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Tissue Factor Activity is Increased in a Combined Platelet and Microparticle Sample from Cancer Patients

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Abstract

Background—Cancer patients have an increased risk of thrombosis. Tissue factor (TF) antigen and TF activity associated with microparticles in plasma is elevated in patients with various types of cancer. Of these two measurements, TF activity is considered superior to TF antigen levels because the activity more closely reflects the ability of TF to initiate coagulation. Recent studies showed that platelets also express TF.

Objective—To determine the level of TF activity associated with a combined platelet and microparticle sample from cancer patients (n=20) and healthy individuals (n=23).

Methods—TF activity was measured using a two step chromogenic assay and soluble Pselectin was measured by ELISA in healthy controls and metastatic cancer patients.

Results—We determined the composition of a combined platelet and microparticle sample. The sample consisted of platelets, large microparticles (30-200nm) and membrane debris. We compared the TF activity of a combined platelet and microparticle sample from cancer patients with that from healthy individuals. We found that TF activity in a combined platelet and microparticle sample from cancer patients was higher than in samples from healthy individuals (21.5 ± 12.3 pM (n=20) versus 8.6 ± 6.8 pM (n=23), mean $\pm$ SD, $p < 0.001$). Cancer patients also had a higher level of soluble P-selectin compared with controls (18.9 ± 5.5 ng/ml versus 13.2 ± 2.3 ng/ml, $p < 0.001$).

Conclusion—This study indicates that measurement of TF activity in a combined platelet and microparticle sample can be used as a simple assay to determine the level of circulating TF.

Keywords

cancer; microparticles; platelets; tissue factor

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Introduction

Tissue factor (TF) is constitutively expressed on extravascular cells and plays a central role in hemostasis by initiating blood coagulation after vessel injury [1]. TF antigen is also detected in plasma from healthy individuals and patients with various diseases [2-6]. However, the role of this intravascular TF in thrombosis is unclear [7-9]. It has been proposed that TF circulates in blood in an inactive form and is activated at sites of thrombosis [10].

TF has been reported to circulate on microparticles, which are small membrane fragments shed during cellular activation and/or apoptosis [11-13]. These processes may occur in healthy individuals but would be expected to increase in patients with various pathologies. One mechanism to recruit leukocyte-derived TF-positive microparticles to a growing a thrombus is via binding of P-selectin glycoprotein 1 (PSGL-1) to P-selectin on the surface of activated platelets [14,15]. Furthermore, platelets themselves can both rapidly mobilize TF from intracellular pools and synthesize TF [16-18]. Therefore, TF expression by both platelets and microparticles may contribute to thrombosis in various diseases.

People with cancer have an increased risk of thrombosis [19-20]. This risk can be further increased by the presence of central venous catheters and chemotherapy [21]. In cancer patients, the hypercoagulability associated with the disease may be in part due to high levels of circulating TF-positive microparticles [24-27]. Platelets are also activated in cancer patients [28,29]. These cells may contribute to the prothrombotic state of patients in various ways including binding TF-positive microparticles and by providing a prothrombotic surface for the coagulation cascade. In addition, two recent studies reported the presence of elevated levels of platelet-derived, TF-positive microparticles in cancer patients [25,27]. Together, these studies suggest that TF on both microparticles and platelets may contribute to the hypercoagulable state in cancer patients.

In cancer patients no reliable predictive test is currently available to identify individuals at highest risk for thrombosis [30,31]. Therefore, biomarkers of coagulation, such as circulating TF, may be useful in identifying a pre-thrombotic state in patients.

Since activated platelets can express TF, bind TF-positive microparticles and provide a prothrombotic surface, we investigated the level of TF activity associated with a combined platelet and microparticle sample isolated from the blood of healthy individuals and cancer patients.

Materials and Methods

Preparation of Samples

Healthy individuals (Scripps General Clinical Research Center) and cancer patients (Scripps Cancer Center) with various stage IV, metastatic tumors were recruited with informed consent and approval by the Human Subjects Research Committee at The Scripps Research Institute, La Jolla, CA. Patients had primary tumors originating in the lung (9 patients), breast (3 patients), prostate (3 patients), colon (2 patients), pancreas (1 patient), kidney (1 patient), or soft tissue (1 patient) (Table). The majority of the cancer patients (19/20) were on a chemotherapy regimen, which was specific for the type of cancer. These patients had no reported thrombotic events. Blood samples (5mL) were collected using a 21 gauge needle into tubes containing citrate (3.2%; 10:1) and 50µg/mL final concentration of corn trypsin inhibitor (CTI, Hematologic Technologies Inc., Essex Junction, VT; a FXIIa inhibitor that inhibits the intrinsic pathway of coagulation). A combined platelet and microparticle sample from healthy individuals or cancer patients was prepared from 2mL of whole blood by differential centrifugation within 3 hours of collection. Briefly, platelet-rich plasma (PRP) was prepared

from whole blood by centrifuging at $200 \times g$ for 15 minutes at 20°C . Subsequently, the PRP was centrifuged at $20,000 \times g$ for 15 minutes at 20°C . The platelet-free plasma was carefully removed from the pellet and transferred into a clean tube. The samples were then frozen at -80°C .

