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The association between CD24-exposing microparticles, coagulation, thrombosis and prognosis in cancer patients

Ankie Kleinjan
René J. Berckmans
Pieter W. Kamphuisen
Auguste Sturk
Harry R. Büller
Rienk Nieuwland

Submitted
Chapter 3

| ABSTRACT |

Introduction
In cancer patients, microparticles have been associated with development of venous thromboembolism (VTE) and cancer progression. Recently, in a mouse model, cancer cell-derived microparticles exposing P-selectin-glycoprotein-ligand-1 (PSGL-1) and tissue factor (TF) were deposited at a site of vascular injury by binding to P-selectin on activated platelets. Because human cancer cells expose CD24, another ligand of P-selectin associated with tumour biology and poor prognosis, we hypothesize that CD24-exposing microparticles (CD24⁺-MP) may be associated with development of VTE in cancer patients.

Methods
We collected blood from 67 cancer patients and 22 healthy subjects, quantified CD24⁺-MP by flow-cytometry and coagulation markers by ELISA. Based on maximum levels in healthy subjects, the group of cancer patients was divided into “high CD24⁺-MP” and “low CD24⁺-MP”.

Results
Cancer patients had increased levels of CD24⁺-MP compared to controls (median 2.9 versus 1.2 x 10⁵, p<0.001). High and low CD24⁺-MP cancer patients did not show differences in coagulation markers, or in VTE, but the prognosis of high CD24⁺-MP patients was poor (odds ratio 4.6 to die within 15 months; p=0.016) compared to low CD24⁺-MP patients.

Conclusion
CD24⁺-MP, which can be measured non-invasively in peripheral blood, may prove a useful prognostic biomarker.
INTRODUCTION

Exposure of CD24 by cancer cells is a marker of poor prognosis (1-6). CD24, a cell adhesion molecule and one of the ligands of the adhesion receptor P-selectin, supports the binding of cancer cells to activated platelets and endothelial cells (7). The interaction with platelets provides circulating cancer cells with physical protection against recognition and subsequent destruction by the immune system, whereas the binding to endothelial cells promotes extravasation and metastasis (7). Thus, CD24 is associated with cancer growth and metastasis. Cancer growth is also intertwined with coagulation activation, and numerous animal and human studies have shown that cancer promotes coagulation, whereas coagulation promotes cancer growth (8). In 1823, Bouillaud described three cancer patients with fibrin clots in the legs and proposed that these venous thromboses might be related to the malignant disease (9). Approximately 5-10% of all cancer patients will develop VTE during the first year of their disease (10). VTE in cancer patients is associated not only with its related morbidity and mortality, but also indicates a poorer prognosis when compared to cancer patients without VTE (8;11).

Recently, cell-derived microparticles and exosomes have gained a strong clinical interest as being one of the missing links between cancer and coagulation and the development of VTE (12-14). Blood from cancer patients contains elevated levels of circulating microparticles compared to healthy subjects. The vast majority of these microparticles originate from platelets or megakaryocytes, but also microparticles from cancer cells and endothelial cells have been detected (15-18). Because such “blood-borne” microparticles can expose tissue factor (TF), the initiator of coagulation, these microparticles can trigger coagulation and thus are likely to be associated with development of VTE (19). Recently, Thomas and colleagues proposed a new mechanism by which microparticles from cancer cells may promote thrombus formation. They showed that microparticles from cancer cells expose both TF and P-selectin glycoprotein ligand 1 (PSGL-1, CD162), the latter being a ligand of P-selectin. In a mouse model, these microparticles are present in peripheral blood and accumulate at a side of vessel wall injury. Because the accumulation of microparticles is abolished by a monoclonal antibody against P-selectin, they hypothesize that PSGL-1-exposing microparticles from cancer cells interact with P-selectin exposing activated platelets or platelet-derived microparticles present at the damaged vessel wall. This may deposit TF-exposing microparticles originating from cancer cells at a side of vessel wall injury (20).

