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Microparticles

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The Many Faces of Endothelial Microparticles

Françoise Dignat-George, Chantal M. Boulanger

Abstract—Endothelial microparticles (EMP) are complex vesicular structures shed from activated or apoptotic endothelial cells. They play a remarkable role in coagulation, inflammation, endothelial function, and angiogenesis and thus disturb the vascular homeostasis, contributing to the progression of vascular diseases. As a cause or a consequence, elevated levels of EMP were found in plasma from patients with vascular diseases, where they serve as a surrogate marker of endothelial function. More recent data challenged the presumed deleterious role of EMP because they could promote cell survival, exert antiinflammatory effects, counteract coagulation processes, or induce endothelial regeneration. This review focuses on the ambivalent role of EMP in vascular homeostasis. **(*Arterioscler Thromb Vasc Biol*. 2011;31:27-33.)**

Key Words: apoptosis ■ atherosclerosis ■ endothelial function ■ endothelium ■ thrombin ■ thrombosis ■ vascular biology ■ microparticles

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Vascular endothelial cells, like most cells, release different types of membrane vesicles, including microparticles (MP) and exosomes, in response to cellular activation or apoptosis. In addition, apoptotic bodies might be generated during the final steps of programmed cell death. These different vesicles are distinguished from one another on the basis of their subcellular origin, their size, their content, the mechanisms leading to their formation, and, from a practical point of view, how they are obtained.

Endothelial Microparticle Characteristics

Endothelial microparticles (EMP) (≈100 nm to 1 μm in diameter) result from endothelial plasma membrane blebbing and carry endothelial proteins such as vascular endothelial cadherin, platelet endothelial cell adhesion molecule-1, intercellular cell adhesion molecule (ICAM)-1, endoglin, E-selectin, S-endo or αv integrin (Figure 1).[1] Endothelial NO synthase and vascular endothelial growth factor receptor (VEGF-R2) have also been identified on EMP,[2] but there is so far no evidence on whether or not MP endothelial nitric oxide synthase is capable of generating nitric oxide; furthermore, endothelial nitric oxide synthase may also be present on platelet or red blood cell–derived MP. E-selectin (CD62E) is expressed by activated endothelial cells but can equally be found on EMP generated following either tumor necrosis factor-α (TNF-α) activation or growth factor deprivation–induced apoptosis.[3] Identification of endothelial origin of circulating MP relies on the use of specific markers for flow cytometry analysis (see below); unfortunately, except E-selectin and vascular endothelial cadherin, most of them lack exclusive endothelial expression. Endoglin (CD105) is also expressed by activated monocytes/macrophages and bone marrow cell subsets; platelet endothelial cell adhesion molecule-1 (CD31) is present on activated platelets, platelet MP, and leukocyte subsets; S-endo (CD146) has been found on pericytes, tumor cells, and activated T-cells; ICAM-1 (CD54) is also expressed by leukocytes; and αv integrin (CD51) is present on monocytes/macrophages and platelets. Therefore, one must develop strategies combining multiple markers to exclude possible contaminating subpopulations to accurately assess the endothelial origin of MP in biological fluids. EMP accumulate over time in the conditioned medium of cultured endothelial cells, both under basal conditions and following stimulation (see below). In addition, the presence of EMP has been reported in human and murine plasma,[4,5] vitreous fluid,[6] urine (C.M. Boulanger, unpublished data, 2008) and in inflammatory lesions such as the atherosclerotic plaque or ischemic tissues.[2,7] As reported for other MP, EMP externalize phosphatidylserine (PS) and thus bind annexin V in a calcium-dependent manner. However, annexin V–negative membrane vesicles expressing endothelial markers have been identified in human plasma, suggesting that circulating EMP may not all externalize PS or that for yet unknown reasons, PS is unavailable for annexin V binding.[8] The protein composition of EMP highly depends on the stimulus triggering their release, and the identified proteins mostly originate from the plasma membrane, the cytosolic fraction,