In selected individuals, combined platelet and microparticle samples were cytospun and differentially stained with the Diff-quick staining kit (Fisher Scientific, Pittsburgh, PA) to identify leukocytes. We did not observe any monocytes or neutrophils in these samples ($n=5$). A preliminary experiment prepared combined platelet and microparticle samples in the presence of prostaglandin E1 (final concentration 100nM; PGE-1, Calbiochem, San Diego, CA), an inhibitor of platelet activation, or DMSO (0.1%). No difference in TF activity was observed between samples prepared in the absence or presence of PGE-1 (data not shown).

Electron microscopy

For transmission electron microscopy, PRP was fixed with paraformaldehyde (final concentration, 1%) for 5 minutes at 20°C and then centrifuged at $20,000 \times g$ for 15 minutes at 20°C . The resulting pellet was then fixed with 3% glutaraldehyde in 0.2M sodium cacodylate buffer at pH 7.4 for 3 hours and then washed in PBS. The pellets were then treated for 1 hour with 1% osmium tetroxide in 0.1M cacodylate buffer, dehydrated in graded steps of ethanol followed by propylene oxide, and embedded in an Epon/ Araldite mixture. Subsequently, ultrathin transverse sections (60nm) were mounted on copper slot grids coated with parlodion and stained with uranyl acetate and lead citrate for examination on a Philips CM100 electron microscope (FEI, Hillsbrough OR). Images were documented using Kodak SO163 EM film. Negatives were scanned at 600 Dpi using a Fuji FineScan 2750x1 (Hemel Hempstead, Herts., UK).

Measurement of TF activity using a 2 step chromogenic assay

Freeze-thawed combined platelet and microparticle samples were solubilized with 15mM N-octyl- β , D-Glucopyranoside in 25mM Hepes-saline, pH 7.4 at 37°C for 15 min to de-encrypt the TF and then diluted in two volumes of 25mM Hepes-saline, pH 7.4. Thirty μL of the sample was added to 90 μL of reaction mix containing 3nM FVIIa, 100nM FX, 8.33mM CaCl_2 and 0.33mM S2222 (DiaPharma, Columbus, OH). Identical reactions were prepared that contained either an IgG control antibody (select experiments or PBS) or an inhibitory anti-human TF monoclonal antibody [32]. After a 45 min incubation at 37°C , the optical density was measured at 405nm and TF activity (pM) was determined by reference to a standard curve generated using relipidated recombinant TF (American Diagnostica, Greenwich, CT). TF-specific activity was determined by subtracting the amount of FXa generated in the presence of the anti-human TF antibody from the total amount of FXa generated. In a pilot experiment, we did not observe any difference between the addition of an IgG antibody and PBS on the amount of FXa generated (data not shown). Therefore, PBS was used in all other experiments.

P-selectin and D-dimer levels

Soluble P-selectin and D-dimer levels were determined in the plasma using commercial ELISAs (R&D Systems, Minneapolis, MN and Diagnostica Stago, Parsippany, NJ, respectively).

Statistics

Group means were compared by Student's t-test or paired t-test where appropriate. Non-parametric data was analyzed by Mann-Whitney Rank Sum test (SigmaStat). Age can increase coagulation parameters [33]. Therefore, the TF activities and D-dimer levels from the cancer patients and healthy individuals were statistically adjusted before examining any difference

between the two groups using ANOVA (SPSS, v.13). A value of $P < 0.05$ was considered significant.

Results

Combined platelet and microparticle samples from cancer patients have higher levels of TF activity than healthy individuals

We determined the composition of the combined platelet and microparticle samples by electron microscopy. These samples comprised mostly of platelets, large microparticles (size ranges from ~30-200nm) and membrane debris (Fig. 1). We observed a significantly higher TF activity in the combined platelet and microparticle sample from the blood of cancer patients compared with healthy individuals ($p < 0.001$; Fig. 2). Although the patient numbers were low, we observed that patients with colon, pancreatic or lung cancer had the highest TF activities (Table).

