Nanosized blood microparticles

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CHAPTER 9

Summary and General Discussion

Background

Although the intriguing association between cancer and thrombosis has been recognized since the 19th century, the mechanism(s) underlying this association have not been revealed yet. In the past years it has been suggested that microparticles (MPs; also known as microvesicles) derived from various types of cells and formed by budding of cell membranes upon activation and apoptosis may play a role in cancer-associated thrombosis. These MPs still bear antigens from their parental cells (blood cells, endothelial cells, and most likely also tumour cells). In addition they carry the negatively charged phosphatidylserine (PS) on their membrane surface as the result of membrane remodelling during their formation (1;2). Interestingly, MPs bearing tissue factor (TF), the major initiator of blood coagulation, are increased in plasma of patients with certain types of cancer (3-6). Unfortunately, the growing interest to study the role of MPs in health and disease is limited by the lack of well standardized pre-analytical and analytical procedures resulting in a wide variation in the results reported by different research groups (1;2).

The aim of the studies described in this thesis was to develop novel methodology for the sensitive detection and accurate quantification of (subsets of) MPs in their native state and for the analysis of the cellular origin of MPs bearing active TF. In addition, a possible association between MPs bearing active TF and the development of venous thrombosis (VTE) was investigated in patients with multiple myeloma.

Measurement of microparticles

Important factors

Several pre-analytical conditions such as blood collection, centrifugation used to prepare plasma/MPs, and storage (including freezing-thawing) of plasma may influence the outcome of the measurement of MPs. In Chapter 2 of this thesis we discussed that during blood collection, it is necessary to prevent *ex vivo* activation of blood which may lead to inadvertent production of erythrocyte MPs (ErMPs) or platelet MPs (PMPs). We recommend that blood should be drawn by using a large diameter needle, for example a 21-G needle, and that prolonged placement of a tourniquet during venepuncture should be avoided.

Plasma for MP measurements is usually prepared from blood collected in 1/10 volume of sodium citrate (0.105 or 0.129 mol/L). The choice of this anticoagulant is based on international guidelines for routine haematology testing but might be less

optimal for MP measurements. Therefore, more information is needed on the stability of MPs in blood collected in other anticoagulants such as ethylenediaminetetraacetic acid (EDTA) and citrate theophylline adenosine dipyridamole (CTAD) in comparison to that in citrate. Furthermore, the entire process from blood collection until plasma preparation should be rapid and gentle to avoid *ex vivo* activation of blood cells in the collection tube. The removal of platelets from plasma by centrifugation is crucial, but at the same time the centrifugal force used should not trigger inadvertent formation of PMPs.

Preferably, MP measurements should be performed directly in fresh plasma to reduce MP loss and to preserve MP characteristics. However, in multi-centre studies and prospective trials it is often inevitable to freeze and store the plasma samples before performing the analysis. Unfortunately, validated protocols are not yet available for freezing and thawing of plasma to be used for MP analysis. In Chapter 2 we recommend that when frozen-thawed plasma is used for MP measurements, especially PMP and annexin V-positive MP counts should be interpreted with caution.

As mentioned in the general introduction of this thesis, MPs vary in size in the submicron range. Therefore, a method which enables the detection of MPs within this size range is needed to provide an accurate measurement of total MPs in the sample. Current flow cytometers are not able to sensitively detect MPs smaller than 0.5 µm (7). Although there are advances made in developing new generation flow cytometers (5;8), they have not been tested extensively for routine measurements of MPs. Nevertheless, when flow cytometry is chosen as a method for quantification of MPs, some analytical conditions should be standardized. Recently, the Vascular Biology group of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) proposed to standardize the enumeration of PMPs larger than 0.5 µm by using calibrated size standard beads (Megamix beads) to adjust the instrument setting and increase the resolution of the flow cytometer (9).