Although PSGL-1 and CD24 are both ligands of P-selectin, thus far only microparticle-exposed PSGL-1 has been associated with coagulation and thrombosis (7). It is tempting to hypothesize that CD24, in a similar manner to PSGL-1, when present on cancer cell-derived microparticles, may support development of VTE in cancer patients. When this hypothesis is correct, than CD24-exposing MP may be one of the missing links between cancer progression and development of VTE. To investigate this hypothesis, we have analyzed the presence of CD24-exposing microparticles in peripheral blood of cancer patients and studied their association with coagulation activation, thrombosis and prognosis in these patients.
Citrate-anticoagulated blood (0.32%) was collected from 67 unselected cancer patients at the Department of Medical Oncology of the Academic Medical Center, as part of a larger ongoing clinical study. Inclusion criteria were age above 18 years and active cancer, and in all patients the diagnosis of cancer was confirmed by 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 board.

Microparticles were isolated from platelet poor plasma as described previously (21). Microparticles (5 µL) were diluted in 35 µL CaCl$_2$ (2.5 mmol/L)-containing phosphate-buffered saline (PBS). Then, 5 µL allopheocyanin (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, CA). We identify microparticles based on their forward and side scatter and not solely on Ann V exposure.

Fluorescein isothiocyanate (FITC)-labeled IgG$_{1}$, 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) and anti-CD144-PE (resting endothelial cells) were from Beckman Coulter Inc. (Fullerton, CA). Anti-CD62E-PE (activated endothelial cells) was from Ancell Corporation (Bayport, MN). Anti-CD24-PE (one of the P-selectin ligands) and anti-CD105 (endothelial cells) were from Serotec (Kidlington, UK). Anti-CD227 (mucine 1; epithelial or cancer cell origin) was purchased from Pharmingen (San Jose, CA). 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). Prothrombin fragment 1+2 (F$_{1+2}$) and thrombin-antithrombin complexes (TAT) ELISA’s were obtained from Enzygnost (Dade Behring; Marburg, Germany), and D-dimer ELISA from Innovance (Dade-Behring).

All statistical analyses were performed with PASW Statistics, version 17 (SPSS Inc., Chicago, IL, USA). All p-values were corrected for multiple testing using Bonferroni correction. Hence, p-values ≤ 0.0025 were considered statistically significant. Continuous data were expressed as medians with corresponding inter-quartile ranges and between group differences were tested with the Mann-Whitney U test. Differences between dichotomous variables were tested with the Fisher’s exact test. Correlations were tested with the Pearson’s correlation coefficient (2-tailed) or the Spearman’s correlation coefficient for non-parametric data. Low and high CD24$^+$ MP groups were made based on the upper limit of the 95$^{th}$ confidence interval in healthy subjects. Differences in survival between low and high CD24 groups were tested in univariate regression. Kaplan Meier graphs with corresponding log rank testing were used for testing of differences in time dependent survival. The sample size of the study did not allow multivariate (survival)
analysis or adjustment for time between diagnosis and inclusion in the study (‘left truncation’). As this is an exploratory study, no formal power calculation could be done. This present study is a sub-study of an ongoing larger prospective study. In that study the outcome venous thrombosis is being assessed after 6 months. As all patients had a maximum completed follow-up of 15 months at the moment of this sub-study, survival was determined after 15 months.

| RESULTS |

Patients with cancer and healthy subjects
The 67 patients with cancer were heterogeneous concerning type, stage and treatment of cancer. The mean age of the cancer patients was 59 ± 13 years and 54% were male. The cancer patients suffered from pancreatic carcinoma (n=24), gastrointestinal carcinoma (n=17), breast carcinoma (n=15), esophagus carcinoma (n=6), and other types of cancer (n=5: brain, biliary, sarcoma, unknown origin, prostate). Twelve patients had local disease and came for neo-adjuvant therapy. The other patients had locally advanced (n=18) or metastatic disease (n=37). All patients received intravenous chemotherapy via the out-patient clinic; the majority of them for the first cycle (n=47), others for the second (n=8) or third or more cycle (n=12). The mean age of the healthy subjects was 38 ± 10 years, 32% were men.

Microparticles in cancer patients and healthy subjects
Cancer patients had significantly increased levels of CD24-exposing microparticles (CD24+ MP) compared to healthy subjects (median 2.9 versus 1.2 x 10^5 MP/mL, p<0.001). Similarly, median levels of microparticles binding annexin V, i.e. microparticles exposing phosphatidylserine (PS), were higher in cancer patients compared to healthy subjects (5.6 versus 3.1 x 10^6 MP/mL, p<0.001). For levels of other subtypes of microparticles in cancer patients and healthy subjects, see Figure 1.

