

Procoagulant microparticles derived from cancer cells have determinant role in the hypercoagulable state associated with cancer

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Abstract. Hypercoagulability is a common alteration of blood coagulation in cancer patients. However, the procoagulant activity of cancer cells is not sufficient to induce hypercoagulability. The present study was aimed to identify the mechanism with which hypercoagulability is produced in the presence of cancer cells. We focused on the analysis of the procoagulant elements carried by cancer cell-derived microparticles (CaCe-dMP) and we evaluated the impact of microparticles associated with the cancer cells from which they stem on thrombin generation. CaCe-dMP from the cancer cells were isolated from the conditioned medium and analyzed for tissue factor (TF) and procoagulant phospholipid expression. Thrombin generation of normal plasma was assessed by the Thrombinoscope (CAT[®]) in the presence or absence of pancreas adenocarcinoma cells (BXPC3) or breast cancer MCF7 cells supplemented with the respective CaCe-dMP. Both BXPC3 and MCF7 cells express abundant amounts of

active TF. Phosphatidylserine was identified on the surface of CaCe-dMP, unlike the cancer cells themselves. The expression of TFa by the microparticles was significantly higher to that observed on the cancer cells. Culture of the cancer cells with their microparticles resulted in thrombin generation significantly higher as compared to the upper normal limit. In conclusion, cancer cells 'enrich' the microenvironment with procoagulant elements, especially procoagulant microparticles which express TF and procoagulant phospholipids. The association of cancer cells with procoagulant microparticles is necessary for a state of hypercoagulability, at the level of the tumoral microenvironment. The intensity of the hypercoagulability depends on the histological type of the cancer cells.

Introduction

Blood hypercoagulability is a common systemic alteration in patients with cancer that predisposes to thrombosis which has a deteriorating effect on patients' quality of life and survival (1-4). Cancer cells are directly involved in the pathogenesis of thrombosis through several pathways which implicate triggering and enhancement of thrombin generation and imbalance of procoagulant and anticoagulant forces. However, the biochemical basis of the activation of coagulation in cancer patients is still not completely understood. Clinical and experimental evidence support the paradigm that coagulation and tumor growth form a vicious circle. Enhanced generation of serine proteases of blood coagulation, promotes the aggressiveness of cancer cells and vice versa (2). Attention has been focused on tissue factor (TF), a transmembrane receptor protein, which is not only the primary initiator of coagulation but also promotes tumor growth, angiogenesis, and metastasis (5). Cancer patients exhibit heightened levels of circulating procoagulant microparticles that correlate with the risk of thrombosis (6-11).

Tumor cells may express procoagulant activity that can directly induce thrombin generation. In addition, normal host tissues may express procoagulant activity in response to the tumor. Many tumor cells express high levels of TF. In several

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Abbreviations: VTE, venous thromboembolism; TF, tissue factor; MCF7, Michigan Cancer Foundation 7, human breast cancer cells; BXPC3, human pancreatic adenocarcinoma cells; FXII, coagulation factor XII; TFa, tissue factor activity; PPP, platelet poor plasma; MRI, mean rate index; HUVEC, primary human umbilical vein endothelial cells; TG, thrombin generation; UNL, upper normal limit; ttPeak, time to peak; PPL, procoagulant phospholipids; CaCe-dMP, cancer cells derived microparticles; Peak, maximal concentration of thrombin; CAT, cancer associated thrombosis; MP, microparticles

Key words: cancer, thrombin generation, tissue factor, microparticles, hypercoagulability

types of cancer, including breast and pancreatic cancer, TF expression on tumor cells is correlated with grade and tumor progression (12-14). Recent studies from our group showed that the contact of cancer cells with human plasma triggers and enhances thrombin generation in a partially tissue factor-dependent manner (15). Pancreas adenocarcinoma cells BXPC3 and breast cancer cells MCF7 showed that they accelerate the initiation and the propagation phase of thrombin generation but they have different procoagulant potential (15). To some extent, this is due to different amount of TF expressed by the BXPC3 and MCF7 cells. These studies raised the concept that the procoagulant potential varies according to the histological type of cancer cells.

An additional mechanism that amplifies cancer related hypercoagulability is the release of cancer cell-derived procoagulant microparticles (CaCe-dMP) (16). Among the contributing factors, CaCe-dMP has generated considerable interest since the discovery of their pro- and anticoagulant properties, their fibrinolytic activity and their ability to contribute to thrombosis *in vivo* (17,18). It is suggested that CaCe-dMP bearing TF is a trigger for thrombogenesis (6,7,19,20). In addition, TF bearing CaCe-dMP appear to participate in triggering thrombogenesis directly and play an important role in cancer-induced coagulopathy (21). Approximately 50% of MPs in the patients with cancer expressed the tumor antigen mucin 1 on their surface, suggesting that they derive from the tumor (20). Consequently, TF bearing CaCe-dMP could be an important marker in the consideration of prevention or therapy of cancer associated thrombosis.

