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Review Article

Cell-derived microparticles in haemostasis and vascular medicine

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Summary

Considerable interest for cell-derived microparticles has emerged, pointing out their essential role in haemostatic response and their potential as disease markers, but also their implication in a wide range of physiological and pathological processes. They derive from different cell types including platelets – the main source of microparticles – but also from red blood cells, leukocytes and endothelial cells, and they circulate in blood. Despite difficulties encountered in analyzing them and disparities of results obtained with a wide range of methods, microparticle generation processes are now better understood. However, a generally admitted definition of microparticles is currently lacking. For all these reasons we decided to review the literature regarding microparticles in their widest definition, including ectosomes and exosomes, and to focus mainly on their role in haemostasis and vascular medicine.

Keywords

Microparticle, ectosome, haemostasis, vascular pathologies

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Introduction

Blood contains microparticles (MPs) derived from different cell types, including mainly platelets, but also red blood cells, granulocytes, monocytes, lymphocytes and endothelial cells (ECs). Overproduction of MPs has been related to various physiological and pathophysiological conditions such as cell adhesion, apoptosis, immune response, vascular function, vascular remodeling and angiogenesis, haemostasis and thrombosis, cardiovascular diseases, cancer, infections, as well as normal and pathological pregnancy.

About 60 years ago, prolongation of plasma clotting time was observed after high-speed centrifugation, suggesting that sedimentable procoagulant particles are present in plasma and can be removed by centrifugation (1). In 1967, Wolf (2) showed that platelet shedding after activation results in "*platelet dust*".

A population of platelet-derived MPs (PMPs) is generated during platelet activation, whereas other PMPs populations are derived from megakaryocytes during megakaryopoiesis (3–5) or quiescent circulating platelets, or might result from platelet apoptosis (6). MPs from other cells may be released during cell activation, cell injury or following cell activation-independent processes, including senescence and apoptosis. Carrying markers from their parental cells (Table 1), MPs are used as investigation and diagnostic tools (7).

After a general overview of MPs and the processes of their formation, a brief review of the methods to detect MPs will be presented. We will then systematically outline the role of MPs in haemostasis and vascular medicine (Table 2).

Search strategy

Studies whose title and/or abstract contained the terms "microparticles", "microvesicles", "ectosomes" combined with "haemostasis" or "vascular medicine" were searched in the PubMed database until September 23, 2008. Relevant articles were selected according to abstract content. To supplement the search, citations in pertinent review articles were examined (6–9). One-hundred-seventy-seven publications were cited in this review.

Microparticles (MPs): definition

MPs are vesicles that bud off from cells, lack a nucleus, contain a membrane skeleton and are defined by their size and expression on their surface of antigens specific of parental cells (Table 1) (10–12). These phospholipid vesicles are less than

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Table 1: Markers for cell-derived microparticles.

Cellular origin of microparticles	Marker	References
Red blood cell	CD235a	(141, 174)
Leukocyte	CD45	(159, 174)
Granulocyte	CD66b	(141, 159, 174)
Monocyte	CD14	(141, 159, 174)
Lymphocyte	CD4 CD8 CD20	(141, 159, 174) (159, 174) (159, 174)
Platelet	CD31* CD41 CD41a CD42a CD42b CD61 CD62P	(10, 111) (141, 174) (174) (174) (174) (174) (141)
Endothelial cell**	CD31 CD34 CD54 CD62E CD51 CD105 CD106 CD144 CD146	(26, 141, 175) (174) (26, 141) (26, 174–176) (41, 174) (174) (26, 175) (38, 141) (146)

* in association with CD42, ** CD42 negative (114, 141), MPs harbouring a CD62E/CD144/CD146 phenotype are the more likely to originate from endothelial cells (19).

1 µm of diameter. Recently, tissue factor (TF)-bearing MPs have been shown to vary in size from 332 to 501 nm, using impedance-based flow cytometry (13). The minimal size of MPs was defined as 0.1 µm because commonly used flow cytometers are unable to distinguish between smaller particles and the electronic noise. The upper size of MPs was fixed just below 1 µm because a single bigger MP might be difficult to distinguish from MPs aggregates, platelets, or MPs-platelet aggregates. Together with the usage of impedance-based flow cytometry, the development of digital-acquiring flow cytometers and more thinner laser beam has improved the discrimination and characterization of MPs (14).

To reliably define MPs, the terms exosomes and ectosomes need to be introduced. Exosomes originate from multivesicular bodies and exocytosis of endocytic bodies, and ectosomes directly originate from the membrane surface (Fig. 1) (15, 16). In this review, we will mainly use the commonly used term "microparticles", keeping in mind their definition as ectosomes. Some authors use the term "microparticles" without a clear definition or a clear analysis of the origin of the vesicles described in their article. It is therefore possible that in this review the correct definition of MPs is not strictly applied everywhere. A clear discussion about problems and need for a correct definition of all microvesicles has been recently published (17). The vascular biology group within the Scientific Subcommittees of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) has proposed recently to create an international workshop by using calibrated microbeads to clarify confusing data regarding MPs numeration, and the first step will be to establish a normal range for MPs (18, 19). Another point risen during this recent meeting was the discrepancy between the protein or lipid content of MPs and their numbers reported in the literature. It was therefore suggested to express MP concentrations in protein and/or lipid equivalents.

MP formation

The current knowledge on MP formation is mainly issued from experiments performed *in vitro* on isolated or cultured cells. However, mechanisms involved in MP formation *in vivo* remain essentially unknown.

In steady-state, the cell membrane is asymmetric regarding the composition and the distribution of phospholipids in its inner and outer layers: phosphatidylcholine and sphingomyelin are located in the outer layer, while phosphatidylserine (PS) and phosphatidyl-ethanolamine (PE) are present in the inner layer. This asymmetric distribution of phospholipids in the membrane is maintained by a three piece enzyme system: *flippase*, *floppase* and *scramblase* (Fig. 2). Flippase is an aminophospholipid translocase that specifically translocates PS and PE from the outside to the inside of the bilayer membrane. Floppase transports phospholipids from the inner to the outer leaflet. Floppase does not specifically act on transport of aminophospholipids and probably works together with flippase. Scramblase, whose role is thought to be the transportation of phospholipids between the two monolayers of the cell membrane, is inactive in steady-state.

