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Cellular Microparticles In The Pathogenesis Of Pulmonary Hypertension

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Abstract

Pulmonary hypertension (PH) is a fatal disease with no treatment options characterized by elevated pulmonary vascular resistance and secondary right ventricular failure. The etiology of PAH is multiple and its pathogenesis is complex. Although the exact role of cellular microparticles (MPs) remains partially understood, there is increasing evidence to suggest an active role for MPs in PH pathophysiology. Patients with PH exhibited higher circulating levels of MPs compared to controls subjects and *in vitro* or *in vivo* generated MPs can induce endothelial dysfunction, interfere with coagulation pathways or modulate inflammatory phenomenon. Whether or not these new conveyors of biological information contribute to the acquisition and/or maintenance to the altered endothelial phenotype is unexplored in PH and requires further study.

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Key words: *Pulmonary arterial hypertension; Microvesicles; Endothelial dysfunction; Pulmonary vascular remodelling; Cellular cross talk.*

Introduction

Pulmonary hypertension (PH) is characterized by widespread obstruction and obliteration of pulmonary arterioles, leading to a progressive elevation in pulmonary vascular resistance and subsequent right-heart failure and death. The exact mechanisms of pulmonary arterial remodelling that lead to the onset and progression of PH are still largely unclear. However, many disease-predisposing factors and/or contributing factors have been identified, including inflammation, endothelial cell dysfunction, aberrant vascular wall cell proliferation and mutations in the *bone morphogenetic protein-receptor type 2* (*Bmpr2*) gene [1-3].

Precise and dynamic cross talk between cells is critical for cellular behaviour, including for cell proliferation, apoptosis, differentiation, migration and survival, and thus crucial for proper tissue organization and homeostasis; abnormal cellular cross talk can lead to the development of cancer and PH [4, 5]. These well-regulated processes to transfer information between cells occur through direct cell-cell contact, various soluble bioactive factors and through cellular microparticles (MPs) [6, 7].

MPs are plasma membrane vesicle fragments (between 0.1 and 1 μm in size) released from various cell types during activation by agonists or physical or chemical stress, including apoptosis [6, 7]. MPs in body fluids constitute a heterogeneous population, differing in cellular origin (endothelial cells (ECs), platelets, leukocytes and erythrocytes), number, size, antigenic composition and functional properties. Their concentration increases specifically and differently during various pathological states, including cardiovascular diseases (Table 1) [8]. Indeed, patients with PH (Group 1 and 3 with no associated cardiovascular disease) exhibit higher circulating levels of MPs compared to control subjects [9-11]. This increase might indicate a potential involvement of MPs in the disease onset and its progression. This article reviews the evidence supporting potential roles and importance for MPs in the pathogenesis of PH.

General considerations on Microparticles

Detectable levels of MPs of different cellular origin (platelets, red blood cells, leukocytes, endothelial cells) circulate in the plasma of healthy subjects (Figure 1B). Circulating MPs levels increase in a wide range of cardiovascular diseases, including uncontrolled cardiovascular risk factors, stable and unstable atherosclerotic lesions, heart failure, arrhythmias and inflammatory vascular diseases (Table 1) [8]. The presence of MPs in plasma reflects an active balance between MP generation and clearance. Both phagocytic activities present in the liver and the spleen contribute to the removal of MPs from plasma of healthy animals [12, 13]. Changes in circulating levels of MPs might bear important clinical information in healthy subjects and in patients with cardiovascular disorders. MPs display various bioactive substances and receptors on their surface and harbour a concentrated set of cytokines, signaling proteins, lipids, and nucleic acids. The composition of MPs, which subtends their biological effect, is a function of the parent cell they stem from as well as the stimulus used for their generation. Thus, the amount and nature of circulating MPs influence their beneficial or deleterious biological effects in physiological or pathological conditions. Indeed, various experimental evidences reported significant interrelations between circulating MPs (either generated *in vivo* or *in vitro*) and other protagonists of disrupted vascular homeostasis. MPs can modulate vascular reactivity (particularly endothelial dependent relaxation), interfere with coagulation pathways, enhance or decrease inflammatory phenomenon and influence neo-angiogenesis process. A large part of our knowledge regarding MPs is based on data issued from the research on athero-thrombotic disease [14]. However, recent data shed the light on their role as vectors of cellular cross talk with implications in the pathogenesis of non-atherosclerotic cardiovascular conditions, including pulmonary vascular diseases (Table 1).

