Venous thrombosis in cancer patients: Prediction, diagnosis and management
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Cell-derived microvesicles in cancer progression and their application in clinical practice

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|ABSTRACT|

Blood and other body fluids contain a substantial number of cell-derived vesicles, such as microparticles and exosomes. The scientific and clinical interest in these microvesicles has increased considerably due to their multiple specific functions and the growing understanding of their contribution to development and progression of several diseases. Culture supernatants of cancer cell lines were known to contain procoagulant microvesicles already in the 1980’s, and since then the prothrombotic state in cancer patients has invariably been associated with the presence of circulating microvesicles in their blood. Furthermore, a growing body of evidence supports a microvesicle-dependent modulation of cancer cell survival, invasiveness and metastases. Here, we will present an overview of the complex contribution of microvesicles to cancer development and progression. In addition, their role in risk stratification and treatment of cancer patients is discussed.
INTRODUCTION

Compared to healthy controls, cancer patients present elevated levels of circulating cell-derived microvesicles. What are these vesicles and why do cancer patients have higher levels of these vesicles? In the first part of this review the types and origin of different microvesicles and their appearance in oncologic patients will be discussed. The second part summarizes the contribution of microvesicles to cancer-specific properties, such as prolonged and increased cellular survival, invasiveness and the capacity to metastasize. Finally, the potential application of microvesicles in risk stratification of cancer patients and their relevance for anti-cancer treatment will be discussed.

Types of microvesicles

Intracellular vesicle transport is a tightly regulated process which plays an important role in maintaining cellular homeostasis. Vesicles can be released by the cell into the extracellular environment. While some of the extracellular vesicles act rather locally, for example synaptic vesicles and matrix vesicles in bone and cartilage, other vesicles may travel far to execute their actions.

Currently, human body fluids are known to contain at least two and possibly even three different types of cell-derived vesicles: microparticles, exosomes and stem cell-derived particles. All eukaryotic cells, including blood cells, endothelial cells and cancer cells release microparticles into their environment by budding off parts of their outer cell membrane. Based on electron microscopy, microparticles range in size between 100 nm to 1.0 μm (1). They contain intracellular components of the cell of origin, such as mRNA and cytosolic proteins, and expose transmembrane receptors and other proteins on their outer membrane. Sometimes the exposed receptors are unique for a particular cell type or reflect the cellular status during microparticle release, e.g. activation or apoptosis.

Exosomes arise from endosomes, which are formed by plasma membrane invagination. Endosomes release vesicles into their lumen, ‘intraluminal vesicles’. Endosomes containing ‘intraluminal vesicles’ are called multi-vesicular bodies (MVB’s). When membranes of MVB’s fuse with the plasma membrane, the ‘intraluminal vesicles’ are released into the cells’ environment. From thereon, the ‘intraluminal vesicles’ are called exosomes. Exosomes range in size between 30-100 nm and all cell types containing MVB’s can be expected to secrete exosomes. Such cells include haematopoetic cells, tumor cells, and epithelial cells. The protein composition of several exosomes is well known. They contain cytosolic proteins and proteins derived from the membrane of the endocytic compartment (2).

Recently, a potentially new type of (stem cell-derived) vesicle was described. These exosome-sized vesicles expose prominin-1 (CD133), a stem cell marker. Although these vesicles were initially discovered in neural tube fluid of developing mice, they also occur in several body fluids of healthy humans. These prominin-1-exposing microvesicles differ
in protein composition from exosomes, for instance by lacking tetraspannins, and they are believed to play a role in cell differentiation, tissue development and maintenance (3).