Other methods used to study plasma MPs are electron microscopy, capture-based assays and functional assays of isolated MPs. In Chapter 2 we discussed the advantages and disadvantages of these methods. In general, they can be used to study plasma MPs as long as there is standardization of protocols, including the use of appropriate reagents and reference materials, to improve intra- and inter-laboratory reproducibility. We also discussed some novel methods to measure MPs including proteomic analysis, impedance-based flow cytometry, atomic force microscopy (AFM), nanoparticle tracking analysis (NTA), and dynamic light scattering (DLS). At this moment, these methods are not easily applicable in the

clinic since they are time-consuming and labour-intensive. In addition, none of these methods gives information on the functional properties of MPs.

Atomic force microscopy

In Chapter 3 we report the development of a novel method to measure MPs by using AFM. This method is based on the capture of MPs by anti-human CD41 monoclonal antibody (MoAb) coated on a modified mica surface. AFM operated in fluid/liquid tapping mode is used to detect MPs attached to the anti-CD41-coated surface. The advantages of this method are that forces as small as a few picoNewton can be sensitively detected and that sample properties are preserved. In this study, we observed that the binding of MPs to anti-CD41-coated mica seemed to be specific, as a markedly lower number of MPs bound to IgG1 isotype control-coated mica. Also, a linear relationship was obtained between the number of CD41-positive MPs per 100 μm^2 surface and the MP concentration which saturated at a concentration of ~500 MPs per 100 μm^2 surface. We quantified the number of CD41-positive MPs isolated from double-centrifuged fresh platelet-poor plasma (PPP) of 7 blood donors and 3 cancer patients both by AFM and flow cytometry. We found that the numbers of CD41-positive MPs measured by AFM were 1,000-fold higher than those measured by flow cytometry (3-702 x 10^9/L plasma vs. 11-626 x 10^6/L plasma). The MPs detected by AFM have diameters (d_{sph}) ranging from 10-475 nm with a peak at 67.5 nm, which is clearly far below the detection limit of conventional flow cytometry.

We also investigated whether the AFM method can be used to detect TF-bearing MPs (Chapter 4). To this purpose, we coated modified mica with FITC-labelled anti-human TF MoAb to capture MPs isolated from the supernatant of cultured MDA-MB231 cells stimulated with calcium ionophore. TF-positive MPs with diameters (d_{sph}) ranging from 6-296 nm (median: 40.4 nm; mean: 46.4 ± 26.0 nm) were successfully captured on FITC-labelled anti-TF-coated surface. Only few MPs were captured on the mica surface coated with FITC-labelled mouse IgG1 isotype control MoAb. We also found that the numbers of TF-positive MPs measured by AFM were 2,000-fold higher than those measured by flow cytometry in the same sample (8 x 10^6 per µL supernatant vs. 4 x 10^3 per µL supernatant).

To allow the detection of MPs directly in plasma, the use of a combination of microfluidics and AFM was investigated (Chapter 5). Tenfold-diluted EDTA PPP was flown through a microfluidic channel with a controlled pressure driven laminar flow to allow direct contact with anti-human CD41 antibody-coated mica. MPs bearing CD41 antigen were captured on this surface and subsequently detected by AFM operated in fluid tapping mode. The majority of the captured MPs have

diameters (d_{sph}) of 45 nm (range: 30-90 nm), similar to prior results obtained with MPs immediately isolated from fresh PPP. Apparently, the high-speed centrifugation needed to isolate MPs did not influence the size distribution of MPs. Use of the microfluidic system also increased the efficiency of capturing MPs. We applied this microfluidic system to count PMPs in plasma from healthy donors and compared it with a drop method. We detected considerably more CD41-positive MPs per 100 μm^2 surface when plasma was run through the microfludic system than when measured by the drop method. Our preliminary data also showed that there was a linear dose-response curve between the number of CD41-positive MPs per 100 μm^2 surface and the MP concentration.

