Tissue factor procoagulant activity of plasma microparticles is increased in patients with early-stage prostate cancer

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Summary

Tissue factor (TF) plays a critical role in tumour growth and metastasis, and its enhanced release into plasma in association with cellular microparticles (MPs) has recently been associated with pathological cancer progression. We have previously demonstrated significantly elevated levels of plasma TF antigen as well as systemic coagulation and platelet activation in patients with localised prostate cancer. In this prospective study, we used a highly sensitive one-stage clotting assay to measure procoagulant TF-specific procoagulant activity (PCA) of plasma MPs in 68 consecutive patients with early-stage prostate cancer to further explore the relevance of circulating TF in this tumour entity. Automated calibrated thrombography was used to monitor thrombin generation in cell-free plasma samples in the absence of exogenous TF or phospholipids. Compared to healthy male controls (n=20), patients had significantly increased levels of both D-dimer and TF-specific PCA of plasma MPs (p<0.001). Furthermore, MP-associated TF PCA was higher in patients with (n=29) than in those without (n=39) laboratory evidence of an acute-phase reaction (p=0.004) and decreased to normal levels within one week after radical prostatectomy. Overall, we found a significant correlation between TF-specific PCA of plasma MPs and plasma D-dimer (p=0.002), suggesting that plasma MPs contributed to in-vivo coagulation activation in a TF-dependent manner. Thrombin generation in plasma was also significantly increased in patients compared to controls (p<0.01). Collectively, our findings suggest that TF-specific PCA of plasma MPs contributes to intravascular coagulation activation in patients with early-stage prostate cancer and may represent a potential link between hypercoagulability, inflammation, and disease progression.

Keywords

Microparticles, tissue factor / factor VIIa, cancer

Introduction

Activation of coagulation and inflammation are characteristic findings in patients with advanced malignancies, including prostate cancer (1–3). However, there is also convincing evidence that various haemostatic components and inflammatory stimuli play a direct role in carcinogenesis and early tumour progression (4–6). In particular, tissue factor (TF), the cellular receptor for (activated) factor VII (FVII/FVIIa) and principal initiator of blood coagulation, appears to be critically involved in several pathways relevant to tumour growth and metastasis. TF exerts its functions in cancer biology in a coagulation-dependent and -independent manner. The former includes downstream generation of thrombin, which mediates fibrin formation and platelet activation and thus helps circulating tumour cells escape from immune surveillance by natural killer cells (7). The latter includes direct cell signalling of the TF:FVIIa and TF:FVIIa:Fxa complexes via protease-activated receptors (PARs), resulting in enhanced cell proliferation, migration, and tumour angiogenesis (8). Importantly, PAR-mediated cell signalling has been suggested to represent a potential mechanism, by which TF links procoagulant to inflammatory pathways in a murine model of endotoxemia (9).

TF is typically over-expressed on tumour cells and cells of the tumour microenvironment (i.e. tissue macrophages, stromal fibroblasts, and angiogenic endothelial cells). Although the exact role of host-derived TF in tumour biology is not conclusively resolved and most likely context-dependent (10), enhanced TF expression by tumour cells, including those of prostate gland...
origin, is commonly associated with a poor histological differentiation, an increased microvessel density, a high frequency of haematogenous metastasis, and an overall unfavorable patient outcome (11–14). Consequently, continuing efforts are being made to target TF in cancer therapy (6).

TF not only drives cancer progression, but also plays a critical role in the pathogenesis of cancer-associated thrombosis, which is commonly referred to as Trousseau’s syndrome (15, 16). In this regard, particular interest has recently been focused on TF circulating in plasma in association with sub-cellular membrane vesicles, so-called plasma microparticles (MPs). MPs are <1 µm in diameter and released from non-transformed cells (e.g., monocytes, platelets, and endothelial cells) upon activation or induction of apoptosis, whereas spontaneous shedding of MPs is a characteristic feature of most cancer cells (17, 18). Besides TF, exposure of negatively charged phospholipids (i.e., phosphatidylserine) on the outer membrane leaflet contributes to the procoagulant nature of plasma MPs (19).