The present study aimed to identify the mechanism by which hypercoagulability is produced in the presence of cancer cells. We focused on the analysis of the procoagulant elements carried by CaCe-dMP and we evaluated the impact of microparticles associated with the cancer cells from which they stem on thrombin generation. We also explored any potential differences on the prothrombotic potential of the cancer cells and their microenvironment which are related with the histological type of the cancer cells.

Materials and methods

Cell cultures. Adhesive cell lines from human pancreatic primary adenocarcinoma cell line BXPC3 (lot F-11067) was from American Type Culture Collection (ATCC; Rockville, MD, USA). Breast adenocarcinoma cell line MCF7 (Michigan Cancer Foundation-7) was from ATCC (Rockville, MD, USA). Both cell lines were used for thrombin generation experiments in human plasma.

Cells were expanded and cultured as described elsewhere (15). A volume of 100 μ l of cell suspension (50 cells/ μ l) was placed into 96-well plates and cells were cultured and adhered at 37°C in 100% humidified atmosphere with 5% CO₂ for 24 h.

The HUVEC were obtained from Clonetics (San Diego, CA, USA) and cultured in endothelial cell growth media EGM-2 (Clonetics) containing 2% of foetal bovine serum and supplements. Cells of 2nd passage were used in the experiments. Adhesive cultures were developed on 25 cm² culture flasks and adhered at 37°C in 100% humidified atmosphere with 5% CO₂. Cells were used for experiments when they reached

80% confluence. For transfer to the 96-well plates HUVEC (a suspension of 50 cells/ μ l) were treated with the same protocol as that described above for BXPC3 and MCF7 cells.

Normal human plasma. Samples of normal platelet poor plasma (PPP) for thrombin generation experiments were purchased from Stago (ref. no. 00539; Genevilliers, France) in compliance with Helsinki Declaration.

Specific TF activity concentration. The BXPC3 and MCF7 cells were obtained from adhesive cultures as described above. After three washing cycles with PBS, cells were suspended in distilled water (at final concentrations adjusted between 50 and 200 cells/ μ l) and incubated at 4°C for 30 min. Then samples were centrifuged for 30 min at 1,000 x g, afterwards, supernatants were collected and kept frozen at -80°C until measurement of TF activity.

Tissue factor activity (TFa) was measured in normal plasma, the same which was used for thrombin generation experiments; in which cancer cells were suspended. Tissue factor activity (TFa) was assessed with an in-house chromogenic method as described elsewhere (22-24).

Calibrated Automated Thrombogram assay. In each well of the micro-plate, 80 μ l of PPP samples were mixed with saline (20 μ l). Thrombin generation was initiated by adding 20 μ l triggering solution containing CaCl₂ (16.7 mM final concentration) and fluorogenic substrate (Z-Gly-Gly-Arg-AMC, 417 μ M final concentration). Thrombin generation was assessed with the Calibrated Automated Thrombogram assay (Thrombinoscope b.v., Maastricht, The Netherlands) as described elsewhere (25). Among thrombogram parameters we analyzed the mean rate index (MRI), which reflects the rate of the propagation phase of thrombin generation [calculated by the formula $MRI = \text{Peak}/(\text{ttPeak} - \text{lag-time})$]. This parameter includes lag-time, the time to Peak (ttPeak) and the Peak. These parameters of thrombogram as shown in previous studies, reflect the biological activity of cancer cells on thrombin generation, better than the endogenous thrombin potential.

Procoagulant potential of cancer cells assessed with the calibrated automated thrombogram assay. The BXPC3 or MCF7 cells as well as the HUVEC (control experiment) were expanded in the wells of microtiter plates suitable for thrombin generation assessment (as described above). Then, 80 μ l of normal PPP was added in each well and thrombin generation was assessed as described above. In the control experiments, wells were filled with culture medium without cells and treated in the same way as the experiments. Each experiment was repeated several times. Saline (20 μ l) was used in the control experiment. In additional control experiments, thrombin generation was assessed in plasma spiked with increasing concentration of lymphocytes from healthy donors and was compared to thrombin generation obtained after calcification of normal plasma. No significant difference was found between the two experimental procedures (data not shown). In preliminary experiments, we also verified that the culture medium (solution of RPMI, glutamine, penicillin, streptomycin and fetal calf serum) did not influence thrombin

generation process of normal PPP. Thrombin generation was initiated and recorded as described above.

