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Promotion of experimental thrombus formation by the procoagulant activity of breast cancer cells

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Abstract
The routine observation of tumor emboli in the peripheral blood of patients with carcinomas raises questions about the clinical relevance of these circulating tumor cells. Thrombosis is a common clinical manifestation of cancer, and circulating tumor cells may play a pathogenetic role in this process. The presence of coagulation-associated molecules on cancer cells has been described, but the mechanisms by which circulating tumor cells augment or alter coagulation remains unclear. In this study we utilized suspensions of a metastatic adenocarcinoma cell line, MDA-MB-231, and a non-metastatic breast epithelial cell line, MCF-10A, as models of circulating tumor cells to determine the thromobogenic activity of these blood-foreign cells. In human plasma, both metastatic MDA-MB-231 cells and non-metastatic MCF-10A cells significantly enhanced clotting kinetics. The effect of MDA-MB-231 and MCF-10A cells on clotting times was cell number-dependent and inhibited by a neutralizing antibody to tissue factor (TF) as well as inhibitors of activated factor X and thrombin. Using fluorescence microscopy, we found that both MDA-MB-231 and MCF-10A cells supported the binding of fluorescently labeled thrombin. Furthermore, in a model of thrombus formation under pressure-driven flow, MDA-MB-231 and MCF-10A cells significantly decreased the time to occlusion. Our findings indicate that the presence of breast epithelial cells in blood can stimulate coagulation in a TF-dependent manner, suggesting that tumor cells that enter the circulation may promote the formation of occlusive thrombi under shear flow conditions.

Abbreviations

BSA	bovine serum albumin
DIC	differential interference contrast
DMEM	Dulbecco's Modified Eagle Medium
FII, FV, FVII, FVIII, FIX, FX, FXI	coagulation factor II, V, VII, VIII, IX, X, XI, respectively
FBS	fetal bovine serum
GPRP	H-Gly-Pro-Arg-Pro-OH
PAR	protease-activated receptor
PBS	phosphate buffered saline
VTE	venous thromboembolism
TF	tissue factor

1. Introduction

Cancer metastasis is the process whereby cancer cells separate from the primary tumor mass, enter the vascular or lymphatic circulation, exit into a new tissue, and colonize the invaded microenvironment. Metastasis represents a primary cause

of morbidity and mortality associated with many cancers. For instance, although early-stage breast cancer is curable with excision of the primary lesion along with radiation, hormonal therapy and chemotherapy, these treatments are ineffective once a tumor has metastasized. Clinical studies have shown that the presence of micrometastases in bone marrow is associated with the occurrence of clinically overt distant metastasis and death from cancer-related causes, but not with locoregional relapse, in breast cancer patients [1]. Although significant progress has been made in deciphering the molecular and genetic features of epithelial cancers, much is still unknown about the behavior and effects of cancer cells in the fluid phase during transit through the circulation.

Causal association between thrombosis and cancer was first recognized by Bouillard in the 1820s, then developed by Trousseau in the 1860s, who, observing his own disease, described that patients who present with migratory superficial thrombophlebitis are likely to have underlying pancreatic cancer [2, 3]. Since that time, extensive clinical evidence has established the fact that the blood coagulation system is intricately involved in the metastatic process. Poignantly, venous thromboembolism (VTE) complications, including pulmonary embolism, are the second leading direct cause of death of cancer patients, with the risk of VTE elevated from 7-fold to up to 28-fold as compared to non-cancer patients [4, 5]. The median survival of metastatic breast cancer patients who presented with VTE was strikingly short (2 months; range: 1–2) compared with that of metastatic breast cancer patients without thrombosis (13 months; range: 1–44) [6]. Conversely, in patients with symptomatic VTE, the incidence of concomitant diagnosis of cancer that was previously unknown is between 4% and 10%, with the stage of cancer often advanced [7, 8]. With the accumulating evidence that coagulation activation in cancer is critical to the outcome of the disease, there has been increasing interest in elucidating the coagulation and fibrinolytic pathways that promote cancer metastasis and the cellular pathways that promote thrombosis [9–11].

