The involvement of circulating microparticles in inflammation, coagulation and cardiovascular diseases

Paolo Puddu MD, Giovanni M Puddu MD, Eleonora Cravero MD,
Silvia Muscari, Antonio Muscari MD

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Microparticles (MPs) are small vesicles, ranging in size from 0.1 μm to 2 μm , originating from plasma membranes of endothelial cells, platelets, leukocytes and erythrocytes. MPs can transfer antigens and receptors to cell types that are different from their cell of origin. Circulating MPs provide a procoagulant aminophospholipid surface for the assembly of the specific enzymes of coagulation. Both tissue factor and phosphatidylserine are exposed on MP outer membranes. In addition, MPs can play a significant role in vascular function and inflammation by modulating nitric oxide and prostacyclin production in endothelial cells, and stimulating cytokine release and tissue factor induction in endothelial cells, as well as monocyte chemotaxis and adherence to the endothelium. Finally, increased levels of MPs have been found in the presence of acute coronary syndromes, ischemic stroke, diabetes, systemic and pulmonary hypertension, and hypertriglyceridemia. From a practical point of view, MPs could be considered to be important markers of cardiovascular risk, as well as surrogate end points for assessing the efficacy of new drugs and therapies.

Key Words: *Atherosclerosis; Coagulation; Inflammation; Microparticles*

La participation de microparticules circulantes dans l'inflammation, la coagulation et les maladies cardiovasculaires

Les microparticules (MP) sont de petites vésicules de 0,1 μm à 2 μm qui proviennent des membranes plasmiques des cellules endothéliales, des plaquettes, des leucocytes et des érythrocytes. Les MP peuvent transférer des antigènes et des récepteurs à d'autres types de cellules que leur cellule d'origine. Les MP circulantes procurent une surface amphiophospholipidique procoagulante à l'ensemble des enzymes spécifiques de coagulation. Tant le facteur tissulaire que la phosphatidylsérine sont exposés sur les membranes externes des MP. De plus, les MP peuvent jouer un rôle important dans la fonction et l'inflammation vasculaires en modulant la production de monoxyde d'azote et de prostacycline dans les cellules endothéliales et en stimulant la libération de cytokine et l'induction de facteur tissulaire dans les cellules endothéliales, de même que la chimiotaxie monocytaire et l'adhésion à l'endothélium. Enfin, on constate des taux accrus de MP en présence de syndromes coronariens aigus, d'accidents ischémiques cérébraux, de diabète, d'hypertension systémique et pulmonaire ainsi que d'hypertriglycéridémie. Sur le plan pratique, les MP peuvent être considérées comme d'importants marqueurs de risque cardiovasculaire et comme paramètres ultimes auxiliaires pour évaluer l'efficacité de nouveaux médicaments et de nouvelles thérapies.

In 1967, membrane fragments of platelet origin with procoagulant activity were described in human plasma as 'platelet dust' (1,2). This 'dust' consisted of small vesicles (less than 0.1 μm in diameter) capable of promoting coagulation. Subsequently, the release of microparticles (MPs) from endothelial cells (ECs), vascular smooth muscle cells, leukocytes, lymphocytes and erythrocytes has also been shown in vitro. Some of these MP populations have been found in the blood of both patients and healthy individuals.

MPs might play a significant role in the interactions among circulating and vascular cells. Several papers (3-9) have described the possible effects of MPs in regulating vascular function, and their potential physiological and pathological involvement in cardiovascular diseases. In addition, MPs have recently been proposed as new therapeutic targets in the treatment of cardiovascular diseases, in consideration of their prothrombotic and proinflammatory actions (10).

The present review discusses the putative roles played by MPs in inflammation, coagulation and endothelial/vascular function, as well as the possible and importance of MPs in the diagnosis, prognosis and therapy of atherosclerotic diseases.

