Microvesicles and Cancer Associated Thrombosis

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Abstract

Microvesicles (MVs) are small membrane enclosed structures released into the extracellular space by virtually all cell types. Their composition varies according to the cell origin and the stimulus which caused their formation. They harbor functional molecules and participate in intercellular communication. Endothelium, inflammatory cells, and cancer cells produce procoagulant MVs which contribute to cancer-associated thrombosis (CAT) in animal models. The tissue factor (TF) conveyed by these MVs was shown to play a key role in different animal models of experimental CAT. Alternatively, other molecular mechanisms involving polyphosphates or phosphatidylethanolamine could also be involved. In clinical practice, an association between an increase in the number of TF-positive or the procoagulant activity of these MVs and the occurrence of CAT has indeed been demonstrated in pancreatic-biliary cancers, suggesting that they could behave as a biomarker predictive for CAT. However, to date, this association was not confirmed in other types of cancer. Potential causes explaining this limited associated between MVs and CAT are 1) the diversity of mechanisms associating MVs and different types of cancer; 2) a more complex role of MVs in hemostasis integrating their anticoagulant and fibrinolytic activity; 3) the lack of sensitivity, reproducibility and standardization of current methodologies permitting measurement of MVs. Each of these hypotheses constitutes an interesting exploration path for a future reassessment of the clinical interest of the MVs in CAT.

Key words: extracellular vesicles, microvesicles, cancer-associated thrombosis

Introduction

Extracellular vesicles (EVs) constitute a heterogeneous population of small membrane enclosed structures released into the extracellular space by virtually all cell types. They can be divided in subpopulations based on specific characteristics like, biogenesis, size, content and biological function. EVs have been divided in 3 main categories according to their biogenesis. Exosomes are generated by fusion of the multivesicular bodies with the membrane. Ectosomes originate from the budding of cell membranes and are more often referred to as microparticles or microvesicles (MVs) whereas apoptotic bodies result from the late stages of apoptosis (1,2). In this review, we will refer mainly to MVs, small membrane vesicles with a size between 100 and 1000 nm released from activated or apoptotic cells (3). Once released by the cells, MVs circulate in a variety of body fluids and vectorize information to multiple target cells in the body (3). MVs are extremely diverse depending on the inducer triggering their biogenesis and their parent cell. They are considered both as molecular signature of the cells from which they originate and as a depository of important biological information that can be exchanged with neighboring cells (4). Diversity of MVs origin is responsible for a diversity of structures and composition in lipids, proteins and nucleic acid such as mRNA and miRNA resulting in a diversity of functions. Because of the panel of selected molecules they bear, MVs have been involved in different pathophysiological processes such as coagulation, inflammation, angiogenesis or endothelial dysfunction (5,6). This coagulo-inflammatory profile has logically led over the past decades to the question about their involvement in thrombosis. To address the question of the role of MVs in cancer associated thrombosis (CAT), MVs have to be integrated in their complex vascular environment with all partners contributing to thrombus formation including innate immune cells such as polymorphonuclear cells (PMNs) and monocytes, endothelial cells, platelets and also tumor cells. Additional partners resulting from cellular activation also include neutrophil extracellular traps (NETs)

and Polyphosphates (PolyPs) (7). Moreover, MVs originating from normal and tumoral cells contribute to CAT, explaining that both common and cancer specific mechanisms are involved. After a rapid overview of MVs involvement in coagulation and CAT and their association with the development of clinical thrombosis, we will address the current causes potentially explaining why MVs were not always found associated to CAT including: 1) the diversity of CAT mechanisms, 2) the more complex role of MVs in hemostasis integrating their anticoagulant and fibrinolytic activity and 3) the lack of sensitivity, reproducibility and standardization of current methodologies allowing to measure MVs in clinical situations.

MV involvement in coagulation and CAT

MVs are procoagulant because they provide a catalytic surface for the assembly of different clotting factor complexes. Their procoagulant properties mainly rely on the exposure of anionic phospholipids, especially the phosphatidylserine (PS) on the external leaflet of the membrane after a calcium-dependent cellular activation (6). PS allows the binding of coagulation factors at the MVs surface by their carboxylglutamic acid-rich (GLA)-domains. A critical transporter with scramblase activity responsible for the PS exposure was identified as an anoctamin, the calcium-dependent channel TMEM16F (8). Accordingly, mutated form of TMEM16F lead to a defective PS membrane exposure and MVs shedding which is associated with a bleeding disorder in rare Scott Syndrome patients (9).