Using functional and/or antigen-based detection systems, recent studies have implicated TF-bearing MPs in various cancer-associated clotting disorders such as venous and/or arterial thromboembolism (20–22), de-compensated disseminated intravascular coagulation (DIC) (23), or low-grade systemic coagulation activation (24, 25). Increasing experimental evidence emerges that hemostatic perturbances in cancer patients are, at least in part, controlled by defined genetic events in molecular tumourigenesis, including activating and inactivating mutations of oncogenes and tumour suppressor genes, respectively (26). In particular, using a colorectal cancer model, Yu et al. (27) have demonstrated that activation of K-ras and inactivation of p53 resulted not only in increased tumour cell TF expression, but also in enhanced release of TF-bearing MPs, potentially linking circulating TF to pathological cancer progression.

We have previously demonstrated significantly elevated levels of plasma TF antigen in patients with early-stage prostate cancer, a finding that was associated with increased markers of systemic coagulation and platelet activation (28). Furthermore, we have recently described a patient with advanced prostate cancer and bleeding symptoms due to de-compensated DIC, in whom effective anti-hormonal treatment was paralleled by resolution of overt coagulopathy and a significant decline in TF-specific procoagulant activity (PCA) of plasma MPs (23). Because these observations suggested an important biological role of circulating TF in prostate cancer, we conducted this prospective study on MP-associated TF PCA in patients with early-stage prostate cancer to further explore the relevance of circulating TF in this common and still life-threatening tumour entity. Realising that measurement of MP-associated TF PCA is not a standardised technique, we first used an established in-vitro model of bacterial endotoxemia to generate monocyte-derived, TF-positive MPs and characterise our various assay systems (29).

Materials and methods

Patients
All patients with prostate cancer admitted to the Department of Urology at the University Hospital Hamburg-Eppendorf, Germany, for radical prostatectomy in 2007 and 2008 were eligible for participation in the study. Inclusion and exclusion criteria were as previously described (28): patients were to have histologically proven prostate cancer by means of fine-needle biopsy with no clinical evidence for locally advanced or distant metastatic disease. Patients with any medical condition potentially affecting coagulation assessment, such as surgical procedures, including prostate biopsy, within four weeks of admission, acute or chronic infections, advanced hepatic or renal failure, inflammatory disorders, thromboembolic complications, or significant cardiovascular disease, were excluded from the study. Any anti-coagulant or anti-platelet medication was an exclusion criterion.

Preoperative plasma levels of fibrinogen (normal range, 1.8–3.5 g/l) and C-reactive protein (CRP, <5 mg/l) were used to stratify patients into those with and those without laboratory evidence of an acute-phase reaction. Healthy male controls (mean age, 56 ± 9 years) were either staff members or recruited from the hospital’s local blood bank. The study protocol for the ex-vivo analysis of plasma microparticles was approved by the local ethics committee of Hamburg, Germany.

Blood collection and storage
After obtaining informed consent from patients and controls, venous blood was drawn into 1/9 volume of 0.32% (0.105 M) trisodium citrate and centrifuged for 18 minutes (min) at 2,880 x g (4,000 rpm) to obtain cell-free plasma. Plasma aliquots were snap-frozen in liquid nitrogen and stored at –80°C until analysis.

In a subgroup of patients (n=10), blood samples were obtained both before and five to seven days after surgery, in which case the patients had not received any heparin for thromboprophylaxis in the preceding 48 hours (h).

