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Risk factors of thrombosis in cancer : the role of microparticles
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Case -control study identifying
microparticle-associated tissue
factor activity as a biomarker
of cancer-specific thrombosis

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Submitted

Abstract

Objective

Cancer-related venous thrombosis (VT) is associated with poor survival. Microparticles (MP) released from cells bearing active tissue factor (TF), may be involved in the pathogenesis of thrombosis.

Methods and results

We designed a case-control study in which unselected cancer patients with VT ($n = 51$) were matched according to several criteria (age, sex, disease type and stage, and cancer-specific treatment) with a corresponding set of control cancer cases without VT ($n = 49$). MP were isolated from blood and MP-TF activity was measured as factor VIIa-dependent factor Xa generation (normal value < 273 fM Xa/min). Low MP-TF activity was found in 49 controls (median 150 fM Xa/min, range 23-535) and in 27 cases with known thrombosis risk factor, *i.e.* chemotherapy or compression of veins by the tumor (median 131 fM Xa/min, range 19-506). By contrast, high MP-TF activity was detected in the 24 cases with cancer-specific coagulopathy (median 1,131 fM Xa/min, range 395-12,333). Median survival was significantly shorter in patients with elevated MP-TF activity (3.5 months) than in patients with normal MP-TF activity (10 months).

Conclusions

Our results show both shorter survival time and markedly elevated TF activity associated with circulating MPs as features of cancer-specific thrombosis. We propose that TF-containing MPs could serve as a biomarker of cancer-specific coagulopathy and disease progression.

Introduction

Cancer is associated with venous thrombosis (VT) (1,2), which often precedes the diagnosis of cancer and cancer patients who develop VT have a worse survival than cancer patients with the same type of tumor who do not develop VT (3). The strong association with particular malignancies, and the more aggressive course of the disease in those who develop VT, points to a tumor-specific cause of the observed hypercoagulability in patients with cancer. However, the vast majority of cancer patients will not develop VT, while the occurrence of VT is particularly associated with certain types of malignancy. Although elevated plasma levels of markers of coagulation activation have been observed in cancer patients (4), studies failed to demonstrate a difference in clotting factor profile between cancer patients who developed thrombosis and those who did not. The pathogenesis of the hypercoagulability in cancer has not been elucidated. Cancer patients who are exposed to chemotherapy treatment are known to develop VT, most likely mediated by endothelial damage caused by the individual cytotoxic agents. Young male patients with germ cell tumors for instance, are only at increased risk for development of VT during the period of chemotherapy treatment (5), but not prior to diagnosis of their tumor or after treatment.

Tissue factor (TF), a transmembrane-receptor protein and initiator of coagulation, plays a central role in the theory that clotting and tumor growth form a vicious circle, in which hypercoagulability facilitates the aggressive biology of cancer and vice versa (6;7). TF acts both as receptor and co-factor for factor VIIa and thus initiates the (extrinsic) pathway of coagulation (8). Complex formation of TF with factor VIIa also triggers intracellular signals involved in angiogenesis, cell migration and inflammation (9). Expression of TF is associated with poor differentiation of tumors, increased angiogenesis and poor prognosis (10).

Microparticles (MP), membrane microvesicles produced during activation or apoptosis of cells (11), have been isolated from blood of healthy individuals. Recent data indicate that MP are cellular effectors involved in cell-cell cross talk and multiple MP-cell interactions seem to occur, indicating that MP may act locally at the site of origin as well as at a distance. Alterations in the numbers and origin of blood MP have been suggested to be involved in promoting thrombus formation in various clotting disorders and inflammatory conditions (12;13). MP may support prothrombinase activity (14) and express TF activity (15;16) and MP isolated from various patient populations have been reported to support in vitro coagulation (17;18). Small clinical studies demonstrated that

MP levels are elevated in individuals with thrombosis (19), whereas impaired membrane vesiculation in Scott syndrome is accompanied by decreased numbers of circulating MP and a bleeding tendency (20). MP isolated from human plasma, injected into rats were highly thrombogenic, and this effect was abolished by pre-incubation of MP with anti-tissue factor antibody.

