Nanosized blood microparticles

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CHAPTER 1

General Introduction

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Background

The association between cancer and venous thromboembolism (VTE) is bidirectional. Starting with the observations of Bouillaud (1) and Trousseau (2), it was discovered that cancer patients are more prone to develop thrombosis, whereas thrombosis may be the harbinger of the presence of cancer. Epidemiological studies by Sorensen (3) and Blom (4) indicated that the incidence of cancer is increased among patients diagnosed with acute VTE. About 10% of patients with an episode of idiopathic VTE will develop cancer within a few years after the diagnosis (5;6). Cancer patients who developed thrombosis also have a poor survival compared to those who did not develop thrombosis (7).

Microparticles

Microparticles (MPs) are nanosized membrane vesicles, also named microvesicles, which are formed by budding of cell membranes upon activation or apoptosis and released into body fluids, such as blood, urine and synovial fluid. In the past, they have been considered to be inert cell debris (“platelet dust”) (8).

MPs still bear antigens derived from their parental cells. By using specific antibodies it was shown that the majority of MPs detected in blood originates from platelets (9;10). Other blood cells such as leucocytes, erythrocytes, endothelial cells and also malignant cells all may shed MPs (11). MPs have been shown to contain DNA, RNA, and recently also miRNA (12;13).

Most MPs expose the negatively charged phosphatidylserine (PS) on their membrane surface as a result of plasma membrane remodeling during formation from their parental cells (11;14;15). The exposure of PS on MPs provides a catalytic surface which promotes the assembly of procoagulant proteins and stimulates coagulation reactions (16;17). The PS content of MPs may vary according to their cellular origin, the mechanism by which they have been formed, and several pre-analytical conditions (18;19).

The presence of MPs in the blood of healthy persons is well documented, and elevated numbers of specific subsets of MPs have been reported in vascular disorders, cancer, and auto-immune diseases (11;16;20). In recent years, there has been a growing interest to study the role of MPs in physiology and pathology. However, individual studies use a wide variation of pre-analytical and analytical procedures, which all may affect the outcome of the MP measurement (21;22).

Procoagulant microparticles in cancer-associated thrombosis

Several studies have been conducted to identify the mechanism underlying the association between cancer and thrombosis (23-25). In various studies, an association between (active) tissue factor (TF)-bearing MPs and thrombosis in cancer patients has been found. This suggests that perhaps the release of MPs bearing TF by tumor cells and host cells into the blood may trigger thrombosis (9;26-28). TF has a major role in the initiation of blood coagulation. TF is found predominantly in the adventitial and much less in the medial layers surrounding blood vessels. Fibroblasts, adipocytes, and pericytes have also been shown to constitutively express TF. Normally, TF is not expressed by cells within the vasculature. However, after *in vitro* or *in vivo* stimulation by various agonists smooth muscle cells, endothelial cells, and peripheral blood mononuclear cells (monocytes and neutrophils) can express TF. TF binds to coagulation factor VII/VIIa (fVII/VIIa) to form a bimolecular complex that functions as the primary initiator of blood coagulation *in vivo*. In the presence of calcium ions (Ca^{2+}) and negatively charged phospholipids (PL), the TF/fVIIa complex activates fX into fXa which subsequently leads to the formation of thrombin and insoluble fibrin. Thrombin also activates platelets which together with fibrin form a stable clot (29).

One of the processes of tumor metastasis is the invasion of tumour cells through the vessel wall into the blood. This invasion of tumour cells causes vascular damage which triggers the expression of TF on cells within the vasculature leading to exposure of TF to the blood (30). The presence of a tumour may also elicit a host response that leads to induction of TF expression in monocytes and possibly endothelial cells and to the shedding of MPs bearing TF (31). In addition, some tumour cells express TF themselves (32) and these cells may shed MPs bearing TF (33;34). Figure 1 illustrates this hypothetical explanation of the role of MPs bearing TF in cancer-associated thrombosis.

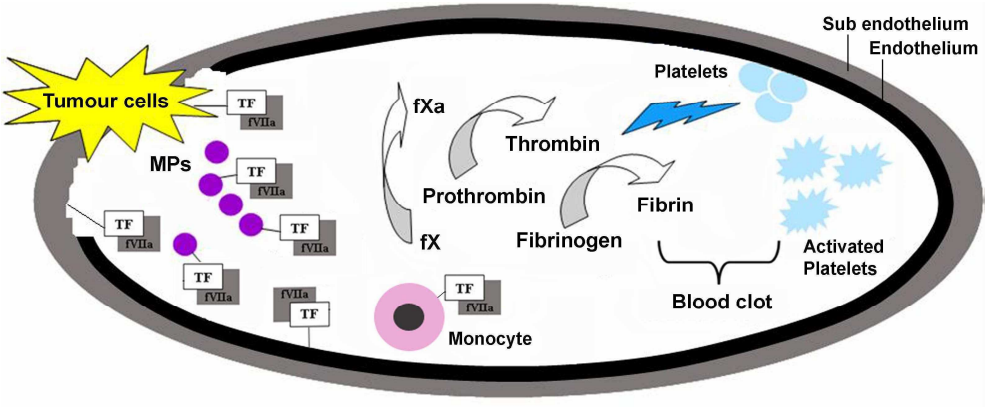

Figure 1. Hypothesis of the role of MPs bearing TF in cancer-associated thrombosis