Measurement of MP-associated TF procoagulant activity
Cryopreserved plasma samples were thawed in a water bath at 37°C, and 1-ml aliquots were subsequently centrifuged for 1 h at 16,100 x g in an Eppendorf microcentrifuge to separate cellular MPs from the plasma compartment. MP pellets were washed once in phosphate-buffered saline (PBS) and resuspended in 5 mM CaCl2, times until fibrin clot formation were recorded and calibrated against a standard curve obtained by serial dilutions (1:10–1:105) of lipiddiated recombinant human full-length TF (rhTF1–263) (Innovin™, Dade-Behring), showing a linear correlation in a log-log plot with R2>0.99. The 1:10 dilution of the TF standard, which clotted NHP in 22 ± 1 seconds (s), was arbitrarily defined to contain 100,000 units (U) of TF. All assays were carried out as triplicates in the presence of 30 µg/ml inhibitory TF monoclonal antibody (mAb) (45059; American Diagnostica) or control IgG (Sigma), and results were expressed as units of TF PCA per ml of plasma (U/ml). The lower detection limit of this assay for rhTF1–263 (33 kDa) was <5 pg/ml (<150 fM), and the intra- and inter-assay coefficients of variation were <10% (23).

In some experiments, MPs were incubated with 500 nM annexin V (Sigma) to block phosphatidylserine-dependent PCA. In
other experiments, plasma samples were centrifuged at 100,000 x g instead of 16,100 x g to isolate cellular MPs.

**Induction of TF-positive MPs in whole blood by endotoxin**

Citrate-anticoagulated whole blood from healthy volunteers was incubated for 18 h at 37°C in the presence and absence of 10 µg/ml *Escherichia coli*-derived lipopolysaccharide (LPS) (Sigma). Whole blood was centrifuged and plasma separated as described above. Plasma recalcification times were measured in the KC10 coagulation instrument after adding 50 µl of 0.1 M CaCl₂ to 300 µl of plasma in the presence of 30 µg/ml inhibitory TF mAb or control IgG.

**Calibrated automated thrombography**

Thrombin generation was measured in cryopreserved cell-free plasma samples by calibrated automated thrombography (CAT) using the Techno thrombin™ thrombin generation assay (TGA) (Technoclone) on a BioTek™ FLx800 TBI fluorometer: 40 µl of plasma were mixed with 60 µl of substrate containing 1 mM Z-G-G-R-AMC fluorogenic peptide and 15 mM CaCl₂. Thrombin generation was monitored over a 2-h period without the addition of exogenous TF or phospholipids. For analysis of cancer cell-derived membrane vesicles, adherent prostate cancer cell lines (PC-3, LNCaP, and DU145) were grown until 75–80% confluence, washed three times in PBS, and cultured for another 24 h in fresh medium. Thereafter, supernatants were harvested and detached cells removed by centrifugation for 10 min at 400 x g (1,500 rpm). Membrane vesicles were subsequently isolated from cell-free culture supernatants as described above and resuspended in NHP depleted of intrinsic phospholipids by centrifugation for 1 h at 16,100 x g. All TGA measurements were carried out as quadruplicates, and the intra- and inter-assay coefficients of variations were <20%. In some experiments, inhibitory TF mAb or control IgG (30 µg/ml) were added to plasma samples before CAT was performed.

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**Figure 1: Characterisation of TF-positive MPs after incubation of whole blood with LPS.**

A) Citrate-anticoagulated whole blood was incubated with LPS (10 µg/ml) or PBS for 18 h at 37°C before plasma microparticles (MPs) were isolated by high-speed centrifugation (16,100 x g) and assayed for procoagulant activity (PCA) as described under Materials and methods (mean ± SD, n=3–7). B) Following incubation of whole blood with PBS or LPS, TF antigen was measured in plasma and plasma supernatants by ELISA (mean ± SD, n=3). C) Calibrated automated thrombography (CAT) was used to monitor thrombin generation in plasma after incubation of whole blood with PBS (left panel) or LPS (right panel). CAT was performed in the presence of inhibitory TF monoclonal antibody (mAb) or control IgG. High-speed centrifugation for 1 h at 16,100 x g completely abolished thrombin generation in plasma supernatants. Results are representative of three independent experiments.
Measurement of plasma TF antigen

TF antigen was measured in cell-free plasma using a commercially available enzyme-linked immunosorbent assay (ELISA) (Imubind™; American Diagnostica) according to the manufacturer’s instructions. As a minor modification, 1% instead of 0.1% Triton X-100 was added to sample buffer, which has been shown to result in improved TF antigen recovery (23).