Successive mechanisms initiate MP (*ectosome*) formation during cell activation or other cell processes including apoptosis and senescence (Fig. 3):

1. Calcium is released by the endoplasmic reticulum.
2. Calcium inactivates flippase and activates floppase and scramblase, inducing the loss of phospholipids asymmetry between the inner and the outer leaflets. Contacts between aminophospholipids and cytoskeleton are then disrupted.
3. In addition, calcium release leads to activation of two enzymes: *calpain* and *gelsolin*. Calpain hydrolyzes actin-binding proteins that decreases association of actin with membranes glycoproteins (20, 21), while gelsolin (only in platelets) is involved in the cleavage of the actin capping proteins (22).

Protein anchorage to the cytoskeleton is therefore disrupted, resulting in membrane budding and microparticles shedding (23).

The main mechanism of PMPs formation is therefore calpain dependant. Nevertheless, calpain-independent microparticles formation can occur, such as when platelets are stimulated *in vitro* with collagen and thrombin in absence of stirring (21) or when cells are stimulated with the complement proteins C5b-9 (24).

MPs enriched in PE and PS exposed on their outer surface are released. MPs isolated from blood (mainly PMPs) comprise 60% phosphatidylcholine, 20% sphingomyelin, 9% PE, the remainder being minor quantities of other phospholipids (25). However, not all MPs exhibit PE and PS on their surface. For example, EC MPs originating from activated ECs are different in their lipid composition than those derived from apoptotic ECs. Indeed, annexin V binding sites are modestly but significantly

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Table 2: Cell-derived microparticles in some diseases.

Clinical condition	Cellular origin	Consequences	MP level in blood	References
Bleeding and thrombotic disorders				
Castaman's syndrome	platelet	bleeding predisposition	↓	(94, 95)
Scott's syndrome	platelet	bleeding predisposition	↓	(30, 99, 100)
Stormorken's syndrome	platelet	bleeding predisposition	↑	(101)
Venous thromboembolism	platelet, endothelial cell	thrombogenicity	↑	(102, 104)
Heparin-induced thrombocytopenia	platelet	thrombogenicity	↑	(106–108)
Antiphospholipid antibodies syndrome	platelet, endothelial cell	possible thrombogenicity	↑	(41, 109–112)
Thrombotic thrombo-cytopenic purpura	platelet, endothelial cell	possible thrombogenicity	↑	(113, 114)
Paroxysmal nocturnal haemoglobinuria	platelet	possible thrombogenicity	↑	(115–117, 177)
Sickle cell disease	platelet, red blood cell, endothelial cell, monocyte	possible thrombogenicity	↑	(119, 121)
Cardiovascular diseases				
Hypertension	monocyte, platelet, endothelial cell	possible thrombogenicity	↑	(133–135)
Hyperlipidemia	endothelial cell	possible thrombogenicity	↑	(145)
Atherosclerosis	monocyte, platelet, endothelial cell	possible thrombogenicity	↑	(140, 141, 144)
Acute coronary syndrome	platelet, endothelial cell	thrombogenicity	↑	(146–149)
Infectious diseases				
Sepsis	platelet, endothelial cell	thrombogenicity in some circumstances	↑	(156–159)
HIV infection	lymphocyte	possible thrombogenicity, transfer of infection	↑	(5, 165, 166)
Prion diseases	platelet	transfer of infection	↑	(34, 168, 169)

increased on ECs only in apoptosis, and are preferentially enriched on EC MPs derived from apoptotic ECs (26).

Despite a recent proteomic characterization of tumoral lymphocyte MPs (27), the chemical composition of MPs remains poorly described. MPs contain various proteins inherited from their parental cells and a membranous skeleton. Thereby, their origin can be identified by the presence of cell-specific surface antigens (Table 1). Differences between plasma- and platelet-derived MP proteins have been established by proteome analysis: they are constituted by proteins involved in apoptosis and iron transports (28). The protein content of MPs depends also on the way of activation of their parental cells. T-cells activated via the T-cell receptor are enriched in cluster differentiation (CD) 3ε- and CD3ξ-chains, but not when activated by ionomycin plus *p*-methoxyamphetamine hydrochloride (29). Similarly, PMPs activated by thrombin or collagen express glycoprotein (GP) IIb-IIIa complexes (leading to the binding of PMPs to fibrinogen) whereas those produced by platelets activated with complement do not (30). Other components of MPs have been recently described, such as mRNA (31, 32), prions (33, 34), contractile proteins such as thrombosthenin (35).

Methods for the detection and isolation of MPs

A wide variety of methods are used to measure, quantify, and phenotype MPs from blood samples or cell culture supernatants. For this reason, critical evaluation and standardization of the different methods used by each laboratory are necessary to reliably compare studies together. The ISTH SSC Working Group on Vascular Biology recently addressed this question to worldwide laboratories (19). An interesting forum has been recently published about this issue (10). Most methods use flow cytometry although the use of classical flow cytometer is subject to caution, as discussed by Bruce and Barbara C. Furie (13). The small size of MPs enhances the difficulties for their detection and quantification. In flow cytometry analysis, MPs appear close to the electronic noise, together with cellular debris, in the first quarter of the light scatter in logarithmic scale. In addition, it is impossible to discriminate by their size two particles of different types using the light scatter because the signal of a big event is not always larger than that of smaller events (36). This is due to the laser wavelength (488 nm) of common flow cytometers that is comparable to the size of most MPs (ranging between 50 and

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1,000 nm). Becker et al. (36) have used calibrated beads from 3–8 µm with a commonly used Becton Dickinson FACSCalibur, and observed that the beads could not be sized better than ± 2 µm. To overcome this technical limitation, Bruce and Barbara C. Furie (13) have modified a commercially available impedance flow cytometer (dielectric characterization). The modifications comprise: a slower flow speed, an electronic enhancement for a better sensitivity and lower noise, an optimization of fluid management and an ultrafiltration of buffers. With these changes they were able to measure 520 nm beads with an accuracy of ± 20 nm.

Analysis of MPs by solid-phase capture (annexin V or antibodies) has the advantage to combine MPs quantification with measurement of MPs procoagulant activity (37). However, MPs cannot be always detected as annexin V-binding events. This is illustrated in patients with sickle cell disease whose endothelial- and monocyte-derived MPs, and not PMPs, are either detected or not by annexin V (38). It has even been reported that the concentration of circulating MPs is 30 times higher than the concentration of MPs that are detected by annexin V (39).