Circulating Microparticles as Regulators of Endothelial Dysfunction

Endothelial dysfunction is a pivotal element in the development and progression of PH, irrespective of disease origin [15]. The dysfunctional endothelium displays to varying degrees an imbalanced production of several mediators (vasoconstrictors versus vasodilators; activators versus inhibitors of smooth muscle cell growth; proinflammatory versus anti-inflammatory signals; prothrombotic versus antithrombotic substances) leading towards an excess of vasoconstriction, smooth muscle hyperplasia and pulmonary vascular remodeling [1-3]. In addition, disturbances of the normal balance between endothelial cell proliferation and endothelial cell apoptosis have been reported in idiopathic PAH [16, 17]. Indeed, accumulating evidence supports the concept that increased endothelial apoptosis at early stage and decreased endothelial apoptosis at later stages of the disease could contribute to PAH [18]. Consistent with this hypothesis, we have recently reported that primary pulmonary endothelial cells generated from PAH lung specimens exhibit various intrinsic abnormalities and present a modified pro-proliferative, apoptotic-resistant phenotype [4, 17, 19]. Although we have shown that increased activity of the FGF2 autocrine loop is among the mechanisms needed to acquire this altered endothelial phenotype in PAH [17], the exact nature of pulmonary endothelial cell modification during PAH and the balance between apoptotic and anti-apoptotic phenotypes remain only partially understood. In addition, alterations of endothelial cell monolayer integrity in mice, such as a selective disruption of the endothelial peroxisome proliferator-activated receptor (PPAR)γ or of the endothelial BMPR2 signaling, are sufficient to predispose to PH [20, 21].

Different groups have reported increased concentrations of different subsets of circulating endothelial MPs (EMPs) in PH patients compared to controls subjects [9-11], supporting the presence of an increased structural damage with enhanced cellular activation and/or apoptosis. Indeed, the levels of PECAM+ and VE-cadherin+ EMPs were also correlated with several hemodynamics pulmonary vascular remodeling parameters [10] (Figure 1) and inflammation biomarkers [11]. The etiology of the disease appears also to

influence the MPs levels: Amabile et al. reported that PAH patients had higher levels of EMPs compared to chronic pulmonary disease related PH subjects [10], whereas Diehl et al. observed higher levels of EMPs bearing E-selectin in thrombo-embolic PH compared to non-thrombo-embolic PH subjects [11].

The origin of these circulating MPs (systemic vs. pulmonary endothelium) is still a matter of debate. Although a trans-pulmonary gradient of CD105+ (endoglin) EMPs has been observed, suggesting a local release of MPs within the pulmonary vascular bed, no significant change in CD31+ (PECAM), CD144+ (VE-Cadherin) and CD62e+ (E-selectin) EMP levels was observed through the pulmonary vasculature [9, 10]. These findings, observed in small samples of patients with various PH origins, could reflect the heterogeneity of the endothelial changes among patients with pulmonary vascular disease, depending on the underlying etiology or the lesion development stage (early vs. late stage). These findings might support the notion that some specific EMP subpopulations could be released by either systemic or pulmonary endothelium. Finally, it cannot be excluded that a lack of sensitivity of the measurement methods for detection of mild variations in concentrations of MPs could also explain these results.

The circulating MPs are essential vectors of biological informations and are involved in cellular communication through a paracrine or endocrine action [22]. Cellular effects of MPs are directly influenced by the various antigens harbored at their surface, which is a function of the cellular origin and the stimulus involved in the vesiculation process [8]. *In vitro* or *in vivo* generated MPs can induce endothelial dysfunction, interfere with coagulation pathways or modulate inflammatory phenomenon [8]. Accordingly, Tual-Chalot *et al*. reported that circulating MPs isolated from hypoxia-induced PH rats could affect the vascular tone [23]. They showed that these MPs, mainly of platelet and erythrocyte origin, impaired the endothelium-dependent vaso-relaxation in pulmonary arteries *ex vivo* and decreased the nitric oxide (NO) production by pulmonary endothelial cells through increase in oxidative stress [23]. The authors observed comparable results in aorta and pulmonary arteries [23].

Taken together, all these experimental findings support the notion that circulating MPs could contribute to both initiation and/or amplification of the pulmonary endothelial dysfunction during PH and encourage further studies of the precise pathogenic mechanism underlying this phenomenon. Moreover, we cannot exclude that the generation of MPs and their migration to the systemic circulation could mediate a cross-talk between pulmonary and systemic endothelium and therefore explain the peripheral endothelial dysfunction observed during PH [21, 24, 25].

