Pathophysiological significance of procoagulant microvesicles in cancer disease and progression

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Summary
Microvesicles (MV) are submicrometric membrane fragments (0.1 to 1 µm), released from the plasma membrane of activated or apoptotic cells. They are characterized by most of the antigenic profile of the cells they originate from, and by the presence of procoagulant phospholipids at their surface. MV are detectable in the peripheral blood of mammals and considered as efficient effectors in the haemostatic or thrombotic responses, able to remotely initiate or amplify beneficial or deleterious processes, depending on the circumstances. Variations in their level and phenotype make them relevant pathogenic markers of thrombotic disorders and vascular damage. To date, MV are recognized as mediators of communication allowing cells to influence a target present in the local microenvironment as well as to distant sites. The mechanisms by which MV interact with target cells are still unclear, but a number of studies suggest involvement of MV-cell fusion or ligand-receptor interactions. More importantly, MV have been shown implicated in horizontal transfer of genetic material. This review focuses on the role of MV in the context of cancer, and their possible part in cancer associated thrombosis.

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Aft er cell stimulation, rapid phosphatidylserine (PS) and phosphatidylethanolamine egress occurs in the outer leaflet of plasma membrane, followed by a slow influx of phosphatidyicholine and sphingomyelin to the inner leaflet, producing a transient phospholipid mass imbalance in the outer leaflet at the expense of inner one. This phospholipid overload leads to “bleb” formation (8, 73). The resulting surface tension is resolved through the release of membrane fragments called microvesicles (MV) or microparticles. Although the term is neither specific nor fully descriptive, it has been used for 30 years and remains standard in the current literature (22).

In general, MV are phospholipid vesicles of 0.1–1 µm in diameter derived from the eukaryotic cell plasma membrane as the result of an active membrane remodelling process. A wide spectrum of MV phenotypes correlating with the cellular origin and type of stimulation can be observed. It is well known that media obtained from growing cell cultures and analyzed by fluorescence-activated cell sorter contain MV, further identified by electron microscopy. The proportion of MV circulating in peripheral blood increases during

- cell injury,
- inflammation,
- thrombosis, and
- platelet activation.

Thus, MV levels are elevated in the blood of patients suffering from infection or cardiovascular disorders (27, 46, 68). Moreover, in patients with severe inflammatory syndrome, endothelial MV were significantly increased (48). MV are also secreted by tumour cells (TMV) explaining why they circulate at high level in venous blood of cancer patients (23, 32, 60). MV are considered to have pathophysiological importance in relation with the haemostatic system because they expose procoagulant anionic phospholipids such as PS in a similar fashion as activated cells, chiefly activated platelets. The loss of membrane phospholipid asymmetry is related to a number of pathophysiological mechanisms. In haemostasis, the exposure of PS at the platelet surface promotes coagulation cascade reactions. This remodelling, subsequent to intracellular Ca2+ increase, is thought to be induced by thrombin/collagen combination in physiology or by the complement attack complex C5b9 in inflammation for instance. MV not only carry accessible PS but also membrane antigens including adhesion proteins or complexes, which can be active, and other procoagulant entities such as tissue factor (TF), when they stem from TF+ cells (59).

TF, the main trigger of the coagulation cascade (39), is implicated in cellular signalling (57) and gene expression (13, 56) and represents a molecular link between the vascular and haemostatic systems. TF can be horizontally transferred via MV and induce signals transduction (9). Due to their plasma membrane origin, MV can be vehicles for protein anchored to specific lipid membrane domains (rafts). Lipid-raft proteins have been found in MV shed from blood cells such as platelets monocytes and red blood cells (17, 58). Of importance, the procoagulant properties of TMV can take part in a complex orchestration of signals delivering growth-promoting stimuli to neighbouring or remote cells, inducing cell signalling and transferring functional material. MV have been recognized in the latest years as true mediators of intercellular communication.