FUNCTIONAL CHARACTERISTICS OF MPs AND MOLECULAR BASIS OF THEIR FORMATION

MPs are phospholipid- and protein-rich submicron particles. These fragments, originating from plasma membranes of eukaryotic cells, typically contain cell surface proteins and cytoplasmic components of their cell of origin (11,12), ranging in size from 0.1 μm to 2 μm . More precisely, vesicles larger than 100 nm in diameter originating from plasma membranes are usually called MPs, while smaller vesicles originating from the endoplasmic reticulum are described as 'exosomes'. Finally, larger particles (greater than 1.5 μm) containing nuclear components are called 'apoptotic bodies' (3,13,14).

MPs are released from cell membranes by triggers such as cytokines, thrombin, endotoxins, hypoxia and shear stress, capable of inducing activation or apoptosis (3). It is unclear whether the mechanisms underlying MP formation during these two events are identical (9).

The activation of platelets by different agonists promotes platelet aggregation and secretion, as well as membrane vesiculation and MP release. Thrombin, collagen and adenosine diphosphate bind specific transmembrane receptors that, through changes in second messenger

Department of Internal Medicine, Aging and Nephrological Diseases, University of Bologna and S Orsola-Malpighi Hospital, Bologna, Italy
Correspondence: Dr Antonio Muscari, Department of Internal Medicine, Aging and Nephrological Diseases, University of Bologna – S Orsola-Malpighi Hospital, Via Albertoni, 15, 40138 Bologna, Italy. Telephone 39-051-636-2280, fax 39-051-636-2210, e-mail antonio.muscari@unibo.it
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concentrations, can modulate cellular responses (15,16). Alternatively, the intracellular concentration of the second messenger can be directly changed by agents such as calcium ionophores. The increase in intracellular levels of calcium ions leads to the activation of calpain, with subsequent degradation of cytoskeletal proteins. This mechanism is believed to play a putative role in MP formation (15-17).

In the apoptotic pathway, caspase-3 plays an important role, activating the rho-associated kinase, resulting in the release of apoptotic membrane vesicles (18).

The proteinic composition of MPs reflects the composition of the cell membrane from which they are released. This includes constitutively expressed antigens, and antigens that have been induced on the parent cell by the activating or apoptotic triggers, leading to MP release (19).

The composition and distribution of constitutive cell membrane phospholipids are highly specific. Phosphatidylserine (PS) and phosphatidylethanolamine are mainly sequestered in the inner leaflet of the plasma membrane, while phosphatidylcholine and sphingomyelin are mainly located in the outer membrane layer (11,20). This asymmetric distribution is essential for biomembrane function and is under the control of a complex transmembrane enzymatic balance that involves enzymes such as gelsolin (present only in platelets), aminophospholipid translocase, floppase, scramblase and calpain (7). During cell activation and the subsequent increase of Ca^{2+} concentration in the cytosol, plasma membranes are modified and phospholipid asymmetry is compromised. In particular, the loss of phospholipid asymmetry results in the exposure of PS on the outer cell surface. Because PS efficiently binds coagulation factors (21), it leads to a prothrombotic state (22-25). Furthermore, following cellular activation, the cytoskeleton undergoes several changes. Spectrin and actin are cleaved, and protein anchorage to the cytoskeleton is disrupted. Thus, an increase in bleb formation takes place and bleb-generated MPs are released into the circulating blood. It has been shown that platelet MPs (PMPs) express P-selectin, the integrin glycoprotein (GP) IIb/IIIa, platelet endothelium adhesion molecule-1 (PECAM-1 [CD31]), CD63, CD42a and CD42b (7,15,26). GP IIb/IIIa blockade inhibits platelet PS exposure by potentiating translocase and attenuating scramblase activities (27). Endothelial cell MPs (EMPs) express CD31, CD34, CD51, CD54, CD146, E selectin and endoglin, and bind von Willebrand factor (28). CD4, CD3 and CD8 are present at the surface of leukocyte MPs (29,30).