Besides PS, the presence of the coagulation initiator tissue factor (TF) on subsets of MVs also significantly contributes to their procoagulant activity. TF was initially identified on MVs after the observation that tumor cells release procoagulant vesicles (10,11). PS and TF are not independent each other, indeed TF needs anionic phospholipids to be fully activated. It

is the concentration of the same restricted surface of both molecules which lead to highly procoagulant MVs. Incorporation of TF in PS+MVs is tightly regulated (12–16).

Once release in the circulation, TF positive MVs (TF+MVs) participate in a transfer of information between cells at the crossroad between inflammation and thrombosis. Indeed, it was demonstrated that MVs released by activated endothelial cells induce TF-dependent pathways on monocytes (17) whereas activation of monocytes generate TF+MVs which induce a switch of the endothelium phenotype towards a proinflammatory profile. This profile is associated with the release of inflammatory cytokines, TF expression and release of new TF+MVs which can in turn transferred TF to platelets or activate monocytes and increase their TF expression (18). Consequently, TF expression on cells or MVs which initially do not express TF, is enabled by different mechanisms of membrane fusion (19) or endocytosis recycling (20).

The procoagulant potential of MVs depends on their cellular origin and the stimuli which triggered their formation. Platelet MVs (PMVs), the main source of MVs present the blood circulation (21,22) are natively lacking TF-dependent procoagulant activity (23). When observed, the TF activity on PMVs may result from the fusion of endothelial or monocyte-derived TF+MVs with activated platelets as described above. Moreover, the PS-rich environment generated by PMVs may amplify the TF+MVs activity from MVs from another source. It was also showed that PMVs represent an appropriate surface to activate the contact phase pathway (24). Similarly, erythrocyte-derived MVs (EryMVs) trigger the coagulation independently of TF but in a contact phase-dependent manner (25).

Among leucocytes, monocyte-derived MVs (MoMVs) represent a major component of the TF+MVs circulating pool (26). As recently reviewed by Halim et al (27), TF on MoMVs can be upregulated by several factors including LPS (lipopolysaccharide) and proinflammatory cytokines such as TNFα and Interleukin (IL)-1β. Interestingly, it was

recently shown that the different human monocyte subsets generated MVs with different procoagulant potential after stimulation by IL-33 (28). In contrast to MoMVs, granulocyte-derived MVs (Gran-MVs) generated only a low level of TF activity, if any, while this property may be observed in pathological situations (29), although MoMVs transfer to granulocytes cannot be excluded (30).

Endothelial-derived MVs (EMVs) harbor PS and TF-dependent procoagulant activity in response to a variety of stimuli including cytokines, complement, and LPS and therefore contribute to the circulating pool of TF+MVs in clinical situation where the endothelium is activated (5). Cancer MVs represent another set of procoagulant MVs. Cultured human tumor cell lines of different origins express variable levels of TF and release cancer MVs with various procoagulant potentials in their supernatant (31,32). As illustrated for breast cancer, according to the cell line, mechanisms involve TF-independent pathways which promote platelet activation and aggregation and/or TF-dependent thrombin generation (33).

Evidence of MVs involvement in thrombus formation *in vivo* is supported by different studies using endogeneously formed or exogeneously injected MVs and mice mouse models of thrombosis. The pioneering work by Furies's group in 2003 showed that MoMVs rapidly accumulate at the site of injury in a laser injury model of small cremaster muscle arteries (34,35). This result was reproduced in various murine model of arterial and venous thrombosis with different sources of MVs (36–40).

MVs association with the development of clinical thrombosis

Cancer patients have an increased risk of thrombosis, especially venous thromboembolism (VTE), which differs according to the type of cancer (31,41). Although cancer is known as a hypercoagulable state, the pathogenesis is not totally elucidated. TF bearing MVs has been

assessed as a risk factor for cancer related VTE in several studies. The 5 main retrospective studies (31,42–45) that analyzed the link between TF+MVs and VTE demonstrated increased levels of circulating TF+MVs(measured by flow cytometry and functional assays) in cancer patients with VTE compared with cancer patient without VTE, with the highest values found in patients with pancreatic cancer.