In conclusion, AFM operated in fluid tapping mode can sensitively detect and accurately quantify subsets of MPs by using specific MoAbs. In combination with microfluidics MPs can be directly measured in plasma, thus, reducing time between venepuncture and MP measurement and also preventing MP loss because of washing steps in the isolation procedure. Importantly, as this method relies on the use of an antibody for the detection of MPs, a specific and high-affinity antibody is required to capture a specific MP subset on the mica surface. Furthermore, to assess the precise number of MPs in a given sample, the availability of an external standard (e.g. MPs with a known concentration) will be essential.

Cryo-electron microscopy

Conventional electron microscopy (EM) has been used to study the morphology of isolated MPs. However, the sample preparation and imaging techniques in conventional EM require dehydration, chemical fixation, and staining of specimens and are, therefore, less suitable to study the morphology of MPs directly in plasma. We investigated whether cyro-EM can be used to study the size and morphology of submicron particles (MPs and exosomes), and also to estimate the number of these particles in a fresh plasma sample without the use of staining or chemical fixation procedures. We used fresh plasma (platelet-rich plasma/PRP, PPP, platelet-free plasma/PFP), isolated MPs, and also (thrombin activated-) isolated platelets. These samples were directly applied onto an EM grid and vitrified for cyro-EM measurement (Chapter 6).

In Chapter 6 we observed that platelets were more activated in fresh citrate PRP than in EDTA PRP, although clearly less than in samples of thrombin-activated platelets. Therefore, to reduce *in vitro* activation of platelets and PMP formation, blood collection in EDTA is recommended for the study of plasma MPs. Most of the submicron particles found in plasma of healthy volunteers were spherical electron dense particles resembling lipoprotein particles. Low numbers of electron lucent

particles with a discernable lipid bilayer (lipid vesicles) were also found. These could be MPs, exosomes or both. Similar lipid vesicles were found in samples of thrombin-activated isolated platelets and isolated MPs. All types of particles have about the same size distribution (median: 30 nm; range: 25-260 nm). Because MPs form only a minority of all submicron particles present in fresh plasma, we have to consider the limitations of techniques used to measure MPs directly in plasma. Finding ways to label MPs and/or exosomes directly in plasma will be essential for further studies of these particles by for instance NTA and cryo-EM.

Immuno-magnetic beads capture

There is not yet a method which provides information on the cellular origin of the plasma MPs carrying active TF. In Chapter 7 we report on the development of an immuno-magnetic beads method to capture a defined subset of MPs from plasma by using a specific biotinylated antibody and streptavidin magnetic beads.

This method was optimized for the capture of PMPs from plasma of healthy volunteers. Plasma was incubated with biotinylated anti-human CD41 MoAb before streptavidin magnetic beads were added. After application of this mixture to the column, positive (containing the selected subset of MPs) and negative (containing the depleted plasma) fractions were collected and used directly for the isolation and analysis of MPs for specific antigens and TF activity.

By using flow cytometry we showed that biotinylated anti-human CD41 MoAb specifically binds and saturates the surface antigen CD41 of PMPs. The streptavidin magnetic beads did not interfere with the binding of anti-CD41 and anti-CD61 antibodies to PMPs. After separation, there were no CD41-positive MPs and only few CD61-positive MPs in the negative fraction, indicating that almost all PMPs (CD41-positive MPs) were captured. This capture was CD41-specific as most of CD41/CD61-positive MPs were recovered in the negative fraction of the biotinylated IgG1-beads separation.

Using biotinylated anti-CD14 MoAb at the same concentration as anti-CD41 MoAb, CD14-positive MPs were captured from normal plasma spiked with MPs isolated from the supernatant of LPS-stimulated isolated monocytes (MoMPs). TF activity was found both in the positive and negative fractions indicating that both CD14-positive and -negative MoMPs have active TF. This seems in agreement with our finding that not all active TF carrying MoMPs isolated from the supernatant of LPS stimulated monocytes were CD14-positive.

