Cellular origin and coagulant activity of tissue factor exposing microparticles in cancer patients

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Submitted
ABSTRACT

Introduction
Tumours release tissue factor-exposing microparticles (TF-MP), which have been associated with venous thrombosis in cancer patients. Recently, however, TF-MP number and coagulant activity were disproportionate in tumour-bearing mice. Therefore, we investigated the clinical relevance and relationship between TF-MP number and coagulant activity in cancer patients (n=209).

Methods
Patients were categorised as having low (n=196) or high number of TF-MP (n=13; ≤ or > 95th percentile), and having low (n=141) or high TF-MP coagulant activity (n=63; ≤ or > 13% TF-dependent prolongation of clotting time).

Results
Of the patients with high TF-MP, nine had low TF-MP coagulant activity. Of the 63 patients with high TF-MP coagulant activity, 60 had low number of MP-TF. No correlation is present between TF-MP number and coagulant activity (r=0.029, p=0.69). TF-MP coagulant activity is associated with poor survival (hazard ratio 2.5; p=0.004), whereas number of TF-MP is not. Of the patients with high number of TF-MP, only 5 had tumour-derived TF-MP.

Discussion
In conclusion, most TF-MP in cancer patients are non-coagulant, and only a minority originate from the tumour. Because TF-MP number and coagulant activity are almost mutually exclusive, we postulate that two forms of TF-MP circulate in cancer patients. TF-MP coagulant activity might become a prognostic biomarker.
INTRODUCTION

Venous thromboembolism (VTE) is a frequent complication in cancer patients and the second leading cause of in-hospital mortality (1). The procoagulant state is characterised by elevated plasma levels of activated factor VII and tissue factor (TF) (2). Already in 1981, a pioneering publication initiated a discussion about microparticles (MP) and their possible contribution to the procoagulant state in cancer patients (3). Dvorak and colleagues demonstrated that several cancer cell lines release coagulant plasma membrane vesicles (i.e. MP), which range in size between 15 to 800 nm. They postulated that MP (i) provide phospholipids and thereby a surface for assembly of tenase and prothrombinase complexes, and (ii) exhibit a ‘thromboplastin-like’ activity, i.e. TF activity (3). Later studies confirmed both hypotheses (4;5). Therefore, TF, originally thought to be solely present hidden in and behind the vascular wall, is now known to be circulating on cell-derived MP as well (6).

In vivo, cancer patients have increased number of TF-exposing MP compared to healthy controls (7-10). Furthermore, the TF-MP dependent coagulant activity is increased in cancer patients compared to healthy controls (11;12). Cancer patients with VTE have higher number of TF-exposing MP than cancer patients without VTE (9;13;14), as well as a higher TF-MP dependent coagulant activity (14;15). Therefore, number and coagulant activity of TF-exposing MP have both been proposed as predictive biomarkers for VTE in cancer patients (12;14).

Two aspects, however, remain to be elucidated. First, the relationship between number of TF-exposing MP and TF-MP dependent coagulant activity is unclear, as also indicated by a recently published animal study (16). TF can be present in a non-coagulant form, and TF has other functions such as induction of angiogenesis and signal transduction (17). Hence, TF exposed on circulating MP is not necessarily procoagulant. Therefore, more insight into the relationship between the number of TF-exposing MP and TF-MP coagulant activity may provide clinically relevant information as it helps to select the most promising biomarker for predicting VTE in cancer patients: i.e. either number or coagulant activity of TF-exposing MP.