**Assessment of microvesicles**

At present, no generally accepted definition of the various types of cell-derived vesicles exists. Not only theoretical issues, but especially methodological problems hamper the achievement of consensus. Thus far, flow cytometry, electron microscopy, western blotting, enzyme-linked immunosorbent assay (ELISA) and proteomics have all been used to study vesicle origin and composition. Isolation and purification of a single type of vesicles from body fluids and in fact even from (in vitro) conditioned cell culture media have proven to be difficult since contamination, substantial losses of (sub-) populations and altering of vesicle function(s) occur (4;5). Furthermore, different types of vesicles overlap in size with each other or with small cells such as platelets, and the (protein) composition of different types of vesicles may show similarities. No general consensus exists on the preferred technique for determination. This is relevant for the interpretation of results of different study groups. Although in the literature microvesicles are mostly subdivided in subtypes, we will use the general term microvesicles in this review.

**Microvesicles in cancer patients**

In 1946, Chargaff reported a subcellular factor in cell-free human plasma that promoted clotting (6). By using a combination of gradient centrifugation, electron microscopy and coagulation assays, Wolf et al. showed in 1967 that this procoagulant ‘Platelet Factor 3 activity’ could be attributed to subcellular particles originating from blood platelets (7).

The presence of microvesicles in cancer patients has been noticed already in the late 1970s’ (8). In 1981, culture supernatants of cancer cells were shown to contain microvesicles, which contributed to contribute to the procoagulant state observed in cancer patients (9). In 1993 and thereafter, the high risk of thrombosis in cancer patients has partly been attributed to microvesicles (10;11). This assumption has been strengthened by recent studies showing that cancer patients not only have higher levels of circulating microvesicles compared to controls, but also that cancer patients suffering from venous thrombosis present higher levels of microvesicles than cancer patients without venous thrombosis. Some microvesicles express tissue factor that can induce coagulation and especially these vesicles are relatively common among cancer patients with venous thrombosis. Zwicker and colleagues measured tissue factor-bearing microvesicles in twenty-two cancer patients with venous thromboembolism and in twenty-eight age, stage, gender and diagnosis-matched controls without thrombosis. The first group of patients was more than four times as likely to have detectable levels of tissue factor bearing microvesicles in their blood. These investigators also compared cancer patients with venous thromboembolism to patients with an idiopathic venous thromboembolism without cancer. Again, cancer patients had significantly more circulating tissue factor bearing microvesicles (12). Similar
findings were reported by Tesselaar et al. who found an 18-fold higher level of tissue factor bearing microvesicles in cancer patients with thrombosis compared to cancer patients without venous thromboembolism (13). Thus, levels of tissue factor bearing microvesicles are significantly higher among cancer patients compared to controls and seem associated with an increased risk for venous thromboembolism in cancer patients.

After reviewing the different types, the assessment and the appearance of microvesicles in oncologic patients, we will now focus on their role in cancer progression.

| ROLE OF MICROVESICLES IN CANCER PROGRESSION |

**Cellular survival**

*Escape from apoptosis*

Formation of microvesicles may shift the balance between apoptosis and cellular survival towards the latter. In a pioneering study published in 1988, Sims and co workers showed that exogenous stress triggers formation of microvesicles. When human platelets were incubated with the complement C5b-9 complex, microvesicles were released that contained 1000-fold higher levels of the C5b-9 protein as compared to what was present in the releasing cell. The release of microvesicles triggered by exogenous stress protected the cells from lysis and directly contributed to cellular survival. This mechanism was called ‘complement resistance’ (14). Indeed, cancer cells use this release to escape from complement-induced lysis (15;16).

More recently, several studies have shown that cells also release microvesicles as a protective mechanism against intracellular stress. In nucleated mammalian cells, caspase 3 is one of the main executioner enzymes of apoptosis. Microvesicles with substantial amounts of caspase are present in culture supernatants of viable cell cultures (17;18). Concurrently, caspase 3 is absent in the cultured cells. Various investigators have postulated that cells may escape from apoptosis by releasing caspase 3-containing microvesicles, thus preventing accumulation of the potentially dangerous caspase 3 within the cells. Recently, this hypothesis was strengthened by the finding that cells indeed accumulate caspase 3 and undergo apoptosis when the release of microvesicles is inhibited (19). Taken together, the release of microvesicles also supports cellular survival by removing internal stress. There is also evidence that caspase 3 may modulate the release of microvesicles. The MCF-7 human breast cancer cell line lacks functional caspase 3 due to a deletion in exon 3. These cells do not or hardly release any microvesicles, but their release is restored when cells are transfected with a construct encoding caspase 3 (20). Since these microvesicles also contain caspase 3, it seems that caspase 3 in microvesicles contribute to its own removal (A.N. Böing, unpublished observation).