In the future the beads capture method can be used to investigate the source of MPs carrying active TF in plasma of patients with cancer by using cell- or other tumour-specific antibodies such as anti-MUC1 (breast and pancreatic cancer cells) (4;5), anti-FasL (melanoma cells) (10), anti-EGFRvIII (glioblastoma cells) (11), and anti-CXCR-4 (leukaemic cells) (12).

MP-associated TF activity in patients with multiple myeloma

For the measurement of MP associated tissue factor activity (MP-TF activity) freshly isolated MPs are supplemented with excess FVII(a) and FX in the presence of calcium, after which the formation of FXa is measured with a specific chromogenic substrate using either a one-stage (4;6;13) or two-stage approach (14;15). These two different approaches were discussed in detail in Chapter 2 of this thesis. To asses MP-TF activity in multiple myeloma (MM) patients, we used the one-stage approach (Chapter 8).

Chemotherapy in combination with anti-angiogenic drugs in multiple myeloma is associated with a high risk of thrombo-embolic complications (16;17). We measured MP-TF activity in MPs isolated from PFP of 122 patients with newly diagnosed MM before and after chemotherapy to investigate whether MP-TF activity is associated with VTE. MP-TF activity levels of untreated patients were significantly higher than those in healthy volunteers [17.6 fM FXa/min (IQR 8.6-33.2) versus 4.1 fM FXa/min (IQR 2.3-6.6), P<0.001]. None of the patient characteristics or laboratory measurements (sex, age, disease stage, beta-2 microglobulin, albumin, calcium, haemoglobin, platelets) correlated with the MP-TF activity levels.

MP-TF activity prior to the start of treatment was not different between patients who developed a VTE during follow-up (n=15) and those who did not (n=107). In 75 patients in whom plasma was obtained before and after chemotherapy, MP-TF activity decreased significantly (from 17.4 [10.2-32.8] to 12.0 [7.0-18.5] fM Xa/min, P=0.006). This reduction was observed in all ISS stages of disease suggesting that the tumour load is not associated with MP-TF activity levels. The MP-TF activity levels also decreased irrespective of the therapeutic regimen [vincristine, adriamycin, and dexamethasone (VAD), thalidomide, adriamycin, and dexamethasone (TAD), or bortezomib, adriamycin, and dexamethasone (PAD)]. MP-TF activity remained, however, elevated in patients who developed VTE (15.1 [10.3-25.2]), in contrast to patients not developing VTE (11.4 [7.0-25.2], P<0.001).

In future studies MP-TF activity should be assessed more frequently during induction treatment of MM to investigate whether MP-TF activity has a pathogenetic role in VTE in MM patients.

General Discussion

Thus far there is no general consensus on the size and number of MPs due to discrepancies between the results of MP measurements reported in the literature. We are aware that these discrepancies are not only caused by differences between the methods used to measure MPs. Pre-analytical procedures such as blood collection, plasma/MPs preparation, and storage are some of the important factors which might influence the number, morphology and characteristics of MPs. The use of EDTA instead of citrate as anticoagulant for blood collection to reduce *in vitro* activation of platelets and PMP formation (Chapter 6) is preferred for the study of plasma MPs. We also have an indication that the high-speed centrifugation used to isolate MPs has no effect on the size distribution of MPs (Chapter 5). Eventually, it is essential to set up guidelines for these pre-analytical procedures to improve intra- and inter-laboratory reproducibility.

In the past, MPs were defined by having a size distribution in the micrometer range, whereas now we found them to be in the nanometer range (Chapter 3, 4, 5, and 6). This is explained by the technical limitations of the methods previously used to characterize MPs. For example, methods such as flow cytometry with a detection threshold of 0.5 µm and conventional EM for which dehydration, chemical fixation, and staining of specimens are needed for sample preparation, are less suitable for detection and characterization of MPs. Furthermore, there are no suitable reference materials (standards) to calibrate and adjust the settings of the methods for MP analysis (18). Currently, new methods such as AFM, NTA, and cryo-EM allow detection and characterization of MPs in the nanometer range and in their physiological state. However, these methods are not yet suitable for use in the clinic as they are low throughput, labour-intensive and still need to be optimized and validated (e.g. accuracy, precision, specificity, detection limit and quantification, linearity and range, robustness). Therefore, improvements are needed, for example by using automated sample handling systems, standardized reagents, reference materials for calibration and settings, and an external standard to obtain absolute numbers of MPs. Ultimately, a small MP measurement device which uses just a pinprick of blood and provides results in less than 30 minutes (lab-on-a-chip) would be desirable for a routine test.

