Venous thrombosis in cancer patients: Prediction, diagnosis and management
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Chemotherapy and anti-angiogenic drugs affect composition and coagulant phenotype of cell-derived vesicles in cancer patients

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| ABSTRACT |

Introduction
In cancer, cell-derived vesicles may contribute to the prothrombotic state, cancer progression, and resistance of cancer cells to chemotherapy. Therefore, in an exploratory study, we determined the effects of therapy on the coagulant activity and composition of circulating vesicles.

Method
Blood was collected from 11 patients with glioma and 5 patients with lung cancer, at different time points before and after start of two different chemotherapy regimens, in glioma combined with an anti-angiogenic agent. Procoagulant activity was studied in a fibrin generation test, and endothelial and tumour derived microparticles were determined by flow-cytometry.

Results
Treatment did not affect the overall procoagulant activity of vesicles in cancer patients (p=0.39). Levels of endothelial microparticles (CD62E+) tended to increase in the glioma (p=0.18), but not in lung cancer patients (p=0.41). Baseline levels of microparticles bearing the receptor for vascular endothelial growth factor (VEGFR-1) were increased in cancer patients compared to healthy subjects (p=0.012), and VEGFR-1-exposing microparticles decreased by 85% after anti-angiogenic therapy in glioma patients (p=0.021). Finally, in two lung cancer patients the concentration of mucine-exposing microparticles increased two days after chemotherapy.

Discussion
Chemotherapy and anti-angiogenic therapy lead to specific changes in the composition of circulating microparticles, especially with regard to endothelial- and tumour-derived microparticles. These changes are markedly different between the two patients groups and even between subjects within one group, suggesting that such microparticles may be associated with prognosis or response to treatment.
INTRODUCTION

Cell-derived vesicles (microparticles and the smaller sized exosomes) are present in all body fluids that have been investigated so far (1-3). In cancer patients, microparticles can also directly originate from tumour cells (4-6). Circulating microparticles in cancer have gained much interest, as they may play a role in the procoagulant and prothrombotic state in cancer patients, and can even contribute to cancer progression (7;8). Microparticles are procoagulant, as they provide phospholipids necessary for the assembly of coagulation factors, and, second, they may carry tissue factor (TF), the initiator of coagulation (9;10). In vivo, the concentration of TF-exposing microparticles and their coagulant activity is increased in cancer patients who have or will develop venous thromboembolism (VTE) (11-15). Furthermore, microparticles can modulate angiogenesis and the immune response, thereby contributing to development of tumours with a more aggressive phenotype (8;16;17). Therefore, microparticles constitute an attractive future target for therapy and have potential applications as prognostic biomarkers (12).

The relationship between chemotherapy and microparticles is complex. First, the shedding of microparticles may contribute to removal of intracellular substances which are detrimental to the cancer cell. Examples are vesicles containing increased concentrations of apoptotic proteins or chemotherapeutic drugs (18-21). Second, chemotherapy and agents that inhibit angiogenesis are one of the main causal factors for the development of VTE in cancer patients. In a large cohort study, the odds ratio for VTE increased from 4.1 for cancer patients without chemotherapy to 6.5 for patients receiving chemotherapy (22). The aetiology remains largely unknown; postulated mechanisms include direct endothelial damage and changes in blood composition, such as decreases in the levels of anticoagulant proteins, including protein C, or increases in the concentration of procoagulant clotting factors (23-25). In vitro, cisplatin and daunorubicin induce the release of microparticles by endothelial cells. These microparticles support coagulation by binding coagulation factors rather than by exposing coagulant TF (26;27). To which extent chemotherapy affects the composition and procoagulant phenotype of microparticles in vivo, however, is unclear. Furthermore, whether or not all coagulant activity is exclusively associated with microparticles, or also with smaller types of vesicles such as exosomes, is debated.

Therefore, in the present explorative study we investigated the effects of different chemotherapy regimens on the procoagulant activity and the composition of circulating microvesicles, the latter with a focus on endothelial and tumour derived microparticles.