A second example showing how the release of microvesicles protects cells from intracellular stress comes from studies demonstrating an association between microvesicle release and multi-drug resistance. Shedden and colleagues quantified the membrane shedding-related gene expression and observed that chemo-insensitive cancer cell lines express more
membrane shedding-related genes. Furthermore, the released vesicles contained high levels of the chemotherapeutic agent doxorubicin (21). Perhaps the most convincing evidence that the release of microvesicles contributes to resistance to chemotherapy and therefore to cancer cell survival comes from the study of Safeaei and colleagues who demonstrated that upon incubation of cancer cells with cisplatin, the microvesicles of the cisplatin-insensitive cells contained 2.6-fold more cisplatin than those released from the sensitive cells (22).

Escape from immune surveillance

A more indirect way to improve cancer cell survival is to suppress the immune response via formation of microvesicles bearing immune modulatory molecules. Microvesicles from various cancer cells expose Fas ligand (FasL, CD95L), a ligand of the death receptor Fas (CD95), which triggers T-cell death and diminishes the function of adaptive immune cells. For instance, Fas ligand-exposing cancer cell-derived vesicles induced apoptosis of T-cells in vitro (23;24). Kim and colleagues reported a modest correlation between lymph node infiltration and tumor burden and the numbers of circulating Fas ligand-exposing microvesicles in blood from twenty-seven patients with oral squamous cell cancer (25). Vesicles from lymphoblastoma cells, which expose latent membrane protein-1 (LMP-1), another immune suppressing transmembrane protein, inhibited leukocyte proliferation. This finding may explain the observed inhibition of T-cell proliferation in patients with Epstein-Barr Virus-associated tumors (26;27). Microvesicles not only suppress effector T-lymphocytes but also target antigen presenting cells (dendritic cells). Valenti et al. showed that cancer cell-derived vesicles are able to fuse with the membrane of monocytes, impairing their differentiation to antigen presenting cells (28).

Finally, cancer cells may hide from the immune system by mimicking the host environment. In the before mentioned study of Tesselaar et al, a low number of microvesicles was present that stained positive for MUC1, a cancer cell antigen, as well as glycoprotein IIIa (integrin β3), which is abundantly exposed on platelets and platelet-derived microvesicles. Based on these data, the authors suggested that such microvesicles resulted from fusion of cellular vesicles from malignant epithelial cells and platelets (13). Alternatively, platelet-derived microvesicles were shown to transfer (platelet-derived) integrins to membranes of breast and lung cancer cells (29;30). Thus, cancer cells can fuse with non-cancer cell-derived microvesicles and receive lipids and membrane-specific proteins which would protect them from immune surveillance. Apparently, the release of microvesicles after such membrane fusion would also result in the occurrence of microvesicles with a “mixed phenotype”. Figure 1A summarizes the effects of microvesicles on cellular survival.

Invasive growth and metastasizing

Environmental degradation

Degradation of the extracellular matrix (ECM) is essential for tumor growth (31). Microvesicles expose and contain several proteases, including matrix-metalloproteinase (MMP)-2 and...
Microvesicles in cancer patients