Measurement of plasma D-dimer

D-dimer plasma concentrations were quantified using the Innovance™ D-dimer test on a BCS™ coagulation analyser (Dade Behring). A D-dimer level of <500 µg/l is considered within the normal range.

Flow cytometric analysis of TF on cancer cells and MPs

Suspensions (1 x 10⁶ cells in 200 µl PBS) of prostate cancer cell lines (PC-3, LNCaP, and DU145) were incubated with 20 µl of phycoerythrin (PE)-conjugated TF monoclonal (clone, HTF-1; BD Pharmingen) or isotype-matched control antibody (Immunootech) for 20 min at room temperature in the dark. After extensive washing with PBS, cell-associated fluorescence was analysed on a FACSCalibur™ flow cytometer (BD Biosciences) counting 10,000 events. For analysis of TF antigen on MPs, cellular MPs were isolated from cryopreserved plasma samples as described above, and total numbers of TF-positive MPs were enumerated using the PE-conjugated TF monoclonal (HTF-1) in comparison to the IgG-PE control antibody. Calibration microspheres (Molecular Probes) measuring 1.1 µm and 1.9 µm in diameter were used for size exclusion and sample flow standardisation, respectively: during acquisition of a predefined number of 1.9-µm microspheres, all TF-positive events within the 1.1-µm gate (forward scatter) were counted (28, 30).

Table 1: Clinical and histopathological characteristics of patients with localised prostate cancer (n=68).

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Measurement of IL-6 and IL-8
Commerially available ELISAs were used to quantify antigen concentrations of IL-6 (Quantikine™ HS) and IL-8 (Quantikine™; both R&D Systems) in plasma samples. The mean minimum detectable doses were 0.04 pg/ml for IL-6 and 3.50 pg/ml for IL-8.

Statistical analysis
Normally distributed data were presented as mean ± standard deviation (SD), and differences between groups were analysed using the two-sided Student’s t-test. Non-Gaussian distributed data were presented as median and (inter-quartile) range and analysed using the Wilcoxon rank-sum (Mann-Whitney U) test. Correlation coefficients (r) between normally and non-Gaussian distributed data were calculated according to the methods of Pearson and Spearman, respectively. Throughout the study, statistical significance was assumed when p<0.05.

Results
Isolation and functional characterisation of LPS-induced MPs
An in-vitro model of endotoxin-induced monocyte TF expression and MP generation was used to characterise the various assay systems employed in this study. Incubation of citrate-anticoagulated whole blood for 18 h with 10 µg/ml LPS, a dose previously shown to induce phosphatidylserine (PS) membrane exposure on monocytes (31), resulted in a significant shortening of plasma recalcification times from 791 ± 137 to 154 ± 49 s (n=7), indicating the release of PCA into the plasma compartment. Addition of an inhibitory TF mAb to LPS-treated plasma samples prolonged clotting times from 154 ± 49 to 417 ± 131 s, suggesting that a substantial fraction, but not all, of the released PCA was TF-dependent. Accordingly, isolation of plasma MPs by high-speed centrifugation (16,100 x g) revealed an 11-fold increase in TF-specific PCA (defined as the PCA in the presence of TF mAb subtracted from the PCA in the presence of control IgG) in LPS-treated compared to PBS-treated samples (Fig. 1A). Addition of annexin V also largely abolished the PCA of isolated MPs, emphasising the critical role of PS in both TF activation and promotion of key enzymatic reactions relevant to the intrinsic and common coagulation pathways (e.g. functional assembly of the tenase and prothrombinase complexes). In this in-vitro model, high-speed centrifugation (16,100 x g) was as effective as ultracentrifugation (100,000 x g) in isolating MP-associated TF PCA (not shown).