Microvesicles and thrombosis

Hypercoagulability

Hypercoagulability is documented in virtually all kind of cancers, albeit at different rates, and is the second leading cause of death in cancer patients (51). Activation of the coagulation system generates thrombin, as well as promoting the formation and subsequent deposition in blood vessels of fibrin, thereby generating a process of disseminated intravascular coagulation, which
is partially controlled by the fibrinolytic system. Proteins that regulate the fibrinolytic system, plasminogen activators such as u-PA and t-PA, as well as their receptors, can be expressed by tumour cells. Modulation of this pathway interferes with tissue remodeling and tissue proliferation (71). Furthermore, cancer cells are characterised by high procoagulant properties (53), and overexpress TF (70). TF critical haemostatic role is under the control of its expression normally restricted to some extravascular cells resulting in the separation from its ligand FVII(a) until the vascular integrity is preserved. TF overexpression in many cancers cells (20, 51) and shedding of TF+-MV from cancer cells in the circulation (26, 66) seem to play a role in
- cancer-related coagulopathy (Trouseau's syndrome),
- angiogenesis, and
- disease progression (45).

The presence of TF+-MV in venous blood of cancer patient has been correlated with the pathogenicity of cancer (17, 55, 66). In giant-cell carcinoma patients, high amounts of TF+-TMV strictly associate with a hypercoagulable state (18). Coagulation activation is not only attributable to TF harboured by MV as the role of anionic phospholipids has to be emphasized, especially PS. Surface-exposed PS strongly propagates the coagulation process by facilitating the assembly and activation of tenase and prothrombinase complexes. Furthermore, accessible PS at the MV surface is an important cofactor for TF activity providing the indispensable negatively-charged surface promoting thrombin generation. C6 glialia cells support all procoagulant reactions leading to robust thrombin formation, and this ability results from concomitant TF and PS exposure at their surface (21). In line with this, MV shed from activated endothelial cells were able to trigger and induce TF-dependent thrombin formation in vitro and thrombus formation in vivo. In contrast, MV from non-activated endothelial cells had no coagulant properties (1).

Other studies have correlated the activation state of cells with the sorting of plasma membrane components in MV. Monocyte-derived MV can bind to activated platelets through a ligand-receptor mechanism implicating P-selectin glycoprotein ligand-1 (PSGL-1) on MV and P-selectin on platelets. These MV were differentially enriched in CD45, TF and PSGL-1 suggesting that they arise from different membrane compartments. Moreover, monocyte-derived MV increase the proteolytic TF-FVIIa complex on platelets (17). Other proteins as synexin, sorcin and flotillin implicated in lipid-raft organization have been associated with vesicular shedding (58). These studies relate the heterogeneity of rafts with the mechanism of vesicle release from cholesterol-rich domains, introducing a novel aspect of the dynamics and organization of the plasma membrane.

Many speculations have been done onto the hypercoagulable properties of TF shed from cancer cells but it seems clear that the pool of TF+-TMV could interact with platelets, endothelial as well as stromal cells producing local blood coagulation and thrombosis. In venous blood, MV from different origins are subjected to ligand-receptor interactions or mechanisms of membrane fusion, allowing mixture of antigens and modulation of their functionality. It is still unclear whether MV present in plasma of cancer patients staining for cancer specific markers derived exclusively from cancer cells, but it is largely accepted that even if these MV are secreted by monocytes, endothelial platelets or stromal cells, the shedding is influenced by the presence of tumour. In our observations, in the context of tumour microenvironnement, TF activity carried by MV is related to the genetic state of cancer cells (70). These studies established a close relationship between coagulopathy, angiogenesis and genetic progression of the tumours. It has been proposed that TF could be implicated in tumour development and metastasis. They would facilitate the invasion of new zones by transformed cells, according to the antigens they carry at their surface. Shedding of TF, a bioactive lipid, vehicle by TMV was able to induce pro-angiogenic activities on endothelial cell migration, invasion, and tube formation in vitro, and neovascularization in vivo (31). The pro-an-
The pro-angiogenic activity of PMV was confirmed in vivo in a rat chronic myocardial ischemia model (12). Although large amounts of MV were required to stimulate cell invasiveness in vitro, much lower concentrations stimulated tube formation. Moreover, in cultured human endothelial cells (HUVEC), high counts of endothelial MV affected several parameters of angiogenesis, and a possible mechanism may be the increased oxidative stress, which leads to apoptosis. In fact, the rate of MV-induced apoptosis of HUVEC correlated strongly with the parameters of altered angiogenesis (42). An in vivo study revealed that, under physiological conditions, low counts of endothelial MV do not affect the endothelium (44). However, higher amounts of circulating endothelial MV associated with different vascular diseases may have important pathophysiologic effects on endothelial and blood cells, and thus directly contribute to pathogenesis.