MPs can be characterized by the detection of the different cell surface antigens (Table 1). These antigens reflect their origin and activation method. For example, T-cells MPs express CD4 (29). Beside the presence on MPs of parental protein (such as CD41 on both PMPs and platelets, and CD4 on both helper T-cells and their derived MPs [40]), MPs can express molecules that have been upregulated or translocated by cell activation or apoptosis (41). Thus, PMPs express at their surface P-selectin and GP 53 originating both from intracellular membranes (30, 42). To the contrary, some parental protein are not expressed by MPs derived from them. As an example, T-cells-derived MPs lack CD28 and CD45 despite that those proteins are present on T-cells.

MPs can be phenotyped or functionally analyzed directly from whole blood (43–45), with the advantage to let the MPs in a physiological environment, without pre-analytical steps. The blood must be drawn in tubes containing citrate as anticoagulant and studied within two hours. Paraformaldehyde fixation is impossible due to its action on cell membrane. Other methods include the isolation of PMPs from plasma (platelet-rich, -poor, or -free plasma) using different centrifugation steps. Their disadvantage is the production of variable results between laboratories, leading to a loss of MPs, or activation of platelets. Furthermore, the freezing of samples before analyses is not recommended due to the fact that the freezing itself generates MPs (46). MPs levels appear to be gender-specific and modulated by the menstrual cycle (47) and the circadian rhythm (48).

MPs can be generated *in vitro* and subsequently injected in animals and detected by intravital microscopy (49–51). PMPs can be produced by activation of platelets by classical agonists (52, 53). The potency to generate PMPs is variable depending on the used agonist (epinephrine < adenosine diphosphate (ADP) < thrombin < collagen < thrombin + collagen < A23187). Other agonists can cause the release of PMPs, such as desmopressin (54), and the membrane attack complex of the complement which induces rapid depolarization of the membrane, and the subsequent recovery of the membrane potential follows the shedding of PMPs (55, 56). To isolate MPs from leukocytes or ECs, calcium ionophore A23187 or specific cellular activators can be used. ECs produce MPs after thrombin, histamine, interleukin (IL)-6, granulocyte macrophage colony stimulating factor (GM-CSF) and thrombopoietin stimulation (57–59). Fur-

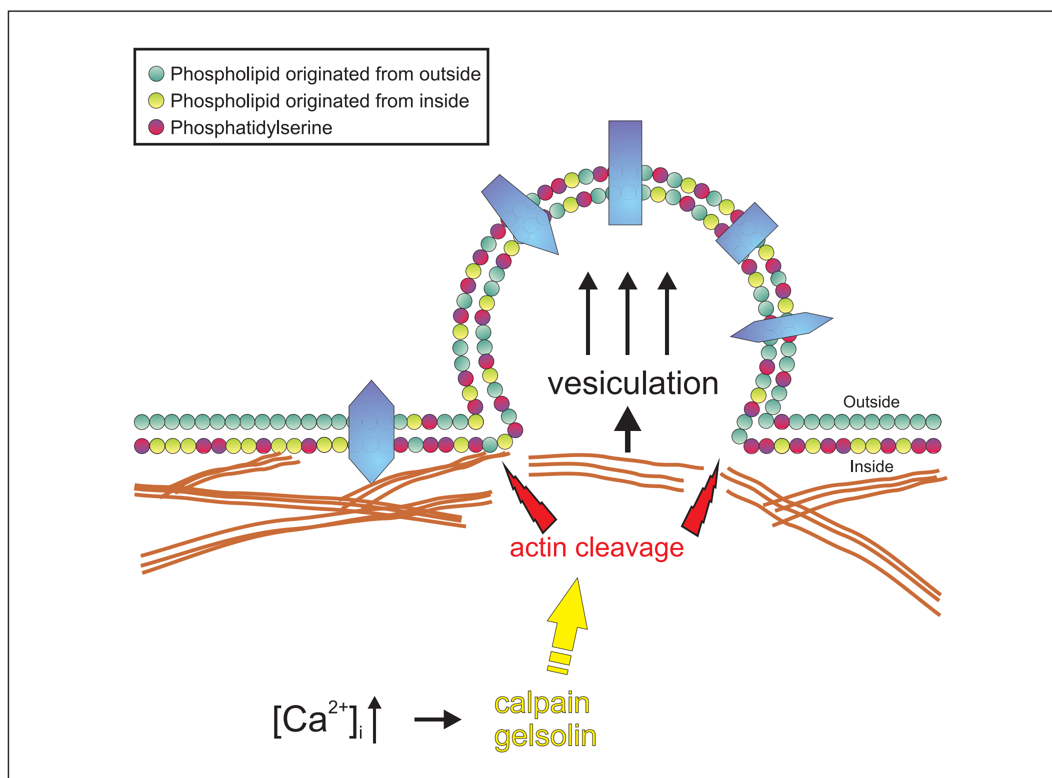

Figure 1: Ectosomes are formed by structural rearrangement of the cytoskeleton, budded off the cell membrane. Exosomes are formed by first the invagination of endosomes. Inward blebbing of the endosomal membrane forms intraluminal vesicles. This vesicle-containing endosome is called multivesicular body. Finally the content of the multivesicular body is released by its fusion with the plasma membrane.

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thermore, cell apoptosis induces MPs shedding, as observed in apoptotic lymphocytes induced by phytohaemagglutinin (60). Somehow, MPs generated *in vitro* could have a different phenotype than MPs produced *in vivo*.

Circulating MPs

Circulating MPs in blood originate from different cells (i.e. red blood cells, granulocytes, monocytes, lymphocytes, platelets and ECs) and their blood levels result from the balance between their rates of release from cells and their clearance from the circulation. Changes in MP levels in circulating blood may be due to some pathological conditions (Table 2). PMPs are the most abundant, representing about 70–90% of all circulating MPs (61). Aminophospholipids are exposed on their surface, as in activated platelets. The involvement of MPs in blood coagulation was first suspected by Wolf in 1967 (2) who described microvesicles < 0.1 µm and microvesicle aggregates as "*platelet dust*". Recently, Heijnen et al. (62) have shown that platelets release MPs by membrane shedding as well as exosomes from α-granules and multivesicular bodies.

A population of PMPs is generated during platelet activation, whereas other PMPs populations are derived from megakaryocytes during megakaryopoiesis (3–5), quiescent circulating platelets or might result from platelet apoptosis (6). Using transmission electronic microscopy, Warren and Vales first evidenced the release of MPs following platelet stimulation by collagen (53) or adhesion to vessel wall (63). George et al. (52) identified MPs generated after platelet thrombin stimulation, that are positive for the platelet-specific integrin $\alpha_{IIb}\beta_3$. They also found that platelet MPs are 10-fold more abundant in serum than in plasma, implying that PMPs are produced in the course of clotting (52).