In a previous study (7), we found that patients with adenocarcinoma arising from breast and pancreas, who presented with VT, had markedly elevated MP-TF activity and poor survival, compared to patients with the same type of cancer who did not develop thrombosis. The question then arises whether MP-TF activity is involved in the pathogenesis of cancer-related coagulopathy regardless of the origin of the tumor. To circumvent the need for a large sample size and cohort stratification we addressed this question by performing a case-control study in which MP-TF activity was analyzed in unselected cases with various types of malignancies who presented with a first episode of thrombosis. Cancer patients with VT were matched according to several criteria (age, sex, type of cancer, stage of the disease and subsequent cancer-specific treatments) with a corresponding set of control cancer cases without VT. Thus, pairs of patients (case and control) and consequently both groups of patients had identical a priori life expectancies.

Methods

Case- control design

In a case-control study design, we studied MP-TF activity in 100 cancer patients. Between mid 2003, and mid 2006, a total of 51 consecutive cancer patients who experienced a first episode of deep venous thrombosis of the leg or arm or pulmonary embolism and who did not have a family history of thrombosis were studied. VT was ascertained by echo Doppler and/or spiral computed tomography (CT).

For each cancer patient with VT, one eligible control was enrolled in the study who was matched for age \pm 2 years, sex, type of cancer, stage of the disease and type of cancer-specific treatment, including the same chemotherapy regimen and previous cancer-specific treatments. To avoid genetic stratification, control cancer patients were matched for ethnicity and geographical area. We included 49 cancer patients as controls. One control was not matched for sex. For two cases with an adenocarcinoma of the lung we were not able to identify an appropriately matched control. Exclusion criteria were a

personal or family history of thrombotic disease, any other serious disease such as diabetes or renal insufficiency or use of anticoagulants.

Cancer patients who presented with VT were stratified based on the presence or absence of a known pre-specified risk factor for the development of VT, which were defined as: presence of compression of (large) veins due to tumor masses, administration of anticancer therapy i.e chemotherapy, hormonal therapy or antiangiogenic therapy, the presence of an indwelling venous catheter, and recent surgery or immobilization.

The Medical Ethics Committee approved investigation of blood MPs in patients with various types of cancer and different stages of their disease. All individuals gave informed consent. Thirty seven healthy subjects served as controls for blood cell count and MP measurements.

Patients

The diagnosis, classification and staging of the different types of cancer were based on standard diagnostic evaluation, including CT and magnetic resonance imaging of the body and histopathological examination of tumor tissue specimens. All cancer patients were staged using the final version of the American Joint Committee on Cancer (AJCC) staging system and followed-up in our department of Clinical Oncology with regular intervals until death or end of study (May 2007) with no patients lost to follow-up, thus enabling us to precisely assess the time of death. In all cases, mortality was due to cancer-specific death.

The 51 consecutive cancer patients who presented with thrombosis suffered from different malignancies: 27 had gastro-intestinal adenocarcinomas (esophageal carcinoma, colorectal carcinoma, pancreatic carcinoma and cholangiocarcinoma), 12 had genitourinary tract tumors (renal cell carcinoma, prostate carcinoma and germ cell tumors of the testis), 8 had adenocarcinoma originating from various other organs including ovary, breast, lung, and adrenal gland, 2 had squamous cell carcinomas of the head and neck and 2 patients had an osteosarcoma (Table 1). Forty-one (80%) of the patients who presented with thrombosis had an adenocarcinoma originating from different organs, whereas 10 patients did not have an adenocarcinoma (2 patients with squamous cell carcinoma, 6 with a malignant germ cell tumor of the testis and the 2 patients with osteosarcoma).