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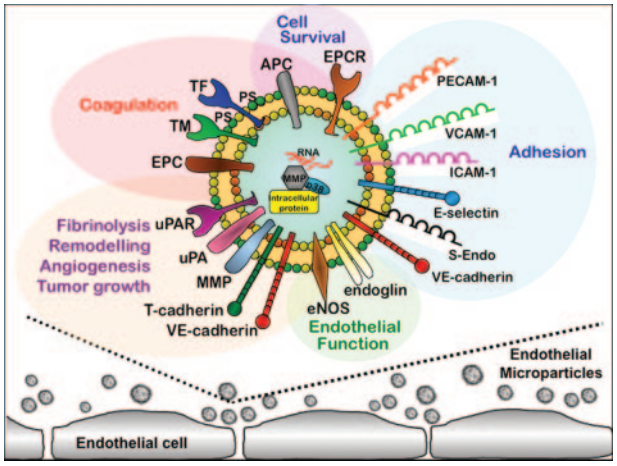

Figure 1. Schematic representation of the panel of molecules conveyed by EMP and the associated biological effects. EPCR indicates endothelial protein C receptor; PECAM-1, platelet endothelial cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; E-selectin, endothelial selectin; S-Endo, CD146/melanoma cell adhesion molecule; VE-cadherin, vascular endothelial cadherin; eNOS, endothelial NO synthase; MMP, matrix metalloproteases; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; EPC, endothelial protein C; TM, thrombomodulin.

the cytoskeleton, or mitochondria.[9] Unfortunately, there is so far no information regarding the lipid composition of EMP. Interestingly, MP, including those of endothelial origin, contain nuclear material such as DNA, RNA, and microRNA, which they transfer to target cells.[10–12] In most cases, the biological role of MP nuclear material remains to be determined, but Deregibus et al have shown that mRNA horizontal transfer from EMP to endothelial cells promotes angiogenesis following Akt activation and endothelial nitric oxide synthase expression.[10]

Endothelial Apoptotic Bodies and Exosomes

Exosomes (<100 nm in diameter) are produced in multivesicular bodies during endocytosis and they play a role in antigen presentation. Unlike MP, they do not externalize PS and they express specific exosomal markers such as Lamp1, CD63, and TSG101; they also contain RNA and microRNAs.[13] A recent study suggests that endothelial exosomes might be involved in vascular development as they incorporate and transfer Dll4 to neighboring endothelial cells, conferring a tip cell phenotype resulting in an inhibition of Notch signaling and an increased branching formation.[14]

Apoptotic bodies are larger than MP or exosomes and are characterized by externalized PS and, unlike MP, a permeable membrane facilitating propidium iodide staining of the nuclear material they contain.[15] Several reports indicate that apoptotic bodies are passive cargos delivering their nuclear content (oncogenes, DNA, microRNA) to phagocytes by horizontal transfer,[15,16] and thus they share this specific property with EMP.[10] Indeed, endothelial apoptotic bodies favor in vitro the differentiation of human endothelial progenitor cells[15] and stimulate the incorporation of Sca-1+ progenitor cells in atherosclerotic lesions by upregulating CXCL12 production following transfer of microRNA-126, thereby enhancing plaque stability.[17] Endothelial apoptotic bodies also circulate in human blood, and their content in micro-RNAs is altered in diabetic patients and possibly in patients with cardiovascular diseases.[11]

In summary, endothelial cells release distinct membrane vesicles, including MP, which are likely to have different impacts on neighboring cells. The rising interest for their specific physiopathological role should promote a better identification of these vesicles. A significant number of earlier studies need to be revisited, as they attributed biological effects either to exosomes, MP, or apoptotic bodies, without demonstrating the purity of the membrane vesicle preparation used.

Mechanisms of EMP Formation

The current knowledge of mechanisms of endothelial vesiculation derives mainly from experiments in isolated or cultured endothelial cells documenting their capacity to generate MP after activation by a variety of stimuli (Figure 2). Combes et al firstly described the generation of EMP from human umbilical vein endothelial cells stimulated by TNF-α.[4] Besides TNF-α, other inflammatory cytokines[18] and also bacterial lipopolysaccharides, reactive oxygen species,[18] plasminogen activator inhibitor,[19] thrombin,[20] camptothecin,[21] C-reactive protein,[22] and uremic toxins[23] are able to induce in vitro EMP generation. Interestingly, endogenous nitric oxide