The main prospective studies are listed in table 1. The first one supporting the hypothesis that high levels of TF+MVs activity predicts VTE in pancreatic cancer was performed by Khorana et al (46). Since this initial work, several other prospective studies, measured TF-MVs to evaluate the relationship between TF+MVs and future development of VTE. A positive association was found in studies using reported methodologies (43,47–49) including the most recent studies (50–52) whereas it was not found in other large studies (53–55). A recent meta-analysis on the relationship between TF+MVs and cancer patients with VTE concluded that the increased presence of TF+MVs represents an increased risk for cancer patients VTE with an overall odds ratio (OR) of 1.76 (56). However, due to the heterogeneity of study design, methods to measure TF-MVs, cancer origins and causes of death, more well-designed studies and more comprehensive adjustments for confounders are needed to confirm this association. Indeed, it is most likely that the results of the aforementioned studies are primarily driven by pancreaticobiliary cancer.

From these clinical studies, it seems that MVs have a real potential to predict CAT. However, most of these studies have limitations including a low number of patients, and the fact that few of them rely on multicenter prospective designs. More importantly these studies display a broad methodological heterogeneity and lack of standardization. We will now address the potential causes explaining that MVs are not always associated to CAT including: 1) the diversity of CAT mechanisms, 2) the more complex role of MVs in hemostasis

integrating their anticoagulant and fibrinolytic activity and 3) the lack of sensitivity, reproducibility and standardization of current methodologies allowing to measure MVs in clinical situations.

MVs and the diversity of cancer associated thrombosis mechanisms

Over the past years, a special attention has been given to CAT and the underlying mechanisms linking MVs and the thrombus formation (Figure 1). Several mouse models of cancer-associated thrombosis have been used with different mouse strains, tumors, tumor sites and thrombosis models (40). As a result, it seems that the importance of MVs and the mechanisms involved may vary according to the cancer origin (57). Consequently, the relevance of measuring MVs as a biomarker of CAT may similarly vary according to the cancer origin. Moreover, the exact nature of what should be measured on these MVs may also not be restricted to TF.

Pancreatic cancer was the most studied, due to its high rate of CAT (58). The role of pancreatic cancer-derived MVs in thrombosis has been first studied with the model of Panc02 pancreatic tumor cell lines. Using the ferric chloride mesentery vessel model and an ectopic pancreatic cancer mice model, a first study demonstrated that MVs released by PancO2 cell are detectable in mice peripheral circulation and accumulated in developing thrombus. Thrombus formation was reduced by inhibition of P selectin suggesting a P-selectin / PSGL-1 interaction may be invoved (59). In this model, thrombus growth induced by both exogeneously infused tumor-MVs and endogenous MVs, was prevented by inhibition of platelet activation by clopidrogrel and by blocking αvβ1/β3 integrins, suggesting that tumor-MVs bind to the thrombus on activated platelets in an integrin-dependent manner (60). Injection of the PancO2 derived MVs in the inferior vena cava (IVC) stenosis models

significantly increased the number of mice developing a thrombus but also the thrombus size (61). Interestingly, when MVs with low TF were injected, significantly less mice developed a thrombus, showing that TF from tumoral MVs is essential to their thrombus promoting activity. It was also recently shown that infusion of tumor-MVs resulted in the activation of platelets both *in vitro* and *in vivo* as suggested by the accumulation of platelets in the lung (62). Thus TF+MVs enhance VTE both by increasing fibrin formation but also by activating platelets. The direct demonstration of the role of TF on MVs originating from the tumor cell itself has recently been confirmed in a recent study performed with human pancreatic BxPc-3 grown orthotopically in nude mice (Figure 1A). In the IVC stenosis model, inhibition of human TF reduced the clot size in tumor-bearing mice but did not affect clot size in control mice. These results demonstrate that TF+MVs released from pancreatic tumors are responsible for increased clot size in tumor bearing mice (63). Interestingly, inflammation is a further putative pathway that could provide the trigger driving the procoagulant response with a particular relevance for the development of VTE in pancreatic cancer. In this model, high levels of TF+MVs are present and increased leukocyte infiltration contributes to promotes TF pathway and proinflammatory effects. Anticoagulant mechanisms are overwhelmed by the high levels of TF which accumulate in endothelial cells (ECs) and TF is recycled to the EV surfaces with PS, resulting in apoptosis, junctional weakening and subsequent endothelial denudation, resulting in activation of the extrinsic pathway via Xa activation TF+MVs in the context of acute inflammation (64). Beside TF pathway, another alternative novel pathway was also recently reported, using an IVC flow restricted model and strains of genetically engineered mice lacking GP1b or expressing reduced levels of TF (65). The mechanisms are independent of platelets and myeloid cells and involves the synergistic activation of coagulation by pancreatic MVs and host TF. A major contribution of the procoagulant activity of pancreatic MVs was provided by phosphatidylethanolamine (PE) externalization which