Table 1. Demographics and MP-TF activity in 51 cancer patients with VT, 49 cancer patients without VT and 37 healthy subjects

	Cases			Healthy Subjects
	All cases n = 51	Pts with cancer- related VT n = 24	Pts with chemotherapy- or vein compression- related VT n = 27	
Sex (M/F)	28/23	12/12	16/11	25/24
Age (yr)				
median	62	63	61	60
range	30-83	46-77	30-83	20-82
Tumor type				
gastro-intestinal tumors	27	17	10	27
genito-urinary tumors	12	1	11	12
various adeno CA	8	5	3	6
squamouscell CA	2	1	1	2
osteosarcoma	2	0	2	2
Adenocarcinoma	43 (84%)	23 (96%)	20 (74%)	40 (82%)
Distant metastasis	46 (90%)	20 (83%)	26 (96%)	44 (90%)
Survival (months)				
median	7	2	13	12
range	1-48+	1-29	1-48+	1-48+
Platelet counts (10⁹/L)				
median	258	302	227	258
range	44-678	118-678	44-389	123-521
Total MP (10⁶/L)				
median	4.3	4.2	4.7	4.1
range	1.4-15.6	2.2-15.6	1.4-13.5	1.3-13.6
MP-TF activity(fM Xa/min)				
median	168	1,131	131	150
range	19-12,333	395-12,333	19-506	23-535

Laboratory investigations

Blood samples were obtained from cases at the time of VT diagnosis and before the start of anticoagulant therapy and from controls for measurement of MP number and MP-TF activity. Blood samples were collected from healthy subjects as reported previously (7). Plasma samples were coded after collection and stored as described below. The average storage time for plasma of cases, controls and healthy subjects was similar. The samples were analyzed blinded as to case control status.

MP isolation

Blood was collected in 1/10 volume of 3.2 % trisodium citrate (Becton Dickinson, San Jose, CA) and platelet-poor plasma prepared immediately by centrifugation for 20 min at $1550 \times g$ at room temperature. Platelet-poor plasma (source of MP) was carefully removed, snapfrozen in liquid nitrogen and stored at -80°C for future analysis.

MP-containing plasma was thawed in melting ice, centrifuged at $17,570 \times g$ at 20°C , washed, and resuspended in 1/10 of the original volume buffer (154 mM NaCl, 1.4 mM sodium phosphate, 10.9 mM trisodium citrate, pH 7.4). This MP preparation was used for FACS analysis. For the measurement of MP-associated TF activity, MP were washed more extensively to reduce contamination with plasma proteins (0.5% in the final MP preparation) and resuspended in 1/7 of the original volume PBS/citrate.

Enumeration of MP by Flowcytometry

Flowcytometric analysis of MP was performed using APC-labeled Annexin V (BD Biosciences Pharmingen, San Jose, CA). MP were stained with Annexin V and analyzed for 1 minute with Cell Quest software (Becton Dickinson, San Jose, CA), and identified by their characteristic forward and side scatter, and by annexin V binding to anionic phospholipids. Annexin V bound to the majority (>90 %) of circulating MP of patients and healthy subjects. The number of MP/L plasma was calculated as previously described (21).

Tissue factor activity assay

The TF activity in MP preparations (MP-TF activity) was measured in 96 wells plates at room temperature by determining the FVII-dependent factor Xa (FXa) generation as previously described (7). In all samples FXa generation was measured both in the presence and absence of FVII and in the presence and absence of excess polyclonal rabbit anti-

huTF IgG (22) to establish the FVII- and TF-dependence of the FXa generation. Results are reported as MP-TF activity/ ml plasma (fM Xa/min). MP-TF activity is reported as the FVII-dependent, anti-TF sensitive factor Xa generation. The median and mean TF activity in the MP isolated from the plasma of healthy subjects did not differ (129 versus 132 fM Xa/min) and MP-TF activities higher than the mean + 3 SD (> 273 fM Xa/min) were considered to be elevated.