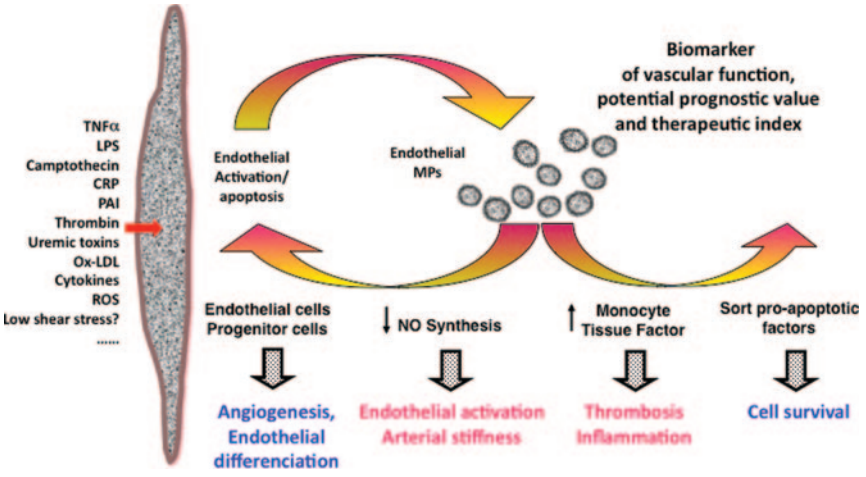

Figure 2. Schematic representation of the different agonists known to augment MP release from cultured endothelial cells and their paradoxical biological functions. EMP might have deleterious effects (shown in red), such as promoting endothelial activation or arterial stiffness or stimulating thrombosis and inflammation. Alternatively, EMP might have beneficial effects (shown in blue) because of stimulation of endothelial differentiation and angiogenesis; in addition, EMP could promote endothelial survival by incorporating caspase-3 during their formation, therefore banning noxious proapoptotic signals from the cellular body. LPS indicates lipopolysaccharide; CRP, C-reactive protein; PAI, plasminogen activator inhibitor; Ox-LDL, oxidized low-density lipoprotein; ROS, reactive oxygen species.

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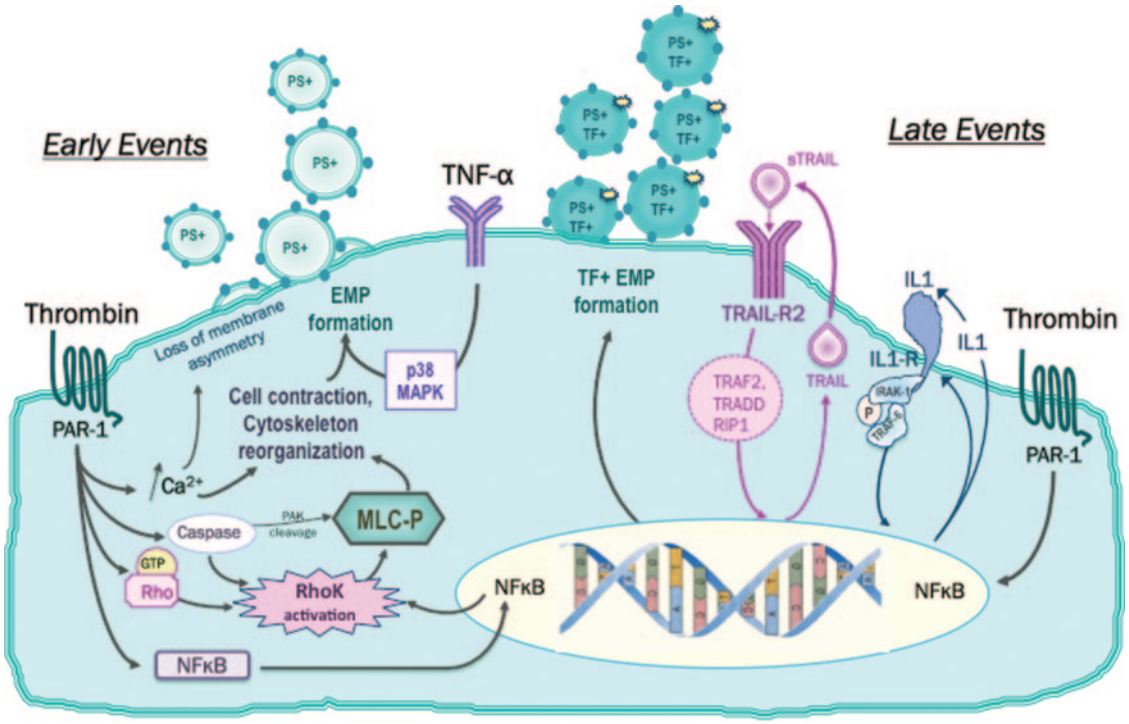