MPs bear antigens of their cell of origin and can transfer these surface signalling molecules to other types of cells. The binding of surface antigens to their specific counter-receptors leads to the activation of intracellular signalling pathways (6). In this way, MPs behave as vectors disseminating biological information to the cells of the vascular compartment, which expose appropriate counter-receptors for the ligands they harbour (31).

METHODS FOR MP MEASUREMENT

MP analysis has the potential to enter the mainstream of clinical testing because it may provide important data for investigating various vascular disorders, such as acute coronary syndromes, venous thrombosis and stroke. However, the wide variety of methodologies used by different laboratories to measure circulating MPs has occasionally provided inconsistent or conflicting results (32), making data analysis and clinical correlations challenging (33).

The main methods of MP detection include flow cytometry, enzyme-linked immunoassays and functional coagulative assays (32,34,35). Lal et al (36) recently developed a new method for the detection of plasma MPs using a fluorescence-based antibody array system that can rapidly identify the cell origin of MPs.

MP counting, as currently performed by flow cytometry, certainly needs to be standardized. In this respect, the preanalytical phases of blood sampling and MP separation according to standardized centrifugation steps (37) are key factors. Robert et al (38) recently developed a strategy for PMP counting with a widely available flow cytometry

instrument (Cytomics FC500; Beckman Coulter Inc, USA), using size-calibrated fluorescent beads in a fixed numerical ratio (Megamix; BioCyte, France). The intra- and interinstrument reproducibility was tested by using annexin and CD41 coexpression to count MPs in previously frozen aliquots of the same platelet-free plasma over four months and in platelet-free plasma from 10 healthy subjects in three independent flow cytometers. Using the three instruments, similar PMP counts were obtained. With the use of this standardized flow cytometry protocol, PMP levels were significantly higher in women than in men. This strategy for PMP count standardization could represent a first step toward multicentre studies, and could also be used for MPs derived from other cell types. However, the measurement of circulating MPs still presents standardization problems and is not yet widely available in clinical practice.

ROLE OF MPs IN COAGULATION

Because PMPs were the first species identified, the studies on the role played by MPs in physiological and pathological conditions have, for a long time, concerned blood coagulation and hemostasis.

Activated platelets and circulating MPs provide a procoagulant aminophospholipid surface for the assembly of the specific enzymes of the coagulation cascade. After activation, MPs exhibit negatively charged phospholipids (chiefly PS) at their surface, which, once in contact with circulating blood factors, allow the local concentrations necessary to achieve optimal thrombin generation as well as efficient hemostasis (39). PS increases the procoagulant activity of tissue factor (TF). TF and PS are both exposed on MP outer membranes and are considered to be the main initiators of the coagulation cascade (8,40). TF is a key player in the onset of blood coagulation (40) because *in vivo* coagulation is initiated when TF binds factor VIIa and catalyzes its activation. TF circulates in plasma, largely on monocyte/macrophage-derived MPs that can bind activated platelets through a mechanism involving P-selectin GP ligand-1 (PSGL-1) on MPs and P-selectin on platelets (41). TF has been identified on leukocyte MPs, EMPs and PMPs (12,19,42-46). Del Conde et al (47) found that MPs derived from monocyte/macrophage cholesterol-rich rafts are selectively enriched in both TF and PSGL-1, and deficient in CD45, suggesting that they arise from distinct membrane microdomains. Interestingly, the shedding of MPs was significantly reduced with depletion of membrane cholesterol. MPs not only bound the activated platelets, but fused with them via PSGL-1, transferring lipids and proteins, including TF, in the plasma membrane. The phospholipids on the surface of MPs from platelets and ECs provide a number of binding sites for factors Va, VIII, IXa and IIa (15,48-50). EMPs express ultra-large von Willebrand factor multimers, which promote and stabilize platelet aggregates (51). These findings provide a mechanism by which blood coagulation can be initiated and propagated on the surface of activated platelets (47). MPs can also contribute to the development of platelet- and fibrin-rich thrombi at sites of vascular injury, through the recruitment of cells and the accumulation of TF (52).