Second, it is important to know the cellular origin of TF-exposing MP in cancer, not only to increase our insight into the pathophysiological background but also to discover potential novel ways to intervene. As many cancer cells express TF (18;19), it is tempting to speculate that all TF-exposing MP and all TF-MP dependent coagulant activity can be ascribed to MP originating from cancer cells. In 2008, Davila and colleagues indeed demonstrated that cancer cells are capable of releasing TF-exposing MP in vivo (20). Other studies have shown that in blood from cancer patients at least part of the TF-exposing MP originate from cancer cells, and they demonstrated that surgical removal of the tumour results in a reduction of the number of TF-exposing MP. These data, however, do not exclude other cellular sources of TF-exposing MP in cancer, as they did not simultaneously check for blood cell origin (11;13). In contrast, Hron et al reported that the majority of the TF-exposing MP in cancer patients are of platelet and monocyte origin, but in this study cancer cell origin was not determined (8).
Therefore, in the present study we evaluated the relationship between the number of TF-exposing MP and TF-MP dependent coagulant activity and determined the cellular origin of circulating TF-MP. To answer these questions, we studied MP and their coagulant properties in plasma of more than 200 unselected cancer patients.

**METHODS**

*Patients*

Citrate-anticoagulated blood (0.32%) was collected from 209 consecutive cancer patients at the Department of Medical Oncology of the Academic Medical Center, the Department of Medical Oncology of the VU University Medical Centre in Amsterdam (the Netherlands) and the Department of Internal Medicine and Ageing in Chieti (Italy). Inclusion criteria were age above 18 years and active cancer, and in all patients the diagnosis of cancer was confirmed by histo-pathology. For comparison, blood samples were collected from 22 healthy subjects. All patients and healthy subjects signed an informed consent and the protocol was approved by the institutional review boards.

*Measurement of TF-exposing MP number and coagulant activity*

Blood was obtained via a single blood withdrawal either directly out of a venous catheter (within 5 minutes after placement) or by vena puncture, via a standardized procedure. Platelet poor plasma (PPP) was prepared by centrifugation at 1560 x g for 20 minutes within 1 hour after blood withdrawal. PPP was stored on melting ice to preserve coagulation factors, and was used within 2 hours in a fibrin generation test (21). Briefly, PPP was recalcified, and the time until fibrin formation, i.e. clotting of the plasma, was monitored in a spectrophotometer in the absence and presence of an inhibitory antibody to activated factor VII (anti-VIIa). The prolongation of the clotting time in the presence of anti-VIIa, which is expressed as % of the clotting time without the addition of anti-VIIa, is a measure for the TF coagulant activity that is associated with MP. Based on previous results, a prolongation of the clotting time in the presence of anti-VIIa above 13% was considered abnormal (22). For some experiments, plasma was ultra-centrifuged at 100,000 g to remove endogenous vesicles, and the clotting time was again measured in the presence and absence of anti-VIIa. Remaining PPP was snap frozen in liquid nitrogen and stored in a -80°C freezer until MP isolation for flow cytometry measurements, as previously described (23).

First, the number of TF-exposing MP (antigen) was determined in all patients as described previously (24;25). Second, the cellular origin of TF-exposing MP was determined in plasma from patients with number of TF-exposing MP above the 95th percentile. MP (5 µL) were diluted in 35 µL CaCl₂ (2.5 mmol/L)-containing phosphate-buffered saline (PBS). Then, 5 µL allophycocyanin (APC)-labeled annexin V was added plus 5 µL of monoclonal or control (isotype-matched) antibody. Samples were analyzed in a fluorescence automated cell sorter (FACS Calibur) with CellQuest software version 4.02 (Becton Dickinson, San Jose, 74
CA). Fluorescein isothiocyanate (FITC)-labeled IgG, phycoerythrin (PE)-labeled IgG, and anti-CD142 (anti-tissue factor (TF))-PE were derived from Becton Dickinson. Anti-CD61-FITC (anti-GP-IIIa; indicating platelet origin) was from Dako (Glostrup, Denmark). APC-conjugated annexin V (binding to phosphatidylserine) was from Caltag (Burlingame, CA, USA). Anti-CD62p-PE (P-selectin; activated platelet origin) was from Beckman Coulter Inc. (Fullerton, CA). Anti-CD24-PE (one of the P-selectin ligands) and anti-CD105 (endoglin; monocyte or cancer cell origin) were from Serotec (Kidlington, UK). Anti-FLT-1 (anti-VEGF-receptor-1; cancer cell origin) was from R&D (Minneapolis, MN) and anti-CD227 (mucine 1; epithelial or cancer cell origin) was purchased from Pharmingen (San Jose, CA).