Thrombin generation in the presence of an anti-TF antibody. In separate experiments, 50 μl of the working suspension of BXPC3 or MCF7 cells (in a volume yielding a count of 100 cells/ μl) were mixed with 50 μl of a solution containing an anti-TF mouse monoclonal antibody 4509 (American Diagnostica, Neuville-sur-Oise, France) or anti-human TF9-10H10 of mouse origin (AbD Serotec, Bio-Rad Laboratories, Steenvoorde, France). An isotype mouse IgG1 (100 $\mu\text{g}/\text{ml}$) or saline were used in control experiments. Cells were incubated with the anti-TF antibody or the isotype IgG1 for 15 min at 37°C and then 20 μl of this suspension were mixed with 180 μl of PPP. The number of BXPC3 and MCF7 cells in plasma assessed for thrombin generation was 50 per μl . The experimental conditions were defined after conducting preliminary experiments on thrombin generation, with variable concentrations of the cells and the anti-TF monoclonal antibody. Cells were used at the lower active concentration in plasma and antibody was employed at the concentration of 25 $\mu\text{g}/\text{ml}$. At this concentration the anti-TF antibody completely inhibited the effect of high TF concentrations on thrombin generation. Impact of anti-TF antibody on thrombin generation were triggered in normal PPP by BXPC3 or MCF7 cells triggered in presence of MP-Reagent® to eliminate any interactions of the phospholipid concentration.

Isolation of cancer cell-derived microparticles. Cancer cell-derived microparticles were isolated from conditioned media, containing 2% of foetal bovine serum and supplements, from confluent BXPC3, MCF7, HUVEC cells by differential centrifugation, as previously described (26). Briefly, culture supernatants (cell conditioned media) were collected and centrifuged at 1,500 x g for 5 min to pellet whole cells and debris. The collected supernatant was re-centrifuged at 15,000 x g for 1 h at 15°C to pellet the CaCe-dMP. The final pellet was re-suspended in 0.5 ml PBS and centrifuged at 2,000 x g for 1 min to remove debris. The clear CaCe-dMP suspension was further centrifuged at 18,000 x g for 30 min at 15°C to pellet MPs. The number of MP was measured with flow cytometry and was standardized for the mixing experiments.

Flow cytometric analysis on cells and cancer cell-derived microparticles. BXPC3 or MCF7 cells were detached using Versene Buffer (0.54 mM EDTA, 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.46 mM KH₂PO₄, and 1 mM glucose, pH 7.4), washed once, and re-suspended in PBS supplemented with 1% BSA (PBS/BSA). Cells suspended in phosphate-buffered saline (PBS), were centrifuged at 300 x g for 5 min. For TF assessment, cells re-suspended in PBS (3x10⁵ cells/25 μl), were incubated with 10 μl of a control mouse immunoglobulin IgG1 (Beckman Coulter, Villepinte, France, reference 731581) or with a mouse monoclonal antibody against human TF (American Diagnostica, product no. 4509), for 30 min at room temperature in the dark. Anti-TF monoclonal antibody and IgG1 isotype control were used at 1/25 and 1/50 dilution, yielding a final concentration of 5 and 7 $\mu\text{g}/\text{ml}$, respectively. After washing twice, cells

were incubated at room temperature with a PE-conjugated antibody goat anti mouse IgG1 (1/50 dilution; Beckman Coulter, ref. no. 731914) for 30 min in the dark. After incubation, cells were washed with PBS and suspended in PBS for tissue factor assessment. Sample data were acquired and analyzed using an FC 500 flow cytometer (Beckman Coulter, Villepinte, France) with CXP Acquisition and CXP Analysis soTFware (Beckman Coulter, Miami, FL, USA). Forward scatter and side scatter of light was set in logarithmic scale.

For the detection of microparticles by flow cytometry, an initial microparticle-size gate was set with the help of calibrating fluorescent 0.8 μm and 3.0 μm latex beads (Sigma, St. Louis, MO, USA). This microparticle gate excludes the electronic background noise through the threshold. In parallel, we used Megamix (American Diagnostic Corp., Hauppauge, NY, USA), a mixture of microbeads of three different sizes (0.5, 0.9, and 3.0 μm) which was developed to confirm the size of the microparticles. Forward scatter and side scatter had a logarithmic gain. The absolute count of microparticles was measured setting the stop condition for TruCount beads at 10,000 events. In order to separate true events from background noise and unspecific binding of antibodies to debris, we defined microparticles as particles that were less than 1.0 μm in diameter, had positive staining for Annexin V and expressed surface antigens (CD31 or CD42 or both). For the measurement of procoagulant phospholipids expression the microparticle suspension was incubated for 30 min with 10 μl FITC-Annexin V or PE-anti-mouse monoclonal antibodies. Annexin V-FITC with phosphate-buffered saline without calcium were used as control. Analyses were performed on a flow cytometer (Navios) using a Megamix bead-calibrated protocol (BioCytex). Preliminary experiment verified that conditioned media containing 2% of foetal bovine serum and supplements without any exposure to the studied cell lines did not contain any detectable amount of microparticles.