Not all TF antigen is able to initiate the coagulation process (35;36). TF with reduced procoagulant activity has been referred to as encrypted. To date, little is known about the encryption status of cell- or MP-bound TF *in vivo*. Interestingly, in the cohorts studied by Tesselaar et al (9), cancer patients with elevated MP-associated TF activity and poor prognosis also had a small fraction of MUC-1-positive MP in their pool of blood MP suggesting tumour MPs bearing active TF as a link between cancer and thrombosis. In addition to its procoagulant activity, TF has cell signaling properties. Formation of the TF/fVIIa complex may activate signal transduction pathways via de proteolytic cleavage of protein-activated receptor 2 (PAR2) promoting tumor growth and tumor angiogenesis (31;37).

Measurement of microparticles

The choice of anticoagulant used for blood collection, the centrifugation speeds used for the preparation of plasma and the isolation of MPs, and the storage of blood and plasma samples are important pre-analytical variables which all may affect the outcome of MP measurements. Blood can be collected in sodium citrate, ethylenediaminetetraacetic acid (EDTA), and citrate theophylline adenosine dipyridamole (CTAD) anticoagulants, but more information is needed on the relative stability of MPs in these anticoagulants (38). After blood withdrawal, blood should be immediately centrifuged to isolate plasma (39). The centrifugation speed applied should remove platelets from plasma to avoid cellular activation of platelets leading to inadvertent production of platelet MPs (PMPs). A direct measurement of MPs in fresh plasma is preferred to reduce MP loss and to preserve MP characteristics (21). When frozen-thawed plasma is used, especially PMP and annexin V-positive MP counts should be interpreted with caution (39-41). For certain MP assays (e.g. fluorescence or electron microscopy, proteomic analysis and some functional MP assays), MPs are isolated from plasma to reduce the plasma protein content and to increase the MP concentration. Nevertheless, isolation procedures include multiple washing steps and will inevitably cause loss of some MPs. Most importantly, it is not known which centrifugation force is optimal to pellet plasma MPs (42).

Different analytical methods and combinations of these have been used to measure the number, concentration, and functional properties of MPs. Flow cytometry, capture-based assays, and functional assays are commonly used methods to measure MPs.

Since flow cytometry uses laser light which typically excites at a wavelength of 488 nm and MPs vary in size below 1 µm in diameter, using flow cytometry the enumeration of MPs with a size of less than 0.5 µm is inaccurate (43;44). To

standardize PMP measurements by flow cytomety in multi-centre studies, the ISTH SSC Working Group on Vascular Biology proposed the use of calibrated beads with sizes of 0.5, 0.9 and 3 µm (Megamix beads) to adjust the instrument settings and increase the resolution of the flow cytometer (45). Recently, a new generation of flow cytometers has been developed (e.g. Gallios from Beckman Coulter, Influx from Becton Dickinson, and Apogee A50 from Apogee Flow System) which seem to have improved scatter performances in terms of resolution and background as they can detect and resolve beads with sizes between 0.3 and 0.5 µm (46). However, plastic beads remain an imperfect model for size calibration of MPs. The scattering intensity of MPs depends on the characteristics of MPs such as shape, refractive index, and absorption which are different from those of the calibration beads (44).

Flow cytometry allows fast enumeration of total MPs by using annexin V. This method is based on the assumption that all blood MPs carry PS on their membrane. However, not all MPs expose PS. For example, in vitro stimulation of endothelial cells by TNF-α generated MPs with low PS exposure (47), platelets treated with thrombin receptor-activating peptide (TRAP) generated both PS-positive and -negative MPs (48), and a human monocytic cell line (THP-1) stimulated with soluble P-selectin-Ig chimera (P-sel-Ig) generated more PS-negative MPs (~60%) than PS-positive MPs (49). Furthermore, the binding of annexin V to MPs is influenced by the calcium concentration and the membrane PS content (50). Therefore, the use of other fluorescent dyes or proteins (e.g. calcein AM, lactadherin, SYTO13) might increase the accuracy of enumeration of total MPs (46).

Specific antibodies conjugated with different fluorochromes are used in flow cytometry measurements to identify and accurately count subsets of MPs. A list of antibodies to distinguish MPs derived from platelets, leucocytes, erythrocytes, and endothelial cells can be found in the publication of the Forum “Measuring circulating cell derived microparticles” (21) and some recent reviews (11;22). Additionally, MPs derived from malignant cells have been detected by the use of antibodies such as MUC1 for breast and pancreatic cancer cells (9;25), FasL for melanoma cells (51), EGFRvIII for glioblastoma cells (52), and CXCR-4 for leukemic cells (53).

