and chloride ions. In addition, the activation of Ca\(^{2+}\)-sensitive K\(^+\) channels (Gardos channels) has recently been implicated in the mechanisms regulating PS exposure in platelets activated by collagen and thrombin. In accordance with this, the lack of procoagulant platelet response observed in one patient with Scott syndrome was partially restored by pretreatment with valinomycin, a selective K\(^+\) ionophore. Another study has suggested that Na\(^+\) loading via Na\(^+\)/H\(^+\) exchange is involved in the regulation of PS scrambling in activated platelets. These results clearly indicate that cytosolic calcium ions levels are not a unique player leading to the collapse of the membrane asymmetry. Whether these mechanisms may have a role in modulating the release of MPs in vivo deserves further scrutiny.

Cytoskeleton Reorganization and Microparticle Formation

Cytoskeleton integrity is believed to participate in the maintenance of membrane asymmetry and cell shape, its reorganization could therefore favor membrane budding in stimulated cells (Figure). Cauwenberghs et al have shown that...
Signal Transduction Pathways and Microparticle Formation

Numerous signal transduction pathways (activation-induced protein tyrosine dephosphorylation, protein phosphorylation, and calmodulin activation) have been implicated in the generation of MPs from activated cells. In a seminal study, Wiedmer and Sims demonstrated that the phosphatase inhibitor vanadate increased the formation of platelet-derived MPs. Interestingly, MP formation was partially inhibited in platelets treated with the protein kinase inhibitor sphingosine, the myosin light chain kinase inhibitor ML-7, and the calmodulin-antagonist W-7 and under conditions of elevated cytosolic concentration of cAMP. Pasquet et al have reported that microvesicle release during platelet activation is specifically associated with µ-calpain activation, increased protein tyrosine phosphatase activity, and decreased tyrosine phosphorylation. Other studies have shown that adhesion-induced procoagulant activity of platelets is regulated by protein tyrosine kinase-dependent Ca2⁺ responses. Altogether, these results clearly indicated that platelet MP formation is influenced by the state of phosphorylation of the platelet cellular compartments and by calmodulin, myosin light chain kinase, and other calmodulin-regulated effectors. Experimental data in human HEL cells have shown that extracellular signal-regulated kinase (ERK) phosphorylation acts as a cornerstone in response to a variety of external stimuli. In HEL cells, the phosphorylation of the 2 isoforms ERK1 and ERK2 is increased during PS exposure induced by the Ca²⁺ ionophore A23187. The reduction of procoagulant activity induced by ERK inhibitors suggests that these proteins are involved in the signal transduction pathways initiated by the increase of Ca²⁺. Nevertheless, ERK phosphorylation induced by Ca²⁺ occurs in a cell-specific manner, requiring, for example, an external Ca²⁺ influx in Jurkat cells but not in platelets. It is difficult to reconcile current data in the context of a procoagulant cell response, as ERK phosphorylation and membrane scrambling leading to PS exposure are independent mechanisms occurring in platelets.

Yan et al have recently reported that inhibition of basal cAMP-dependent protein kinase activity resulted in an increased platelet MP formation and PS exposure. Pretreatment of platelets with the cAMP-elevating agent forskolin abolished thrombin plus collagen-induced MPs formation and PS exposure and obviously decreased calcium ionophore-evoked MPs shedding. These results imply that protein kinase plays key roles in the regulation of platelet MPs formation and PS exposure. Signal transduction pathways and MP formation have been studied in other cell lineages and under various stress conditions. Curtis et al have shown that the pharmacological inhibition of p38 mitogen-activated protein kinase in human aortic endothelial cells, resulted in a 50% reduction of tumor necrosis factor α-induced endothelial MPs. These findings implicate p38 mitogen-activated protein kinase signaling as significant and selective in the formation and maturation of endothelial MPs.

Interestingly, in a subproteomics analysis of B-lymphoblasts from a patient experiencing Scott syndrome, Imam-Sghiouar et al failed to find any differential expression of tyrosine-phos-
phorylated proteins that can account for the lack of exposure of procoagulant PS at the exoplasmic leaflet of plasma membrane.