There is evidence that MP-TF activity is increased in cancer patients who develop (cancer-associated) VTE (4;6). Further investigations are needed to learn whether

MPs bearing active TF in cancer patients are indeed the cause of thrombosis. Furthermore, to understand the mechanism(s) of the association between cancer and thrombosis, it is important to identify whether MPs bearing active TF come from the cancer cell itself or not; in the latter case it is important to identify the cell of origin (monocytes, endothelial cells, platelets) and the mechanism by which the active TF is produced.

Finally, further research is needed to investigate how MPs are precisely formed. According to a widely accepted hypothesis MPs are shed by cells as a result of loss of membrane phospholipid asymmetry during processes such as activation and apoptosis, exposing PS on the outer cell surface. This hypothesis is supported by the rare bleeding disorder, Scott syndrome. This syndrome is characterized by a decreased scramblase activity which results in reduced expression of PS and reduced MPs shedding (1;2). Interestingly, the existence of MPs that do not expose PS challenges the hypothesis of MP formation (19-22). It remains a matter of debate whether the existence of PS-negative MPs is the result of the limitations of the current methodology. Annexin V is widely used as a probe of PS exposure in methods to detect and quantify MPs such as flow cytometry and annexin V capture-based assays. However, the binding of annexin V to MPs depends on the calcium concentration and the membrane PS content (23;24). Another possible explanation for the finding of PS negative MPs might be that they are formed by multiple fusion events between plasma membrane components with cell debris or small endosomal secreted vesicles (exosomes) (25). Alternatively, MPs might be released from the plasma membrane as a result of destabilization of the actin cytoskeleton via αIIbβ3 signalling which does not require rearrangement of phospholipids distribution (26).

In conclusion, MPs are no longer regarded as cellular debris or experimental artefacts, but in fact MPs may participate in processes of coagulation, inflammation and tumorigenesis. It is of interest to investigate from which cells MPs are formed, why they are formed and how they interact with other cells in the body. Therefore, standardisation of pre-analytical and analytical procedures for the measurement of MPs is urgently needed to understand their presence and to elucidate their physiological function and role in diseases. In the future the measurement of MPs will contribute to the development of MPs as diagnostic and perhaps predictive (bio)marker in various diseases.

References

(1) Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. Blood Rev 2007;21:157-171.

(2) Burnier L, Fontana P, Kwak BR, Angelillo-Scherrer A. Cell-derived microparticles in haemostasis and vascular medicine. Thromb Haemost 2009;101:439-451.

(3) Hron G, Kollars M, Weber H, Sagaster V, Quehenberger P, Eichinger S, et al. Tissue factor-positive microparticles: Cellular origin and association with coagulation activation in patients with colorectal cancer. Thromb Haemost 2007;97:119-123.

(4) Tesselaar ME, Romijn FP, van der Linden IK, Prins FA, Bertina RM, Osanto S. Microparticle-associated tissue factor activity: a link between cancer and thrombosis? J Thromb Haemost 2007;5:520-527.

(5) Zwicker JI, Liebman HA, Neuberg D, Lacroix R, Bauer KA, Furie BC, et al. Tumor-derived tissue factor-bearing microparticles are associated with venous thromboembolic events in malignancy. Clin Cancer Res 2009;15:6830-6840.

(6) Tesselaar ME, Romijn FP, van dL, I, Bertina RM, Osanto S. Microparticle-associated tissue factor activity in cancer patients with and without thrombosis. J Thromb Haemost 2009;7:1421-1423.