Whether MPs are procoagulant *in vivo* is not a completely resolved issue, but several data suggest that MP-mediated coagulation may be clinically significant. For instance, an association between the number of circulating MPs and the risk of thromboembolic complications has repeatedly been demonstrated (9).

The procoagulant activity of MPs can be quantified using the thrombin generation test. In this system, MPs supply the procoagulant surface, while TF and plasma provide the necessary coagulation factors. By adding calcium ions, coagulation factors bind to MPs to initiate coagulation. In this assay, the generation of thrombin is dependent on the presence and activity of MPs, and in their absence, no coagulation would occur (9).

ROLE OF MPs IN INFLAMMATION AND VASCULAR FUNCTION

EMPs might directly lead to the development of endothelial dysfunction. In fact, *in vitro* experiments have shown that EMPs

TABLE 1
Microparticle (MP) involvement in cardiovascular diseases

Condition	MP type	References
Atherosclerosis	Plaque MPs	73–75
Coronary endothelial dysfunction	Apoptotic MPs	76,83
Acute coronary syndromes	Procoagulant MPs	29,69
Acute ischemic stroke	EMPs	77–79
End-stage renal failure	EMPs	80
Hypertension	EMPs, PMPs	71
Pulmonary hypertension	Procoagulant MPs	81
Type 2 diabetes	TF exposing PMPs	82,83
The metabolic syndrome	Circulating MPs	84
Hypertriglyceridemia	EMPs	70

EMPs Endothelium-derived MPs; PMPs Platelet-derived MPs; TF Tissue factor

might regulate vascular tone by modulating both nitric oxide (NO) and prostacyclin production in ECs (5). Moreover, the oxidized phospholipids in the MPs released from ECs exposed to oxidative stress may be particularly active in causing monocyte adherence to ECs and activation of neutrophils (53,54).

A further key feature in atherogenesis is leukocyte adhesion to ECs, with subsequent transendothelial migration of leukocytes (55). Specific adhesion molecules on ECs interact with ligands that are present not only on leukocytes, but also on leukocyte-derived MPs. Mesri and Altieri (56,57) suggested that leukocyte MPs stimulate cytokine release and TF induction in ECs by activating a signalling pathway involving the tyrosine phosphorylation of c-Jun NH₂-terminal kinase-1. This may lead to increased proinflammatory and procoagulant activity in ECs.

High shear stress-induced activation of platelets and the addition of PMPs may also enhance the expression of cell adhesion molecules, and the production of cytokines in the human monocytic leukemia cell line (THP-1) and ECs (58,59). Moreover, PMPs may deliver arachidonic acid to ECs, with consequent upregulation of intercellular adhesion molecule-1 and subsequent monocyte adhesion (60). PMPs can also promote leukocyte-leukocyte aggregation (61), as well as monocyte chemotaxis (60) through the transformation of MP arachidonic acid into the proinflammatory and vasoconstricting thromboxane A₂ (62). However, PMPs may also induce the endothelial production of cyclooxygenase-2 and of the vasodilating prostacyclin. Finally, PMPs contain a significant amount of RANTES (Regulated on Activation, Normal T cell Expressed and Secreted), an inflammatory chemokine, and can deposit it on activated ECs, triggering monocyte adhesion on these cells (63). By means of these mechanisms, PMPs can modulate the inflammatory and vasomotor response (64). However, although MPs may have deleterious, as well as beneficial effects on vascular function in vitro, there is not yet any direct evidence that they play a significant role in vascular dysfunction in vivo. Specific studies are needed to address this question.

ROLE OF MPs IN CARDIOVASCULAR DISEASES

Although in vitro studies have suggested various possible molecular mechanisms leading to MP formation (2,7,14), the precise mechanisms of in vivo MP generation remain unclear. Moreover, it is also unclear whether increased MPs are a cause or a consequence of vascular disease states because cardiovascular disease-related factors, such as metabolic disturbances, cytokines and, possibly, infectious agents, can trigger MP production.