supported robust generation of Xa (Figure 1C). Disrupting PE dependent activation of factor X suppressed pancreatic MVs induced DVT without causing changes in hemostasis (65). In terms of therapy, this study confirms the observation that low molecular weight heparins are effective for prevention and treatment of CAT (66,67) but also suggests that oral actor Xa inhibitors may also be even more effective and tolerated (68).

In other cancers, TF is not necessary the major pathway by which MVs can promote VTE. For example, in prostate cancer, a recent work using mice knock-out for FXII, FXI and kininogen showed that these mice were protected against pulmonary embolism by the infusion of prostate cancer-derived MVs (69). These tumor-MVs also exhibit polyphosphates which suggests that tumor generated polyphosphate-rich MVs may activate the contact pathway (Figure 1B), favoring the thrombus formation (70). In brain cancers, platelet aggregation is a mechanism by which brain tumors may release podoplanin (PDPN) positive MVs in the circulation, that could activate circulating platelets (Figure 1D), resulting in increased VTE in patients with brain cancer (71–73). Additionally, mammary carcinoma derived EVs induce the formation of NETs which may represent another mechanism linking MVs and thrombosis (74). Thus according to the type of cancer, TF-independent mechanisms associated with MVs can also be involved in CAT and thus represent a potential cause explaining why an association between the biomarker TF+MVs and CAT was not always found.

A more complex role of MVs in hemostasis

Beside their well-documented procoagulant activity, MVs vectorize anticoagulant and fibrinolytic molecules which may balance the resulting effect of MVs in the hemostasis equilibrium (Figure 2).

Anticoagulant molecules have been described at the surface of MVs originating of cells which may be activated in the tumoral environment such as platelets, leukocytes and endothelial cells. Anticoagulant proteins S and C, as others vitamin K-dependent molecules, can bind to the MVs surface on anionic PS residues and directly inhibit the procoagulant activity of platelet MVs (75–77). The endothelial protein C receptor (EPCR) (78) and thrombomodulin (79) have been reported not only on endothelial-derived MVs generated in vitro but also among circulating MVs in patients (80,81). Regulatory molecules such as TFPI (TF pathway inhibitor) was detected on leukocytes and endothelial MVs (82). Thus, the thrombogenicity of MVs depends on a balance between the pro- and anti-coagulant molecules which may vary among various disease conditions. For instance, the TF/TFPI balance of MVs has been evaluated in 26 patients with cancer from various origins compared to 92 healthy control volunteers. As a result, the ratio of TF/TFPI MVs was significantly increased in the cancer patients due to a significant decrease of the percentage of TFPI+ MVs (83). The same team also recently showed that this balance is modified by chemotherapy drugs. Indeed, a significant increase in the circulating MVs TF/TFPI ratio was found after the first 24 hours of doxorubicin treatment (84). Consistently, tumoral MVs derived from MDA-MB-231 cells stimulated with high-dose doxorubicin display a strong decrease in the TFPI levels resulting in a six-fold increase in their procoagulant activity (82). Interestingly, in these studies, the critical variable was the level of the anticoagulant molecule on the MVs surface. These results stress/emphasize the importance of taking into account both the procoagulant and the anticoagulant molecules on the MVs surface to better reflect their global effect. In practice, the presence of these anticoagulant molecules such as TPFI may impact in the resulting MVs procoagulant activity measured by functional assays. Indeed, the inhibition of TFPI increases their sensitivity to procoagulant MVs (85,86). However, how the balance of TF/TFPI on

circulating MVs is modified by the pre-analytical and analytical protocols remains to be clarified.