Statistical analysis. Non-parametric Mann-Whitney test was applied to control changes in thrombogram parameters in the presence or in the absence of cancer cells in plasma as well as in the different experimental conditions described above. Results are shown as mean \pm SD. The level of statistical significance was set at 0.05. The inhibition of thrombin generation (TG) was calculated by the formula: Inhibition of TG = (1-TGcells/TGcontrol) %. Two-sided values of P<0.05 were considered as statistically significant. SPSS statistical soTFware package was used for statistical analysis.

Results

After the initial screening of several cancer cell lines, the BXPC3 and MCF7 cancer cell lines were selected because they were from different organs (pancreas and breast) and induced significantly different intensity of thrombin generation. For technical reasons it was not possible to obtain cells from healthy pancreatic or breast tissue. So we used primary human umbilical vein cells (HUVEC) as normal control experiment.

In preliminary experiments we found that after 24 h of incubation, the BXPC3 cells were at 70% confluence and the MCF7 cells were at 80% confluence. In these conditions the

two cancer cell lines gave similar intensity of thrombin generation. We also ruled out any potential interference of trypsin on cell activity by assessing thrombin generation either with cells which were moved by the flasks mechanically or by exposure to trypsin. We also confirmed that the concentration of fetal bovine serum (ranging from 1 to 10%) and the incubation time of cells in the plaque (ranging from 5 to 72 h) did not significantly influence their procoagulant potential on thrombin generation. For the studied cancer cell lines, a plateau effect on thrombin generation was observed at cell numbers equal or higher than 50 cells/ μ l (data not shown). Cells were used in the experiments only if the apoptotic cell number was lower than 2% of the whole cells count.

Enhancement of thrombin generation by BXPC3 and MCF7. As previously showed, the contact of cancer cells with normal PPP resulted in a significant increase of the Peak and the MRI and a reduction of the lag-time and ttPeak as compared to normal PPP without cancer cells. At equal numbers of cells the MCF7 had less potent procoagulant activity as compared to the BXPC3 cells. The parameters of thrombogram assessed in the presence of HUVEC were not significantly different as compared to the control experiment (PPP without cells) (Table I).

Both BXPC3 and MCF7 cells expressed significantly higher levels of TFa (1.42 \pm 0.10 and 0.82 \pm 0.08 pM, respectively) as compared to the normal plasma and the HUVEC (0.20 \pm 0.05 and 0.23 \pm 0.02 pM, respectively; $P < 0.05$). The BXPC3 cells expressed significantly higher levels of TFa as compared to the MCF7 cells ($P < 0.05$). Data are summarized in Table II.

Exposure of tissue factor and phosphatidylserine by cancer cells. Flow cytometry analysis was performed to BXPC3 and MCF7 cells. The labeling of cancer cells by an anti-TF antibody showed the presence of TF on the membrane of cancer cells. Fluorogenic intensity was significantly higher on BXPC3 cells than on MCF7 cells. The mean index of fluorescence (MIF) was 395 \pm 132 and 95 \pm 20 for BXPC3 and MCF7 cells, respectively. In the same experiment on HUVEC traces of TF were detected (MIF=5 \pm 10). Data are summarized in Table II.

The proportion of cells carrying TF was slightly higher in BXPC3 than MCF7 cells (95 \pm 13 vs. 78 \pm 10%, respectively; $P = 0.001$). A significantly lower number of HUVEC expressed TF (10 \pm 2%). The labeling of cancer cells with Annexin V documented the absence of procoagulant phospholipids on the surface of the BXPC3 or MCF7 cells or the HUVEC (control experiment). The study by a functional test did not detect phospholipids on these three types of cells.