TMV can activate endothelial cells by delivering glycoproteins involved in protein-protein interactions and catalysis. CD147/Basigin/EMMPRIN (extracellular matrix metalloproteinase inducer) is a membrane-spanning molecule highly expressed in tumour cells and one of the promising actors implicated in this kind of transcellular protein activation. EMMPRIN may be involved in promoting neo-angiogenesis as shown by Millimaggi and colleagues. In fact, TMV can induce an angiogenic phenotype in HUVEC in vitro, and this event is strictly correlated with the relative EMMPRIN enrichment of TMV (44). As well as TMV, PMV can also support angiogenesis in vitro in lung cancer cell lines. PMV stimulate the expression of pro-angiogenic factors like MMP-9, MT1-MMP (membrane type 1-MMP), VEGF, IL-8, or hepatocyte growth factor (HGF) and support adhesion of Lewis lung carcinoma cells to fibronogen. In addition, they transfer CD41 to the cancer cell plasma membrane, stimulate their proliferation, show chemotactic capacity and induce phosphorylation of MAP kinases. Importantly, PMV stimulate lung carcinoma cells in their metastatic spread to the lung (35).

Although it is not clear what is the primary signal inducing platelet activation and subsequent PMV shedding, these data indicate a strong link between the coagulation system and metastatic spreading and growth. Moreover, high plasmatic levels of PMV, VEGF (vascular endothelial growth factor), IL-6 and RANTES were detected among patients with gastric cancer, suggesting that the presence of high plasmatic levels of PMV could be a predictive marker for metastatic lesions (32). Developmental endothelial locus-1 (DEL-1) has been found on MV shed from mesotheloma cells that can act as a strong proangiogenic factor and promote vascular development in the neighborhood of the tumour (24).

The role of MV in intercellular communication and antigen transfer is particularly important. In this context, MV are vectors of biological information of a cellular type towards another one located in a proximal environment or in distal tissues.

**Horizontal transfer between cells via microvesicles**

MV not only transport oncogenic receptors but contain genetic material (mRNA, DNA) that can be transported towards proximal or distal acceptor cells. The integration of DNA or mRNA in target cells can occur by phagocytosis of MV, especially in a context where highly differentiated epithelial cells can become phagocytic. The genetic exchange of information via apoptotic bodies was shown between prostate cancer cells (16). Fibroblasts, monocytes or endothelial cells put into contact with apoptotic bodies originating from cells carrying EBV virus, integrated viral DNA and expressed specific EBV markers (25). The transfer of functional HIV DNA through apoptotic bodies towards cells without CD4, CCR5 and CXCR4 receptor expression was also shown (62). Such a mechanism allows the horizontal transfer of oncogenes between eukaryotic cells (10). MV are able not only to transfer antigens or RNA and thus modify the phenotype of the target cells but also to induce phosphorylation of particular proteins (63). Evidence for horizontal transfer of mRNA between cells via MV comes also from studies by Ratajczak and colleagues who have shown for the first time the capability of MV to reprogram a target cell. In this study, murine embryonic stem cell-derived MV (ES-MV) induce reprogramming of hematopoietic progenitors cells (HPC) (54). This allowed the authors to hypothesize that ES-MV could enter HPC as a kind of physiological ‘lypososmes’ and increase their pluripotency. MV derived from endothelial progenitor cells (EPC) are able to trigger angiogenesis in endothelial cells by horizontal transfer of mRNA (19).

This process could directly take part in tumoural transformation since it requires the accumulation of a series of genetic changes and could be related to the important genetic heterogeneity of tumour cells. Recently, an elegant demonstration of cell-to-cell transfer of oncogenic material has been published by Al-Nedawi and colleagues (3). In glioblastoma, a subset of cancer cell populations expressed Wnt and mutant EGFR (EGFRvIII). EGFRvIII ex-
pression in indolent glioma cells induced TMV shedding staining positive for EGFRvIII. They observed an intercellular transfer of EGFRvIII via TMV to EGFRvIII lacking cells able to induce a change in the expression of EGFRvIII-related genes, morphological transformation and an increase anchorage-independent growth.