(7) Furie B, Furie BC. Cancer-associated thrombosis. Blood Cells Mol Dis 2006;36:177-181.

(8) Lacroix R, Robert S, Poncelet P, Dignat-George F. Overcoming limitations of microparticle measurement by flow cytometry. Semin Thromb Hemost 2010;36:807-818.

(9) Lacroix R, Robert S, Poncelet P, Kasthuri RS, Key NS, Dignat-George F. Standardization of platelet-derived microparticle enumeration by flow cytometry using calibrated beads: results of ISTH SSC collaborative workshop. J Thromb Haemost 2010;8:2571-2574.

(10) Andreola G, Rivoltini L, Castelli C, Huber V, Perego P, Deho P, et al. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. J Exp Med 2002;195:1303-1316.

(11) Al Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nat Cell Biol 2008;10:619-624.

(12) Kalinkovich A, Tavor S, Avigdor A, Kahn J, Brill A, Petit I, et al. Functional CXCR4-expressing microparticles and SDF-1 correlate with circulating acute myelogenous leukemia cells. Cancer Res 2006;66:11013-11020.

(13) Garcia RP, Eikenboom HC, Tesselaar ME, Huisman MV, Nijkeuter M, Osanto S, et al. Plasma levels of microparticle-associated tissue factor activity in patients with clinically suspected pulmonary embolism. Thromb Res 2010;126:345-349.

(14) Tilley RE, Holscher T, Belani R, Nieva J, Mackman N. Tissue factor activity is increased in a combined platelet and microparticle sample from cancer patients. Thromb Res 2008;122:604-609.

(15) Manly DA, Wang J, Glover SL, Kasthuri R, Liebman HA, Key NS, et al. Increased microparticle tissue factor activity in cancer patients with Venous Thromboembolism. Thromb Res 2010;125:511-512.

(16) Zangari M, Saghafifar F, Mehta P, Barlogie B, Fink L, Tricot G. The blood coagulation mechanism in multiple myeloma. Semin Thromb Hemost 2003;29:275-282.

(17) Zangari M, Elice F, Fink L, Tricot G. Thrombosis in multiple myeloma. Expert Rev Anticancer Ther 2007;7:307-315.

(18) Chandler WL, Yeung W, Tait JF. A new microparticle size calibration standard for use in measuring smaller microparticles using a new flow cytometer. J Thromb Haemost. 2011 Jun;9(6):1216-1224.

(19) Jimenez JJ, Jy W, Mauro LM, Soderland C, Horstman LL, Ahn YS. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. Thromb Res 2003;109:175-180.

(20) Aras O, Shet A, Bach RR, Hysjulien JL, Slungaard A, Hebbel RP, et al. Induction of microparticle- and cell-associated intravascular tissue factor in human endotoxemia. Blood 2004;103:4545-4553.

(21) Perez-Pujol S, Marker PH, Key NS. Platelet microparticles are heterogeneous and highly dependent on the activation mechanism: studies using a new digital flow cytometer. Cytometry A 2007;71:38-45.

(22) Bernimoulin M, Waters EK, Foy M, Steele BM, Sullivan M, Falet H, et al. Differential stimulation of monocytic cells results in distinct populations of microparticles. J Thromb Haemost 2009;7:1019-1028.

(23) Tait JF, Gibson D. Phospholipid binding of annexin V: effects of calcium and membrane phosphatidylserine content. Arch Biochem Biophys 1992;298:187-191.

(24) Connor DE, Exner T, Ma DD, Joseph JE. The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib. Thromb Haemost 2010;103:1044-1052.

(25) Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. Trends Cell Biol 2009;19:43-51.

(26) Cauwenberghs S, Feijge MA, Harper AG, Sage SO, Curvers J, Heemskerk JW. Shedding of procoagulant microparticles from unstimulated platelets by integrin-mediated destabilization of actin cytoskeleton. FEBS Lett 2006;580:5313-5320.