Procoagulant phospholipids expressed by cancer cell-derived microparticles. Cancer cells in culture spontaneously release procoagulant microparticles into the conditioned medium. Flow cytometry assessment using Annexin V labeling showed the presence of procoagulant phospholipids at both BXPC3 and MCF7 derived microparticles, which showed similar MIF (30 \pm 4 and 49 \pm 6, respectively; $P > 0.05$). The percentage of phosphatidylserine expression was the same in both BXPC3 and MCF7 derived microparticles (56 \pm 3 and 57 \pm 5%). No

Table I. Variability of the pro-coagulant effect of cancer cells on thrombin generation of normal human plasma. Thrombin generation was triggered by addition of CaCl₂.

	lag-time (min)	tt-Peak (min)	Peak (nM)	MRI (nM/min)
MCF7 cells	6.1 \pm 0.9 ^a	9.6 \pm 1.1 ^a	121 \pm 22 ^a	34 \pm 6 ^a
BXPC3 cells	4.1 \pm 1.1 ^{a,b}	6.9 \pm 1.3 ^{a,b}	199 \pm 13 ^{a,b}	71 \pm 7 ^{a,b}
HUVEC	8.6 \pm 1.1	15.2 \pm 1.4	86 \pm 11	13 \pm 4
Control	9.6 \pm 1.2	16.3 \pm 1.5	118 \pm 9	17 \pm 4

^a $P < 0.05$ versus HUVEC; ^b $P < 0.05$ vs. MCF7.

Table II. Tissue factor activity (measured by a clotting based assay) and TF expression (measured by flow cytometry) of the cells and the respective microparticles.

	TFa (pM)	TF expression (MIF)
BXPC3 cells	1.42 \pm 0.1 ^a	395 \pm 132 ^a
BXPC3 MPs	33 \pm 2 ^{b,c}	491 \pm 24 ^{b,c}
MCF7 cells	0.82 \pm 0.08 ^a	95 \pm 20 ^a
MCF7 MPs	4.6 \pm 0.08 ^{b,c}	378 \pm 25 ^{b,c}
HUVEC	0.23 \pm 0.02 ^a	5 \pm 10
HUVEC MPs	0.34 \pm 0.1	8 \pm 2
normal PPP	0.2 \pm 0.05	0

^a $P < 0.05$ vs. HUVEC; ^b $P < 0.05$ vs. HUVEC MPs; ^c $P < 0.05$ vs. the respective cells. Data are means \pm SD of 5 experiments.

procoagulant phospholipids were detected on microparticles derived from HUVEC (Fig. 1).

Tissue factor expression by cancer cell-derived microparticles. Flow cytometry analysis showed the presence of TF on microparticles from BXPC3 and MCF7 cells (MIF: 491 \pm 24 and 378 \pm 25, respectively; $P > 0.05$) (Fig. 1). Only traces of TF were detected on HUVEC derived microparticles (MIF=8 \pm 2). The proportion of carrier cells was identical for BXPC3 and MCF7. Data are summarized in Table II.

TF expressed by CaCe-dMP had procoagulant activity. The levels of TFa expressed by BXPC3 and MCF7 derived microparticles were 33 \pm 2 and 4.6 \pm 0.5 pM, respectively; $P = 0.001$. The levels of TFa expressed by HUVEC-derived microparticles was 0.34 \pm 0.1 pM. Data are summarized in Table II. The CaCe-dMP expressed approximately 20-fold higher levels of TFa as compared to the respective cells.

Effect of cancer cell-derived microparticles on thrombin generation. The addition of CaCe-dMP to normal PPP led to a significant increase of the Peak and MRI, as well as a reduction of the lag-time and ttPeak in comparison to the control (Table III). This effect was more important for BXPC3 than MCF7 derived microparticles. No significant effect was observed for HUVEC-derived microparticles which did