Hashimoto et al. have recently investigated whether the platelet expression of toll-like receptor 4, the receptor for Gram-negative bacterial lipopolysaccharide, may influence the release of MPs. Acute direct activation of toll-like receptor 4 increased phosphorylation of p38 mitogen-activated protein kinase and decreased production of prothrombotic PS and P-selectin-positive MPs in response to TRAP, the thrombin receptor agonist peptide. Therefore, reduced platelet reactivity and procoagulant potency could result in vitro from toll-like receptor 4--driven prestimulation. Another line of evidence questioning the role of initial platelet conditions in the promotion of membrane remodeling comes from transgenic mice overexpressing the platelet purinoreceptor receptor-operated calcium channel P2X₁. Indeed, the perfusion of whole blood from P2X₁-overexpressing mice over collagen fibers under shear results in increased P2X₁-dependent aggregation and PS exposure.

Whatever the initial mechanism leading to PS exposure and MP release may be, the generation of circulating thrombin contributes to the autoamplification loops by targeting resting platelets and endothelial cells, ultimately leading to an enhanced release of deleterious MPs in the vessel. It has recently been demonstrated that thrombin can also increase the expression of cell-associated and soluble form of TRAIL, a cytokine belonging to the tumor necrosis factor family. The TRAIL/Apo2L complex has been shown to mediate the release of endothelial-derived MPs by initiating the recruitment of adaptor proteins and the activation of nuclear factor-kB. Inflammatory mediators secreted after tumor necrosis factor treatment (intercellular adhesion molecule-1) or upregulated by thrombin (intercellular adhesion molecule-1, interleukin-8) can also contribute to the autocrine pathways that amplify endothelial vesiculation. Indirect evidence also suggests that interleukin-1 and its receptor may play a role in the amplified generation of MPs from thrombin-treated human microvascular endothelial cells. Therefore, additional complexity is brought by the cross-talk of elements involved in the tuning of endothelial cell membrane remodeling. Together, these findings indicate that the relative contribution of the pathways involved in the tuning of membrane remodeling is dependent on both the cell lineage and the type of stress, some of them being linked to the mechanisms of acute cell response.

**Blebs, Pores, and Microparticle Formation**

Traditionally, the transblayer movement (flip-flop) of PS has been suggested to be the key mechanism in the induction of membrane lipid asymmetry. However, a temporary, localized disordering of the lipid bilayer structure with the formation of transient membrane pores may serve as another pathway for movement of lipid molecules from one leaflet of the membrane to the other (Figure), ultimately leading to membrane disruption. Following the formation of hydrophilic pores, PS has access to both leaflets of the membrane. The formation of membrane blebs is another mechanism putatively involved in PS externalization. In this regard, Elliott et al. have shown that Ca²⁺-stimulated PS exposure in B cells is strain variable, ABCA1 independent, and both preceded by and dependent on a decrease in lipid packing. Following stimulation with Ca²⁺ ionophore, K⁺ and Cl⁻ ions leave the cells that shrinks, shedding appearing as the consequent membrane distortion. The importance of this work lies in the fact that changes in the plasma membrane organization may occur by a translocase-independent mechanism. Whether this MP-forming mechanism may serve as a novel target that can be moved toward the clinic deserves further study.

**Platelets, Apoptosis, and Microparticle Formation**

Before cells undergo nuclear apoptotic DNA fragmentation, they show early cell-membrane asymmetry and expose PS. Importantly, PS exposure on the cell surface during apoptosis occurs on a different time scale than platelet activation. In cells that show inducible tissue-factor expression, apoptosis promotes a high-dependent procoagulant activity because of PS expression. There is convincing evidence that the membrane of platelet-derived MPs arising from megakaryocyte fragmentation through an apoptotic process also retain procoagulant properties. Of note, platelets contain several key regulators of apoptotic cell death, including caspases and different members of the Bcl-2 protein family. Previous studies have shown that the apoptotic machinery may contribute to the agonist-driven PS exposure by platelets. For example, one prosurvival member of the Bcl-2 protein family, termed Bcl-xl, plays a crucial role in the maintenance of platelet viability. Interestingly, a decline in Bcl-xl levels occurs in stored platelets and is associated with an increase in PS exposure and platelet procoagulant activity. The activation of apoptotic signaling in platelet can include both death receptors and mitochondrial pathways. These cytotoxic signaling pathways result in activation of effector caspases.

Another recent study has shown that the platelet apoptosis machinery and the classical calcium-driven rapid agonist pathways, although both able to promote PS exposure, are distinct. The coexistence of different signaling pathways triggered either by activation or apoptotic stimuli is not restricted to platelets. The differences related to PS exposure in MP generated from apoptotic or activated endothelial cells may reflect distinct mechanisms leading to the release of MPs (see above).