PMPs can be produced by various stimuli, including platelet agonists, calcium ionophore, complement-binding proteins, or high shear. After stimulation by the thrombin-receptor agonist peptide (TRAP), MPs from 0.1 µm to 1 µm and exosomes from 40 to 100 nm are released (62). Stimulation of platelets activates intracellular calpain, and calpain inhibitors impair MPs release (21). The incubation of platelets with phosphatidylinositol-4,5-bisphosphate (PIP_2) inhibits PMPs formation by agonists, suggesting a possible role for calpain in degradation of phosphatidylinositol 4-phosphate (PIP) kinases thereby preventing the increase of PIP_2. However, a calpain-independent pathway has also been proposed, because PMPs are released following exposure of platelets to complement C5b-9, even in presence of calpain inhibitors (24).

PMPs are generated under certain blood flow conditions as well (64). High shear stress in severe atherosclerotic arteries activates platelets, generating PMPs, whereas normal shear stress does not (65). Patient blood before and after abdominal surgery has been studied *in vitro* under high and low shear stress rate. Interestingly, platelet aggregation induced by both high and low shear stress rates are enhanced in the blood of post-surgery patients (66). In another study, formation of PMPs is only increased under high shear stress rate, after surgery (66).

In addition, platelet adhesion to immobilized von Willebrand factor (VWF) under fast flow conditions, engages a mechanism for the generation of MPs. This results in the deposition of proco-

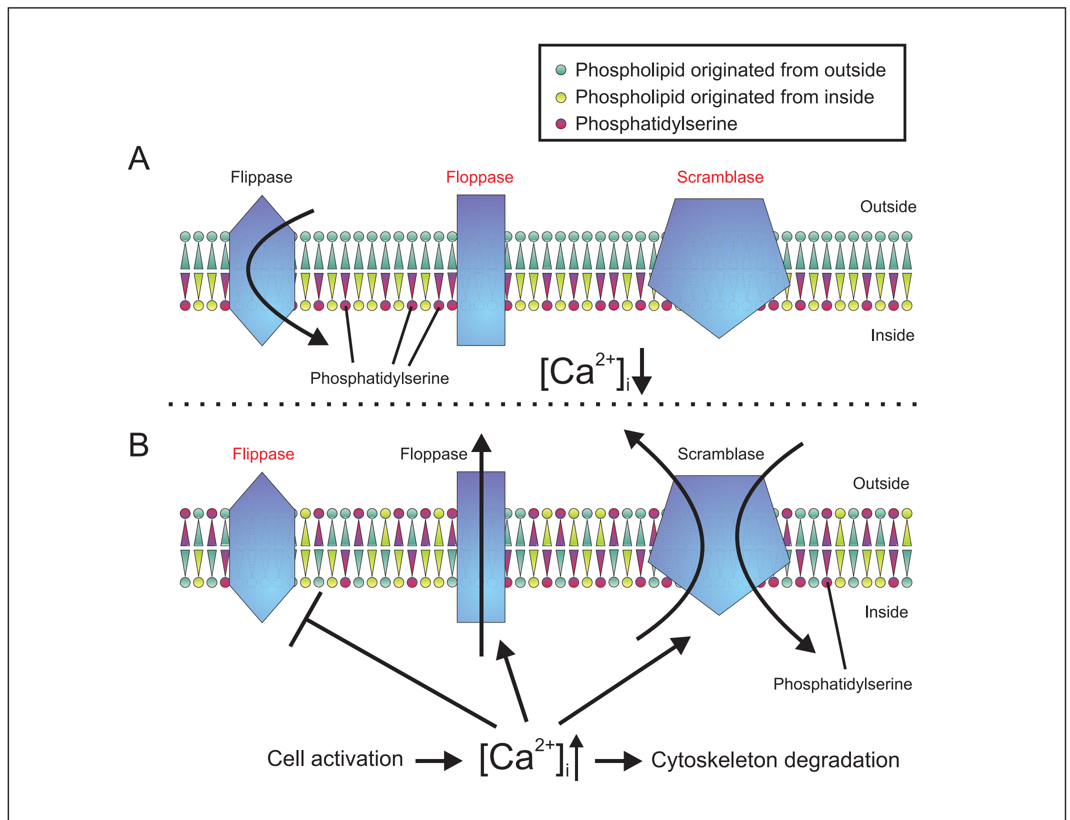

Figure 2: Maintenance of the asymmetric distribution of phospholipids in the membrane by a three piece enzyme system: flippase, floppase and scramblase. A) Resting cell, characterized by a low cytoplasmic calcium concentration and active flippase. B) The formation of microparticles (MPs) is associated with the loss of the plasma membrane asymmetry. This is due to the exposure at the outside surface of phosphatidylserine (in red), as a consequence of the activation of floppase and scramblase and inhibition of flippase by an increase of intracellular calcium concentration.

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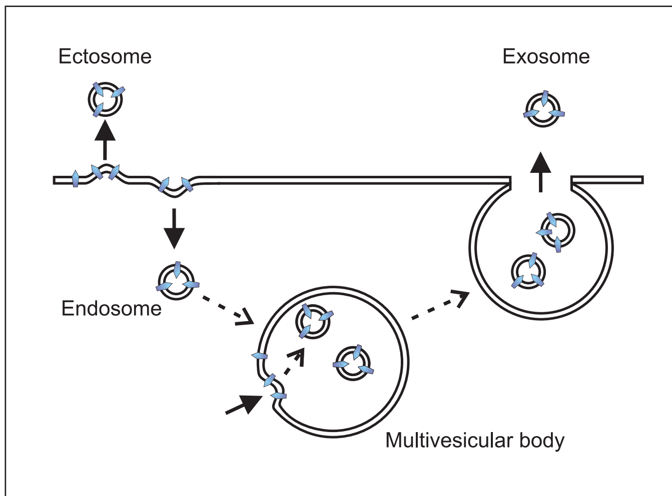

Figure 3: Microparticle formation. Intracellular calcium increases, inducing the loss of phospholipid asymmetry between the inner and the outer leaflets, and activating calpain and gelsolin which leads to membrane-actin dissociation and cleavage of actin filaments, respectively. This results in disruption of contacts between aminophospholipids and cytoskeleton.

agulant structures that are not removed even under extreme flow conditions, as encountered in severely stenosed arteries (67). This mechanical release of PMPs is dependent on the interaction of vWf with glycoprotein (GP) Ibα, and the resulting procoagulant PMPs enhance thrombus formation.