Statistical Analysis

We compared means and proportions and constructed confidence intervals based on normal and binomial distributions. Kaplan-Meier curves were constructed to assess survival and Cox models were used to calculate hazard ratios. All analyses were performed using SPSS for Windows, version 14.0 (SPSS Inc, Chicago, IL)

Results

Patient characteristics

In Table 1 characteristics of the 100 cases and controls, and 39 healthy subjects are shown. None of the patients (cases or controls) received hormonal therapy or antiangiogenic therapy which is known to be associated with increased risk for VT. None of the patients had an indwelling venous catheter, recent surgery or immobilization. Of the 51 cases who developed thrombosis, 24 patients did not have any pre-specified risk factor for the development of VT like chemotherapy or tumor related vein compression. In these 24 patients the occurrence of thrombosis was assumed to be attributable to a cancer-specific coagulopathy. In the remaining 27 patients the VT was attributable to chemotherapy ($n = 22$) or tumor related vein compression ($n = 5$; Table 1). There was no difference with respect to age or presence of distant metastases between cases with cancer-specific coagulopathy or chemotherapy c.q. vein-compression-related thrombosis (Table 1). Furthermore, platelet levels were within the normal range and did not differ between cases and controls, although platelet counts were somewhat lower in the cases with a pre-specified risk factor for thrombosis (Table 1).

Forty-six (90%) of the patients who presented with thrombosis had distant metastases at the time of VT and blood collection (Table 1). The percentage of patients with an adenocarcinoma was higher in the 24 cases without (96%) than in the 27 cases with a pre-specified risk factor for thrombosis (74%, Table 1).

Number of MP and MP-associated TF activity in healthy subjects and cancer patients

Table 1 shows the concentrations of circulating MP in cases and controls. The total number of MP did not differ between the cases and controls, but was more than 2-fold higher than in healthy subjects (Table 1). The median MP-TF activity in the 49 cancer patients without VT (controls) was 150 fM Xa/min, range 23-535 fM Xa/min (Table 1; Figure 1). The majority of these patients had a normal MP-TF activity. The four control patients (8%), who had slightly elevated MP-TF activity (290, 350, 336 and 535 fM Xa/min) all received chemotherapy treatment. The median MP-TF activity in the 51 cancer cases with VT was 168 fM Xa/min, range 19-12,333 fM Xa/min (Table 1).

The median MP-TF activity in the 27 cases with chemotherapy treatment or vein compression as pre-specified risk factors for thrombosis was 131 fM Xa/min, range 19-506 (Table 1, Figure 1). Twenty three had normal MP-associated TF activity levels, whereas 4 had elevated MP-TF activity (288, 342, 355 and 506 fM Xa/min) and all 4 received chemotherapy.

In contrast, all 24 cases with VT only related to the malignancy had markedly elevated MP-TF activity (median MP-TF activity 1,131 range 395-12,333 fM Xa/min) (Table 1; Figure 1). The 2 patients with lung adenocarcinomas for whom no control could be found, had MP-TF activity of 574, respectively 1812 fM Xa/min. MP-TF activity was 10.2-fold higher in these 24 cases than in the 27 cases with pre-specified risk factors or patients without VT (HR=10.24 CI 95 6.24-16.35). There was no association between MP-TF activity, the total number of MP, nor with platelet counts in the groups of cancer patients.

Survival of cancer patients

Twenty eight (28 %) of the 100 cancer patients were still alive at the end of follow-up with a median follow-up of 35 months (range 12-48+ months). All deaths were cancer-specific. Survival in 51 cancer cases with VT was 7 months (range 1-48 months), and in 49 cancer controls 12 months (range 1-48+ months) (Table 1).

Median survival in patients with elevated MP-TF activity ($n = 32$) was 3.5 months (range 1 to 43 months) and significantly shorter when compared to that of patients with non-elevated MP-TF activity (median 10 months, range 1 to 48+).

Survival of 24 of the 51 cases in which thrombosis seemed directly related to the presence of their malignancy was much shorter (median 2 months, range 1-29) than that of 27 of the 51 cases with a pre-specified risk factor for thrombosis (median 13 months,

range 1 - 48*) and of the 49 matched cancer controls without thrombosis (median 12 months, range 1 - 48*) (Table 1; Figure 2).