Figure 3. Schematic representation of the molecular mechanisms controlling the release of endothelial MP release on stimulation of PAR-1 receptor with thrombin or following TNF-α activation. The early phase of the thrombin response leads to the release of endothelial MP externalizing phosphatidylserine and involves caspase, Rho-kinase, and nuclear factor-κB (NFκB) activation; the late phase promotes the release of EMP expressing TF, which requires activation of NFκB and the expression of proinflammatory cytokines such as interleukin-1 (IL-1) and TRAIL/Apo2L, which then both fuel EMP release. MAPK indicates mitogen-activated protein kinase. PAR-1 indicates protease-activated receptor-1.

dampens the release of EMP on stimulation with C-reactive protein by a mechanism involving tetrahydrobiopterin[22] (Figure 3). Although the precise mechanisms involved in EMP release in vivo remain unknown, the strong inverse relationship between arterial shear stress and circulating EMP levels in patients with end-stage renal failure suggests that low shear stress levels stimulate EMP release.[24]

EMP release into extracellular fluid is the consequence of plasma membrane budding due to disruption of both phospholipid membrane asymmetry and cytoskeleton protein reorganization. It is generally admitted that remodeling of membrane phospholipids is a universal feature of cells undergoing activation or apoptosis, leading to exposure of PS on the outer leaflet as a consequence of the calcium-dependent dysregulation of scramblase, floppase/ABC1 and translocase/flippase activities.[25–27] Very few studies have analyzed the molecular mechanisms controlling MP release by endothelial cells. A study based on gene profiling analysis has identified an original pathway induced by thrombin, depending on the nuclear factor-κB activation, and involving the Rho-kinase ROCK-II activation by caspase 2 in the absence of cell death[20] (Figure 3). This mechanism proceeds with 2 steps: a first phase that occurs early after thrombin binding to its receptor protease-activated receptor-1 (PAR-1) and a second phase that depends on transcriptional events mediated by thrombin and involving TRAIL/Apo2L, a cytokine belonging to the TNF-α superfamily.[28] The transcription factor nuclear factor-κB was required for both early and late production of EMP by thrombin-stimulated cells. As additional targets of thrombin-induced EMP shedding, gene profiling study also identified interleukin-1 and interleukin-1Ra, which further recruit adaptor proteins TRAF6 and IRAQ1, which activate a signaling pathway leading to the amplification of EMP release.[29] Consequently, the inflammatory mediators regulated by thrombin represent an autocrine pathway that amplifies endothelial vesiculation (Figure 3). Whether or not intracellular pathways regulating EMP release are related to the general inflammatory response remains an open question. A recent study identified p38 mitogen-activated protein kinase as a critical pathway in the production of proinflammatory EMP[30] (Figure 3). In this study, EMP release triggered by TNF-α activation increased the release of soluble ICAM-1 secretion from endothelial cells, thus providing a paracrine loop enhancing the endothelial response to inflammation.

EMP as Vascular Injury Markers

Currently Available Methods to Numerate Circulating EMP Levels

Flow cytometry has been largely used to quantify EMP levels in clinical samples, although this widely used methodology suffers from limitations according to MP probing, sizing, and counting in a standardized manner. However, by reference to the other available methodologies, important recent improvements have maintained flow cytometry highly competitive to detect MP. First, the latest generation of instruments has greatly improved flow cytometry performance to detect MP of the smallest size (extending the limit to 0.1 μm). Second,