**Membrane Remodeling, Calcium, and Tuning of Different Signaling Pathways**

During apoptosis, the exposure of PS is a relatively early event, taking place before DNA degradation and the loss of membrane integrity. Although PS exposure is a downstream event during the apoptotic cascade, evidence suggests that caspases do not exert a direct effect on the floppase activity. Accordingly, floppase activation in certain cell types proceeds efficiently even in the presence of broad spectrum caspases inhibitors that block downstream event such as DNA degradation. It is noteworthy that the kinetics of PS exposure and MP release differ significantly in cells entering apoptosis compared with those undergoing activation. Although shedding of MPs occurs on the hour time scale in apoptosis, platelet activation...
results in a rapid exposure of PS and the release of MPs is observed within a few minutes.102

The question of whether apoptosis and procoagulant PS exposure share the same molecular pathways remains a matter of debate. The exact role played by calcium ions in the mechanisms of PS exposure occurring in apoptotic cells is still incompletely understood. Interestingly, the removal of extracellular Ca\(^{2+}\) has been shown to prevent PS exposure in Jurkat cells undergoing apoptosis, whereas chelation of intracellular Ca\(^{2+}\) did not.103 Another study has shown that Ca\(^{2+}\) flux—and not a net change in intracellular Ca\(^{2+}\) levels—is essential for the activation of the scramblase activity in apoptotic cells.104 It is worth noting that the loss of PS asymmetry does not always result in the activation of the apoptotic machinery.105 In light of these findings, it has recently been suggested that PS exposure can occur through at least 2 different pathways.76 The first is strictly calcium ion dependent and is amplified by the mitochondrial depolarization, whereas the second (the apoptotic pathway) occurs independently of intracellular Ca\(^{2+}\) concentration changes.76 This possibility fits well with the observation that PS exposure occurs in Scott lymphoblasts during apoptosis.100 A previous study has shown that inhibition of caspase-3 leads to a blockade of PS exposure and MP shedding in platelets. Conversely, other platelet responses (α-granule secretion, shape changes, and aggregation) remained unaffected.74

Mitochondrial Permeability Transition Pore and PS Exposure

In recent years, studies of murine platelets lacking key mitochondria-associated proteins have demonstrated the role played by mitochondrial permeability transition pore (mPTP) in the regulation of PS exposure. For example, the cyclophilin D knockout mice (lacking a key component of the mPTP) show marked defects in PS externalization and prothrombinase activity after platelet stimulation.101 Conversely, platelets from mice lacking Bax and Bad (2 critical sensitizers of the mPTP) have a normal rate of PS exposure following stimulation.76 Therefore, mitochondrial membrane depolarization appears to be an integral event leading to exposure of PS in platelets exposed to thrombin and collagen.102,103 The mPTP is a channel that causes the loss of the mitochondrial at high Ca\(^{2+}\) mitochondrial concentration.104 When opened, mPTP allows the release of proapoptotic into the cytosol (Figure).105 Importantly, the mitochondrial permeability transition appears to be an important checkpoint for the shedding of platelet-derived MPs.106 The engagement of the mitochondrial-dependent pathway is characterized by cytochrome c release, a prolonged increase in cytosolic Ca\(^{2+}\) concentrations, the processing of caspases 0 and 3 by calpains and is accompanied by the externalization of PS, even in the absence of extracellular calcium ions. The reciprocal influences between the signaling pathways regulating membrane remodeling deserve further investigation. However, a large body of evidence points to a role for mitochondria as the key regulators in PS exposure and MP shedding. Very intriguingly, the Scott syndrome trait locus recently identified in dogs includes the canine homologues of several mitochondria-associated proteins and apoptosis regulators.53

In line with our findings in one human Scott patient,107 tetracaine (a local anesthetic agent that triggers mitochondrial depolarization) induces PS exposure in the canine Scott syndrome to an extent similar to that in canine control platelets.43 Although platelets from Scott syndrome dogs show a loss of mitochondrial potential following exposure to thrombin and collagen, PS externalization does not occur.

A recent study has focused on the reciprocal importance of the increase of cytosolic calcium ions and the mitochondrial membrane depolarization in the induction of PS exposure.108 The results demonstrated that—regardless of the loss of membrane mitochondrial permeability—PS exposure is triggered by a marked increase in cytosolic calcium ions concentrations. Challenging previous observations, these findings clearly indicate that PS exposure can occur even without loss of membrane mitochondrial permeability. In turn, evidence in rabbits suggests that the loss of membrane mitochondrial permeability in aged platelets can take place without PS exposure.109 Taken together, these results indicate that the increase of cytosolic calcium ions concentration rather than mitochondrial membrane depolarization is the critical event leading to PS exposure.