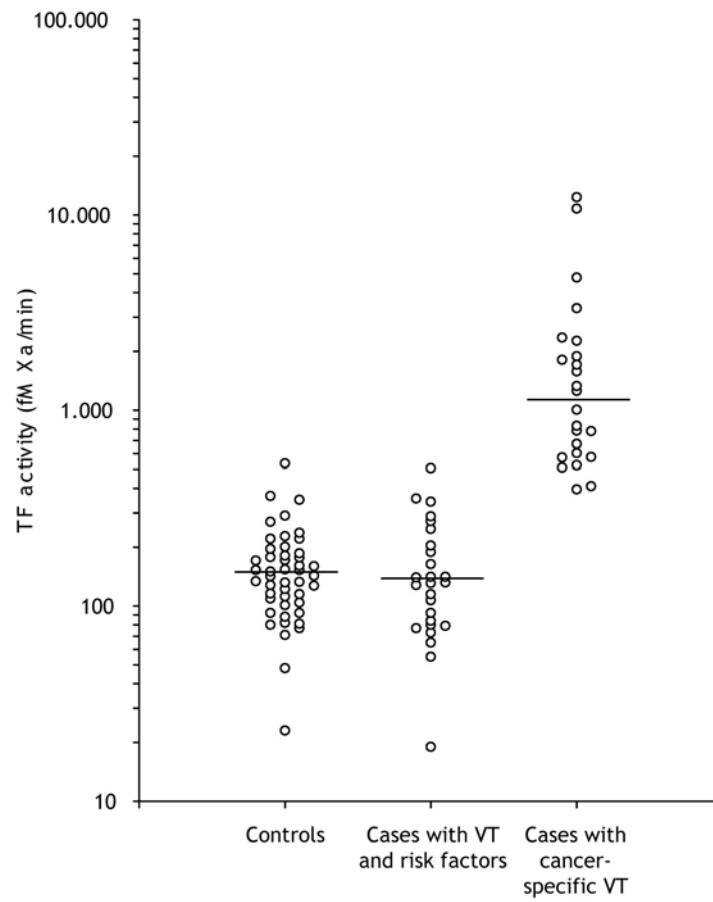


Figure 1. MP-associated TF activity in cancer controls without VT and cancer cases with VT. MP-associated TF activity in 49 cancer patients without VT (Controls), 27 cases with pre-specified risk factors for VT (Cases with VT and risk factors) and 24 cases with VT only related to the malignancy (Cases with cancer-specific VT).

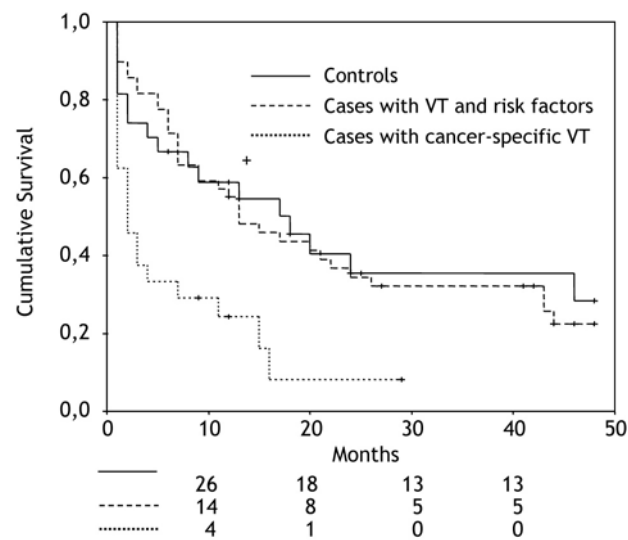


Figure 2. Kaplan-Meier survival curve of patients with cancer. Cumulative survival of 49 control cancer patients without VT (Controls), 27 cases with VT attributable to pre-specified thrombosis risk factors, either chemotherapy or tumor-related venous compression (Cases with VT and risk factors) and 24 cases with cancer-specific coagulopathy (Cases with cancer-specific VT). A cross indicates censored patients still alive at the time of analysis of the survival data. The likelihood of survival was reduced for cases with cancer-specific coagulopathy (Cases with cancer-specific VT) as compared to cases with chemotherapy or tumor-related vein compression as cause for coagulopathy (Cases with VT and risk factors) and control cancer patients (Controls).