Incubation of whole blood with LPS also resulted in a significant increase in plasma TF antigen (68 ± 48 vs. 406 ± 148 pg/ml) (Fig. 1B). Centrifugation for 1 h at 16,100 x g or 100,000 x g similarly lowered the LPS-induced elevated TF antigen levels in sample supernatants, suggesting that most, if not all, of the LPS-induced plasma TF antigen was associated with cellular MPs. Using calibrated automated thrombography we also measured thrombin generation in plasma samples after incubation of whole blood with PBS or LPS (Fig. 1C). Treatment with LPS resulted in a shortening in lag phase and an increase in both peak thrombin generation and the endogenous thrombin potential (= area under the thrombin generation curve). All three parameters were normalised by the addition of inhibitory TF mAb. It is noteworthy that thrombin generation was completely abolished in plasma supernatants of both PBS- and LPS-treated samples fol-

Figure 3: Levels of plasma D-dimer and MP-associated TF PCA are increased in patients with localised prostate cancer.
A) Levels of plasma D-dimer were quantified in 68 patients with clinically localised prostate cancer and in 20 healthy male controls (56 ± 9 years). Horizontal bars indicate mean D-dimer levels (p-value is according to Student’s t-test). B) TF-specific procoagulant activity (PCA) of microparticles (MPs) isolated from cell-free plasma by high-speed centrifugation was measured in healthy male controls (n=20) and patients with localised prostate cancer (n=68) as described under Materials and methods. Based on plasma fibrinogen and C-reactive protein levels, patients were grouped into those with (+ APR, n=29) and those without (- APR, n=39) an acute-phase reaction. Horizontal bars indicate mean TF PCA levels (p-values are according to Student’s t-test). C) In the whole study population (n=88), there was a significant correlation between levels of plasma D-dimer and MP-associated TF PCA. The correlation coefficient (r) is according to the method of Pearson.
lowing centrifugation for 1 h at 16,100 x g, most likely due to efficient elimination of active phospholipids (i.e. cellular MPs) (Fig. 1C). In summary, these findings suggest that high-speed centrifugation represents an appropriate methodology for the isolation of MP-associated TF PCA following incubation of whole blood with LPS.

Prostate cancer cells release procoagulant membrane vesicles

Besides tissue macrophages and circulating monocytes, tumour cells are a potential source of MP-associated TF PCA in cancer patients. The prostate cancer cell line, LNCaP, exhibited strong expression of TF antigen on the membrane surface as assessed by flow cytometry (Fig. 2A). Spontaneously shed membrane vesicles were isolated from culture supernatants by high-speed centrifugation and assayed for TF PCA using the one-stage clotting (Fig. 2B) and thrombin generation assay (Fig. 2C). In both assay systems, significant TF-specific PCA of isolated membrane vesicles was demonstrated. Similar results were obtained using the PC-3 and DU145 prostate cancer cell lines (not shown), suggesting that transformed epithelial cells of prostate gland origin are capable of initiating the TF-dependent coagulation pathway via the release of procoagulant MPs in vitro.

MP-associated TF PCA in patients with localised prostate cancer

A total of 68 consecutive patients with clinically localized prostate cancer were included in the study (Table 1). Demographic, clinical, and histopathological patient characteristics were similar to those of our previous study cohort (28).

Compared to healthy male controls (n=20), patients had laboratory evidence of low-grade DIC, as indicated by elevated levels of plasma D-dimer (Fig. 3A). Patients also had significantly increased levels of TF-specific PCA associated with plasma MPs (582 ± 335 vs. 292 ± 74 U/ml) (Fig. 3B). Based on plasma fibrinogen and CRP concentrations, patients were stratified into those with (n=29) and those without (n=39) laboratory evidence of an acute-phase reaction (APR). Of the 29 patients in the APR group, 27 (93%) had elevated fibrinogen levels (maximum level, 5.3 g/l) and 11 (38%) had elevated CRP levels (maximum level, 25 mg/l). Both parameters were elevated in nine patients (31%). Compared to patients without an APR, patients in the APR group had significantly increased levels of MP-associated TF PCA (713 ± 416 vs. 484 ± 218 U/ml) (Fig. 3B). The difference in TF-specific PCA of plasma MPs between healthy male controls and patients without an APR was also significant (p<0.001).