Toward a Pharmacological Control of PS Exposure and Microparticle Shedding?

The pharmacological control of flip-flop and MP shedding remains a challenging issue because of the high number of intricate pathways leading to membrane remodeling. Some of these pathways are crucial to tissue homeostasis, whereas others are involved in the cellular response to acute stress (eg, blood leakage). Additional lines of evidence in support of this complexity are derived from the understanding of the effects of cyclosporin A both in vitro and in vivo. Cyclosporin A, a ligand for cyclophilin D, is known to block mPTP opening.104 When cyclophilin D–null platelets are exposed to Ca\(^{2+}\) ionophores, cyclosporin A does not inhibit PS externalization.99 Accordingly, PS exposure is triggered by a marked increase in cytosolic Ca\(^{2+}\) concentrations in platelets regardless of a loss of membrane mitochondrial permeability.108 In any case, cyclosporin A holds promise for the pharmacological modulation of MP shedding in the clinical setting of kidney transplantation because treatment with cyclosporin A results in an earlier reduction of endothelial-derived MPs levels than in patients treated with tacrolimus/mycophenolate.110 The above evidence indicates that different molecular pathways (caspase-dependent mechanisms, increased mitochondria permeability, sustained increase in intracellular calcium ions) can mediate membrane lipid scrambling and ultimately lead to PS exposure. Importantly, these mechanisms might have different sensitivity to pharmacological agents. The exact role played by transmembrane ion transport or transient transmembrane pores in phospholipid scrambling remains to be established. Notably, PS exposure may also represent a signal for platelets clearance at the steady state. This possibility is supported by experimental data showing a reduced adhesive function and an enhanced clearance of senescent platelets from the bloodstream that may actually lead to a reduced prothrombotic potential.76
Besides their procoagulant activity, MPs may play a regulatory role in vascular remodeling and fibrinolysis, which may ultimately have therapeutic implications. In this regard, Podor et al. have demonstrated that the vimentin-dependent localization of active plasminogen activator inhibitor-1 on the surface of activated platelets and platelet-derived MPs is suggestive of an intricate role played by MPs in the context of atherothrombosis. Of note, endothelial MPs also support plasmin generation by expressing the urokinase-type plasminogen activator and its receptor, enabling urokinase-type plasminogen activator–driven fibrinolytic cross-talk in situ. In addition, they are able to convey metalloproteinases that could contribute to vascular remodeling by influencing the properties of endothelial progenitor cells.

Given the noxious role of procoagulant MPs in a variety of inflammation-induced coagulopathy disorders (malaria, sepsis, heat stroke, and others), the control of MP release may be a relevant pharmacological target. Another attractive pharmacological target is represented by Orai1 inhibitors. It is likely that specific targeting of Orai1 may reduce platelet procoagulant activity and thrombus formation when the pathophysiological role of TF is limited. In contrast, the blockade of Orai1 may not affect the bleeding risk following wounds given the predominance of the TF pathway. It should, however, be emphasized that the possible existence of 2 pathways that lead to PS translocation and exposure in platelets—which is certainly protective from a phylogenetic point of view—could hamper the development of potent pharmacological modulators.

Conclusions and Perspectives

Although circulating MPs are increased in patients with atherothrombotic and inflammatory disorders, this does not necessarily endorse their invariably harmful nature. MPs are ubiquitously shed from cell membranes, and the intercellular exchange of protein and RNA-containing MPs is an important mode of cell-cell communication. Despite theoretical advantages of novel MP-targeted treatment strategies in atherothrombotic disorders, some can potentially be harmful to the patient. However, the absence of bleeding symptoms in heterozygous Scott subjects clearly indicates that the pharmacological modulation of the release of procoagulant MPs may have an important therapeutic impact in the management of atherothrombotic vascular disorders. At present, the most important obstacle in the field of MP-modulating drugs seems to be carefully targeting MP release to particular cell types at an optimal level, so as to achieve a beneficial action and to limit possible adverse effects. Unfortunately, the genetic cause of Scott syndrome remains elusive. Pharmacological manipulation of the biochemical defect of Scott syndrome will provide an alternative or complementary approach to treatment of atherothrombosis through the modulation of procoagulant activity of MPs. In addition, the contribution of the recently discovered calcium entry pathways to the regulation of platelet procoagulant activity represents another attractive target for pharmacological modulation because platelets are the main providers of circulating procoagulant MPs in thrombotic disorders.
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82. Morel et al. Cellular Mechanisms of MP Formation...


Cellular Mechanisms Underlying the Formation of Circulating Microparticles
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