Finally, measurement of circulating EMPs might also represent a potential new prognostic tool in patients with PH. Preliminary data reported that baseline values of circulating CD62e+ MPs, measured before initiation of any specific therapy, predicted occurrence of adverse outcome (death+ readmission for right heart failure) in a small cohort of patients with mixed etiologies of PH [26]. Whereas these results were not observed for other EMP subsets (CD144+ and CD31+ EMPs) and should be confirmed in a larger population sample, they are in line with data reported in coronary artery disease [27], heart failure [28] or chronic end-stage renal failure [29] and thus confirm their potential role as a biomarker for outcome prediction in patients with cardiovascular diseases [30].

Circulating Microparticles as Regulators of Inflammation

It is now well established that inflammation plays an important role in PH in humans: histological studies of the PAH lung specimens demonstrate a prominent perivascular infiltration composed of macrophages, dendritic cells, T and B lymphocytes, and mast cells; circulating levels of certain cytokines and chemokines are elevated including interleukin (IL)-1β, IL-6, IL-8, CX3CL1, CCL2, CCL5 and tumor necrosis factor (TNF)-α serum levels, and these may correlate with a worse clinical outcome. Evidence supporting a role for inflammation in the progression of the disease have been reviewed recently by Price *et al.* [31]. However, the initial process triggering inflammation onset remains unknown.

Cellular MPs are key actors in the inflammatory process because they contribute to the endothelial production of various pro-inflammatory cytokines and chemokines. MPs have been shown to stimulate the release of IL-1ß, IL-6, CCL2, and induce the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin [32-35]. In addition, MPs provide a preferential substrate for non-pancreatic secretory phospholipase A2 (sPLA2) and thus a source of lysophosphatic acid, a potent pro-inflammatory mediator and platelet agonist [36]. Furthermore, a recent study has shown that MPs from septic shock patients increase the expression of endothelial and inducible nitric oxide synthase (iNOS), cyclooxygenase-2, and nuclear factor-κB in heart and lung [37]. In addition, Densmore *et al.* have demonstrated that endothelium-derived MPs sequestration in the lung is capable of initiating inflammatory cascades at pathophysiologically relevant concentrations [38, 39]. Altogether, these findings support the notion that circulating MPs could actively contribute to the initiation and/or amplification of the pulmonary vascular inflammation in the pathogenesis of PH.

Procoagulant Properties of Microparticles

Thrombosis is a key mechanism in onset and progression of cardiovascular diseases, including venous thromboembolic disease, atherosclerosis and PH. Chronic thromboembolic PH results from the obstruction of the pulmonary arteries by clot fragments migrated from deep venous thrombosis [40]. On the other hand, PAH pathological specimens often display thrombotic lesions in the absence of clinical or pathological evidence of pulmonary embolism [41], suggesting an *in situ* clotting phenomenon [42]. Furthermore, PH is associated with a hypercoagulable phenotype [43] that includes vascular up-regulation of tissue factor [44] and an increase in circulating levels of Von Willebrand factor or fibrinopeptide A [45].

MPs are involved in thrombus generation by different mechanisms. MPs can provide the anionic phospholipid surface necessary for the assembly of clotting enzyme complexes (VIIIa and Xa) promoting thrombin generation. In some cases, MPs also directly expose

tissue factor, the main cellular initiator of blood coagulation, at their surface. Furthermore, MPs can also harbour other functional membrane or cytoplasmic effectors (such as selectins, GPIIbIIIa, GPIb, von Willebrand factor, arachidonic acid, thromboxane A2, etc.) that can promote prothrombotic responses [22]. Therefore, MPs can promote thrombin generation *in vitro,* but also favour thrombus propagation *in vivo* [46] or transfer their procoagulant potential to target cells. The thrombogenic potential of MPs has been demonstrated for several subpopulations, including platelets [47], endothelial [48, 49] or leukocytes derived MPs [50].

Most of our knowledge regarding MPs' procoagulant activity derives from data observed in atherosclerosis, cardiac surgery and conditions associated with cardiomyocyte inflammatory stimulation, such as cardiac ischemia.

An increase in circulating global procoagulants as well as TF+ MPs (MPs bearing tissue factor) levels has been reported in patients with PAH [9]. Furthermore, the levels of TF+ MPs and von Willebrand factors were correlated. Interestingly, the highest levels of TF+-MPs were observed in patients with functional class III-IV and/or the weakest 6-min test performance, suggesting a potential correlation between MPs and functional status. Comparable results were observed in patients with recurrent deep venous thrombosis, a potential trigger for acute pulmonary embolism development [51] .