**Statistical analysis**

All statistical analyses were performed with PASW Statistics, version 17 (SPSS Inc., Chicago, Ill, USA). Most variables showed a non-parametric distribution, these data were expressed as medians with corresponding inter-quartile ranges (IQR) and between group differences were tested with the Mann-Whitney U test, unless indicated otherwise. Variables with a parametric distribution were presented as mean ± standard deviation. Differences between dichotomous variables were tested with the Fisher’s exact test. Correlations were tested with the Pearson’s correlation coefficient or the Spearman’s correlation coefficient for non-parametric data.

Survival of the patients was determined after a follow-up of 6 months. Differences in survival were tested in univariate regression. Kaplan Meier graphs with corresponding log rank testing were used for assessing differences in time-dependent survival and for calculating the hazard ratio (HR) with the relative 95% confidence intervals (CI) for death. Adjustment for confounders was done using Cox regression, if allowed, by the size of the groups and number of events.

**RESULTS**

Number of TF-exposing MP and TF-MP dependent coagulant activity were measured in a cohort of 209 cancer patients (98 gastro-intestinal, 59 pancreatic, 23 breast, 17 lung, 5 prostate cancer, 2 ovarian cancer, and 5 other types). In Table 1, the patient characteristics are summarized. Briefly, 98 of the 209 patients suffered from metastasized disease (47%), patients had a mean age of 60 ± 11 years, and 75 patients were women (36%). For comparison, TF-exposing MP were also measured in 22 healthy subjects, of whom 15 women and 7 men, with a mean age of 38 ± 9 years.

**TF-exposing MP antigen and origin**

The number of TF-exposing MP were increased in cancer patients compared to healthy subjects (median: 2.0 vs. 0.40 x 10^4 /mL, p=0.01; Figure 1A and Table 1). Thirteen patients had number of TF-exposing MP above the 95th percentile, i.e. 1.0 x 10^5. These patients did
not differ from the patients with TF-exposing MP below the 95th percentile in number of chemotherapy cycles they already received. The cellular origin of TF was determined in the patients with TF-exposing MP above the 95th percentile using antibodies against cell type specific CD antigens. There was a marked variation with regard to the cellular origin of the TF-exposing MP between patients, with two distinctive patterns (Table 2).

In 5 patients, cellular origin of the TF-exposing MP was variable, including 67% and 44% of the TF-exposing MP staining with two independent tumour cell markers, 66% staining with a platelet marker, and 59% with a monocyte marker (all medians). Two of
these patients suffered from pancreatic cancer and died during follow-up, the three other patients had gastro-intestinal, lung and breast cancer. The median total number of TF-exposing MP was $1.8 \times 10^5$ /mL (IQR 1.4-3.1).

In the second group of patients, the cellular origin of only a minority (< 25%) of the TF-exposing MP could be established (Table 2). Most of these TF-exposing MP originated from platelets. These patients suffered from gastro-intestinal (3), pancreatic (2), lung (2) and breast cancer. Two patients died during follow-up. The median total number of TF-exposing MP was $0.89 \times 10^5$ /mL (IQR 0.64-2.4).