**Microvesicles and the tumour microenvironment**

The tumour is not only a pathology associated to a single alteration of cells but to an orchestrated process in cell communication. The tumour consists on a variegated ensemble of communicating cells. All cells within the tumour collaborate in order to establish a favourable environment to the cancer development called microenvironment. All cells forming the tumour microenvironment as fibroblasts, immune cells, endothelial cells and adipocytes are instrumental. It seems clear in this context that not only cancer cells influence their environment, but host tissue, on its part, has an essential role in the pathogenic balance. This balance plays a central role in the sprout or eradication of cancer cells. So far, data concerning influence of different cell types in the establishment of tissue microecologies are mainly available for soluble factors. Only in the latest years, attention focused on MV as possible vehicles of biological information between cells. Molecules harboured by MV have been implicated in alteration of cancer cells behaviour, induction of angiogenesis, stromal matrix metalloproteinase expression and consecutive extracellular matrix remodelling, as well as in modulation effects from immune recovery. In this context has been proposed a role for TMV as vehicle of EMMPRIN in tumour stroma interactions. EMMPRIN vesiculation on TMV has been shown dependent on MEK1/2 phosphorylation, protein kinase C and Ca\(^{2+}\) mobilization. EMMPRIN-TMV were responsible for MMP transactivation in peritumoural stromal cells and involved in the progression of malignancy (61). Moreover, since activation of the coagulation system is associated with an increased risk of developing cancer (43), and thrombin induces proliferation and inhibition of apoptosis in tumour cells, the implication of TF in the shaping of the tumour microenvironment seems effective. Again, TF-MV can play a role in this context. Although the exact role of the host pool of TF is poorly studied, it has been suggested that in absence of TF in cancer cells, the host TF became critical for tumour development. Thus, the role of TF pool, both on cancer and stromal cells, has been related to the establishment of a favourable niche for cancer cell homing and proliferation (17, 52).

To date, data concerning the mutual effect of MV in cancer-stromal cells axis were missing. In this context, focusing on fibroblast implication, recent observations from our laboratory suggest a mechanism by which cancer and normal cells communicate via mutual shedding of MV. Our data demonstrate that MV have the potential to alter normal cell function and increase tumourigenicity, in particular, in promoting a favourable microenvironment for cancer cells by stimulating their migration and invasion, supporting the development of cancer (14). Figure 1 gives a schematic presentation of the role of procoagulant MV in the intercellular communication between cancer cells and their local microenvironment.

**MV in tumour defence**

**Escape from the immune system**

Cancer cells are believed to mould microenvironment components and affect the immune system mainly by pathways involving cell-to-cell contacts and the release of suppressive soluble factors. Moreover, tumours are able to successfully escape the host immune system losing surface antigens to become invisible to immune cells by the release of immune suppressive MV and/or exosomes. Tumour cells can also release exosomes, small membrane vesicles secreted into the extracellular compartment by exocytosis, which may be involved in the sampling of antigens to antigen presenting cells or as decoys allowing the tumour to escape.
immune-directed destruction. Many proteins are present in exosomes secreted by tumour cells (from cytoskeleton, heat shock proteins, involved in membrane transport and function). It is important to underscore, that most of the works report results obtained with a mixture of MV and exosomes. Indeed, when MV carry Fas-ligand, they could trigger apoptosis of cytotoxic T lymphocytes expressing Fas in an essential way, increasing the possibility of tumour cells to escape from the immune system (4).

It has been recently shown that TMV, derived from human tumours (such as melanoma and colorectal carcinoma), harbouring bioactive FasL and TRAIL, induce apoptosis in activated tumour-specific T cells (34). The induction of apoptosis and TCR (T cell receptor) alterations in effector T cells could be reproduced with MV isolated from plasma of cancer patients, which may help to explain the high frequency of apoptotic or CD3-zetaneg lymphocytes that are often found in the peripheral circulation of these patients (65). Natural killer cells are not spared from these negative influences, as they lose their cytolytic potential through the down-modulation of perforin expression, upon contact with TMV (36). Proteomic analysis of MV underlined that although several molecules are shared between MV of different cellular origins, MV functionality seems to be determined by their specific protein content. Due to their antigenic profile, MV can affect immune cell behaviour.