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microbeads calibrated in various sizes offer new options for standardized counting. For probing, EMP detection still presents the limitations of rare events detection and lacking fully specific antigens, and this holds true for all experimental approaches attempting to quantify EMP. CD144 was proposed as one of the most specific, though less sensitive, markers for EMP detection. Others strategies to counteract these difficulties have been developed as the combination of multicolor antibodies (CD31+/CD41−, CD31+/CD42b−, CD105+/CD45−) to improve the specificity and monochrome composite markers to improve the sensitivity (CD144+CD105+, CD146+CD105+).[31]

EMP Circulating Levels in Atherothrombosis: Potential Biomarker With Prognostic Value

Detectable levels of MP of different cellular origin circulate in the plasma of healthy subjects. Obviously, the presence of MP in plasma reflects an active balance between MP generation and clearance, but their respective contributions to circulating levels have not been thoroughly investigated, possibly because of scarce information regarding mechanisms regulating MP clearance from the blood.[32,33] Although MP of endothelial origin represent a sparse population of circulating MP, changes in their plasma levels might carry important clinical information in healthy subjects and in patients with cardiovascular disorders.[1] In patients presenting a characterized endothelial dysfunction, levels of circulating EMP are inversely correlated with the amplitude of flow-mediated dilatation, independently of age and pressure.[34–37] Furthermore, acute endothelial injury such as that induced by secondhand smoke rapidly impairs endothelial function and increases circulating EMP in young healthy subjects.[38] Therefore, EMP emerge as a new surrogate marker of endothelial health.

So far, only a few studies have investigated the prognostic potential of the measurement of EMP plasma levels. In patients with acute ischemic stroke, EMP levels are associated with lesion volume and clinical outcome, but there was no report about clinical events during follow-up.[39] In patients with pulmonary hypertension, circulating levels of EMP expressing E-selectin predict the 1-year outcome.[40] In subjects with high risk of coronary heart disease, baseline levels of EMP expressing vascular endothelial cadherin predicted outcome, independently of Framingham score and of C-reactive protein (CRP) and brain natriuretic peptide levels.[41] Similar findings were observed in chronic renal failure, where high values of $CD31^{+}CD41-$ EMP were independent predictors of cardiovascular death, whereas other MP plasma subpopulations had no prognostic value.[42] These data suggest that EMP levels may be used in the future as a biomarker for stratification of patients and identification of subjects with a high risk of developing cardiovascular complications. Recently, an interest in multimarker strategies combining EMP with endothelial progenitor cell levels has emerged from the literature as an integrative marker of vascular health. A change in the ratio of EMP to endothelial progenitor cells may reflect an imbalance between endothelial damage and repair that could be useful to identify patients with damaged vasculature.[43,44]

Medication affects MP plasma levels, although their real impact on cell blebbing and MP release is difficult to distinguish from the benefit resulting from a better control of pathological process and risk factors that would also ameliorate cellular injury and in turn affect MP levels. Antioxidant agents, such as vitamin C, improve circulating EMP levels in patients with diabetes and dyslipidemia after myocardial infarction.[45] The effect of statins on EMP release remains controversial: on one hand, clinically relevant concentrations of statins appear to stimulate in vitro endothelial detachment and MP release by inhibiting prenylation[46]; on the other hand, in vitro data have shown that 3-hydroxy-3-methylglutaryl–coenzyme A reductase inhibitors (statins) may have an antiinflammatory effect on endothelial cells, leading to a decreased release of EMP following inhibition of the Rho-kinase pathway.[47] Several beneficial therapies were reported to reduce circulating MP levels in cardiovascular disorders. For example, patients with type 2 diabetes who were treated with the calcium antagonist nifepidine showed a reduced level of endothelial cell–derived MP.[48] Administration of benidipine, another channel calcium blocker, decreased concentrations of EMP in hypertensive patients with type 2 diabetes.[49] Moreover, diabetic patients treated with eicosapentaenoic acid showed a significant decrease of their EMP plasma concentrations.[50] In patients with metabolic syndrome, pioglitazone administration diminished EMP levels.[51] Intravitreal anti–vascular endothelial growth factor injection decreased vitreous EMP shed following proliferative diabetic retinopathy.[6] Thus, these studies bring new insights into the understanding of the mechanisms of EMP generation and the development of associated diseases.