Table 2. Cellular origin of TF-MP in the two subgroups of cancer patients as detected by flow cytometry

<table>
<thead>
<tr>
<th>Cellular origin</th>
<th>Total cellular origin ≥ 100% (n=5)</th>
<th>Total cellular origin &lt; 25% (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour: CD227</td>
<td>67% (33-71) 1</td>
<td>1.0% (0.5-5.4)</td>
</tr>
<tr>
<td>Tumour: CD24</td>
<td>44% (27-70)</td>
<td>1.5% (0.0-2.9)</td>
</tr>
<tr>
<td>Platelet: CD61</td>
<td>66% (50-71)</td>
<td>22% (2.1-28)</td>
</tr>
<tr>
<td>Monocyte: CD14</td>
<td>59% (36-76)</td>
<td>1.2% (0.0-3.9)</td>
</tr>
<tr>
<td>Granulocyte: CD66b</td>
<td>4.0 (1.6-15)</td>
<td>0.58 (0.48-1.7)</td>
</tr>
<tr>
<td>Erythrocyte: CD235</td>
<td>3.5 (2.8-7.5)</td>
<td>1.9 (0.57-3.3)</td>
</tr>
<tr>
<td>TF-dependent coagulant activity (%) 2</td>
<td>0.23 (0.0-12)</td>
<td>10 (3.3-24)</td>
</tr>
<tr>
<td>Number of TF-exposing MP</td>
<td>$1.8 \times 10^5$ (1.4-3.1)</td>
<td>$0.89 \times 10^5$ (0.64-2.4)</td>
</tr>
</tbody>
</table>

1 Depicted in this table is the proportion (% of total; median, IQR) of the total number of TF-exposing MP (per individual patient) which labelled double positive with the mentioned markers; 2 TF-dependent coagulant activity is measured in a fibrin generation test by comparing the clotting time in the presence and absence of anti-VIIa. The prolongation (in %) of the clotting time in the presence of antibody, compared to the clotting time in the absence of antibody, is a measure for the amount of TF-MP dependent clotting.

Coagulant activity of TF-exposing MP

Cancer patients and healthy subjects did not differ in TF-MP dependent coagulant activity (median 5.9% versus 2.5%, p=0.63; Figure 1B). A prolongation of the TF-MP dependent clotting time above 13% in the presence of anti-VIIa was observed in 63 of the 203 cancer (31%; 95% CI 25-37%), compared to 5 of the 22 healthy subjects (23%; 95% CI 7.8-45%; p=0.30).

The TF-MP dependent clotting time was not associated with the number of TF-exposing MP ($r=0.029$, p=0.69; Figure 2). When comparing patients with high (>95th percentile) and low (<95th percentile) number of TF-exposing MP, respectively 3 of the 12 (25%; in 1 patient the activity measurement failed) and 60 of the 192 (31%) had TF-MP dependent clotting activity. Similarly, when comparing the patients with high (>13%) and low (<13%) TF-MP dependent clotting activity, respectively 3 of the 63 (4.8%) and 9 of the 141 (6.4%) had high TF-exposing MP number. This, together with Figure 2, indicates that there are two distinct forms of circulating TF-exposing MP, one non-coagulant form of TF exposed on circulating MP which can be detected by flow cytometry, whereas the coagulant form of TF-exposing MP is below the detection limit of flow cytometry.
Chapter 6

Survival

The number of TF-exposing MP did not differ between patients who died between inclusion and 6 months of follow-up, and those who did survive during this period (median levels respectively 2.4 vs. 1.8 x 10^4; p=0.43). At univariate analysis, the survival of patients with number of TF-exposing MP above and below the 95th percentile was similar (hazard ratio 1.5; 95% CI 0.38 – 5.8; Figure 3).

Figure 1. Number of TF-exposing MP per mL (A) and coagulant activity of the TF-exposing MP (B) in healthy subjects and cancer patients. In both figures, the open dots indicate the patients with a high number of TF-exposing MP. In (B) the majority of these open dots (n=7) are at the level of the X-axis which makes them barely visible.

Figure 2. Correlation between number of TF-exposing MP antigen and TF-MP dependent coagulant activity. On the X-axis the number of TF-exposing MP (i.e. antigen) are shown, on the Y-axis TF-MP dependent clotting time (percentage of control, i.e. the clotting time determined in the absence of antibody to antIVIIa). For overview, the Y-axis is logarithmical; r=0.081, p=0.253. The patients with the highest number of TF-exposing MP are depicted in open dots. In 5 patients, TF-MP coagulant activity measurement failed, among them one patient with a high number of TF-exposing MP.