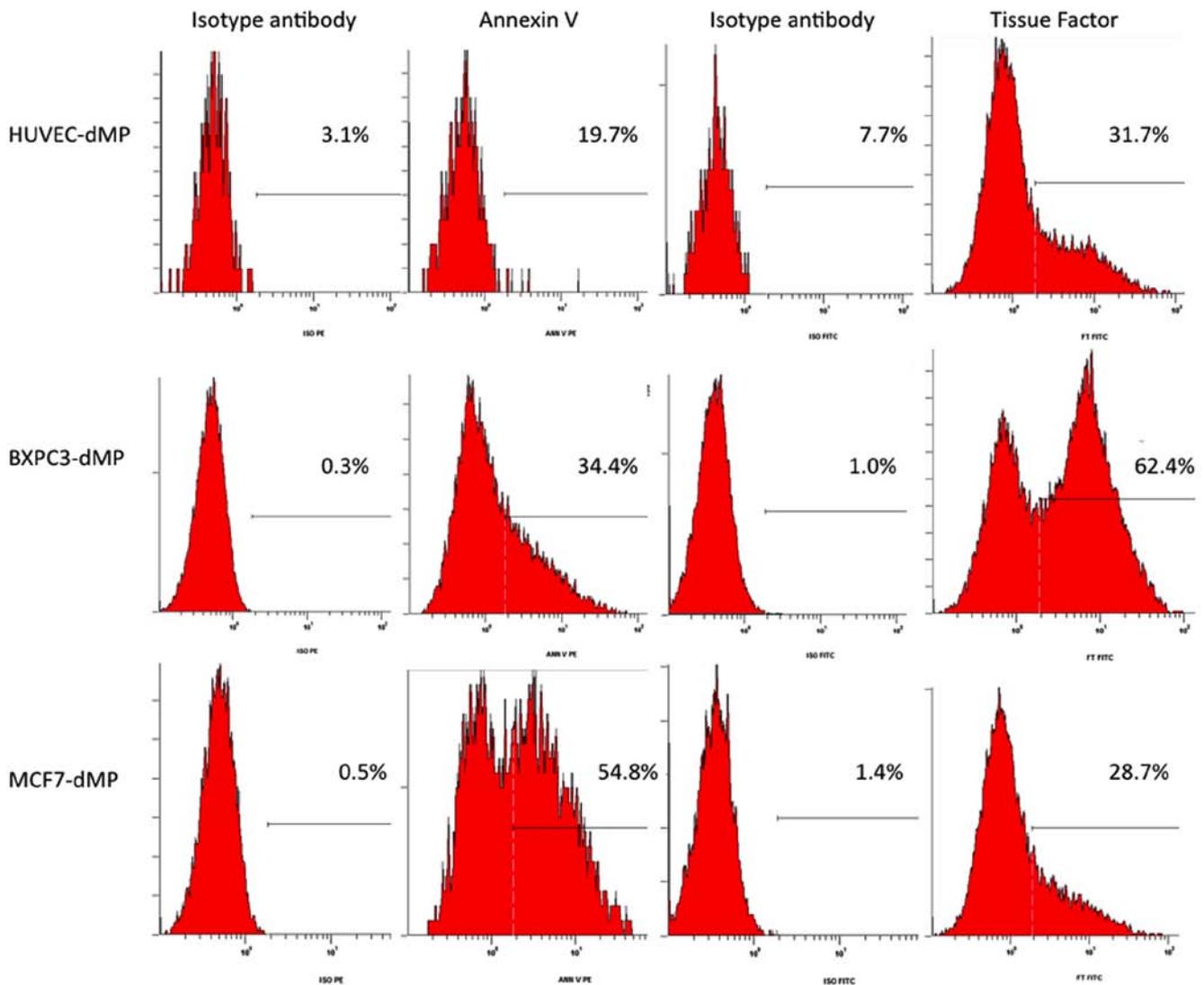


Figure 1. Flow cytometry analysis for Annexin V and tissue factor (TF) expression on BXP3 and MCF7-derived microparticles compared with those from HUVEC. Representative results of 1 out of 5 experiments.

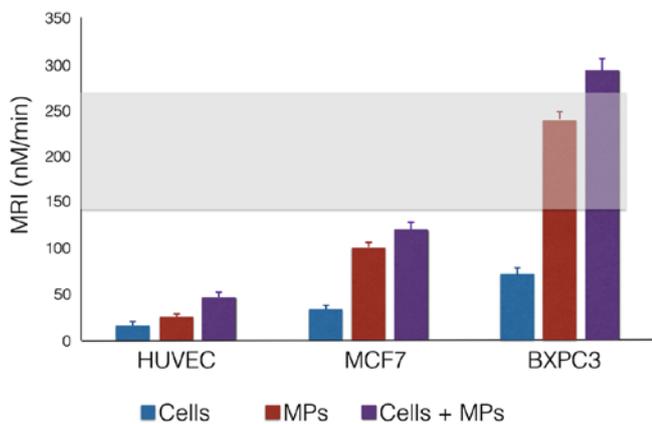


Figure 2. Impact of combination of cancer cells and microparticles on thrombin generation. The gray depicts the normal range of MRI. Data are means \pm SD of 5 experiments.

not show any significant effect on thrombin generation as compared to the control.

Effect of the association of cancer cells and cancer cell-derived microparticles on thrombin generation. Thrombin generation induced by cancer cells in the presence of CaCe-dMP from the same cell type in normal PPP showed a significant increase in Peak and MRI, as well as a reduction of the lag-time and ttPeak as compared to the control experiment (Table III). A significant variability in the effect of CaCe-dMP of different types of cancer cells on thrombin generation in human plasma was observed. At an equal number of cells, MCF7 derived microparticles had lower procoagulant activity than BXP3 derived microparticles. Under the same conditions, the parameters of the thrombogram in the presence of HUVEC (control) did not differ significantly from the control experiments without cells or in the presence of microparticles (Table III).

Thrombin generation triggered by cancer cells in association with their respective microparticles amplified the procoagulant efficiency of BXP3 and MCF7 cancer cells. In contrast to cancer cells, the normal cells (HUVEC) in association with HUVEC-derived microparticles had no effect on thrombin generation. The association of cancer cells with their homologous microparticles resulted in a significant

Table III. Impact of CaCe-dMP on thrombin generation in normal PPP.