Platelets stimulated by calcium ionophore produce MPs comprising integrins, proteins present in α-granules (i.e. fibrinogen, platelet factor 4, thrombospondin, β-thromboglobulin) and cytoskeletal proteins (actin, talin) (21, 68). In contrast, MPs generated from platelets stimulated with dibucain, a local anesthetic reported to activate cytoplasmic calpain, do not contain protein normally found in α-granules (20). Nearly 90% of the total platelet activating factor (PAF) is found in PMPs after platelet stimulation.

Clearance of MPs

The mechanism of MPs clearance from the circulation is not known. Platelets have a life span of about 10 days, contrasting with that of PMPs of which is about 30 minutes in mice (6), or even less than 10 minutes in rabbits (69). These MPs could be cleared from the circulating blood by phospholipases (70), by direct mechanisms such as PS exposure and subsequent phagocytosis, or by indirect mechanisms such as opsonization by proteins such as growth arrest-specific gene 6 product (GAS6), protein S and complement (71).

PMPs are opsonized by C3b, the most abundant complement protein. C3b-bearing PMPs are subsequently bound to complement receptors on red blood cells and delivered to phagocytes in the liver and spleen (6). Also, red blood cell-derived vesicles are removed from the circulation by scavenger receptors, as is the case for aged platelets that undergo phagocytosis by macrophages. Kupffer cells in the liver thus contain large amounts of haemoglobin after injection of isolated red blood cell-derived vesicles (72).

Procoagulant MPs

A surface area unit of PMP has approximately 50- to 100-fold higher procoagulant properties than an identical surface area unit of an activated platelet (73). Thus, the usually accepted role of MPs is to promote coagulation. This is principally due to the presence of TF, the principal initiator of coagulation, exposed on the surface of MPs. Regardless the stimulus, about 25% of the procoagulant activity in blood is associated with MPs derived from activated platelets (74). TF on MPs is either in a latent (or "encrypted") form lacking coagulant activity or in an active form capable to initiate blood coagulation (or "de-encrypted") (75, 76). Encryption mechanisms may comprise dimerization (77), lipid reorganization (78) and cellular secretion of TF-rich granules (79). One of the disulfide bonds in TF may be labile allosteric and thereby can be cleaved or formed with effects on the function of TF itself. These conformation changes in TF are caused by a protein disulfide isomerase (80, 81) which is released from activated endothelial cells and platelets at the site of injury (82). Conformation changes in TF resulting from the formation of a disulfide bond allow the complex between TF and activated factor VII to bind to and activate factor X (80, 81).

Giesen et al. (83) quantified a TF activity in human blood and identified circulating and thrombi-associated TF-bearing cells. This blood-borne TF is thrombogenic. Another study shows that TF-positive MPs originate from monocytes (49). As TF-positive monocytes do not participate in the first phase of thrombus formation, the presence of TF in the thrombus, shown by intravital microscopy, could have a MP origin. *In vitro*, monocyte-derived microparticles (MMPs) expressing TF and P-selectin glycoprotein ligand-1 (PSGL-1) accumulate in thrombi of wild-type but not of *P-selectin*$^{-/-}$ mice. The presence in the blood of MMPs containing both TF and PSGL-1 allows TF-positive MMPs to bind to P-selectin bearing platelets within the thrombus. Thus, the delivery of TF by MPs inside the developing thrombus is dependent on PSGL-1 and P-selectin (49). Consequently, in mice lacking PSGL-1 or P-selectin, TF accumulation appeared minimal (49). Beside MMPs, PMPs can be TF-positive as well. Therefore, TF can transfer from monocytes and possibly polymorphonuclear leukocytes (PMNs) to platelets (83, 84). The reason why TF-positive MPs accumulate very quickly in the thrombus, as compared to TF-bearing leukocytes, is probably due to their very small size. Consequently, they can access the P-selectin-expressing platelets in the center of the thrombus, before the luminal exposition of P-selectin for leukocyte adhesion (50).

The integrin $\alpha_{IIb}\beta_3$ is another thrombogenic marker. It allows PMPs to bind to immobilized and soluble fibrinogen, and coaggregate with platelets. This mechanism is inhibited by blockers of $\alpha_{IIb}\beta_3$ (85, 86). PMPs can be also enriched in β2-glycoprotein I and P-selectin in some pathological conditions (87).

MPs can affect endothelial biology

Circulating blood contains a variety of MPs derived from platelets, leukocytes and ECs. MPs are able to activate ECs and moreover to transfer chemokines to endothelium. Effects of PMPs have been most extensively studied. Thus, the stimulation of ECs by PMPs, *in vitro*, results in cytokines release and ex-

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pression of adhesion molecules (88). Furthermore, it has been recently demonstrated that PMPs contain substantial amounts of regulated on activation, normal T-cells expressed and secreted (RANTES). This pro-inflammatory cytokine can be deposited on activated endothelium, such as may be found on atherosclerotic lesions. Thus, this transcellular delivery of RANTES promotes leukocyte recruitment to murine atherosclerotic carotid arteries (89). Beside RANTES, PMPs can deliver arachidonic acid in a transcellular manner (90). Indeed, PMPs are capable of inducing cyclooxygenase-2 production by ECs, which at their turn, then activate platelets (90). In addition, ECs deliver arachidonic acid to PMPs. This arachidonic acid is subsequently metabolized to thromboxane A_2, that induces artery contraction (91). Methacholin-induced contractions were even increased by preincubation with PMPs (91). Finally, endothelial MPs affect endothelial nitric oxide (NO) synthesis but do not change NO synthase expression (92, 93).

MPs in some physiological and pathological conditions

Scott's syndrome and related platelet disorders

The importance of PMPs for haemostasis is illustrated by a defect in their production in patients with Castaman's and Scott's syndromes that cause a bleeding predisposition. Patients with Castaman's syndrome have a bleeding tendency despite a normal platelet prothrombinase activity (94, 95), whereas patients suffering from Scott's syndrome have an additional defect in platelet prothrombinase activity (30). The importance of calcium for MPs shedding is underlined by the defect in store-operated calcium entry (96, 97) in Scott's syndrome. In this bleeding syndrome, red blood cells (98), B-lymphoblasts (96), platelets (99) are defective in PS externalization and have reduced capacitative calcium entry, without mobilization deficiency, inducing a defect in the calcium-triggered scramblase, with bilayer asymmetry conservation. A missense mutation in the adenosine triphosphate (ATP)-binding cassette transporter A1 implicated in PS translocation has been identified (100). Stormorken's syndrome, also called "inverse Scott's syndrome", is characterized by a bleeding tendency associated with a constitutive activation of platelets with elevation of circulating PMPs (101).