Recently, quantum dots, nanocrystals of inorganic semiconductors with a diameter of 2–8 nm (200–10000 atoms), have opened new possibilities for antibody labelling (54;55). Quantum dots have significant advantages over common fluorochromes (for example, fluoroscein, phycoerythrin or allophycocyanin), such as brighter fluorescence and resistance to photobleaching (54). Additionally, quantum dots

have relatively narrow emission spectra giving less spectral overlap in standard multicolor immunophenotyping by flow cytometry (55). In the near future, the use of quantum dots-labeled specific antibodies may improve the sensitivity of flow cytometry detection of MP subsets (44).

Capture-based assays for MP measurement rely on an antibody or annexin V which specifically binds a subset of MPs from plasma. Captured MPs can then be quantified/characterized by using a second probe (e.g., peroxidase conjugated-antibody) (56) or by the measurement of the concentration of negatively charged phospholipids supporting prothrombinase activity (57;58). These capture-based assays enable a high-throughput measurement of subsets of MPs but do not provide direct enumeration of total MPs in plasma. Other disadvantages are interference by soluble antigens and lack of information on the size distribution of MPs (59).

The measurement of functional properties of plasma MP is limited at the moment to the measurement of MP-associated TF activity. Such assays are based on the fVII-dependent fXa generation in isolated MPs (9;27). The TF activity of MP (MP-TF activity) is defined as the fVII dependent and anti-TF sensitive formation of fXa. The assay provides information on MPs bearing active TF. A commercial two-stage chromogenic assay (Actichrome® TF from American Diagnostica) has also been used to measure TF activity in plasma (58;60). However, the observed fXa generation was found to be independent of exogenously added fVIIa and/or TF, and not inhibited by anti-fVIIa (61). Although the measurement of MP-associated TF activity provides information on MPs bearing active TF, it still fails to identify the cellular origin(s) of these MPs.

Aims of this thesis

MPs have important physiological and pathological roles in blood coagulation, inflammation and tumor progression (11;16). In recent years MPs also have been recognized to participate in important biological processes (15), such as in signaling and in the horizontal transfer of their membrane and/or cargo molecules, which are enriched in specific proteins and mRNAs. However, studies of MPs have been hampered by the lack of methods for the sensitive detection and accurate quantification of MPs. Furthermore, there is no method available to asses the origin of MPs bearing active TF in plasma. Thus, one of the aims in this thesis was to develop a new methodology for the sensitive detection and accurate quantification of MPs (MP subsets). Preferentially, such a method would also allow the determination of the size distribution of these MPs in their native state. In addition, efforts have been made to develop a method for analyzing subsets of MPs bearing

active TF. Finally, TF activity was measured in MPs isolated from plasma of multiple myeloma patients and its possible association with the development of VTE was investigated.

Outline of this thesis

Chapter 2 is a literature based review of pre-analytical and analytical issues in the analysis of blood microparticles. This review discusses pre-analytical variables related to plasma and MP preparation which may affect the outcome of MP measurements. In addition, several analytical issues in commonly used MP assays and some novel approaches for the detection and characterization of MPs are briefly discussed.

In Chapter 3 atomic force microscopy (AFM) is proposed as a new methodology for MP measurement. The use of AFM operated in fluid tapping mode is compared with the widely used conventional flow cytometry to quantify the number of CD41-positive MPs isolated from fresh plasma and to determine their size distribution. Finally, it was investigated whether this new methodology also can be used to detect MPs bearing TF antigen (Chapter 4).

To prevent loss of MPs during the isolation procedure, it would be preferable to use blood plasma directly for the MP measurements. Therefore, the use of microfluidics in combination with AFM was explored for the direct detection, quantification, and size determination of CD41-positive MPs in plasma. Results of this study are reported in Chapter 5.

Although conventional transmission electron microscopy (TEM) has been used in the past to study the morphology and membrane composition of MPs, this method requires steps such as dehydration, chemical fixation, and staining of specimens which all may affect MP morphology. This method is also not suitable for direct detection of MPs in plasma. For that reason, the use of cryo-TEM for visualization of submicron particles (MPs and exosomes) directly in plasma was studied. Chapter 6 deals with the results of cryo-TEM imaging of platelet-rich, platelet-poor, and platelet-free plasma isolated from citrate and EDTA anticoagulated-blood. Results are compared with images of submicron particles isolated from plasma and in the supernatant of thrombin activated-isolated platelets.

Up till now the cellular origin of MPs bearing active TF in blood is unknown. Chapter 7 presents the development of an immuno-magnetic beads method to directly capture/deplete selected subsets of MPs from plasma. By using biotinylated antibody and streptavidin coated magnetic beads, CD41- and CD14-

positive MPs were captured from plasma. Fractions containing captured and depleted CD41-/CD14-positive MPs were analysed for MP-associated TF activity.

Multiple myeloma (MM) is one of the malignancies associated with an increased risk of VTE complications. Chapter 8 reports the results of MP-TF activity measurements in patients with newly diagnosed MM before and after chemotherapy and discusses whether MP-TF activity levels found in these patients are associated with VTE.

All results presented in this thesis are summarized and discussed in Chapter 9.

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