The precise molecular mechanisms governing these biological effects of MPs are still under investigation, but several elements support the notion that procoagulant MPs might interfere with the coagulation process in PH patients and promote the disease development in different ways. High levels of procoagulant circulating MPs could favour recurrent deep venous thrombosis [52], which will in turn create silent pulmonary embolism and ultimately lead to post-thromboembolic PH [40, 53]. On the other hand, the plexiform lesions and the dysfunctional endothelium could release procoagulant MPs, which could lead to propagation of the thrombotic process among the pulmonary vessels. The resulting local thrombosis could worsen the microvascular obstruction and thus increase pulmonary artery pressure

(PAP). This hypothesis is supported by the results from Bakouboula *et al*., who observed an increase in the concentration of the global number of procoagulant MPs between the jugular vein and the wedged pulmonary artery, suggesting a local release of procoagulant microvesicles [9]. However, the authors reported no significant transpulmonary gradient of TF+ MPs, which might be explained by the small size of the group (n=20) and the heterogeous etiologies of PH [9]. Moreover, proteomics analysis in experimental models of post thromboembolic PH have shown that this condition was associated with an increased expression of coagulation proteins carried by circulating MPs [54].

Potential implications of MPs in PH pathophysiology and remaining questions

In the light of the above-mentioned data, the involvement of MPs in PH pathogenesis seems to be, at least, plausible. Experimental and clinical data suggest multiple roles of MPs in the progression of the disease and one might hypothesize a possible global scheme (Figure 1C). Pro-coagulant MPs can enhance local and systemic thrombogenicity leading to pulmonary artery clot. Moreover, pro-inflammatory MPs could also increase endothelial inflammation, local cytokine and growth factors release. Furthermore, MPs might also favor endothelial cell proliferation. Finally, circulating MPs can enhance endothelial dysfunction, decrease NO release and deteriorate the vasodilation/vasoconstriction humoral balance. Altogether, these effects can support pulmonary vessel remodeling and vascular obstruction and should encourage further *in vivo* and *in vitro* studies to precise their pathogenic mechanisms. In addition, a very recent study has reported that epoprostenol could inhibit human platelet-leukocyte mixed conjugate and platelet microparticle formation in whole blood, demonstrating that treatments might interfere with MPs and some of their biological role [55]. Since our knowledge on microparticles properties *in vivo* might also be influenced by the current methods used for their isolation and their measurements, there is a need to

establish a consensus regarding the standard centrifugation method to prepare poor platelets plasma from whole blood samples in order to determine MPs phenotypes and levels. Nowadays, the appropriate method for MPs measurement is still a matter of debate [56]. Flow cytometry methods have been widely used during the past 20 years, need small samples volumes and allow complete phenotypical analysis. However, flow cytometry doesn't analyze MPs function and is limited by its poor sensitivity, resulting from the biophysical and optical properties of laser interaction with small vesicles (<500 μm). The "capture" and other purely biological methods inform on MPs function but don't provide any direct quantification. Dynamic light scattering, atomic force microscopy, automated flow through single-particle optical microscopy or impedance based flow cytometry represent promising methods for the future, allowing more accurate MPs quantification, but these methods are not currently adapted for clinical research as they are limited by their cost or their availability.

In addition to these potential pathogenic roles of MPs in PH, MPs are well-known modulators of different stages of angiogenesis. These particular effects of MPs depend on the stimulation and activation status of the cell from which they originate from and also on their concentration [57, 58]. Indeed, MPs from platelets display pro-angiogenic properties by promoting capillary-like structures and pro-angiogenic factor production [59]. By contrast, lymphocyte- and endothelial-derived MPs exhibit pro- or anti-angiogenic properties depending on the stimuli used for their production [57, 60-63]. These additional properties of MPs reinforce the idea that they could be potential key actors in PH pathogenesis in the early-, middle- and/or late-stage of the disease.

Whether or not these new conveyors of biological information contribute to the acquisition and/or maintenance to the altered endothelial phenotype is not fully explored in PH and requires further study. Thus, several questions remain regarding their nature and true importance in this disease. What are the compositions of MPs in PH and what are their functions? What causes increased amounts of circulating MPs in PH? Are they beneficial or

deleterious in PH? Are they useful for disease recurrence, prognosis, and monitoring of therapeutic efficacy? By which potential mechanism(s) do MPs initiate and propagate PH?