The role of MVs in hemostasis is further complicated by the presence of fibrinolytic activity. Initially, proteolytic molecules such as metalloproteinases and the urokinase(uPA)/uPA receptor have been reported on MVs from tumoral cell lines in vitro (87–89) and from ascites from patients with ovarian cancers (90–92). Tissue-type plasminogen activator (t-PA) was also identified on MVs derived from a prostate cancer cell line (93). This tumoral MV proteolytic activity was first associated with an increased cellular invasiveness and metastasis (94–96). It was shown that hsp90alpha is secreted in small EV and interacts with tPA and its receptor annexin II in invasive cancer cells and contributes to their invasive nature by enhancing the conversion of plasminogen to active plasmin (97). Finally, the complete panel of molecules allowing MVs to efficiently generate plasmin and to vectorize a fibrinolytic activity was found to be present at their surface. This was first demonstrated on MVs from endothelial origin in vitro (98,99) and extended to leucocyte MVs (100). The plasmin generation capacity involves the binding of plasminogen on a specific receptor such as alpha-enolase and its activators such as uPA or tPA on receptors specific to surface of the MVs (101). Interestingly, this MV-dependent activity was found on circulating MVs of patients including cancer patients with acute promyelocytic leukemia (100,102). The fibrinolytic potential varies according to the cellular origin and the vesicular subsets. Comparison of the thrombotic and fibrinolytic activity of exosomes and MVs from several tumor cell lines showed that exosomes and/or MVs from most cell lines (MCF7, NB4, MDA-MB-231) with the exception of the A549 cell line, displayed fibrinolytic activity toward a pure fibrin clot, while only exosomes from MDA-MB-231 cells could degrade a fibrin clot formed in plasma (103). Interestingly, this fibrinolytic potential could be transferred from cell

to cell by exosomes. Indeed, the incubation of MDA-MB-231 cell–derived exosomes with A549 cells increased plasmin generation by these cells (103).

Plasmin generation is a highly regulated process controlled by a balance between activators and inhibitors. The plasminogen activator inhibitor (PAI-1) has been detected on tumoral MVs surface. MVs derived from leukemic mast cells inhibit the process of fibrinolysis by increasing the expression and secretion of PAI-1 (104). It has been also recently shown that the active oncogene EGFRvIII increased the concentration of TF and PAI-1 in prostate cancer derived MVs increasing their procoagulant activity (105).

Presence of both procoagulant and profibrinolytic activity at the surface of MVs lead us to propose that the resulting effect on bleeding or thrombotic events will be oriented as a function of the balance between the MV-dependent procoagulant and fibrinolytic activities (106,107). This concept of the coagulo-fibrinolytic balance of MVs was recently evaluated in the context of the fibrinolytic imbalance associated with septic shock. In a preliminary study, we showed, that the MVs fibrinolytic activity was significantly higher in septic shock patients who survived compared to those who died and that this MV-dependent activity was inversely correlated with multiorgan failure scores and ischemic markers (108). However, to what extent the fibrinolytic side may counterbalance the procoagulant side and play a significant role in the cancer-associated thrombosis pathophysiology is still largely unknown. Similarly, whether the coagulo-fibrinolytic balance of MVs is a better predictor of the occurrence of thrombosis in cancer patients rather their procoagulant activity alone, remains to be evaluated with suitable methodologies.

Methodological considerations

The reported limited performance of MVs as biomarker to accurately predict the risk of thrombosis in cancer patients, may also originate from methodological issues.

Pre-analytics remains an important source of artifacts. Recommendations have been made but they are not always easy to apply in clinical studies (109,110). Moreover, several functional assays require MV isolation from the body fluids before performing the test. For many years, ultracentrifugation has been used. However, the centrifugation step significantly decreases the reproducibility of the assays and may impede inter-laboratory comparisons. Immunocapture on magnetic beads may represent a useful alternative (108).

The two main approaches which have been applied to evaluate the potential of MVs biomarker of cancer associated thrombosis are: 1) the antigenic detection of procoagulant molecules at the surface of MVs (111), or 2) the functional capacity of MVs to generate factor Xa, thrombin or a clot. Combined strategies have also been developed (112) (Table 2).