	Pool	HUVEC	BXPC3 cells	MCF7 cells	MPs HUVEC	CaCe-dMP BXPC3	CaCe-dMP MCF7	HUVEC + MPsHUVEC	+CaCe-dMP BXPC3	+CaCe-dMP MCF7
Lag-time (min)	8.9±1.6	7.7±0.9	4.8±0.6	7.5±0.7	8.2±0.9	2.2±0.6 ^f	6.1±0.4 ^e	7.9±0.9	1.2±0.5 ^c	5.1±0.7 ^b
tt-Peak (min)	14.6±1.3	12.1±0.9	5.7±0.8	9.3±1.0	12.3±1.4	3.3±0.4 ^f	8.2±0.8 ^e	11.7±0.9	2.6±0.6 ^c	6.9±1.1 ^b
Peak (nM)	136±11	150±11	220±12	179±11	142±11	380±14 ^f	210±11 ^e	166±10	410±12 ^c	220±10 ^a
MRI (nM/min)	24±9	35±10	244±13	99±12	32±10	247±14 ^f	100±13 ^e	45±10	294±11 ^a	120±12 ^b

^aP<0.05, ^bP<0.01, ^cP<0.001 cells without MPs vs. cells + MPs; ^dP<0.05, ^eP<0.01, ^fP<0.001 Pool without MPs vs. Pool + MPs. In all experiments, normal PPP was mixed with MP reagent and thrombin generation was triggered by CaCl₂ (n=5).

Table IV. Effect of an anti-TF antibody on the generation of thrombin in the presence of MPs of BXPC3, MCF7 and HUVEC (n=3).

	Thrombin generation of MPs in PPP in presence of anti-TF antibody with MP reagent (no TF/PPL 4 μM)											
	Lag-time (min)			tt-Peak(min)			Peak (nM)			MRI (nM/ml)		
	MP of HUVEC	MP of BXPC3	MP of MCF7	MP of HUVEC	MP of BXPC3	MP of MCF7	MP of HUVEC	MP of BXPC3	MP of MCF7	MP of HUVEC	MP of BXPC3	MP of MCF7
Pool	11.1±0.9	2.2±0.6	6.1±0.4	17.1±0.9	3.3±0.4	8.2±0.8	146±11	380±14	210±11	35±10	247±11	100±13
Pool + TF9-10H10	13.2±1.3	9.2±1.0 ^b	8.7±0.7 ^a	20.3±0.9	12.3±0.9 ^b	13.6±1.8 ^a	132±12	98±10 ^b	121±12 ^b	22±8	31±9 ^b	26±7 ^b

^aP<0.05, ^bP<0.01, MPs without anti-TF vs. MPs with anti-TF. TF9-10H10, anti-human-TF antibody.

increase in thrombin generation Peak and MRI, as well as a reduction of lag-time and ttPeak as compared to the control. At an equal number of cells, MCF7 derived microparticles had a lower procoagulant activity as compared to BXPC3 derived microparticles. Under the same conditions, the parameters of the thrombogram in the presence of HUVEC did not differ significantly from the control experiments (Fig. 2).

Addition of an anti-TF monoclonal antibody in PPP in the presence of CaCe-dMP or HUVEC derived microparticles significantly increased the lag-time and ttPeak with decreased MRI and Peak of thrombin compared to the assay without anti-TF antibody. In the presence of BXPC3 derived MP, inhibition of thrombin generation by the anti-TF antibody was 74% as compared to the control experiment (without any antibody addition). The anti-TF antibody also partially reversed the thrombin generation triggered by MCF7 cells (42%; P<0.05 vs. BXPC3 derive microparticles). The anti-TF antibody reduced by 10% thrombin generation triggered by HUVEC (Table IV).

Discussion

Cancer associated thrombosis is a common complication of malignancy representing the second most frequent cause of death in cancer patients (1,2). However, the pathophysiology CAT is not entirely understood. Microparticles produced by tumour cells and their microenvironment generate considerable interest since they modulate blood coagulation and fibrinolysis

and may contribute to thrombosis (17,18,27-31). The link between cancer and hypercoagulability is surrounded by some critical questions: i) Are the mechanisms of CAT specific for the type of cancer? ii) Is there any link between this cancer type-specific thrombosis with the biological properties of the cancer cells? iii) Which are the implications of blood borne cancer-derived microparticles on the hypercoagulable state?