Multifaceted Roles of EMP

Effects of EMP in Thrombosis and Inflammation

A direct consequence of PS expression on EMP results from the ability of PS to bind and activate coagulation factors, conferring a procoagulant potential to EMP. In addition to PS exposure, EMP harbor tissue factor (TF), the initiator of the extrinsic coagulation pathway.[4] The capacity of EMP to mediate thrombin generation was first demonstrated using cultured endothelial cells by the reduction of the clotting time of normal plasma incubated with increasing amounts of EMP released in vitro. The thrombogenic activity of EMP was then confirmed by the demonstration that EMP triggered TF-dependent thrombin formation in vitro and thrombus formation in vivo.[52] Moreover, TF-positive EMP expressing endothelial adhesive molecules can bind to other cell types, such as monocytes, and transfer bioactive TF in vitro.[53] A possible TF transfer from TF-bearing EMP to activated platelets could be also involved in this procoagulant response, because such a mechanism was reported for TF-exposing leukocyte MP.[54] Although convincing studies support a role for hematopoietic-derived TF bearing MP in thrombus formation, the potential contribution of vessel wall–derived TF MP in vivo is still controversial and continues to be explored.

Experiments in cultured endothelial cells indicated that EMP release correlated with interleukin-6 release, implying a close relationship between endothelial vesiculation and classic inflammatory pathways of cytokine production.[30] In addition, interaction between EMP and naïve endothelial cells triggers proinflammatory responses assessed by upregulation of ICAM mRNA expression and soluble ICAM shedding

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from targeted cells. This paracrine effect of MP was influenced by conditions of EMP generation because it could not be observed using EMP from unstimulated endothelial cells. Thus, these data integrate EMP as both a cause and a consequence of the inflammatory response. Therefore, EMP appear as bioactive vectors amplifying the bidirectional relationship between inflammation and thrombosis.

In vivo, the contribution of endothelial cells to the circulating pool of TF positive MP has been documented in human diseases, including sickle cell anemia and endotoxemia.[8,55] Procoagulant EMP have also been found in atherosclerotic plaques[7,56] and in patients with acute coronary syndrome.[57] Thus, by exposing PS and TF, EMP behave as biological vectors potentially contributing to a procoagulant potential. However, recent studies have demonstrated that EMP can also expose endothelial protein C receptor and exhibit anticoagulant properties,[58,59] suggesting that EMP participate in the tuning of the procoagulant/anticoagulant equilibrium. Moreover, EMP also behave as a surface supporting plasmin generation by expressing the urokinase-type plasminogen activator and its receptor,[60] which confer on them fibrinolytic properties with a pivotal role in clot dissolution.

Role of EMP in Endothelial Cell Survival

Abid Hussein et al first documented the potential contribution of EMP in endothelial cell survival by showing that EMP release could protect endothelial cell apoptosis by diminishing levels of caspase-3 in cultured endothelial cells resulting from trapping caspase-3 in MP.[61] Thus, endothelial-derived MP contribute to the sorting of several proapoptotic factors preventing cell detachment and apoptosis (Figure 2). In a more recent study, it was demonstrated that statins improve the overall condition of the remaining vascular endothelium by facilitating this EMP release in vitro.[46] Nevertheless, the effect of statins on EMP release is still a matter of debate.[47,62] Finally, EMP carrying endothelial protein C receptor and activated protein C (APC) could also promote cell survival by induction of cytoprotective and antiinflammatory effects[58] (Figure 1).