Survival

The number of TF-exposing MP did not differ between patients who died between inclusion and 6 months of follow-up, and those who did survive during this period (median levels respectively 2.4 vs. 1.8 x 10^4; p=0.43). At univariate analysis, the survival of patients with number of TF-exposing MP above and below the 95th percentile was similar (hazard ratio 1.5; 95% CI 0.38 – 5.8; Figure 3).
TF-MP coagulant activity was higher in patients who died (10%; IQR 0.0-24%) compared to surviving patients (0.05%; IQR 0.0-14%; p=0.023). Patients with a TF-MP dependent clotting time above 13% had a more than two fold higher risk of dying compared to those with a normal test (hazard ratio 2.5;95% CI 1.3 – 4.7; p=0.004). After adjustment for stage of disease and the time between start of chemotherapy and blood withdrawal, TF-MP coagulant activity remained associated with a worse prognosis (HR 2.1;95% CI 1.1 – 4.0).

**DISCUSSION**

This study demonstrates that there are likely to be at least two forms of TF associated with circulating MP in cancer patients. One form of MP-TF is coagulant, but is present in minute quantities, whereas the other form of TF circulates in relatively high quantities but lacks coagulant activity. These findings agree with data from small series of patients with cancer in which the number of TF-exposing MP were not associated with TF-MP coagulant activity (11;20;26). Although Khorana et al. earlier showed an association between number and coagulant activity of the TF-exposing MP, these results seem to be markedly influenced by two patients with VTE who had extremely elevated number of TF-exposing MP (14). More recently, Wang and colleagues injected cells from two different human pancreatic cancer cell lines in a mouse model, and observed that the number of TF-exposing MP are comparable in mice injected in the pancreas with either HPAF-II or HPAC cells, whereas the MP-associated TF-mediated coagulant activity is high only in mice receiving HPAF-II cells but almost absent in mice receiving HPAC cells (27). So, similar to our present study in humans there is no relationship between the number of TF-exposing MP and TF-MP dependent coagulant activity. The question then is what we can learn from this discrepancy.

First, the patients with the coagulant but (at least by flow cytometry) non-detectable form of TF illustrates that either an extremely low number of coagulant TF-exposing MP must be
present, or that coagulant TF is exposed on vesicles, e.g. exosomes (21), which have a diameter <100 nm and are therefore simply too small to be detected by even state-of-the-art flow-cytometers (28). We tested this second option by removing MP from plasma samples of patients showing the strongest (> 50%) delay of clotting time in the presence of anti-VIIa. After removal of MP by conditions of high-speed centrifugation sufficient to pellet MP but insufficient to pellet smaller vesicles such as exosomes, the MP-depleted plasma samples were unable to clot after recalcification (clotting time > 3600; n=10; data not shown). Therefore, the TF-MP coagulant activity is likely to be associated mainly with MP and not with smaller types of vesicles.

Second, the TF-MP dependent coagulant activity in patients with number of TF-exposing MP above the 95th percentile requires attention. Given the fact that TF-dependent coagulation is associated with (below) threshold number of TF-exposing MP in cancer (Table 2), it seems unlikely that patients who have high number of coagulant TF-exposing MP will be able to survive. Indeed, previous studies showed that injection of coagulant TF-exposing MP triggers massive venous thrombosis in an experimental model (29;30), and thus far we have reported only one patient who had high number of coagulant TF-exposing MP. This patient suffered from meningococcal septic shock and died from disseminated intravascular coagulation (31). Furthermore, it is unlikely that these high numbers of coagulant TF-exposing MP circulate in vivo, as they are cleared with high efficiency (32).