METHODS

Patients from two cohorts were included in this study. In the first study, patients with non-small cell lung cancer stage IIIb or IV were hospitalised to receive their first gift of chemotherapy. They received either a combination of cisplatin and gemcitabin on
day 1, and gemcitabin on day 8, or they received daily radiotherapy combined with cetuximab daily (Table 1). Blood was obtained just before start of the chemotherapy (day 1), 1 hour after the chemotherapy was administered, at day 2, at day 8 just before the new gift, and at day 22 just before the new gift. Exclusion criteria were the use of anticoagulants in therapeutic doses and previous chemotherapy within one year before inclusion. The institutional review board of the Academic Medical Center in Amsterdam approved the protocol, and all patients provided written informed consent.

In the other cohort, patients with histologically confirmed intracranial high grade glioma (World Health Organization grade III or IV) and evidence of tumour recurrence at baseline magnetic resonance imaging were included. They were treated with continuous dose-intense temozolamide (Temodal® (Schering-Plough, Houten, The Netherlands), daily 50 mg/m2, orally, continuously), bevacizumab (Avastin®, 10 mg/kg i.v., every 21 days, defined as one cycle) and dexamethasone. All patients provided written informed consent, and the trial was approved by the institutional review board of the Academic Medical Center and the Dutch Central Committee on Research investigating Human Subjects (ISRCTN23008679). For details about the study design please see the original publication (28). Blood was withdrawn at day 0, 3, 22 and 84. For comparison of baseline coagulant activity and microparticle composition, age and sex matched controls were included, and blood was withdrawn once. For analyses of differences between baseline composition between healthy controls and cancer patients, we further extended the group with a cohort of 45 patients and 22 healthy subjects, described previously (15). Briefly, these patients were heterogeneous with respect to type and stage of cancer.

Citrate-anticoagulated blood (0.32%) was collected by venapuncture with a loosely applied tourniquet through a 19-gauge needle using a Vacutainer system. To obtain platelet poor plasma (PPP), blood was centrifuged at 1550 g, 20 minutes at 20°C. Plasma was kept on ice until further measurements, or snap-frozen in liquid nitrogen and stored at -80 °C until use.

The procoagulant activity of microparticles was determined using a fibrin generation test (3). Briefly, PPP was recalcified, and the time until fibrin formation was determined in a spectrophotometer both in the absence and presence of an inhibitory antibody to activated factor VII (anti-VIIa) and activated factor XII (anti-XIIa). The prolongation of the clotting time (CT) in the presence of anti-VIIa, which is expressed as a percentage of the CT in the absence of anti-VIIa, is a measure of the TF coagulant activity associated with microparticles. The CT in the absence of any antibody is a measure for the inherent ability of the plasma to stimulate coagulation, as a resultant of the interaction between coagulation factors and all microparticles and smaller vesicles (exosomes). For some experiments, plasma was centrifuged in an eppendorf centrifuge at 18,890 g for 30 minutes and the CT was again measured in the presence and absence of antibodies. Centrifugation at this speed removes microparticles, but not exosomes, and is therefore an appropriate way to measure the contribution of exosomes (3).

Total microparticles and cellular origin of the microparticles was determined as follows. Microparticles (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 incubated for 5 minutes at room temperature, after which 900 µL of PBS/Ca buffer was added. Samples were analyzed in a fluorescence automated cell sorter (FACS Calibur) with CellQuest software version 4.02 (Becton Dickinson, San Jose, CA). Fluorescein isothiocyanate (FITC)-labeled IgG, phycoerythrin (PE)-labeled IgG, and anti-CD142 (anti-TF)-PE were derived from Becton Dickinson (San Jose, CA). Anti-CD61-FITC (anti-GP-IIIα; indicating platelet origin) was from Dako (Glostrup, Denmark). APC-conjugated annexin V (binding to phosphatidylserine) was from Becton Dickinson (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), anti-CD105 (endoglin; monocyte or cancer cell origin) and IgM 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). Anti-CD62E-PE (E-selectin; activated endothelial cell origin) was obtained from Ancell (Bayport, MN, USA), CD133 (stem cells, glioblastoma cells) from MACS Miltenyi Biotec (Bergisch Gladbach, Germany) and CD144 (endothelium) from Alexis (Lausen, Switzerland). For calculation of total number of microparticles with certain cell-specific markers, we corrected for a-specific labelling (i.e. fluorescence of the control antibodies).