The plasmin generation capacity of EMP[60] confers on them a pivotal role in maintaining vascular patency. Indeed, the proteolytic potential of the plasminogen activation system affects the angiogenic potential of endothelial progenitor cell in vitro.[60] By conveying plasmin, EMP activate matrix metalloproteases, which are involved in the extracellular matrix degradation and the release of growth factors that play a crucial role in tissue remodeling, angiogenesis, and cancer spreading. Other in vitro studies provided evidence that EMP convey angiogenic messages supported by proteases belonging to the matrix metalloprotease family[63] and also by a horizontal transfer of mRNA, able to activate a proangiogenic program in endothelial cells.[10] These findings were confirmed in vivo by a recent study demonstrating that EMP derived from ischemic muscle promote in vitro endothelial proliferation and in vivo postnatal vasculogenesis.[2] However, the involvement of EMP in angiogenic response and vascular repair is still controversial. For example, Mezentsev et al demonstrated that EMP reduced endothelial proliferation, as well as formation of new vessels in vitro.[64] Taraboletti et al reported that MP isolated from human umbilical vein endothelial cells promoted formation of capillary-like structures by endothelial cells in low concentrations, whereas high levels of EMP abolished angiogenesis.[63] Finally, a recent study showed that T-cadherin is present on EMP and is responsible for angiogenic behavior on target endothelial cells via homophilic interactions and induction of Akt phosphorylation.[65] Thus, EMP might influence the endothelial regeneration via 2 mechanisms: they could directly interact with endothelial cells and promote vascular regeneration or they might activate endothelial progenitor cells, supporting endothelial repair.[66] Whether or not EMP affect endothelial progenitor cells mobilization remains an open question. Therefore, the proangiogenic role of EMP and the associated mechanisms need to be further elucidated and will open new perspectives for MP pathological implications.

Are EMP Friends or Foes?

In conclusion, EMP can be considered complex structures displaying a large repertoire of endothelial molecules and biological functions, depending on their composition. When the data are taken together, the involvement of EMP in vascular homeostasis appears to be more complex than initially thought. EMP can play a major role in inflammation, thrombosis, and angiogenesis. However, depending on the pathological context, the mechanisms and sites of formation, EMP could have favorable effects to maintain vascular homeostasis. These paradoxical functions might result from EMP composition, as proteomic analysis has shown that one third of the proteins found on EMP are specific to the stimulus initiating their release, not only demonstrating the plasticity of these vesicles but also revealing the complexity of the mechanisms governing their formation.[9] However, it remains to be firmly established that these different EMP "phenotypes" are also found in vivo, and it remains to be determined whether or not they differentially affect the biological effects of EMP.

The initial vision was that MP were noxious, supporting proinflammatory, procoagulant potential and inhibiting vascular repair. Increased levels of MP of endothelial origin in various pathologies, such as atherothrombosis, vasculariti, and sepsis, also support this noxious potential. However, recent data have brought to light the potential beneficial effect of EMP on endothelial integrity, such as stimulation of vascular repair, control of cell death mechanisms or cytoprotective activities supported by APC, or induction of adaptive immunity. All these regulatory functions have been delineated mostly using in vitro systems, and obviously, their in vivo relevance remains to be fully demonstrated.

EMP were first considered to have a role in sepsis as conveyers of deleterious biological information for endothelial function, triggering blood coagulation and death. Actually, EMP could have beneficial effects during sepsis by harboring functional endothelial protein C receptor and APC. Moreover, APC positive EMP are able to inhibit the release of procoagulant MP and display cytoprotective effects on endothelial cells through the reduction of apoptosis.[67] These recent data confirm the complex, ambivalent role of EMP in each physiological process, as they can either promote or inhibit coagulation, inflammation, or angiogenesis, and they

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may consequently orientate the evolution of the disease associated with increased release of EMP.

Promotion of angiogenic processes by EMP may have both beneficial and deleterious effects. EMP could be endogenous survival signals responsible for vascular repair in ischemic tissues. However, promotion of angiogenic response may also have deleterious effects in cancer spreading, proliferative diabetic retinopathy,[6] or atherosclerotic plaque destabilization by promoting intraplaque neovascularization.[68] These examples suggest that involvement of EMP in vascular homeostasis is more complex than initially thought and demonstrate that further studies are needed to dissect the mechanisms involved.

In summary, EMP express a large repertoire of endothelial molecules and biological functions that are related to their potential involvement in the tuning of vascular homeostasis. The notion that MP are new conveyors of biological information in vascular biology is an exciting prospect. However, proof of this concept remains to be fully established in vivo and poses important challenges: first, to develop animal models to better understand the pathophysiological role of MP; second, to extend our knowledge on the mechanisms controlling endothelial vesiculation and MP release; third, to decipher the mechanisms governing MP tethering and binding to target cells; and finally, to manipulate MP generation by pharmacological approaches, a critical step in answering the question of whether or not MP are good or deleterious. There is no doubt that future in vivo and in vitro studies will delineate when and how EMP play Dr Jekyll or Mr Hyde.

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Disclosures

None.

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