It is well established that cancer cells express TF; the major trigger of blood coagulation (8,9,32). Using an original and validated experimental system that allows the study of thrombin generation triggered directly by cancer cells we investigated if the release of microparticles by cancer cells modify the procoagulant potential of plasma. We previously demonstrated that the pancreas adenocarcinoma cells BXPC3 express significantly higher amounts of TF as compared to the breast cancer cells MCF7 and the HUVEC cells (15). The levels of TF expressed by each type of cancer cells are correlated with their effect on thrombin generation (15). We recently showed that cancer cells, i.e. BXPC3 and MCF7 activate blood coagulation via TF as well as via the activation of FXII (33). However, the procoagulant activity of cancer cells is not sufficient to induce hypercoagulability (33). Cancer cell-derived TF can activate coagulation by binding the serine protease FVII/VIIa to form an activating complex that promotes thrombin generation close to the tumor (17). Microparticles released by tumor cells could exhibit negatively charged phospholipids and TF which enhance thrombin generation (34-37). Zwicker *et al* observed elevated levels of TF-positive microparticles in the plasma

of patients with pancreatic, breast, colorectal, ovarian, and non-small cell lung cancer (9,38). Furthermore, approximately 50% of microparticles in the patients with cancer expressed the tumor antigen mucin 1 (MUC-1) on their surface, which suggests that they derived from the tumor.

In order to estimate the specific role of CaCe-dMP in coagulation activation initiated by cancer cells we isolated the microparticles present in the conditioned medium from the two cancer cell lines (BXPC3 and MCF7) and normal cells (HUVEC). On these microparticles, we first studied the expression of procoagulant phospholipids and TF using Annexin V and an anti-TF antibody using flow cytometry method. We also determined the expression of TFa using a clotting based assay. Subsequently, we studied thrombin generation induced by cultures of BXPC3 or MCF7 cells in the presence of the corresponding microparticles (isolated from the respective conditioned medium). Phosphatidylserine was identified on the surface of CaCe-dMP, unlike the cancer cells themselves. Microparticle-derived MCF7 cells exhibited lower amounts of phosphatidylserine as compared to BXPC3 derived microparticles.

Both BXPC3 and MCF7 cells express abundant amounts of TF. The TF density at the membrane of the BXPC3 cells and the BXPC3 derived microparticles was significantly higher than that measured at the membrane of the MCF7 cells and MCF7 derived microparticles. This difference was directly correlated with the difference in the procoagulant potential of BXPC3 and MCF7 cells. Expression of TFa by BXPC3 cells was significantly superior as compared to MCF7 cells. The expression of TFa by the microparticles was significantly higher to that observed on the cancer cells, in a ratio of approximately 30 for BXPC3 and 5 for MCF7.

In summary, CaCe-dMP provide abundantly procoagulant phospholipids and active TF allowing the initiation and amplification of thrombin generation. Culture of the cancer cells with their microparticles resulted in a significant acceleration of the initiation phase and amplification of the propagation phase of thrombin generation. In accordance to the experiments with the corresponding cancer cells the BXPC3-derived microparticles showed higher procoagulant potential as compared to the microparticles derived from MCF7 cells. Noteworthy, excessive increase of thrombin generation at levels which correspond to a hypercoagulable state were observed when BXPC3 cells and the corresponding microparticles were combined. Microparticles, when they are cell-free present in the plasma, result in a significant increase in thrombin generation as compared to the control (normal PPP without MPs). This effect is more pronounced for the BXPC3 than the MCF7 CaCe-dMP; a phenomenon that we can relate to the expression of TF.

In this study, we demonstrated that TF specifically released from tumor cells exhibits procoagulant activity *in vitro*. We found that the procoagulant activities derived from cancer cells *in vitro* was mainly associated with MP. The procoagulant activity associated with these MP was completely dependent on TF (and PS), as it was abolished by anti-TF (or Annexin V). These results indicate that it is important to measure levels of MP TF activity, and not simply levels of PS-positive MPs. To the best of our knowledge, the present study demonstrates for the first time that cancer

related hypercoagulability is the resultant of the combined procoagulant effect of cancer cells with the procoagulant microparticles and active TF which are released by the cancer cells themselves. In addition, we show that the intensity of the hypercoagulability is related with the histological type of the cancer cells. This concept is valuable for the cancer cells as well as for the cancer cell derived elements present into the microenvironment.

In conclusion, all these data show that: i) cancer cells by themselves do not possess sufficient power to generate a state of hypercoagulability; ii) cancer cells 'enrich' the microenvironment with procoagulant elements, especially procoagulant microparticles which express both TF and procoagulant phospholipids, iii) the association of cancer cells and microparticles is necessary for a state of hypercoagulability, at the level of the tumor microenvironment and that the intensity of this hypercoagulability depends on the histological type of the cancer cells.

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