It was suggested that MPs can spread proinflammatory and procoagulant mediators throughout the body in response to a stimulus, including activation and apoptosis, contributing to the severity of the disease (9). On the other hand, Agouni et al (65) reported possible beneficial effects of MPs in a mouse model of endothelial dysfunction. After injection in mice, MPs from human activated/apoptotic

T-lymphocytes were able to stimulate NO production from ECs, enhancing endothelium-dependent coronary vasodilation. Furthermore, the same MPs reversed endothelial dysfunction in a model of mouse coronary arteries subjected to ischemia/reperfusion. This effect was mediated by the morphogen sonic hedgehog (Shh), a modulator of NO production carried by MPs that is also involved in embryonic and adult development. Thus, MPs might exert beneficial or deleterious effects for the vascular wall depending on their cellular origin, the stimuli involved in their cellular generation and the clinical setting (66,67).

Nevertheless, owing to the complex procoagulant and proinflammatory activities of MPs, research has mainly been focused on their possible role in cardiovascular diseases (9). There is evidence that MP levels are increased in patients with cardiovascular diseases and risk factors, including acute coronary syndromes, diabetes, hypertension and hypertriglyceridemia (29,68-72) (Table 1).

Human atherosclerotic plaques contain MPs released during cell activation or apoptosis. Plaque MPs bear TF activity and expose PS, a major determinant of their procoagulant activity (73,74). Leroyer et al (75) demonstrated that plaque MPs originate from macrophages, erythrocytes and smooth muscle cells, whereas circulating MPs are mainly derived from platelets. MPs were more abundant and thrombogenic in plaques than in plasma. However, the study showed that most of the circulating MPs do not originate from ruptured plaques, but are generated within the blood compartment or at the blood-vessel interface.

Atherosclerosis is initiated and propagated by EC dysfunction. Werner et al (76) recently showed that EC apoptosis is independently involved in the pathogenesis of endothelial dysfunction, and circulating CD31⁺/annexin V⁺ apoptotic MPs positively correlated with the impairment of coronary endothelial function, independent of classic risk factors.

Elevated levels of MPs with procoagulant potential are present in the circulating blood of patients with recent clinical signs of coronary plaque disruption and thrombosis (29). EMPs are associated with high-risk coronary lesions, including multiple, irregular lesions, those with an eccentric appearance and those with thrombi (69). Thus, EMPs may be a useful marker for the risk of acute coronary events.

High levels of circulating EMPs were also found in patients with acute ischemic stroke (77-79).

Furthermore, circulating EMPs are closely associated with vascular dysfunction in patients with end-stage renal failure (80).

Preston et al (71) suggested that EMPs and PMPs increase in severely hypertensive patients.

MPs bearing TF and endoglin as well as vascular cell adhesion molecule-1 and chemoattractant protein-1 were elevated in patients with pulmonary arterial hypertension compared with controls (81). It was even suggested that MPs might prove to be valuable tools in determining the severity of pulmonary hypertension.

In patients with uncomplicated type 2 diabetes mellitus (DM), Diamant et al (82) found elevated numbers of TF-exposing PMPs. Although this MP-associated TF did not show any procoagulant activity, it might play a role in other processes such as angiogenesis, cell growth and signal transduction. Patients with DM also showed elevated levels of EMPs (83). In particular, CD144 EMP levels were significantly higher in DM patients with coronary artery disease (CAD) than in those without CAD, and allowed the identification of a subpopulation of DM patients who had CAD without typical chest symptoms.

In patients with the metabolic syndrome, circulating MPs of various origin are increased and impair endothelial function (84).

Ferreira et al (70) evaluated the possible relationship between levels of EMPs and changes of postprandial hypertriglyceridemia in healthy normolipemic subjects after a single high-fat meal. In these subjects, the high-fat meal led to a significant elevation of plasma EMPs, suggesting structural endothelial damage caused by triglycerides, followed by the impairment of endothelial function (85-87).