All statistical analyses were performed with PASW Statistics, version 18 (SPSS Inc., Chicago, Ill, USA). Most variables showed a non-parametric distribution, and the corresponding data are expressed as median with inter-quartile ranges (IQR). Between group differences were tested with the Mann-Whitney U test, unless indicated otherwise. Differences between dichotomous variables were tested with the Fisher’s exact test. Repeated measures were tested using the Friedman’s test. Correlations were tested with the Spearman’s correlation coefficient for non-parametric data. A p-value below 0.05 was considered as statistically significant.

RESULTS

Patient’s characteristics
In total 11 patients with glioma and 5 patients with lung cancer were included in the present analysis, 9 men and 7 women. For the patients with glioma, 11 age and sex matched controls were added. Blood was collected at various moments before and after start of chemotherapy (Table 1). Results are presented first for the entire group of cancer patients, and then the remarkable findings (if any) in glioma and lung cancer patients separately.

Coagulant activity of microparticles and exosomes at baseline
At baseline, before chemotherapy, the microparticle-dependent CT was 599 seconds in the entire group of cancer patients (IQR 468-680). For comparison, the CT was 918 seconds (680-
1056) in the matched healthy subjects (p=0.069). Prolongation of the CT in the presence of anti-VIIa or anti-XIIa was 3.9% (0.0-16) and 0% (0.0-3.6%), respectively, and was not different from the prolongation observed in healthy subjects (p=0.138 and p=0.260, respectively).

Three patients, two with glioma and 1 with lung cancer, had a detectable procoagulant activity after high-speed centrifugation, a procedure known to remove most of the microparticles but insufficient to pellet exosomes. In one of these plasma samples, clearly a TF/VIIa-dependent coagulant activity was present. All other plasma samples did not clot within one hour (CT > 3600 s) after removal of the microparticles, indicating that in these samples the procoagulant activity is exclusively associated with microparticles.

No differences in baseline coagulant activity were observed between glioma and lung cancer patients.

**Coagulant activity of microparticles and exosomes after treatment**

Consecutive samples for the measurement of coagulant activity were available for 13 patients with glioma and lung cancer, the other 3 patients had insufficient plasma. Neither the microparticle-dependent CT nor the VIIa/TF-dependent clotting showed significant responses to chemotherapy in the whole group of cancer patients (p=0.39 and p=0.28, respectively), same applied for the glioma and lung cancer patients separately; Figure 1A to 1D). Of the 7 patients with glioma and 3 patients with lung cancer who did not have a

### Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Non-small cell lung cancer patients (n=5)</th>
<th>High grade glioma patients (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (SD)†</td>
<td>42 (17)</td>
<td>62 (4.3)</td>
</tr>
<tr>
<td>Female sex</td>
<td>2/5 (40%)</td>
<td>5/11 (45%)</td>
</tr>
<tr>
<td>Treatment</td>
<td>Daily radiotherapy + daily cisplatin (n=2)</td>
<td>Continuous dose temozolamide, bevacizumab every 21 days and dexamethason (n=11)</td>
</tr>
<tr>
<td></td>
<td>Cisplatin + gemcitabine / pemetrexed, 3 weekly cycles (n=3)</td>
<td></td>
</tr>
<tr>
<td>Measurement intervals</td>
<td>Day 1: before (t=1) and 2 hours after chemotherapy (t=2)</td>
<td>Day 1: before start therapy (t=1)</td>
</tr>
<tr>
<td></td>
<td>Day 2 (t=3)</td>
<td>Day 3: after treatment (t=2)</td>
</tr>
<tr>
<td></td>
<td>Day 8 (t=4)</td>
<td>Day 22 (t=3)</td>
</tr>
<tr>
<td></td>
<td>Day 22 (t=5)</td>
<td>Day 84 (t=4)</td>
</tr>
<tr>
<td>Overall survival†</td>
<td>76 (13-148)</td>
<td>21 (5.2-62)</td>
</tr>
<tr>
<td>Progression free survival†</td>
<td>51 (8-148)</td>
<td>12 (2.4-23)</td>
</tr>
<tr>
<td>Co-morbidities</td>
<td>COPD (n=3), cardiovascular disease (n=2)</td>
<td>Cardiovascular disease (n=2), hypertension (n=1)</td>
</tr>
<tr>
<td>Development of VTE during study</td>
<td>None</td>
<td>Symptomatic DVT (n=1)</td>
</tr>
</tbody>
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Characteristics of the two groups of cancer patients. †Age in years and ‡survival in weeks. Abbreviations: standard deviation (SD); chronic obstructive pulmonary disease (COPD); venous thromboembolism (VTE); deep vein thrombosis (DVT)
detectable coagulant activity in microparticle-depleted (i.e. exosome containing) plasma at baseline, in 6 plasma samples a coagulant activity was detectable in the microparticle-depleted plasma at one of the moments after start of the treatment. In the 3 remaining patients, the CT of the microparticle-depleted plasma remained above 3600 s. Addition of an inhibitory antibody to human factor XII did not affect the microparticle-dependent CT in patients after chemotherapy more than at baseline (data not shown).

The numbers of TF-exposing MP were low and unaffected by chemotherapy (p=0.72; Figure 1E and F). In one patient with glioma a distinct population of TF-exposing microparticles double stained for CD144, VE-cadherin, a transmembrane protein which is exclusively present on endothelial cells (Figure 1G). The endothelial origin of these vesicles was further confirmed by a double labelling with CD144 and CD62E (E-selectin), a marker of activated endothelial cells. Overall, the number of TF-exposing microparticles was inversely associated with the microparticle-dependent CT (r=-0.30; p=0.03), but not with the VIIa/TF-dependent prolongation of the CT as measured in the presence of anti-VIIa (r=0.084; p=0.55).

One glioblastoma patient developed a deep vein thrombosis during the study on day 22. The microparticle-dependent CT decreased from 648 seconds (before chemotherapy) to 565 seconds at day 3, to 450 seconds at day 22, with a concurrent prolongation of the TF/VIIa-dependent CT from 0 (before chemotherapy), 1.7% (day 3) to 12% (day 22). In the exosome fraction, a procoagulant activity was detectable at day 22, and this activity was independent from activation of TF/VIIa.

**Number of annexin V-binding and P-selectin exposing microparticles**

At baseline, levels of annexin V-binding microparticles (as an estimate of the total levels of coagulant microparticles) were 2.4 x 10⁶/mL, which was comparable to controls (1.7 x 10⁶/mL, p=0.32). Chemotherapy induced no consistent changes in the numbers of circulating annexin V-binding microparticles, with levels immediately after chemotherapy of 2.0 x 10⁶/mL (1.1–3.0 x 10⁶/mL; p=0.57).

At baseline, P-selectin exposing microparticles, i.e. microparticles originating from activated platelets and not from megakaryocytes, were significantly increased in the entire group of cancer patients, when compared to healthy subjects, 5.9 x 10⁴ per mL (1.4–19 x 10⁴/mL) versus 2.1 x 10⁴/mL (0.4–7 x 10⁴/mL; p=0.044). Chemotherapy did not lead to consistent increases or decreases in levels of P-selectin-exposing microparticles (p=0.59), and the same applied to changes in brain and lung cancer patients separately.