In the whole study population (n=88), there was a modest, albeit statistically significant correlation between plasma D-dimer and TF-specific PCA of plasma MPs (r=0.32, p=0.002) (Fig. 3C), suggesting that higher levels of MP-associated TF PCA were more likely associated with higher levels of plasma D-dimer. For instance, deriving a robust cut-off value of 662 U/ml from the control population (mean ± [5 x SD]), we determined that patients with MP-associated TF PCA above this cut-off (n=16) had higher levels of D-dimer (492 ± 137 µg/l) than patients (n=52) with MP-associated TF PCA below this cut-off (426 ± 194 µg/l). Although this difference did not reach statistical significance, our findings collectively suggest that TF-specific PCA of plasma MPs is elevated in patients with early-stage prostate cancer and correlates with molecular markers of systemic coagulation activation (D-dimer) and inflammation (fibrinogen and/or CRP).

Elevated MP-associated TF PCA decreases after radical prostatectomy

In a subgroup of 10 patients, plasma MPs were analysed both before (day –1) and five to seven days after surgery (day 5–7) (Fig. 4A). Mean levels of TF-specific PCA of plasma MPs were 570 ± 348 U/ml before and 267 ± 140 U/ml after radical prostatectomy (p=0.006) (Fig. 4B). In particular, in all three patients presenting with MP-associated TF PCA levels above the aforementioned cut-off value (>662 U/ml), such levels normalised (i.e. decreased to <662 U/ml) after surgery (Fig. 4B), suggesting that, in this representative subgroup of patients, elevated levels of MP-associated TF PCA were indeed related to the presence of prostate cancer.

Increased thrombin generation in patients with localised prostate cancer

Using calibrated automated thrombography, we measured thrombin generation in cell-free plasma samples of a randomly chosen cut-off value (713 ± 416 vs. 484 ± 218 U/ml) (Fig. 3B). The difference in TF-specific PCA of plasma MPs. A) Microparticles (MPs) were isolated from cryopreserved plasma samples obtained from 10 patients with clinically localised prostate cancer on the day before (day –1) and five to seven days after radical prostatectomy (day 5–7). Means were assayed for procoagulant activity (PCA) as described under Materials and methods (mean ± SD, n=10). B) Individual data pairs for TF-specific PCA of plasma MPs before and after radical prostatectomy. Horizontal bars indicate mean TF PCA levels (p-value is according to paired Student’s t-test).
selected subgroup of patients with localised prostate cancer (n=37) in the absence of exogenous TF or phospholipids. Compared to those of healthy male controls (n=20), plasma samples of patients showed increased thrombin generation, as evidenced by a significant shortening in lag phase (LP) and a significant increase in both peak thrombin generation (PT) and the endogenous thrombin potential (ETP) (Fig. 5A). However, addition of an inhibitory TF mAb to plasma samples did not significantly change mean values of the various thrombin generation parameters (not shown), and we did not observe any differences in LP, PT, or ETP between patients with (n=19) and those without (n=18) an acute-phase reaction (not shown). To shed light on this seemingly surprising finding, we measured thrombin generation in two plasma samples from a previously described patient with advanced prostate cancer and de-compensated disseminated intravascular coagulation (DIC) (23). At presentation (sample #1), TF-specific PCA of plasma MPs was increased 193-fold over the mean level of controls. During follow-up and resolution of overt coagulopathy (sample #2), there was still a 15-fold increase in MP-associated TF PCA with evidence of significant systemic coagulation activation. Addition of inhibitory TF mAb to sample #1 prolonged LP from 16 ± 2 to 26 ± 2 min, whereas LP remained essentially unchanged in sample #2 (Fig. 5B). These findings suggest that thrombin generation in patient-derived plasma samples (+ TF mAb) may only be sensitive to dramatically increased levels of MP-associated TF PCA.