Among antigenic assays, flow cytometry (FCM) was used for the detection of procoagulant molecules such as PS with annexin V or lactadherin and TF with specific antibodies. Over the past few years, significant improvements have been made regarding the sensitivity of FCM to detect single vesicles with a diameter of < 300 nm which have further established this methodology as the most promising tool for routine enumeration of EV subsets (113,114). However, the lack of sensitivity of the current flow cytometers to characterize small MVs and to detect small amount of molecules at the MVs surface still impede this evaluation. Moreover, most FCM studies did not take into account the presence of other active PLs, such as PE or the presence of physiological inhibitors regulating the procoagulant activity such as TFPI. As an interesting alternative, we measured fibrin+ (positive) MVs by FCM in pancreatic and colorectal cancers. The hypothesis was that as fibrin is the final product of the coagulation cascade, the level of fibrin+MVs may directly reflect the activation state of the blood coagulation cascade. As a result, fibrin+MVs were

significantly increased in patients with thrombosis (39). Other antigenic methods have been recently proposed such as the detection of TF+MVs by scanning confocal microscopy (115).

Regarding functional tests, a first group of methods focuses on the PS-dependent procoagulant activity of MV:

1. PS contribution can be evaluated measuring a PL-dependent coagulation time after dilution in a PL-depleted plasma and activation with factor Xa (FXa) and calcium. Thus, the only PLs present in the reaction mixture are provided by the patient sample. This test, initially described by Exner et al. (116), is commercially available (XaCT test, Haematex, Australia; STA-Procoag-PPL, Stago, Paris, France) and can be fully automated on routine hemostasis instruments.
2. Other assays combine solid-phase capture of MVs on annexin V and thrombin generation (Zymuphen-MP ELISA kit, Hyphen Biomed, Neuville-sur-Oise, France) (117). The generation of thrombin after adding prothrombin, FXa, FVa and calcium, is measured via cleavage of a chromogenic thrombin substrate producing absorbance at 405 nm. A standard curve of known MVs concentration is used to convert the absorbance into nM PS. This test may be adapted to quantify different subsets of MVs substituting annexin V by various capture antibodies. However, the sensitivity of these methods is limited by a heterogeneous and passive sedimentation of MVs and a limited contact surface.
3. The Calibrated Automated Thrombogram assay was originally developed by Hemker et al. for measuring thrombin generation in platelet-rich or platelet-poor plasma in a variety of clinical settings (Calibrated Automated Thrombogram, Thrombinoscope BV, Maastricht, NL and Stago) (118). To evaluate the PS contribution of MVs, thrombin generation is initiated by the addition of a reagent containing tissue factor and a minimal amount of PLs (platelet poor plasma reagent). Thrombin generation is

monitored with a thrombin fluorogenic substrate. The use of a thrombin calibrator for each individual plasma corrects for donor-to-donor differences in color of plasma, inner filter effects and substrate consumption. This assay is sensitive to the contact phase activation. Alternatively, plasmatic MVs can be pelleted, washed and re-suspended in MV-free plasma. The reproducibility of the measurement will decrease, but the measurement of thrombin generation will not be affected by soluble factors, the presence of anticoagulants or a coagulopathy.

A second group of assays focuses on the measurement of TF-dependent procoagulant activity of MVs :

1. The calibrated automated thrombogram assay can be modified to be more sensitive to TF. Thrombin generation in platelet free plasma or purified MVs spiked in MV-free plasma is initiate in presence of a "MP reagent" which contents only PLs and no TF. This assay is sensitive to the contact phase activation. Even if this test is more sensitive than the Zymuphen-TF (described below) in a Neisseria meningitidis-stimulated whole blood model (119), high concentrations of TF+MVs are necessary to be detectable probably because of a masking effect due to the high concentration of PLs added in the test.
2. Most of the studies evaluating the potential interest of MVs as a biomarker of the cancer-associated thrombosis have measured procoagulant MVs with FXa generation assays as a read-out of TF-FVIIa activity (120). These assays have been performed using either antibody-mediated MVs captured on coated plate or isolation of the MVs by high-speed centrifugation. In the commercially available assay (Zymuphen-TF, Hyphen Biomed), MVs are captured using an anti-TF antibody which does not interfere with TF activity. Unfortunately, no anti-TF inhibitor antibody is incorporated during the measurement of FXa. It was found that this assay has reduced specificity

and sensitivity compared with an “in-house” assay that isolates MVs using centrifugation (121). Two slightly different "in house" assays have been reported for the measurement of TF-MVs activity after isolation of MVs by high speed centrifugation. MVs are washed then resuspended in buffer before measuring FXa. Both assays use commercially available Dade thromboplastin reagent Innovin as a standard. FVII is added with synthetic PLs in the "kinetic assays" (42) whereas FVIIa is used without adding PLs in the endpoint assay (46). A moderate correlation was found ($r=0.61$, $P<0.001$) between the two assays in 54 pancreatic cancer patients (54). Several ongoing developments are improving the sensitivity of these assays (122,123).