Venous thromboembolism

Both EC MPs and EC MP-monocyte conjugates are elevated in patients with venous thromboembolism and are key events in thrombogenesis (102). In addition, high PMP levels combined with high D-dimer and P-selectin levels correlate with the diagnosis of deep venous thrombosis. This could be the result of procoagulant PS and TF exposure on MPs. Nevertheless, MPs carry TF pathway inhibitor (103) and can thus either favor or protect against intravascular thrombosis. In contrast, selectins recruit MPs and amplify thrombosis in an animal model of inferior vena cava thrombosis (104).

Heparin-induced thrombocytopenia (HIT)

Following heparin administration, platelet factor 4 (PF4), which is found in the platelet α-granules and is released following platelet activation, binds to heparin to form a PF4-heparin complex. (105). The immune complexes that form through antibodies directed against the PF4-heparin complex activate platelets through the Fc receptor (106) and generate MPs with procoagulant properties initiating thrombin generation.

Sera of HIT patients induce MPs formation *in vitro* in an heparin-dependent manner (107). These microparticles have sizes ranging from 0.1 μm to 1 μm (108).

Antiphospholipid antibody syndrome

The endothelium is the target of antiphospholipid antibodies (aPL), responsible for the antiphospholipid antibody syndrome (APS). Higher titers of EC MPs are found in patients with APL and in patients with systemic lupus erythematosus (SLE) with aPL but not in patients with SLE or thrombotic disease without aPL (109). *Ex vivo,* APS plasma induces shedding of MPs by cultured ECs (41). Furthermore, EC MPs and PMPs are higher after myocardial infarction in patients with aPL than without aPL (110). The risk of thrombosis for patients with aPL has been recently associated with platelet activation rather than endothelial injury (111). In this study, PMPs are more elevated in aPL patients with thrombotic disorders than without. Finally, β_2 glycoprotein I, a plasma glycoprotein that interacts with negatively charged phospholipids and that is the major antigen for aPL, has a significant physiologic role in the budding of phospholipid membranes (112).

Thrombotic thrombocytopenic purpura (TTP)

Increased levels of PMPs that express calpain on their surface are found during acute and chronic phases of TTP (113). Moreover, EC MP levels are also elevated (114). In this study, the plasma of TTP patients clearly induces MP generation when co-cultured with brain and renal microvascular ECs, together with a 13-fold increase of integrin expression.

Paroxysmal nocturnal haemoglobinuria

Patients with paroxysmal nocturnal haemoglobinuria (PNH) have very high levels of MPs in plasma (115). However, these MPs do not seem to originate from red blood cells, although haemolysis often occurs in PNH and red blood cell-derived MPs are enriched in CD59 and CD55 (116). Indeed, very high titers of PMPs are detected in the plasma of PNH patients. Such PMPs could be responsible for thrombosis in these patients, since platelet activation by C5b-9 complex has been reported in PNH (117). Assembly of C5b-9 complex on the surface of platelets results in dense and α-granule secretion and MPs release (118).

Sickle cell disease

Sickle-cell anemia (SCA), in addition to being associated with chronic haemolysis resulting in a mild to moderate anemia, may be complicated by arterial or venous thrombosis (119, 120).

Circulating MPs have been detected in patients with SCA. They originate from platelets (121, 122), red blood cells (123), ECs as well as from monocytes (38). Total blood MPs are more elevated in the crisis phase of the disease than in steady-state. A fraction of MPs originating from ECs and monocytes are TF-positive, which could contribute to the thrombotic manifestations of the disease.

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Angiogenesis

Contradictory data have been reported regarding the effect of MPs on angiogenesis. This effect depends on the origin of the MPs: PMPs were described as pro-angiogenic whereas lymphocyte- and endothelial-derived MPs were reported as both pro- or anti-angiogenic.

PMPs enhance chemotaxis of ECs through Boyden chambers and induce tube formation of ECs. Platelet compounds, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and heparanase, mediate different stages of angiogenesis (124). Cultured human peripheral blood mononuclear cells (PBMCs) stimulated with PMPs enhance the growth of endothelial progenitor cells cultured with VEGF. In addition, phospholipids released from platelets induce EC migration and tube formation and inhibition of phosphoinositide-3-kinase markedly inhibits the induction of tube formation, chemotaxis and proliferation of ECs by PMPs (125). Thus, PMPs stimulate proliferation of human ECs in a dose-dependent manner, and inhibit their apoptosis (125). Local injections of PMPs after left coronary artery ligation in the rat increases the number of capillaries in the ischaemic region (126). These results demonstrate that PMPs might have beneficial effects.

Low concentrations of endothelial-derived MPs have been reported as pro-angiogenic through the matrix metalloproteinase activity that they harbor. Indeed, remodelling of the extracellular matrix is essential for angiogenesis. To this aim, ECs produce matrix metalloproteinases, that are stored in secretory granules (127). Those matrix metalloproteinases-containing vesicles are released by ECs and this event is enhanced by angiogenic factors (128). In sharp contrast, high concentrations of endothelial-derived MPs have been reported as anti-angiogenic as they decrease the formation of capillary-like structure by the production of reactive oxygen species (ROS) (129).

Lymphocyte-derived MPs may have pro-angiogenic effects via their capacity to favor NO release from endothelial cells (130). On the other hand, these MPs may have also anti-angiogenic effects due to the development of an oxidative stress associated with a reduced release of NO from endothelial cells (131). Indeed, lymphocyte-derived MPs generated *in vitro* by actinomycin D suppress aortic ring microvessel sprouting through VEGF receptor type 2 down-regulation and increased ROS production (132). In addition, these MPs impair vascular proliferation, migration and survival.

Hypertension

Hypertension is associated with platelet activation and increased PMPs levels compared to normal individuals with a correlation between MPs levels and blood pressure (133). This indicates that hypertension can generate PMPs even though shear stress rates are only moderately elevated. Similarly, pulmonary hypertension is associated with MPs from various origine (platelets, leukocytes and endothelial cells), with EC MPs predicting haemodynamic severity of the disease (134, 135).