Studies have demonstrated an association between elevated levels of circulating tissue factor (TF) and thrombosis in cancer patients [12]. TF is a key protein in the initiation of blood coagulation, assembling with the proteolytic enzyme activated factor VIIa (FVIIa) on blood cell membranes with exposed negatively charged phosphatidylserine. Exposure of phosphatidylserine promotes the assembly of the tenase complex, where the TF–FVIIa complex catalyzes the activation of FIX and FX to FIXa and FXa, respectively [13]. The serine protease, FXa, goes on to assemble with the coagulation protein cofactor, FVa, to form the prothrombinase complex, which catalyzes the generation of thrombin (FIIa) from prothrombin (see review by Mann *et al* [14]). The primary procoagulant functions of thrombin are the cleavage of soluble fibrinogen to insoluble fibrin and the activation of platelets via the cleavage of protease-activated receptors (PARs) [15]. Additionally, thrombin also stimulates its own generation through the activation of FXI and the cofactors FV and FVIII, leading to rampant thrombin generation [14, 16]. In this study, we aimed to characterize the molecular pathways by which epithelial cells that originate from breast tumors promote coagulation factor activation and occlusive clot formation under physiologically relevant shear conditions.

2. Materials and methods

2.1. Reagents

Recombinant TF (Dade Innovin) was purchased from Siemens Healthcare Diagnostics (Deerfield, IL). Recombinant inactivated FVIIa (FVIIai) was obtained from Enzyme Research Laboratories (South Bend, IN). A FITC-conjugated anti-TF antibody was from LifeSpan BioSciences (Seattle, WA) and a neutralizing anti-TF antibody (clone D3H44) was from Genentech (South San Francisco, CA). The FXa inhibitor, rivaroxaban, was obtained from Bayer Healthcare (Leverkusen, Germany) and the direct thrombin inhibitor, hirudin, was obtained from CIBA-Geigy Pharmaceuticals (Horsham, UK). Annexin A5 was purchased from AnaSpec (San Jose, CA). H-Gly-Pro-Arg-Pro-OH (GPRP) was from Calbiochem (Darmstadt, Germany). Dulbecco's Modified Eagle Medium (DMEM) for MDA-MB-231 and MCF-10A cells, fetal bovine serum (FBS), horse serum, cholera toxin and recombinant trypsin (TrypLE) were from Invitrogen (Carlsbad, CA). Fibrillar equine collagen was from Chrono-log (Havertown, PA). All other reagents were purchased from Sigma-Aldrich (St Louis, MO) or previously described sources [17].

Purified human thrombin was fluorescently labeled at the active site with N^{a}-[(acetylthio)acetyl]-D-Phe-Pro-Arg chloromethyl ketone and 5- (and 6)-iodoacetamido-2′,7′-difluorofluorescein (OG488-iodoacetamide) as described in [18].

2.2. Collection of human blood and preparation of plasma

Blood was drawn from healthy volunteers by venipuncture into a one-tenth volume of sodium citrate. Platelet-poor plasma was prepared by centrifugation of citrated whole blood (0.32% w/v sodium citrate) at 2150*g* for 10 min. Plasma from three donors was pooled and stored frozen at −80 °C until use.

2.3. Cell preparation for experiments

MDA-MB-231 and MCF-10A cells were a kind gift from Dr Tlsty (University of California, San Francisco, CA). Cells were detached with TrypLE for 30 min at 37 °C, pelleted at 150*g* for 5 min, washed with serum-free DMEM, and resuspended to a concentration of 2×10^{6} mL^{-1} in serum-free DMEM.

2.4. Clotting times and OG-488 thrombin binding

Clotting times of pooled human plasma were measured with a KC4 Coagulation Analyzer (Trinity Biotech, Bray, Co. Wicklow, Ireland). Plasma samples were treated with antibodies or inhibitors to TF, FXa, thrombin, or phosphatidylserine for 3 min at room temperature, followed by incubation with vehicle, MDA-MB-231, or MCF-10A cells for 3 min at 37 °C. Clotting was initiated by the addition of

16.7 mM $CaCl_2$ and the clotting time (recalcification time) was recorded, as described in [17].

For OG-488 thrombin-binding experiments, plasma was incubated with OG-488 thrombin (1 μM) and the fibrin polymerization inhibitor, GPRP (10 mM) before the addition of MDA-MB-231 or MCF-10 cells (2×10^5 mL^{-1}). Coagulation was triggered with 16.7 mM $CaCl_2$ and plasma samples were taken 5 min later. Samples were imaged with differential interference contrast (DIC) and fluorescence microscopy on a Zeiss Axiovert 200 M microscope as described in [19].

2.5. Flow cytometry

MDA-MB-231 or MCF-10A cells (1×10^6 mL^{-1}) were washed with PBS prior to incubation with a FITC-conjugated anti-TF antibody (1 μg mL^{-1}) for 30 min at room temperature. Following labeling, cells were analyzed on a FACSCalibur flow cytometer with CellQuest acquisition and analysis software (Becton Dickinson, Franklin Lakes, NJ). Unlabeled cells served as negative controls.