Alternatively, a more global assay has been proposed monitoring the fibrin generation (FGT; fibrin generation test) after incubating purified MVs by high speed centrifugation in a plasma in presence of anti-TF or anti-FXII blocking antibodies (124,125). Clotting is initiated by addition of calcium and fibrin formation is monitored by kinetic measurement of the optical density at 405 nm.

Data comparing all these methods to measure the procoagulant potential of MVs are lacking. Moreover, FCM is the only method which benefits from a standardization effort so far which lead to different tools to reduce the inter-laboratory variability (123,124). This work is currently ongoing in a collaborative workshop on the standardization of EVs combining the experts from three international societies: International Society on Extracellular Vesicles (ISEV), International Society on Advancement of Cytometry (ISAC), and the International Society on Thrombosis and Haemostasis (ISTH). However, development of international standards is still needed to compare the results in the field. In a timely manner a new ISTH workshop, comparing the sensitivity and the specificity of assays to measure TF-MV in plasma samples will start this year. Results of these studies will certainly provide important

information to progress toward the optimal methods to measure MVs and standardization tools mandatory for robust multicenter studies in the cancer associated thrombosis field.

Conclusions

Up to now, several issues explain why MVs are not systemically found associated with CAT, including the diversity of mechanisms associating MVs and different types of cancer, the complex role of MVs in hemostasis integrating their anticoagulant and fibrinolytic activity and the limited sensitivity, reproducibility and standardization of current methodologies allowing to measure MVs. Continuation of the technical development for measuring MVs, standardization of methods in combination with efforts to define the most interesting antigenic or functional target to be measured on MVs in different clinical settings are issues that need to be addressed during the coming years. In addition, modulating MVs with treatment, and the benefits of regulating MVs in the prevention of future cardiovascular events are to be tested in prospective large randomized trials. Altogether, the rapidly growing knowledge on the biology of EV combined with the many technological advances set high expectations for future clinical applications of MVs as a predictive biomarker for CAT.

Patients	Wo VTE / W VTE	Follow-up	MV phenotype	Method	Reference
Locally advanced or metastatic pancreatic cancer	11/02	Every 4 weeks for 20 weeks	TF + MV	Functional assay	Khorana, JTH, 2008 (46)
Cancer Wo VTE	60/5	1 year	TF + MV	Impedance FMC	Zwicker, Clin. Cancer Res., 2009 (43)
Solid and hematological cancer	728/53	2 years	PS + MV	Functional assay	Thaler, Ann. Hematol., 2011 (53)
Cancer Wo VTE	299 (48 pancreatic) / 49 (12 pancreatic)	2 years	TF + MV	Functional assay (chromogenic end point and kinetic)	Thaler, JTH, 2012 (54)
Cancer Wo VTE at study entry	43/5	6 months	Annexin V + MV	FCM	Van Doormaal, Thromb. Haemost., 2012 (47)
Pancreatic Cancer	252/40	10 months	TF + MV	FCM	Hernandez, Thromb. Haemostat., 2013 (55)
Cancer Wo VTE at study entry	43/5	6 months	TF + MV	Functional assay	Van Doormaal, Thromb. Haemost., 2012 (47)
Glioblastoma multiforme	61/11	7 months	TF + MV	FCM	Sartori, Thromb. Haemost., 2013 (48)
Pancreatobilary cancer	117/52	Not specified	TF + MV	Functional assay	Bharthuar, Thromb. Res., 2013 (49)
Pancreatic cancer	65/11	6months – 1 year	TF + MV	Functional assay	Woei-A-Jin, B J Cancer, 2016 (50)
Cancer Wo VTE	608 (90 pancreatic) / 40 (10 pancreatic)	180 days	TF + MV	Functional assay (Fibrin generation and FXa generation	Van Es, Thromb. Res., 2018 (51)
Pancreatic cancer	41/12	1 year	TF + MV	Functional assay	Faille, Oncotarget, 2018 (52)

Table 1 : Role of MVs as prognostic biomarkers of VTE in cancer patients. FCM: Flow-cytometry; FXa: Activated factor X; MV: Microvesicle; PS: Phosphatidylserine; TF: Tissue

factor; VTE: Venous thromboembolism; Wo/W: Without/With. White lines represent studies no associations found between cancer and VTE and grey lines represent studies with a significant association between cancer and VTE.