Discussion

To our knowledge, this is the first case-control study of the causes of venous thrombosis in unselected cancer patients in a single institution.

Markedly increased microparticle-associated TF activity was only observed in those patients in whom the malignant process seemed to be the cause of the hypercoagulability and who had a strikingly poor survival. This suggests a role for MP bearing active TF in triggering thrombotic events and a direct pathogenic mechanism with regard to the release of such MP and their mode of action.

Although it has been suggested that elevated levels of platelets may play a role in cancer-associated thrombosis (23), there was no clear difference in numbers of circulating platelets between the various groups of cancer patients and no association between numbers of platelets and MP-associated TF activity.

The high percentage (96%) of adenocarcinomas among the cases with VT follows from the high incidence of thrombosis for such malignancies. Of all patients referred to our centre about 50% have an adenocarcinoma. An explanation may be that the source of TF are epithelial cancer cells themselves, or that expression of mucins by adenocarcinoma cells or other factors also contribute to the coagulopathy (24;25).

In our population of cancer patients, the high MP-TF activity and poor survival in cases with cancer-related thrombosis is intriguing, particularly since microparticles carrying active TF may play a causative role in the development of thrombosis and perhaps also in the poor survival. In this study we did not address the size and origin of TF positive MP responsible for the MP-TF activity, but the observation that elevated MP-TF activity was found in all patients who presented with cancer-specific coagulopathy which was not attributable to chemotherapy or venous compression, points to a contribution of cancer cells themselves as cause of elevated MP-TF activity. Experimental studies suggest that cancer-specific genetic lesions (e.g. activation of K-ras and inactivation of p53) may impact the level of TF expression in tumor cells and affect the numbers of circulating MPs containing TF originating from cancer cells themselves (26). On the other hand it was proposed that circulating TF originates mainly from TF-expressing stromal cells surrounding the tumor cells (10). Another hypothesis is that tumor-derived MP display enhanced binding to platelet- or other hematopoietic or vascular cell-derived MP, perhaps via mucin-lectin interactions, leading to initiation and propagation of coagulation, fusion of MP and formation of MP rich in decrypted TF.

Cancer patients who develop venous thrombosis have been reported to have a poor prognosis (3;27) with thrombosis as an independent risk factor for survival in cancer patients. Levitan *et al.* (2) demonstrated that the mortality in cancer patients with thrombosis was higher than that of patients with cancer or thrombosis alone. One explanation could be that thrombosis occurs more frequently in patients with advanced disease. However, Blom *et al.* (28) showed that development of thrombosis is a risk factor for dying in lung carcinoma patients independent of the presence or absence of metastases. Recently, Chew *et al.* (29) also reported that VT is an independent predictor of decreased 2-year survival in breast cancer patients and -when stratified by initial

cancer stage- the effect was highest with localized or regional stage cancer compared with patients with metastatic disease.

Elevated MP-TF activity may serve as biomarker for cancer-related thrombosis and poor outcome of the disease but not for thrombosis related to chemotherapy or to tumor compression of veins. The detrimental course of the disease observed in cases without known thrombosis risk factor other than cancer itself, supports the hypothesis that hypercoagulability facilitates the aggressive biology of cancer and vice versa.

This study suggests that MP-TF activity might serve as a biomarker for cancer-related thrombosis and poor prognosis but the mechanism by which MP carrying active TF could contribute to thrombosis in cancer patients remains to be elucidated. The data underscore that occurrence of thrombotic events in cancer patients are not unrelated events, as the increasing likelihood of overt and symptomatic VT is inversely related to worsening of the underlying malignancy.

To further understand the pathophysiology of MP-TF activity in cancer patients with hypercoagulability, additional studies are required to unravel the intriguing relationship between cancer, thrombosis, MP-TF activity and poor prognosis.

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