Atherosclerosis

MPs and particularly PMPs play an important role in atherosclerosis development. They are produced in high-shear-stress conditions (65, 136). The transcellular delivery of the arachidonic acid fraction of PMPs and its subsequent metabolism to thromboxane A_2 activate both adjacent platelets and ECs (90). PMPs up-regulate adhesion molecules in ECs and enhance the production of $CD11_a$ and $CD11_b$ in monocytes and upregulate intercellular adhesion molecule (ICAM-1) in human umbilical vein ECs (137). Furthermore, PMPs enhance EC transmigration. Adhesion of monocytes to the endothelial barrier is an early event in vascular inflammatory syndrome (138), and subsequent development of atherosclerosis (139).

In addition, MPs derived mainly from macrophages, red blood cells and smooth muscle cells are present in atherosclerotic plaques (140, 141). They are more concentrated and thrombogenic in the plaque than in circulating blood, at least partly because they comprise highly thrombogenic smooth muscle-derived MPs (141). Apoptotic processes are more frequent in inflamed zone which are prone to rupture (142). Subsequently to rupture, MPs thus enter the circulation, leading to thrombosis owing to the presence of TF and PS (143). Moreover, inflammation-associated cytokines like thrombopoietin, IL-6 and granulocytes colony-stimulating factor (G-CSF) were shown increase the formation of PMPs under high shear stress (144).

Finally, elevated circulating levels of MPs are associated with several cardiovascular risk factors including hypertension (see previous paragraph), diabetes mellitus, metabolic syndrome and postprandial hypertriglyceridemia (145).

Acute coronary syndrome

Since MPs are located in atherosclerotic lesions, plaque rupture induce MPs shedding into blood flow and may thus trigger thrombosis owing to the presence of TF and PS (136, 145, 146). In a study of patients with acute myocardial infarction, it was demonstrated that procoagulant EC MPs are more elevated than in patients with stable coronary artery disease and controls (146, 147). Moreover, short after myocardial infarction, there was an initial decrease of EC MPs-platelet aggregates compared to stable patients, possibly because these aggregates are sequestered in the infracted vessel. Such phenomenon could also explain that 48 hours after the acute event, EC MPs-platelet aggregates return to levels close to those observed in stable coronary artery disease (148). Furthermore, high levels of EC MPs are associated with high-risk angiographic morphological lesions (149).

Cardiovascular procedures

TF-bearing leukocytes and PMPs appear *in vivo* during cardiac surgery and stimulate coagulation (150). Pericardial blood collected at the end of cardiopulmonary bypass contains significantly more MPs than blood from the systemic circulation (151). Indeed, plasma PMP levels do not increase in patients undergoing coronary artery bypass grafting with cardiopulmonary bypass These MPs are procoagulant and not only originate from platelets or red blood cells, but also from monocytes or granulocytes. The enhancement of active TF-positive MPs could be responsible for the thrombotic complications following bypass heart surgery due to the auto-transfusion of the perivascular blood at the end of the procedure to reduce blood loss. However,

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prothrombotic activity could be also due to the presence in the blood of TF-expressing monocytes (152). Finally, some authors have concluded that PMP formation during cardiac angioplasty might be due to the procedure itself, by showing that PMPs are increased in the coronary circulation from the beginning to the end of the procedure (153).

Sepsis

When the inflammatory response is not sufficient to eradicate a pathogenic organism, a systemic inflammatory response is triggered and eventually leads to organ failure. Activated protein C (APC) has been successfully used in the treatment of sepsis despite its association with serious bleeding (154). APC limits thrombin generation by degrading activated coagulation factors V and VIII, a process enhanced by its cofactor protein S. Protein C binds to endothelial protein C receptor (EPCR), which is expressed on ECs and monocytes (155). These cells, when stimulated by APC, release EPCR-containing MPs, in a dose-dependent manner. EPCR associated with MPs is full-length, in contrast with soluble EPCR that is cleaved by metalloproteases (156). Moreover, the release of EPCR-bearing MPs requires the presence of the protease activated receptor 1 (PAR1). Thus, EC MPs in the circulating blood of septic patients may predict a more favorable outcome. Additionally, the failure of anti- tumour necrosis factor (TNF)-α treatment in sepsis may be at least partially explained by the enhancement of EC MPs release by TNF-α (157, 158).

Consequently, MPs have opposite effects, depending on their origin and shedding mechanisms, presence of anticoagulant (APC) or procoagulant (PS, TF, PSGL-1, VWF). As an example, patients suffering from meningococcal sepsis undergo disseminated intravascular coagulation. These patients have higher titers of MPs than controls, and these MPs support massive thrombin generation *in vitro* (159).

Human immunodeficiency virus (HIV) infection

An increased risk of venous thromboembolic disease in HIV infection has been reported (160, 161), which could be related to procoagulant factors. Notably, the Tat protein of HIV activates ECs through enhancement of TF and E-selectin expression on the EC plasma membrane (162). Deep venous thrombosis can also be attributed to a decrease in anticoagulant proteins. Furthermore, circulating MPs from HIV-infected patients have been shown to reduce endothelial NO synthase expression (163).

Infection also provokes a high degree of apoptosis induced by viral protein R, that is thought to contribute to CD4-positive lymphocyte depletion (164). A study of MPs in the blood of HIV patients shows variable titers of MPs supporting prothrombinase activity. In addition, a clear proportion of PS-positive MPs are CD4-positive. Another study (165) suggests that HIV patients have elevated titers of MPs binding protein S. These MPs are positive for CD42b or CD4 but not for CD8.

Peripheral blood mononuclear cells (PBMCs) can release MPs containing the chemokine (C-C motif) receptor (CCR)5, used by HIV to enter cells. These MPs are taken up by CCR5-negative PBMCs, leading to the possibility of an HIV-entry into these cells (166). It has already been shown that PMPs can transfer arachidonic acid to platelets or ECs (90). Interestingly, the transfer of CCR5 to PBMCs, but not the transfer to T-cells, is inhibited by cytochalasin D, inferring a passive transfer of proteins in the interaction between T-cell and PMPs. In contrast, CCR5 is actively transferred to PBMCs and correctly inserted in the membrane, because RANTES (ligand for CCR5) induces the internalization of the receptor, which is not observed in T-cells. Similar transfer of chemokine (C-X-C motif) receptor (CXCR)4, another receptor for HIV entry, has been demonstrated from platelets to haematopoietic cells by PMPs (167). In a more recent paper, CXCR4 has been shown to be transferred by MPs from platelets and megakaryocytes to the surface of CXCR4-deficient cells, which can restore infectivity by lymphotropic X4-HIV (5). Therefore, it is not possible to rule out the possibility that CD4 transfers from lymphocyte MPs to target cells that makes the latter cells susceptible to HIV infection.