In the present study, the majority of cancer patients with high number of TF-exposing MP exhibit a very low TF-MP dependent coagulant activity. At present, one can only speculate about the function of these TF-exposing MP. The patients in our study with high number of TF-exposing MP did not differ from the other patients with respect to prognosis, cancer type or stage, nor in inflammation as indicated by leukocyte count. Taken together, these data do not support an important contribution of the non-coagulant TF-exposing MP in cancer progression, although the sample size of 13 patients does not allow firm conclusions.

Third, the cellular origin of the non-coagulant TF-exposing MP needs further clarification. In the patients in whom the cellular origin of TF remains mainly unknown, the number of TF-exposing MP may be insufficient to allow the study of their cellular origin by flow cytometry. Alternatively, the cellular origin may be different from those studied but this seems unlikely in view of the broad panel of antibodies used. To study the cellular origin in cancer patients using flow cytometry, a very large population has to be screened, in fact, in our study, in only 5 out of 209 (2.4%) this was possible.

More intricately, some patients showed TF-exposing MP which bound antibodies directed against tumour (epithelial) antigens and antibodies against platelet or monocyte antigens, and total number added up to more than 100%. Theoretically, antibodies labelled with fluorescent probes can form aggregates or antibodies can bind to Fc-receptors. Both explanations, however, seem unlikely because we centrifuge all antibodies before use. Moreover, all antibodies have been titrated and compared to similar concentrations of control antibodies. Furthermore, not all antibodies showed this effect, e.g. the antibody used against glycophorin A (CD235a), i.e. the band 3 protein characteristic for MP originating
from erythrocytes, failed to stain the TF-MP, indicating that the observed effect is specific and is no artefact. Therefore, these data suggest that single TF-exposing MP may indeed expose both tumour and blood cell antigens, possibly due to fusion between MP and/or some cell types. When these cancer cells at their turn shed MP, the MP bear characteristics from both platelets or monocytes and cancer cells. Fusion has evolutionary advantages because receptors (truncated EGFRvIII), genetic material or growth factors can be transferred between cells by vesicles (33;34). For instance, TF-exposing MP from leukocytes can fuse with the membrane of activated platelets, which suggests that this is a way to centralise phospholipid dependent coagulation reactions at the surface of activated platelets (35). To summarize, we cannot relate our findings to previous studies using a similarly broad antibody panel. However, the findings are in line with earlier studies which labelled either tumour or blood cell antigens (8;11;13), and indicate fusion of MP with cells or other MP.

Lastly, the presence of increased TF-MP dependent coagulant activity, but not the number of TF-exposing MP, independently predicts poor survival, which is in line with previous data (15;36). If confirmed, TF-MP dependent coagulant activity may be a useful prognostic biomarker.

Specific aspects of the present study require comment. Flow cytometry has well known limitations for the measurement of microparticle sized vesicles. The measurement is dependent on pre-analytic variables and cannot measure smaller sized vesicles, i.e. exosomes (28;37). However, flow cytometry is a method used widely and much energy has been devoted to standardization of the technique. Furthermore, we could only determine the cellular origin in patients with detectable levels of TF-exposing MP, of which the majority turned out to be non-coagulant rather than coagulant, implicating that the cellular origin of MP exposing coagulant TF remains unclear. Lastly, the healthy subjects were not matched to the cancer patients with respect to age and sex. The comparison between cancer patients and controls was not one of the aims of this study, but controls were included merely for reference.

In conclusion, there are distinct forms of circulating TF-exposing MP in cancer patients: one form of TF is coagulant, but this form seems to be present in minute quantities. A second form of TF can circulate in relatively large quantities and is associated with MP, but lacks coagulant activity. Thus, the true cellular origin of both the coagulant and non-coagulant MP-TF remain to be elucidated. Interestingly, at least part of the non-coagulant TF-exposing MP seem to derive from fused cells or MP, as they bear characteristics from both cancer and blood cells.

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Chapter 6

REFERENCE LIST