Plasma TF antigen and TF-positive MPs

Using flow cytometry and an enzyme-linked immunosorbent assay, we measured total numbers of TF-positive MPs and TF antigen, respectively, in plasma samples of a subgroup of patients with clinically localised prostate cancer (n=37) to investigate, if any of the two parameters would correlate with TF-specific PCA of plasma MPs. However, neither levels of plasma TF antigen (median, 179 pg/ml; interquartile range [IQR], 121–234 pg/ml) nor total numbers of TF-positive MPs (634 x 10^3/ml, 273–996 x 10^3/ml) correlated with MP-associated TF PCA, suggesting that, in patients with early-stage prostate cancer, a variable fraction of the total plasma TF pool circulated as an inactive (i.e. functionally cryptic) variant.

Plasma IL-6 and IL-8 antigen levels

IL-6 and IL-8 are two inflammatory cytokines that have been implicated in prostate cancer progression (32, 33). Because MP-associated TF PCA was elevated in patients with localised prostate cancer and appeared to be correlated with molecular markers of inflammation (Fig. 3B), we also measured systemic levels of IL-6 and IL-8 in a subgroup of patients (n=58) and controls (n=20). Whereas there was no difference in IL-6 antigen levels (2.6 ± 3.3 vs. 2.6 ± 2.1 pg/ml), IL-8 antigen levels were slightly elevated in patients (12.6 ± 9.1 pg/ml) compared to controls (9.1 ± 6.9 pg/ml), but this difference failed to achieve statistical significance (p=0.12). There was also no difference in IL-6 or IL-8 antigen levels between patients with and those without an acute-phase reaction. Collectively, these findings suggest that TF-specific PCA of plasma MPs is not associated with systemic levels of IL-6 or IL-8 in patients with early-stage prostate cancer.

Discussion

In this study, we have demonstrated significantly increased TF-specific PCA of plasma MPs in 68 consecutive patients with early-stage prostate cancer as compared to 20 healthy male controls.

The question of the cellular origin of TF-bearing MPs in cancer patients is intriguing. Experimental and clinical studies have provided direct or indirect evidence that cancer cells constitute an important source of circulating TF-positive MPs in malignancy (20,
21, 23, 27, 30). However, other cells such as monocytes/macrophages, fibroblasts, platelets, and endothelial cells may contribute to the intravascular TF pool. With regard to the plethora of potential interactions between MPs and cells of the vascular system, identifying the exact cellular origin of TF-bearing MPs in cancer may prove elusive (34). TF-specific PCA of plasma MPs may thus be best interpreted as a reflection of the entire tumour burden with its procoagulant, inflammatory, and angiogenic microenvironment rather than of a distinct cell population.

Our previous finding of declining MP-associated TF PCA in a patient with advanced prostate cancer receiving effective anti-hormonal treatment (23), our current observations on spontaneous shedding of TF-positive MPs by a prostate cancer cell line (Fig. 2B) and decreasing levels of MP-associated TF PCA after radical prostatectomy (Fig. 4) as well as several reports on epithelial TF over-expression in primary prostate cancer specimens (11–14) make tumour cells a likely source of TF-bearing MPs in our study cohort. However, stimulation of circulating monocytes by elevated levels of tumour necrosis factor (TNF)-α may represent an alternative pathway of intravascular TF induction and MP generation (35). Although CRP has also been implicated in monocyte TF expression (36), it is unlikely that this acute-phase reactant played a major role in the induction of MP-associated TF PCA in our study, especially, when considering that some of the previously reported effects of recombinant CRP may have been due to contamination with proinflammatory bacterial products (37). Irrespective of the exact cellular source of TF-bearing MPs, our findings support a close interrelation between systemic coagulation activation and inflammation in patients with early-stage prostate cancer.