MMP-9 and its zymogens, and urokinase-type plasminogen activator (uPA). MMPs degrade basement membrane collagens, whereas uPA catalyzes the conversion of plasminogen into plasmin. Plasmin, a serine protease, degrades numerous components of the ECM, including fibrin, and activates various MMP zymogens. Ginestra et al. analyzed vesicle content in ascites-fluids from thirty-three women with different gynaecologic pathologies (19 benign ovarian lesions, 10 ovarian carcinomas, and 4 endometrial carcinomas). They found that malignant tumor fluids contained higher amounts of vesicles compared to benign diseases. Moreover, the microvesicles from benign serous cysts had only minimal lytic activity, whereas those from cancer ascites contained active metalloproteases (32). Furthermore, a correlation was present between the malignant potential of tumors and the microvesicle-associated MMP-2 activity (33). Graves et al., evaluated microvesicles in women with early-stage and late-stage ovarian carcinoma. They reported increased numbers of vesicles in late stage ascites and showed that MMP-2, MMP-9 and uPA activities are primarily concentrated within the microvesicles. Inhibition of MMP-2, MMP-9 or uPA with antibodies nearly abolished the ability of these microvesicles to support tumor invasiveness, which underlines the relevance of this pathway, at least in vitro (34). The increased invasiveness of cancer cells by microvesicle formation is shown in Figure 1B.

Angiogenesis

Fibrin, the end product of the coagulation cascade, plays an important role in tumor growth. Tumor cells can be coated with fibrin to escape from immune detection and attacks, and the fibrin matrix supports the outgrowth of new blood vessels. Microvesicles support coagulation through various mechanisms. They expose negatively charged phospholipids, such as phosphatidylserine, which facilitate binding of coagulation factors and thus formation of tenase- and prothrombinase complexes (35-37). In addition, especially in cancer patients, tissue factor bearing vesicles are present in the peripheral blood, albeit that the cellular origin of this tissue factor is still disputed (38-40). A part of these microvesicles originates from cancer cells and probably contributes to thrombus formation equally to leukocyte-derived vesicles, which also expose tissue factor. Microvesicle-exposed tissue factor, “blood-borne tissue factor”, can be captured by activated platelets adhering at the site of vascular damage and promote coagulation (11;41;42). Furthermore, tissue factor-exposing microvesicles may even fuse with (membranes of) activated platelets, thereby transferring tissue factor to the platelet membrane (43). Figure 1C shows the contribution of microvesicles to fibrin formation.

Tissue factor not only initiates coagulation but also plays a more direct role in angiogenesis. Phosphorylation of the cytoplasmic domain of tissue factor and subsequent downstream signalling events induce angiogenesis. The activation of coagulation by tissue factor generates thrombin which cleaves several protease-activated receptors (PARs), that in turn initiate angiogenesis (44;45). Finally, platelet-derived vesicles stimulate mRNA expression of angiogenic factors in cancer cells (29) and cancer cell-derived...
Chapter 2

A - Cellular survival

- Chemo-therapy → Cancer cell
- C5b9 → Normal cell
- Apoptotic inducer → T-cell

B - Invasiveness

- Chemo-therapy
- C5b9
- Casp3
- FasL

C - Fibrin formation

- T-cell
- Fibrin
- PS

D - Angiogenesis

- T-cell
- FasL
- PSGL
- PS
- TF

E - Metastasizing

- T-cell
- FasL
- PSGL
- PS
- TF

A - Cellular survival
B – Invasiveness
C – Fibrin formation
D – Angiogenesis
E – Metastasizing
Microvesicles in cancer patients

vesicles contain mRNA for growth factors such as vascular endothelial growth factor and hepatocyte growth factor. Baj et al showed that such vesicles fuse with monocytes, transferring their nucleic acids and altering their biologic activity (46;47). Possibly, cancer cell-derived microvesicles transfer mRNA to other cancer cells, enhancing their malignant potential. Intercellular transfer between cancer cells by microvesicles has recently been showed by Newadi et al. They reported intercellular transfer of ongogenic growthfactor-receptor by cancer cell-derived microvesicles altering the phenotype of these cells (48). Figure 1D shows the influence of cancer cell-derived microvesicles on angiogenesis.