On the one hand, TMV can modulate monocyte cytotoxicity through the transfer of CCR6 and CD44v7/8 to monocytes, exerting antiapoptotic effect and activating AKT kinase (6). Moreover, TMV-treated monocytes showed an increased antitumour activity in regard of the enhanced cytotoxicity against tumour cells in vitro (7). On the other hand, in the context of tumour escape, TMV retain a large part of the protein repertoire of the producing cells, including molecules involved in immune suppression and deviation, the additional effects of these organelles on antitumour immune responses would not be a surprise. Indeed, crucial components of the immune response, such as antigen presenting cells, are profoundly affected by the encounter with TMV. These MV not only impair the capacity of circulating CD14+ monocytes to differentiate into functional dendritic cells, but they also skew the differentiation of these cells towards altered CD14+ monocytes expressing low or absent levels of HLA-DR (28, 67). These cells, present in relatively high numbers in peripheral blood mononuclear cells of melanoma patients, exert suppressive activity on lymphocyte proliferation and impair the expression of effector molecules (such as perforin and IFN-γ) in a TGF-β-mediated fashion. The immunosuppressive effects of TMV could potentially be exerted at least on two distinct steps of the process. First, during cross-priming by dendritic cells, with the impaired differentiation of monocytes into dendritic cells, and second, at T cell level, where myeloid suppressor cells release TGF-β, blocking proliferation and effector functions, and with the induction of apoptosis in activated cells (67).

**MV as a drug resistance mechanism**

In some case of cancer, chemotherapy is the only mechanism to counteract cancer cell proliferation. Unfortunately, chemotherapy outcome is related to the susceptibility of cancer cells to drugs leading to disseminated coagulation. Chemotherapeutic treatments could be responsible for endothelial and platelet MV generation in the blood, increasing the risk of venous thromboembolism in cancer patients after cisplatin treatment (50). The resistance of cancer cells to drugs, called chemoresistance, is a major factor limiting the effectiveness of chemotherapy. Tumours can be intrinsically resistant prior to chemotherapy, or resistance may be acquired during treatment by tumours that are initially sensitive to chemotherapy (30). Furthermore, in the process of resistance acquisition, the tumour may become cross-resistant to a range of chemotherapeutic agents, which ultimately leads to treatment failure in over 90% of patients with metastatic disease (37).

Chemoresistance is complex as numerous factors affect sensitivity to drugs, including accelerated drug efflux, drug activation and inactivation, alterations in drug targets, DNA methylation, processing of drug-induced damage, and evasion of apoptosis. In the context of drug efflux, as cancer cells shed MV from their plasma membrane, or TMV, the latter could play a relevant role in the attenuation of drug sensitivity. In fact, there is a strict correlation between MV shedding and anticancer drug resistance. Shedden and colleagues have demonstrated that association between shedding-related gene expression, measured rates of vesicle shedding, and chemosensitivity, together with the observation that doxorubicin associates with shed vesicles, indicates that vesicle release can serve as a mechanism of drug expulsion (60). In this study, MCF-7 cells where treated with a fluorescent doxorubicin in order to follow the localization of this drug within the cells. In a time lapse experiment, the drug, at first exclusively localized to the nucleus, was present in the cytoplasm and in vesicles at the cell margins. Finally, after long incubation time, while a weak nuclear and cytoplasmatic fluorescence was observed, doxorubicin mostly localized to MV at the cell periphery, providing evidence of drug accumulation in TMV.

Moreover, other observations have shown the implication of TMV shed from cells with a different chemoresistance profile, in the binding of chemotherapeutic drugs. This mechanism of drug clearance associated with TMV involves ABC-transporter proteins with a mechanism of binding rather than an active transport (15). It seems clear that TMV clearance in bloodstream of patients with cancer should be an efficient process, at least in part for chemoresistance. Hence, in chemotherapeutic treatments of cancer patients the identification of risk factors associated with thrombosis should include the determination of procoagulant MV on quantitative and qualitative bases in order to better tune chemotherapeutic regimens and anticoagulant prevention (38).

**Conclusion, perspectives**

Organisms are „closed systems“ made of a number of cell types undergoing perpetual death and renewal to maintain homeostasis. In an „economical“ context in terms of ener-
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