Coagulation activation, thrombosis and prognosis in the low and high CD24 group
To study our initial hypothesis that cancer patients with elevated levels of CD24+ MP may have an increased risk of thrombosis, we compared coagulation activation between cancer patients with high (n=14) and low (n=53) levels of CD24+ MP. For the characteristics of the patients in both groups, see Table I. There were no differences between the two groups with regard to the concentrations of coagulation activation markers D-dimer, F, and TAT (p=0.574, p=0.552 and p=0.021, respectively, Table I).

Of the 67 patients, 5 patients developed venous thrombosis within 6 months after blood collection, 4 patients in the low-CD24+ MP group (4/53, 7.5%) and a single patient in the high-CD24+ MP group (1/14, 7.1%; p=0.959). With regard to mortality, however, 8 out of 14 patients (57%) died within 15 months in the high-CD24+ MP group, compared to 12 out of 53 (23%) in the low-CD24+ MP group (odds ratio 4.6, confidence interval 1.3 - 16; p=0.016),
Figure 1. Microparticles in cancer patients and healthy subjects. A. AnnV: annexin V-binding microparticles; CD61: platelet origin; CD24 - one of the P-selectin ligands; CD62E – E-selectin – activated endothelial cells; FLT-1 – VEGF-receptor1 – cancer cell origin; B. CD105 – endoglin – monocyte or tumor origin; CD62p -P-selectin – activated platelet origin; CD227 – mucine 1 – cancer cell origin; CD142 (TF) – tissue factor; CD144 – (resting) endothelial cells. Bars (healthy subjects (black) and cancer patients (grey)) reflect medians and interquartile ranges, with corresponding 5th and 95th percentiles. *: statistically significant with a Bonferroni corrected p-value of ≤ 0.0025. For the sake of overview, the results are shown in two separate graphs. The axes differ between both figures.

see table 1. Thus, patients with high levels of CD24+ MP have a poor prognosis compared to patients with low levels of CD24+ MP. Ten patients out of 15 (71%) versus 27 patients out of 53 (51%) patients had metastatic disease (95% CI for the difference – 0.079 – 0.47; p=0.155), indicating that a difference in stage is not the sole explanation for the difference in prognosis between the low and high CD24+ MP groups. Increasing stages of disease, however, were associated with increasing CD24+ MP levels (p=0.029; Kruskal Wallis test).

Microparticles
To gain more insight in the mechanisms underlying the marked difference in prognosis of the high and low CD24+ MP groups, we looked into more detail at the circulating subtypes of microparticles in both groups, see Table 1 and Figure 2. The numbers of microparticles binding annexin V, often used as a general marker of microparticles, were not different between the patient groups, nor were numbers of P-selectin or TF-exposing microparticles. Compared to the low-CD24 group, however, patients in the high-CD24 group had significantly elevated levels of microparticles exposing E-selectin (CD62E, activated endothelial cells; p<0.001), FLT-1 (VEGF receptor, cancer cell-derived; p<0.001), CD105 (endoglin, monocytes and cancer cells; p<0.001) and a trend towards more CD227 exposing microparticles (mucin, on cancer cells; p=0.038).

In order to explore whether the presence of CD24+ MP is associated with the presence of other subtypes of microparticles in these patients, we studied the relationship in
Table I. Patient characteristics of the high and low-CD24+ MP groups