We measured the level of soluble P-selectin, which has been used as a marker of platelet activation, in the plasma from cancer patients and healthy individuals. We observed significantly higher levels of soluble P-selectin in the plasma from the cancer patients (mean and standard deviation: 18.9 ± 5.5 ng/mL (range: 9.7 – 33.1 ng/mL), $n = 18$; $p < 0.001$) compared with the healthy individuals (mean and standard deviation: 13.2 ± 2.3 ng/mL (range: 7.1 – 19.6 ng/mL), $n = 23$). In addition, the level of D-dimer was significantly higher in the cancer patients (mean and standard deviation: 1.63 ± 1.35 μ g/mL (range: 0.28 – 3.9 μ g/mL), $n=15$; $p < 0.001$) compared with healthy individuals (mean $\pm$ standard deviation: 0.31 ± 0.18 μ g/mL (range: 0.12 – 0.8 μ g/mL), $n=23$ in healthy controls). Together, these data indicate that the cancer patients had greater levels of both coagulation and platelet activation compared with healthy individuals, which is consistent with the literature [28,29,34,35]. We did not observe any relationship between the levels of D-dimer or soluble P-selectin with TF activity in the combined platelet and microparticle samples from the cancer patients.

Discussion

In this study, we observed a higher level of TF activity associated with the combined platelet and microparticle samples from cancer patients compared with healthy individuals. Previous studies have measured increased plasma TF antigen and microparticle -associated TF activities in patients with various malignant disease [24-27]. However, platelets have been overlooked as a potential source of TF in cancer patients. This is despite the recent data showing that platelets can *de novo* synthesize and store TF. In addition, platelets can induce TF activity in monocytes and bind TF-positive microparticles in a P-selectin-dependent manner [14,15,17, 18,36,37]. This suggests that platelets may contribute to the prothrombotic state in cancer patients.

We did not observe a relationship between the TF activity associated with the combined platelet and microparticle sample and either the D-dimer levels or the soluble P-selectin levels. In contrast, a recent study [25] observed a correlation between the levels of TF-positive microparticles and D-dimer [25,35]. This difference may reflect the measurement of TF antigen rather than activity in the latter study. Importantly, a recent study showed that microparticle-associated TF activity correlated with the presence of venous thromboembolism in patients with pancreatic cancer [27]. This indicates that circulating TF activity may serve as a reliable marker for the presence of thrombosis in cancer patients.

Activated platelets play a central role in propagating the coagulation cascade and in the formation of a thrombus [38]. In malignant cancer, the risk of venous thrombosis is greater than in the non-malignant disease [39]. In addition, different types of malignant cancer are

related to a further increased risk of thrombosis [40]. In this study, we observed a range of TF activity associated with the combined platelet and microparticle samples from patients with different cancers. In particular, the highest TF activities were observed in patients with colon, pancreatic and lung cancers, which is consistent with a large population-based, case-controlled study that showed patients with malignant gastrointestinal, lung or hematological cancers had the highest risk of associated thrombosis [40]. Taken together, this suggests that the TF activity associated with the combined platelet and microparticle sample may correlate with the risk of thrombosis in patients with certain types of malignant cancer.

There are some limitations of the study. We did not analyze the levels of TF in platelets alone and microparticles alone. Therefore, we could not determine the relative contribution of platelets and microparticles to the combined platelet and microparticle sample. In addition, we did not measure the platelet count so we do not know if this affects the level of TF activity. Despite these limitations, the results indicate that this simple assay can be used to measure the levels of circulating TF.

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Fig. 1.
A representative image of the enriched platelet and microparticle fraction by electron microscopy. Magnification is 21,000 $\times$. P, platelet. Arrows show microparticles. Scale bar represents 0.5 μ m.

Fig. 2.

A box-whisker plot that shows the TF activity (pM) of the combined platelet and microparticle sample from cancer patients (N=20) and healthy individuals (Controls, N=23). The top and the bottom of the box represent the 75th and 25th percentile, respectively. In the controls, the filled circles represent TF activities from individuals who fall above or below the 75th and 25th percentile. The highest TF activities (above the 75th percentile) were observed in patients with colon, pancreatic or lung cancer.

Table

Sample group characteristics of the cancer patients and healthy individuals.

Population Characteristics			Mean TF Activity ± SD (pM)
Healthy Individuals	N (M: F) Mean Age ± SD (yrs) (Range)	23 (11: 12) 44.0 ± 10.4 (26 - 61)	8.7 ± 6.8
Cancer Patients	N (M: F) Mean Age ± SD (yrs) (Range) Cancer Type (n)	20 (11: 9) 68.5 ± 15.4 (34 - 85) Prostate (3) Lung (9) Renal (1) Breast (3) Pancreas (1) Sarcoma (1) Colon (2)	21.5 ± 12.3 21.1 ± 5.1 19.7 ± 12.2 17.5 17.9 ± 7.0 48.3 2.8 32.8
	Active Chemotherapy (n)	19	
	Presence of Catheter (n)	1	
	Mean WBC count ($\times 10^3/\mu\text{L}$) ± SD	6.7 ± 1.7	
	Mean Platelet count ($\times 10^3/\mu\text{L}$) ± SD	253 ± 113.5 [†]	

[†] All patients had normal platelet counts between 150,000 and 450,000/ μL , except two patients (102,000/ μL and 121,000/ μL). SD, standard deviation.