Because measuring TF-specific PCA of plasma MPs is cumbersome and time-consuming, the question arises of whether any other laboratory parameter could substitute for the quantification of MP-associated TF PCA. Although plasma D-dimer was also significantly increased in patients compared to controls (Fig. 3A), there was only a weak correlation between this parameter and MP-associated TF PCA. This finding is not surprising, considering that pathways other than TF expression on MPs may promote coagulation activation in cancer patients (1, 2, 16). In contrast, our finding that neither plasma TF antigen (as measured by ELISA) nor total numbers of TF-positive MPs (as measured by flow cytometry) significantly correlated with TF-specific PCA of plasma MPs deserves further attention. Striking disparities between the results of functional and antigen-based detection systems have been reported in other studies on plasma TF in cancer (20, 23, 30) and may be explained by the presence of close-reactive, TF-like proteins and/or soluble, biologically less active TF variants (i.e. alternatively spliced TF) (38) and by the mechanisms that potentially control TF activity on plasma membranes. The latter include, but may not be limited to, TF homo-dimerisation, lipid organisation, co-expression of TF pathway inhibitor, and reduction of the Cys88-90-Cys209 disulfide bond in the extracellular TF domain (39).

In addition, membrane-incorporated TF such as TF expressed on cellular MPs may not be sufficiently solubilised by an ELISA’s detergent when present in high quantities in plasma, resulting in significantly reduced antigen recovery (23). Furthermore, it has been suggested that light-scatter flow cytometry may not be an appropriate methodology to enumerate sub-cellular membrane vesicles <1 µm in diameter (22). Although the applied TF ELISA appears to accurately detect differences in MP-associated TF in a controlled in-vitro system (Fig. 1B), results may be more difficult to interpret in a patient-derived plasma sample, especially, since a substantial fraction of the plasma TF antigen may not be associated with cellular MPs in patients with localised prostate cancer (28). In this regard it is noteworthy that neither high-speed nor ultracentrifugation were capable of eliminating TF antigen from plasma supernatants despite the fact that thrombin generation was completely abolished by either means, most likely due to inefficient elimination of procoagulant phospholipids (i.e. cellular MPs) (Fig. 1). It is reasonable to hypothesise that measurement of soluble and MP-associated TF provides complementary information in cancer patients, considering that alternatively spliced TF has recently been implicated in tumour growth and angiogenesis (40). A recent study on patients with locally advanced or metastatic pancreatic cancer found a significant correlation between plasma TF antigen and MP-associated TF activity (41), but this correlation was mainly due to dramatically elevated TF antigen levels (i.e. >300 pg/ml) in two patients experiencing venous thromboembolic complications over the course of treatment. An alternative methodology for the quantification of MP-associated TF antigen involves capturing of plasma MPs by immobilised annexin V and immunologic detection of MP-exposed TF by a peroxidase-conjugated antibody (Hyphen BioMed).

Our thrombin generation data are consistent with a hypercoagulable state in patients with early-stage prostate cancer (Fig. 5A). In the absence of exogenous TF and phospholipids, thrombin generation in plasma significantly depends on the presence and constitution of cellular MPs. In our controlled in-vitro systems (Figs. 1 and 2), addition of inhibitory TF mAb resulted in a prolongation of LP and a decrease in peak thrombin
generation, which was associated with a decrease in the ETP. In the subgroup of 37 patients investigated, mean values for all three thrombin generation parameters were altered in a way to suggest the presence of elevated concentrations of TF-bearing MPs (Fig. 5A). However, addition of inhibitory TF mAb to patient samples had no effect on thrombin generation parameters, suggesting that this assay, at least in cases of only slightly-to-moderately increased levels of MP-associated TF PCA, may not represent an adequate alternative to the analysis of isolated MPs in a standardised (i.e. normal) plasma environment.

It has been suggested that early-stage prostate cancer may not be an optimal tumour entity to study the clinical relevance of circulating TF in malignancy due to its overall favorable prognosis and long latency between surgical intervention and (biochemi-

cal) tumour recurrence (42). Consequently, using a validated nomogram for risk stratification (43), we determined that the five-year probability of progression-free survival was <90% in only nine of the 68 patients (13%), making a statistically meaningful conclusion on the prognostic impact of MP-associated TF PCA in our study cohort impossible at this point. Nevertheless, our findings suggest that quantification of TF-specific PCA of plasma MPs is feasible even in patients with early-stage malignancies and that elevated levels may potentially provide valuable additional information in cancer research and patient care.

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References