<table>
<thead>
<tr>
<th>Low-CD24+ MP cancer patients (n=53)</th>
<th>High-CD24+ MP cancer patients (n=14)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of cancer, stage and events</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>19 (34%)</td>
<td></td>
</tr>
<tr>
<td>Gastro-intestinal</td>
<td>12 (23%)</td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>13 (25%)</td>
<td></td>
</tr>
<tr>
<td>Esophageal</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Other (prostate, sarcoma, unknown origin)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Limited local disease</td>
<td>11 (22%)</td>
<td></td>
</tr>
<tr>
<td>Locally advanced disease</td>
<td>15 (28%)</td>
<td></td>
</tr>
<tr>
<td>Metastatic disease</td>
<td>27 (51%)</td>
<td>0.16</td>
</tr>
<tr>
<td>Venous thrombosis within 6 months</td>
<td>4 (7.5%)</td>
<td>0.96</td>
</tr>
<tr>
<td>Died within 15 months</td>
<td>12 (22%)</td>
<td>0.016</td>
</tr>
<tr>
<td>Coagulation markers and microparticles (median (interquartile ranges))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-dimer</td>
<td>0.77 (0.40-1.4)</td>
<td>1.1 (0.44-2.7)</td>
</tr>
<tr>
<td>F1+2</td>
<td>236 (173-366)</td>
<td>232 (180-287)</td>
</tr>
<tr>
<td>TAT</td>
<td>3.7 (2.9-4.6)</td>
<td>5.7 (3.8-12)</td>
</tr>
<tr>
<td>Ann V binding MP</td>
<td>5.4 x 10^6 (3.4 – 8.4)</td>
<td>6.9 x 10^6 (4.6 – 12)</td>
</tr>
<tr>
<td>CD62p+ MP</td>
<td>2.3 x 10^5 (1.8 – 4.8)</td>
<td>5.6 x 10^5 (1.5 – 8.7)</td>
</tr>
<tr>
<td>TF+ MP</td>
<td>1.7 x 10^4 (0.18 – 3.3)</td>
<td>2.8 x 10^4 (0.41 – 12)</td>
</tr>
<tr>
<td>CD62E+ MP</td>
<td>1.0 x 10^4 (0.0 – 4.0)</td>
<td>4.4 x 10^4 (1.7 – 20)</td>
</tr>
<tr>
<td>CD105+ MP</td>
<td>1.8 x 10^3 (0.71 – 3.1)</td>
<td>8.6 x 10^3 (5.7 – 11)</td>
</tr>
<tr>
<td>FLT-1+ MP</td>
<td>2.0 x 10^2 (0.83 – 3.5)</td>
<td>26 x 10^4 (6.8 – 185)</td>
</tr>
<tr>
<td>CD227+ MP</td>
<td>1.9 x 10^1 (0.0 – 3.5)</td>
<td>4.7 x 10^4 (0.37 – 16)</td>
</tr>
</tbody>
</table>

* This patient with locally limited disease at the moment of blood withdrawal, turned out to be progressive during follow-up and died. AnnV: annexin V-binding microparticles; CD62p+ - P-selectin – activated platelet origin; TF – tissue factor; CD62E+ – E-selectin – activated endothelial cells; CD105+ – endoglin – monocyte or tumour origin; FLT-1+ – VEGF-receptor1 – cancer cell origin; CD227+ – mucine 1 – cancer cell origin

all included cancer patients, so irrespective of their CD24+ MP levels. There is a strong correlation between CD24 and E-selectin (r=0.967, p<0.001). Similarly, there is a strong correlation between CD24 and FLT-1 microparticles (r=0.917, p<0.001). Other significant correlations are present between CD24 and CD227 (r=0.284; p=0.007), CD105 (r=0.584; p<0.001) and P-selectin (r=0.469; p<0.001), but no correlation was found between CD24 and TF (r=0.067, p=0.589) or between CD24 and total number of Annexin V binding microparticles (r=0.077, p=0.535). Numbers of Annexin V binding microparticles were not or to a lesser content correlated with these microparticles, suggesting that the correlations between CD24+ MP are not dependent on total microparticle count.

Leukocyte count at the moment of blood withdrawal was not correlated with CD24+ MP (r=0.057, p=0.636).
In this study, we found that some cancer patients have increased plasma levels of microparticles exposing CD24 (CD24\textsuperscript{+} MP) when compared to healthy subjects. The systemic presence of CD24\textsuperscript{+} MP is not surprising in view of the data in ovarian cancer patients, in which CD24 bearing exosomes have been found in ascites fluid (22;23). A novel observation is that CD24\textsuperscript{+} MP are associated with a lower survival rate in cancer patients, which is in line with the literature pointing to the prognostic value of CD24 expression on cancer cells (1-5;24). This difference in prognosis is not merely a reflection of a difference in stages between both patient groups. Patients with high levels of CD24\textsuperscript{+} MP, however, do not differ from those with low levels of CD24\textsuperscript{+} MP with regard to coagulation activation, nor in the risk of developing venous thrombosis in the near future. Therefore, we could not find supporting evidence for a possible role of CD24 in the prothrombotic state in cancer patients, in analogy to the role of PSGL-1 as proposed by Thomas and colleagues (20). Also, the difference in prognosis between both groups could not be explained by a difference in venous thrombosis risk.