**Endothelial and tumour-derived MP**

First, we explored whether baseline levels of microparticles exposing CD62E (E-selectin; activated endothelium) differed between cancer patients and healthy controls, by combining baseline data of the brain and lung cancer patients with a previously described cohort of cancer patients and healthy controls (15). Levels of CD62E-exposing microparticles were higher in the cancer patients (n=65), with a median of 2.8 x 10⁴/mL
Figure 1. Coagulant activity of microparticles. Panels A, C and E refer to glioblastoma patients (n=9) and panels B, D and F to lung cancer (n=4) patients. Three patients had insufficient plasma. Panels A and B: microparticle-dependent clotting time in response to chemotherapy. Panels C and D: prolongation of the clotting time in the presence of anti-VIIa. The dotted lines indicate a previously determined cut-off (of 13%) above which TF/VIIa-dependent clotting is considered positive; illustrating that this remains relatively constant in time. Panels E, and F: numbers of TF-bearing microparticles as determined by flow-cytometry. Panel G: an example of a glioblastoma patient with TF-bearing microparticles at day 3 after start of treatment. Part of these TF-bearing microparticles were positive for CD144 (endothelial cells; right picture), and part for MUC-1 (cancer cells; left picture). H. a representative example of a glioblastoma patient without these microparticles.
Effects of chemotherapy on vesicles

compared to $0.14 \times 10^4 /mL$ in the healthy subjects ($n=34$; $p=0.0026$). No differences were present between cancer patients and healthy subjects with respect to microparticles bearing CD144, presumably originating from non-activated endothelial cells ($p=0.33$), and total levels of CD144$^+$ microparticles were overall lower (median in patients $0.42 \times 10^4/mL$). In the entire group, no changes in CD62E microparticles as a response to chemotherapy were present ($p=0.41$). Numbers of CD62E-exposing microparticles did show a trend towards an increase directly after the first gift of chemotherapy in the glioblastoma patients ($p=0.18$ for the comparison between the first and second time point; Figure 2A and K), but not in lung cancer patients (Figure 2B).

Baseline levels of microparticles bearing the vascular endothelium growth factor receptor-1 (VEGFR-1; flt-1) were increased in cancer patients when compared to healthy subjects, median levels of respectively $3.5 \times 10^4 /mL$ (0.63-14) versus $1.4 \times 10^4 /mL$ (0.28-2.6; $p=0.012$). In the entire group, no changes in VEGFR-1 microparticles as a response to chemotherapy were present ($p=0.15$). In the glioblastoma patients, combined chemotherapy with angiogenesis inhibition decreased the levels of VEGFR-1-exposing microparticles by 85% (Figure 2C, G and H; $p=0.021$). Again, no clear pattern was visible in the lung cancer patients (Figure 2D). We tried to elucidate the cellular origin of the VEGFR-1-exposing microparticles, but only minor fractions double-stained for CD144 (2.5%), or MUC-1, a marker of normal epithelial cells and tumour cells (<1%).

As CD133 is an established marker of glioblastoma cells (29), the levels of CD133-exposing microparticles in all cancer patients were determined. Levels of CD133-exposing microparticles were negligible in both groups of patients, and there was no trend in the time (data not shown).

Overall, no differences could be observed in the plasma levels of MUC-1-exposing microparticles during treatment, except in two lung cancer patients which showed a clear increase at the third day after start of chemotherapy (Figure 2E, F, I and J).