Assays	Strategy	Target	Method	Preanalytics	EV commercial kit	Reference
Flow cytometry	Antigenic	PS, TF, TF/TFPI	Immunofluorimetry	PFP	No	Poncelet et al Trasci 2015 (113), Nolan et al Platelets 2017 (114)
Flow cytometry	Antigenic	Fibrin	Immunofluorimetry	PFP	No	Mege et al Oncotarget 2017 (39)
Scanning confocal microscopy	Antigenic	TF	Immunofluorimetry	PFP / Centrifugation	No	Hisada et al Thromb Res 2017 (115)
Calibrated Automated Thrombogram	Functional	PS or TF	Thrombin generation	PFP	Yes	Hemker et al, Pathophysiol. Haemost. Thromb. 2002 (118)
STA-Procoag PPL	Functional	PS	Coagulation time	PFP	Yes	Exner et al, Blodd Coagul. Fribinolysis, 2003 (116)
Zymuphen-MP ELISA Kit	Combined	PS	Thrombin generation	Immunocapture (PS) on PFP	Yes	Laroche et al Platelets 2017 (112)
Zymuphen-MP TF	Combined	TF	FXa generation	Immunocapture (PS) on PFP	Yes	Laroche et al Platelets 2017 (112)
Factor Xa generation assays	Functional	TF	FXa generation	Centrifugation	No	Khorana et al, JTH, 2008 (46), Woei-A-Jin, B J Cancer, 2016 (50)
Fibrin generation assay	Functional	PS + TF	Fibrin generation	Centrifugation	No	Berckmans et al, Blood, 2011(125)

Table 2: Procoagulant assay. Different strategies were used: antigenic assay using flow cytometry, functional assay using coagulation time, factor Xa (FXa) generation assay, thrombin generation assay or fibrin generation assay. PFP: Platelet Free Plasma; PS: Phosphatidylserin; TF: Tissue factor; TFPI: TF pathway inhibitor.

Figure 1: Diversity of mechanisms involving MVs in cancer associated thrombosis. MoMVs and MVs originating from the tumor cell itself rapidly accumulate at the site injury, increase fibrin formation and activating platelets(A). Tumor-MVs also exhibit polyphosphates that may activate the contact pathway (B). Pancreatic MVs support robust generation of Xa provided by phosphatidylethanolamine externalization (C). Brain tumors may release Podoplanin (PDPN)-MVs that activate circulating platelets (D). High levels of TF which accumulate in ECs and is recycled to the ECs surfaces (E).

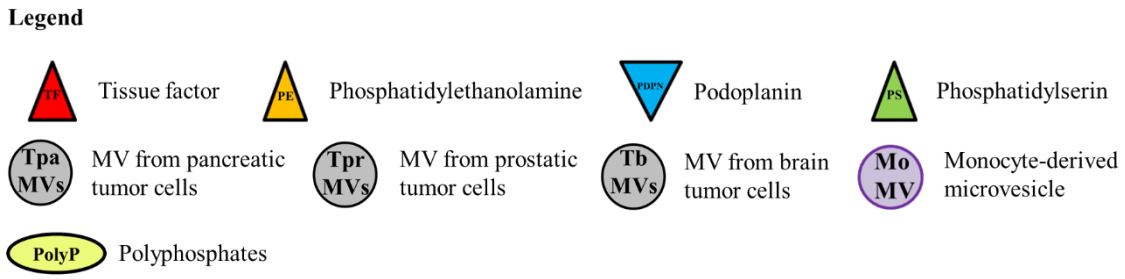

Figure 2: Prothrombotic and antithrombotic molecular pattern of MVs. MVs are initially described to bear procoagulant molecules like phospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE), tissue factor (TF) and polyphosphates. Anticoagulant molecules have been more recently described at the surface of MVs like TFPI, thrombomodulin (TM) that activate proteins C, its cofactor protein S and the receptor EPCR. The role of MVs in hemostasis was complicated by the presence of proteins of the fibrinolytic system : the tissue-type plasminogen activator (tPA) and urokinase (uPA) and its receptor uPAR and their principal inhibitor (PAI-1). MVs can also carry directly plasminogen on its receptors such as α-enolase.

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