What then characterizes the patients with high levels of circulating CD24\textsuperscript{+} MP? Our data reveal that the presence of CD24- microparticles is strongly associated with endothelial activation (E-selectin), angiogenesis (FLT-1), tumor load (CD227) and platelet activation (P-selectin). Whether the presence of CD24 or other cancer cell-derived microparticles is causally linked to progression of disease and death in cancer patients, cannot be concluded from this study. There are, however, extensive in vitro data supporting a causal role. For instance, microparticles and exosomes (even smaller vesicles) can expose or contain active substances such as Fas-ligand, mRNA and matrix metalloproteases, which have various effects on cancer invasion, migration and metastasis (25-27).

The very strong correlation between CD24 and endothelial activation (E-selectin) is intriguing in view of the in vivo data of Myung and colleagues, who showed that CD24

![Figure 2. Survival of high and low-CD24\textsuperscript{+} MP cancer patients.](image-url)
facilitates the rolling of cancer cells on the endothelial wall via binding to E-selectin, and thereby mediates extravasation of cancer cells (28). High plasma levels of E-selectin-exposing microparticles are likely to reflect exposure of E-selectin on activated endothelial cells, which is a prerequisite for extravasation of cancer cells. Alternatively, shedding of microparticles bearing E-selectin could reflect downregulation of this receptor, thereby protecting the organism against extravasation of tumour cells. Of course, there are also several other potential explanations, one being that there is no causal relationship at all, i.e. that both CD62E and CD24⁺ MP are a reflection of a third process such as chemotherapy.

Study design limitations, such as the heterogeneous patient population and the relatively small sample size, hamper the ability to draw definite conclusions. The cohort is heterogeneous with respect to stage, type and treatment for cancer, all parameters which could have affected CD24-MP levels. A rule of thumb for multivariate analysis is that at least 5-10 cases per parameter should be included, and therefore a meaningful multivariate analysis could not be performed (29). Another limitation of the present study is that the healthy subjects were not matched to the cancer patients with respect to age and sex. However, acknowledging these limitations, we aimed to identify promising directions rather than providing a more definitive answer, this in analogy with the phases of biomarker development for early detection of cancer described by Pepe et al (30). Furthermore, flow cytometric analysis of microparticles has some important limitations, such as the lower sensitivity for smaller particles (31). However, the specificity of the detection of subtypes of microparticles by flow cytometry is high, and thus the relative results of measurement of CD24⁺ MP using flow cytometry can be compared between patients, and between patients and controls in the present study. Finally, haematopoietic cells (leucocytes in particular) have also been demonstrated to express CD24⁺ MP, which would explain the very low but not absent numbers of CD24⁺ MP found in the healthy subjects. However, it is unlikely that differences in haematopoietic cells can explain the variations in CD24⁺ MP found in cancer patients, as there was no correlation between leukocyte count (r=0.057) or leukocyte derived microparticles and CD24⁺ MP (data not shown). Also, CD24⁺ MP increased during increasing stages of disease, indirectly supporting that these microparticles derive from cancer cells.

The present study may have relevant clinical implications if confirmed by larger prospective studies. Exosomes and microparticles have attracted great interest as potential biomarkers (32-35). As a potential prognostic biomarker, CD24-MP shows promising features. Information on the presence of these microparticles can be gathered quite simply and non-invasively by one withdrawal of peripheral blood. As these microparticles are likely to originate from cancer cells, they provide insight into the cancer status. In the future this may prove useful, especially when cancer tissue is unavailable.

In conclusion, we found that cancer patients with high plasma levels of CD24⁺ MP neither show signs of coagulation activation nor do they have a higher risk of developing venous thrombosis when compared patient with low plasma levels of CD24⁺ MP. High levels of CD24⁺ MP do reflect a poor prognosis. Furthermore, the strong correlations between
Chapter 3

CD24+ MP and (microparticle-associated) markers of endothelial activation, cancer load and survival indicate that CD24+ MP reflect cancer progression and cell activation.

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| REFERENCE LIST |