Metastasizing

Metastasizing requires increased cellular survival and invasiveness, which are both enhanced by microvesicles. Whether or not microvesicles promote mobilization of tumor cells, however, has not been extensively studied. Some evidence suggests that microvesicles may favor lymphogenous and haematological spread. The expression of Fas ligand by cancer cell-derived microvesicles plays a role in lymph node infiltration (25). Furthermore, activation of platelets by tissue factor bearing vesicles is probably helpful in the haematological spread of cancer cells. Since activated platelets expose P-selectin and cancer cells expose P-selectin ligands such as P-Selectin Glycoprotein (PSGL) and Sialyl Lewis, the cancer cells will be surrounded by platelets and / or P-selectin bearing microvesicles, thus protecting cancer cells from immune surveillance and facilitating their binding to the vessel wall (39;49). The procoagulant properties of cancer-cell derived microvesicles further support intravascular fibrin formation, which in turn facilitates adherence of cancer cells to the vessel wall. Figure 1E presents the contribution of microvesicles to cancer cell migration.

Figure 1. The role of cancer cell derived-microvesicles in cancer progression. A. Cancer cells escape from internal (caspase 3) and external (chemotherapy, complement C5b9 complex, immune attack) stress by releasing microvesicles either containing (caspase 3, chemotherapy) or exposing C5b9 and Fas Ligand (FasL). Thus, the release of microvesicles contributes to cellular survival. 1B. Microvesicles expose and contain several proteases, including matrix-metalloproteinase (MMP)-2 and MMP-9 and its zymogens, and urokinase-type plasminogen activator (uPA). By degrading the extra cellular matrix (ECM), these enzymes facilitate cancer invasiveness. 1C. The membrane of microvesicles facilitates and initiates intravascular coagulation by exposing phosphatidylserine (PS) and tissue factor (TF), respectively. Fibrin protects the tumor against immune attacks and forms a matrix to support angiogenesis. 1D. Cancer cells induce angiogenesis by releasing microvesicles containing mRNA encoding growth factors and by exposure of TF. TF not only initiates coagulation, as shown in figure 1B, but also plays a critical role in angiogenesis. Activation of the cytoplasmatic tail of TF and subsequent downstream signalling events induce angiogenesis. Furthermore, thrombin, the final enzyme of the coagulation cascade, cleaves several protease-activated receptors (PARs), which in turn trigger angiogenesis. 1E. Fas ligand exposing microvesicles enhance lymph node infiltration by killing T-cells. Procoagulant microvesicles facilitate intravascular fibrin formation, thus enhancing hematologic spread. P-selectin glycoprotein ligand-1 (PSGL-1) bearing cancer cell-derived microvesicles contribute to clot formation by binding to P-selectin-exposing (activated) platelets.
FUTURE APPLICATIONS

Although there are potentially many clinical applications of the knowledge about microvesicles physiology, we will limit this discussion to anti-cancer treatment and risk stratification.

Anti-cancer treatment

Cancer cell-derived microvesicles have been used as adjuvant anti-cancer treatment. As described above, cancer cell-derived microvesicles have immunosuppressive activity due to functional alterations induced in T-cells, ranging from apoptosis to defects in T-cell receptor components and function (23-25;50). Cancer cell-derived microvesicles may also facilitate immune attacks (2;51-58). Wolfers and colleagues showed that cancer cell-derived microvesicles transfer tumor antigens to antigen presenting (dendritic) cells, which in turn trigger a T-cell-mediated anti-tumor response (58). In addition, antigen presenting cells may produce microvesicles that prime cytotoxic T-lymphocytes in vivo and eradicate or suppress growth of murine tumors. These autologous dendritic cell-derived microvesicles have been tested in phase I clinical trials in patients with metastatic melanoma (59), advanced non-small cell lung cancer (60) and colorectal cancer (61). All these studies concluded that this therapy is beneficial and safe with some patients experiencing long term stability of disease. Currently, several studies are ongoing to optimize this autologous anti cancer immunotherapy (56;62;63).