Prion diseases

Cellular prion protein (PrP^c) is another GPI-anchored protein (see above, *paroxysmal nocturnal haemoglobinuria*) found primarily in the lipid rafts of neuronal and epithelial cells, but also in platelets and red blood cells. PrP^c is localized in platelet α-granules and released during platelet activation (33). It is present in exosomes but also in ectosomes (or MPs) (34). Thrombin stimulation enhances PrP^c expression on the platelet surface and causes membrane shedding whereas stimulation by calcium ionophore releases MPs devoid of PrP^c. PrP^c is also associated with exosomes derived from α-granules.

The pathological form of PrP^c is PrP^{sc}, an abnormally folded form of the protein. The prion diseases include Kuru disease, a variant of the Creutzfeld-Jakob disease in humans, scrapie in sheep and bovine spongiform encephalopathy. PrP^{sc} has been discovered in exosomes of cultured epithelial and neuroglial cell lines infected with scrapie (168). As PrP^c is released bound to PMPs, it has been suspected that the pathological form PrP^{sc} could be similarly released causing potential infectivity of blood. Indeed, murine blood infected with a variant prion form of the human Creutzfeld-Jakob disease can transmit the infection to mice by platelets and infusion of plasma fractions (169). This observation is particularly important for transfusion medicine because it indicates that infected blood products containing PMPs might transmit prion diseases.

Messenger RNA (mRNA) delivered to target cells by MPs

Beside the transfer of protein (mostly cell surface receptor) or spreading of infective virus or prions to target cells, it has been shown that MPs can transfer mRNA. Therefore, the transfer of CD81-coding mRNA has been observed between leukocytes (31). Moreover, MPs originated from tumor cells can transfer mRNA to monocytes (32). Those tumor cells-derived MPs (TMPs) are carrying mRNA coding for vascular endothelial growth factor, hepatocytes growth factor, IL-8 and surface determinants (CD44H). Surprisingly, TMPs were localized first at the surface of monocytes after exposure of these cells to TMPs but later TMPs were detected intracellularly, somehow, without any

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Abbreviations

ADP, adenosine diphosphate; APC, activated protein C; aPL, antiphospholipid antibodies; APS, antiphospholipid antibody syndrome; bFGF, basic fibroblast growth factor; CCR, chemokine (C-C motif) receptor; CD, cluster differentiation; CXCR, chemokine (C-X-C motif) receptor; EC, endothelial cell; EPCR, endothelial protein C receptor; GAS6, growth arrest-specific gene 6 product; G-CSF, granulocytes colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; GP, glycoprotein; HIT, heparin-induced thrombocytopenia; HIV, human immunodeficiency virus; ICAM-1, intercellular adhesion molecule-1; ISTH, International Society on Thrombosis and Haemostasis; MMP, monocyte-derived microparticle; MP, microparticle; mRNA, messenger RNA; NO, nitric oxide; PAF, platelet activating factor; PAR1, protease activated receptor 1; PBMC, peripheral blood mononuclear cell; PDGF, platelet-derived growth factor; PE, phosphatidyl-ethanolamine; PF4, platelet factor 4; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PMN, polymorphonuclear leukocyte; PMP, platelet-derived microparticle; PNH, paroxysmal nocturnal haemoglobinuria; PrP^c, cellular prion protein; PS, phosphatidylserine; PSGL-1, P-selectin glycoprotein ligand-1; RANTES, regulated on activation, normal T-cells expressed and secreted; SCA, sickle-cell anaemia; SLE, systemic lupus erythematosus; SSC, Scientific and Standardization Committee; TF, tissue factor; TNF-α, tumour necrosis factor alpha; TRAP, thrombin-receptor agonist peptide; TTP, thrombotic thrombocytopenic purpura; VEGF, vascular endothelial growth factor; VWF, von Willebrand factor.

evidence whether these mRNA could be translated in monocytes.

Embryonic stem cells also produce MPs that are highly enriched in mRNA of several pluripotent transcription factors as compared to parental cells. In addition, these mRNA could be delivered to target cells such as haematopoietic progenitor cells and are even translated (170). This mechanism enhances survival and improves expansion of haematopoietic progenitor cells. Furthermore, radiation-injured lung releases MPs that contain pulmonary epithelial cell-specific mRNA, which can enter marrow cells and are further translated (171). Another mRNA transfer has also been shown in angiogenesis (172). This transfer occurs between endothelial progenitors cells and endothelial cells to induce angiogenesis *in vitro* and *in vivo* and this mechanism was reversed by incubation with RNase. Finally, mRNA and even microRNA has been detected in exosomes and were functional (173).

Attempted statements on MPs in haemostasis and vascular medicine

1. MPs are vesicles that bud off from cells, lack a nucleus, contain a membrane skeleton; they are therefore considered as ectosomes (Fig. 1).
2. They express antigens from their parental cells on their surface (Table 1).
3. Their size is comprised between 0.1 µm and 1 µm.
4. They are mainly constituted by proteins and lipids but may also contain other compounds such as mRNA and prions.
5. Current knowledge regarding MPs formation is based on experiments *in vitro*. In contrast, mechanisms of MPs formation *in vivo* is unknown.
6. Circulating MPs are principally of platelet origin, but they also derive from red blood cells, granulocytes, monocytes, lymphocytes and ECs.
7. The blood concentration of MPs needs to be precisely determined, as well as the procoagulant fraction of circulating MPs.
8. Their blood levels result from the balance between their rates of release from cells and their clearance from the circulation. Mechanisms of MPs clearance are poorly understood.
9. Changes in MP levels in circulating blood may be due to some pathological conditions (Table 2).
10. MPs are postulated to play an important role in thrombosis but also in other disorders.

Conclusion

This review points to an absolute necessity to more precisely define MPs. A definition only based on their size is not sufficient and should be completed by a description of their origin or biological effects, such as procoagulant activity. This standardization of methods could resolve the conflicting results described in the literature. Nonetheless, the biological roles of MPs are various. PMPs, that are the most abundant, have been described as thrombogenic. MPs carry also lots of bioactive molecules, even prions and mRNA. Their implication in pathologies like thrombosis, coronary artery disease, HIV infection and sepsis demonstrates the necessity to better understand their formation and activity.

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