MPs AS A THERAPEUTIC TARGET IN CARDIOVASCULAR DISEASES

Due to their procoagulant and proinflammatory effects on vascular walls and target organs (10,88), MPs might also be considered to be a novel therapeutic target in cardiovascular diseases.

In 1998, Nomura et al (89) observed that cilostazol, a selective cyclic AMP phosphodiesterase inhibitor and antiplatelet agent, decreased the levels of PMPs in patients with noninsulin-dependent DM (NIDDM). Subsequently, in hypertensive patients with or without NIDDM, the same group found that treatment with losartan (alone or in combination with simvastatin) significantly decreased the levels of monocyte-derived MPs (90), and PMPs and EMPs (91). Similar results on PMPs and monocyte-derived MPs were obtained in patients with NIDDM treated with ticlopidine (92).

In hypertensive patients, Labiós et al (93) observed that eprosartan significantly reduced blood pressure and normalized the number of MPs after blood shear exposure.

A powerful antiplatelet drug, the GP IIb/IIIa receptor antagonist abciximab also reduces excessive PMP formation and shear stress-induced platelet activation (94). Interestingly, the short-term high-dose administration of vitamin C reduces the number of circulating apoptotic MPs in patients with congestive heart failure and suppresses EC apoptosis in vivo, which might contribute to the beneficial effect of vitamin C supplementation on endothelial function (95).

More recently, Nomura et al (96) found that eicosapentaenoic acid significantly reduced the number of circulating PMPs in hyperlipidemic diabetic patients, contributing to the prevention of vascular complications. This effect was enhanced by the addition of pitavastatin, and is in agreement with the results of a study investigating the favourable effects of n-3 fatty acids on the levels of PMPs and monocyte-derived MPs after myocardial infarction (97).

Because activated peroxisome proliferator-activated receptors (PPARs) can inhibit inflammation and endothelial dysfunction, and may also be effective in the primary prevention of cardiovascular events (98), Esposito et al (99) evaluated the short-term effects of the PPAR-gamma ligand pioglitazone on circulating EMPs in patients with the metabolic syndrome. Pioglitazone reduced circulating EMPs independently of the improvement of insulin sensitivity. Moreover,

experimental data on the PPAR-gamma agonist rosiglitazone showed an inhibition of MP-induced vascular hyporeactivity through the regulation of proinflammatory proteins (100).

Overall, these results suggest that plasma MPs could be a promising target in the treatment of cardiovascular diseases.

CONCLUSIONS

MPs of various origin may be considered to be 'partners in crime' in all crucial steps of atherosclerosis (101). In fact, MPs play a role in inflammation, coagulation, endothelial and vascular function, and apoptosis. MPs could modulate the cross-talk between the cellular elements of the coagulative and inflammatory system, through the transfer of signalling molecules and receptors of their cell of origin to other cell types.

Several studies have shown that MPs are increased in patients with acute coronary syndromes, stroke, diabetes, pulmonary and systemic hypertension, and hypertriglyceridemia. Thus, circulating MPs could be considered to be important markers of cardiovascular risk (102), with prognostic implications. However, the clinical importance of MPs in vascular disease states remains to be fully elucidated because it is unclear whether MPs are a cause or a consequence of these conditions. Conversely, available data from small studies suggest that MPs could also be considered a novel therapeutic target in cardiovascular diseases (10,88).

Recent progress in proteomics has shown that the protein content of lymphocyte MPs is highly influenced by the cell culture medium and type of stimulus used for MP generation (103). Thus, Chironi et al (104) suggested special caution in extrapolating laboratory experimental results to the clinical setting because a given MP type may have different compositions and biological behavioural patterns in vitro and in vivo. Moreover, the different methods of measurement, the lack of standardization and the various types of MPs to be measured may lead to insufficiently reliable results (32). In addition, such methods of measurement are not yet widely available.

Future research is needed before circulating MPs are considered to be of significant clinical interest. Only a complete understanding of the formation, composition, release and mechanisms of action of the various MPs will allow the development of novel approaches in the treatment of atherothrombosis-related diseases (105,106).

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