Microvesicle release itself could be an interesting target of anticancer therapy, i.e. by counteracting the beneficial effects of vesicle release on cellular survival or tumor growth. Some currently used chemotherapeutics impair, at least partially, the underlying mechanisms of microvesicle release, e.g. drugs targeting at Rho-associated coiled coil-containing protein kinases (ROCK) (64). ROCK-I and II are serine-threonine kinases which not only affect cell morphology, migration and adherence, but also contribute to release of microvesicles. (18;65). Rattan and colleagues showed that inhibition of the Rho/Rock pathway resulted in smaller tumor mass in patients with glioblastoma (64). Because vesicle release by cancer cells influences many processes which promote tumor growth, inhibition of vesicle release is a potential target in anti-cancer treatment.

Measurement of protein composition of microvesicles may be useful to monitor the efficacy of anti-cancer treatment. Clayton et al. exposed B-lymphoblastoid cell lines to external stress, i.e. 42 °C for 3 hours (66). Although the number of microvesicles released was comparable to control cells, the protein composition was markedly different. Stressed cells produced microvesicles containing relatively high quantities of heat shock proteins. Since heat shock proteins form complexes with proteins containing one or more production errors, their increased release with microvesicles, could help maintaining cellular homeostasis. Thus, the protein composition of cancer-cell derived microvesicles may reflect the effect of anti-cancer treatment and could be an early biomarker to assess the effectiveness of anti-cancer therapy.
Microvesicles in cancer patients

Risk stratification

Diagnosis

Tumor specific markers, such as mucine in adenocarcinomas, exposed on circulating vesicles, may be useful in the early detection of cancer. In a pilot study by Smalley et al. urine microvesicles were isolated from five healthy individuals and four patients with bladder cancer. Eight proteins were found to be elevated in isolated microvesicles from cancer patients compared to controls (67). Thus, the protein composition of urine microvesicles can potentially be used in early detection of bladder cancer. In future, the protein composition of microvesicles may imply a new marker for the early detection of cancer, for diagnosing tumors of unknown origin, and for monitoring anti-cancer therapy.

Prognosis

Different studies have evaluated the association between the level of microvesicles and survival in cancer patients. In the study of Tesselaar and colleagues, patients with both high microvesicle-associated tissue factor-activity and microvesicle-associated epithelial mucin (MUC1) had a lower survival rate at 3-9 months follow-up compared to those with low tissue factor-activity and no MUC1 expression. After adjustment for other prognostic factors the likelihood for an individual with these two membrane proteins present of survival was 0.42 (95% CI: 0.19- 0.94) (13). In a prospective nonrandomised single-center study in hormone refractory prostate cancer patients the impact of platelet-derived microvesicles on overall survival was assessed. Microvesicles were measured in 43 patients before starting chemotherapy. In patients with a number of platelet-derived vesicles above a cut off level the median overall survival was significantly shorter than in patients with values below that cut off level (68). Kim et al. performed a study in hundred-nine patients with gastric cancer and in twenty-nine healthy controls. Plasma levels of platelet-derived microvesicles were significantly higher in the patients than in healthy controls, and the levels were significantly higher in patients with stage IV disease than those in patients with stage I or stage II/III without a significant difference in platelet number. Platelet-derived microvesicles predicted distant metastasis with a sensitivity and specificity of 93.3% and 91.1% respectively (69). Thus, microvesicles may be used as a predictor of disease stage and survival in cancer patients.

Another potential application of microvesicles, especially those bearing tissue factor, is the prediction of venous thromboembolism (12;13;70). Although cancer patients have 4-5 fold higher risk to develop venous thromboembolism, there are currently no clinical or laboratory criteria to decide which patients warrant primary thromboprophylaxis (71;72). Ongoing studies are evaluating the potential of (tissue factor bearing) microvesicles levels as marker to decide about the appropriateness of primary thromboprophylaxis.
Chapter 2

| SUMMARY |

It is generally accepted that cell-derived vesicles are involved in (patho) physiological processes in humans. This review supports the concept that cancer cell-derived microvesicles facilitate tumor growth and contribute to the risk of venous thromboembolism. The role of the microvesicles in cancer requires further investigation, and additional studies are needed to establish their potential relevance as novel biomarkers to diagnose cancer and cancer progression, potential anti-cancer therapy, or as a new target of anti-cancer treatment.

| REFERENCE LIST |


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