Conclusion

Patients with PH exhibit higher circulating levels of MPs compared to control subjects and it is unclear whether these new conveyors of biological information contribute to the acquisition and/or maintenance to the altered endothelial phenotype or are just a consequence of the disease progression. However, there is strong accumulating evidence that MPs are key protagonists of the disease and they could serve as a worthy biomarker for prognosis stratification and therapeutic efficiency monitoring. Therefore, a better understanding of their importance in the disease is essential and efforts should be made in the future to improve our knowledge by further exploring cellular aspects as well as clinical implications of MPs in pulmonary hypertension.

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Table 1: Microparticle (MPs) involvement in cardiovascular diseases. Adapted from Amabile *et al.* [8]. EMPs indicates endothelial microparticles; PMPs: Platelet microparticles; LMPs: leukocyte-derived microparticles; RBCMPs: red blood cell-derived microparticles; TF: Tissue factor; PS: Phosphatidylserine.

CARDIOVASCULAR RISK FACTORS	
Diabetes	TF-MPs, CD8+MPs, CD66+MPs, PMPs CD51+ EMPs, PMPs, PS+MPs
Severe systemic hypertension	CD31+ EMPs, PMPs
Metabolic syndrome	CD31+ EMPs, CD51+ EMPs
Hypertriglyceridemia	CD31+ EMPs
ENDOTHELIAL DYSFUNCTION (ED)	
Acute endothelial dysfunction	CD31+EMPs, CD144+ EMPs, CD62e+ EMPs
Chronic renal failure with ED	CD31+EMPs, CD144+EMPs, PMPs, RBCMPs, PS+ MPs
Coronary artery disease with ED	CD31+/PS+ MPs
Diabetes with ED	CD144+ EMPs
Obesity with ED	CD31+/42- EMPs
ATHEROSCLEROSIS	
Subclinical atherosclerosis	CD11a+ LMPs
Coronary calcification	CD62e+ EMPs, PMPs
Acute coronary syndrome	PS+ MPs, CD31+ MPs, CD146+ EMPs CD31+ EMPs, CD51+ EMPs, PMPs
Acute coronary syndrome (cont.)	PS+ MPs, CD31+EMPs, PMPs
Stable coronary artery disease	CD31+ EMPs, CD51+ EMPs
Acute stroke	CD105+/144+ EMPs, CD105+/54+ EMPs, CD105+/PS+ EMPs
Cerebrovascular atherosclerosis	CD62e+EMPs, CD31+ EMPs
Peripheral artery disease	PMPs
HEART FAILURE & VALVULAR DISEASE	
Congestive heart failure	CD62e+ EMPs, CD31+ EMPs
Cyanotic congenital heart disease	PMPs
Heart transplant rejection	CD62e+ EMPs, TF+-MPs, FAS-MPs
Pulmonary hypertension	CD31+ EMPs, CD144+EMPs, CD62e+EMPs, CD45+ LMPs CD105+ EMPs, TF+-MPs
Severe Aortic Stenosis	CD62e+EMPs, CD11+ LMPs
ATRIAL FIBRILLATION	PS+ MPs, PMPs
VENOUS THROMBOEMBOLISM	CD31+EMPs, CD62e+ EMPs
OTHER VASCULAR DISEASES	
Inflammatory vasculitis	CD105+ EMPs, CD62e+ EMPs, PMPs PS+ MPs, PMPs
Antiphospholipid syndrome	CD31+/ CD51+ EMPs,
Preeclampsia	CD4+MPs, CD8+MPs, CD66+ LMPs CD31+ EMPs

<u>Figure 1:</u> Relationships between endothelial MPs (EMPs) and pulmonary vascular resistance in patients with pulmonary hypertension (PH) (A; adapted from[10]), the most important known types of circulating MPs, and proposed model for the role of microparticles in the PH pathogenesis (C): high circulating levels of EMPs in patients with PH (Group 1 and 3 with no associated cardiovascular disease) could: 1) contribute, at least in part, to the initiation and/ or perpetuation of the endothelial dysfunction and decreased the nitric oxide (NO) production by pulmonary endothelial cells through increase in oxidative stress; 2) interfere with coagulation pathways; and/ or 3) modulate inflammation through the stimulation of the release of various key cytokines, and/ or via induction of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin at the endothelial cell surface. *EMPs indicates endothelial microparticles; IL, interleukin; CCL2, chemokine ligand 2; ICAM-1, Intercellular Adhesion Molecule-1; VCAM-1, vascular cell adhesion molecule 1; eNOS, endothelial NOS.*