| DISCUSSION |

In this small explorative study we investigated possible changes in procoagulant activity and composition of circulating vesicles as a response to two different treatment regimens in cancer patients. Several aspects and findings of the present study require comment. First, at baseline the procoagulant activity was mainly associated with microparticles, with a role for coagulant TF in a minority of patients. While data need to be interpreted with caution because of the modest sample size; clotting activity, in particular the exosome-dependent, tended to increase after start of treatment which may hint to a role of exosomes in the prothrombotic state in cancer, rather than microparticles. Understanding which types of vesicles are responsible for the coagulant activity will help to clarify the mechanisms underlying the development of VTE. Important for possible future applications of TF-dependent properties of vesicles as predictors for VTE or prognosis, is the finding that the TF-dependent CT remained stable after chemotherapy (Figure 1C and D).
Figure 2. Endothelial and tumour derived microparticles. Panels A, C and E represent patients with glioblastoma; panels B, D and F represent patients with lung cancer. Panels A and B: changes in E-selectin (CD62E)-exposing microparticles; panels C and D: changes in VEGFR-1 bearing microparticles; panels E and F: changes in MUC-1 bearing microparticles. G. Representative example of a patient with glioblastoma, illustrating the major decrease in levels of VEGFR-1 bearing microparticles after start of bevacizumab and temozolamide, and H. a patient lacking such an increase.
The observation of TF-exposing microparticles of endothelial origin (Figure 1G) is an interesting ‘proof of principle’ finding, showing that not only monocytes but also endothelial cells may contribute to intravascular coagulation by releasing microparticles exposing coagulant TF (30). To which extent TF exposed on endothelial vesicles contributes to coagulation activation, however, remains to be determined.

Not unexpectedly, our present data indicate that angiogenesis inhibitors have specific effects when compared to a ‘regular’ chemotherapy regimen, which is also reflected by a clear change in circulating microparticles. One of the major pathways of angiogenesis involves the vascular endothelial growth factor (VEGF) family of proteins and receptors. In this complicated system, VEGF-receptor 1 (flt-1) is one of the receptors for VEGF, which can be present on tumour endothelium, as well as on tumour cells, whereas a soluble form of VEGFR-1 has also been reported (31). To our knowledge, this is the first description of microparticles exposing this receptor in cancer patients, whereas levels of such vesicles are negligible in healthy volunteers. Whether this VEGFR-1 is derived from cancer cells or from the cancer endothelium needs further clarification. Overall, as a mechanism, transport of the VEGF receptor by microparticles in cancer patients could be involved in an angiogenic switch, as has been described for the truncated EGFR receptor (16). Interestingly, bevacizumab, a monoclonal antibody against VEGF, dramatically decreased the concentrations of circulating VEGFR-1-exposing microparticles, whereas chemotherapy without bevacizumab did not. Future studies are needed to establish whether the presence and response to treatment of these microparticles are indeed associated with prognosis.
Furthermore, angiogenesis inhibition lead to increased plasma levels of endothelial microparticles (Figure 2A), which extends the findings of Kuenen and colleagues, who reported that inhibition of the VEGF receptor pathway caused activation of endothelial cells as reflected by increases in soluble E-selectin, von Willebrand antigen and soluble TF (25). The authors concluded that VEGF is an important maintenance factor for endothelium, which may be one of the mechanisms contributing to the high rate of VTE in patients treated with angiogenesis inhibitors.

In lung cancer patients, the increase in microparticles exposing mucine at 2 days after the first gift of chemotherapy is intriguing, especially in view of previous in vitro data showing that gemcitabin decreases the expression of mucine on pancreatic cancer cells, which the authors associated with protection of cancer cells from attacks of the immune system (32). The present data suggest that cancer cells may release mucine-exposing microparticles to remove mucine present on the tumour cell. Alternatively, the appearance of such microparticles may be related to necrosis of the tumour.

To conclude, chemotherapy does not lead to consistent changes in microparticle dependent procoagulant activity, apart from an increase in procoagulant activity of the microparticle-depleted but still exosome-containing plasma. Chemotherapy and angiogenesis agents do lead to specific changes in microparticle composition. After angiogenesis inhibition, levels of E-selectin-exposing microparticles increase, indicating specific endothelial damage and activation. Cancer patients had high levels of microparticles bearing the receptor for VEGF, which decreased after treatment with angiogenesis inhibiting agents. Furthermore, levels of microparticles bearing mucine increase at day 2 after chemotherapy in lung cancer patients. The present study, however, is merely hypothesis generating and cannot link the appearance of these specific microparticles to either a favourable or a non-favourable reaction of the tumour to chemotherapy.

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**REFERENCE LIST**