2.6. Capillary occlusion assay

Glass capillary tubes (0.2 × 2 mm; VitroCom, Mountain Lakes, NJ) were incubated for 1 h at room temperature with 100 μg mL^{-1} fibrillar collagen, blocked with denatured bovine serum albumin (BSA, 5 mg mL^{-1}) for 1 h, and then vertically mounted below a reservoir. The exit of the capillary was immersed in phosphate buffered saline (PBS). Sodium citrate anticoagulated whole blood (0.38% w/v sodium citrate) was incubated with vehicle, MDA-MB-231, or MCF-10A cells for 5 min. Aliquots (500 μL) of treated blood were recalcified by the addition of 7.5 mM $CaCl_2$ and 3.75 mM $MgCl_2$ and added to the reservoir to maintain a prescribed height, yielding an initial wall shear rate of 285 s^{-1} through the capillary, modeled by the following equation as described in [20]:

$$\gamma_{\text{wall}} = -a\left[\frac{\rho_b g(h_c + h_b) - \rho_{\text{pbs}} g h_{\text{pbs}}}{h_c \mu}\right]$$

where γ_{wall} is wall shear rate, ρ_b is the density of the blood, ρ_{pbs} is the density of the PBS, h_c is the height of the capillary tube, h_b is the height of the blood in the reservoir, h_{pbs} is the length of the capillary which is submerged in PBS, g is the acceleration due to gravity, μ is the viscosity of blood, and $2a$ is the width of the capillary. The time to occlusion of the capillary was recorded over an observation time of 60 min.

2.7. Statistical analysis

Data are presented as mean ± SEM. For paired data, statistical significance between means was determined by the paired Student's *t*-test. For all other data, one-way ANOVA with the Tukey *post hoc* test was employed to determine statistical significance between means. Significance differences for all statistical tests required $P < 0.05$.

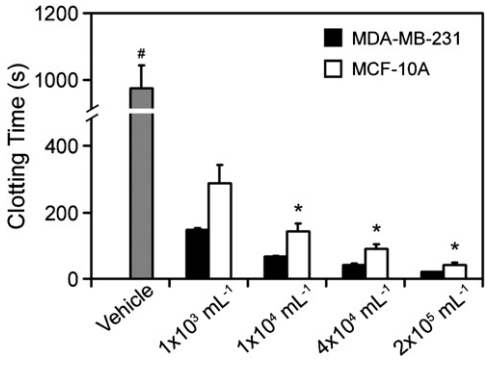

Figure 1. Breast epithelial cells promote coagulation. Human sodium citrate-anticoagulated plasma was incubated with vehicle or suspensions of cultured MDA-MB-231 or MCF-10A cells (1×10^3–2×10^5 mL^{-1}) for 3 min at 37 °C. Coagulation of plasma was initiated by recalcification using 16.7 mM $CaCl_2$ (final concentration) and clotting times were recorded on a coagulometer. Data are reported as mean ± SEM, from six to eight experiments. In comparison to vehicle, clotting times were significantly shortened at all MDA-MB-231 or MCF-10A cell numbers, $^{\#}P < 0.05$. $^{*}P < 0.05$ versus the corresponding MDA-MB-231 cell concentration.

3. Results

3.1. Epithelial MDA-MB-231 and MCF-10A cells promote coagulation

To investigate the relationship between metastastic cancer cells and coagulation, we first developed a model of coagulation in the presence of breast epithelial cells lines. In this work, we utilized two cultured epithelial cell lines derived from human breast tissue differing in its metastatic potential. MDA-MB-231 is an immortalized human metastatic breast cancer cell line originally derived from a pleural effusion of a patient with metastatic adenocarcinoma of the breast [21]. MCF-10A is an adherent, immortal, non-transformed human mammary epithelial cell line that arose spontaneously from cells that were originally derived from a patient with fibrocystic changes [22]. We used a plasma recalcification assay to measure the effects of these epithelial cells on coagulation. The clotting of pooled human plasma was initiated by the addition of 16.7 mM $CaCl_2$ and the clotting time (recalcification time) was measured. Our data demonstrate that, in comparison to vehicle controls, the presence of either MDA-MB-231 or MCF-10A cells significantly decreased clotting times in a cell number-dependent manner (figure 1). At the same cell concentration, the metastatic cell line, MDA-MB-231, accelerated coagulation of plasma more effectively than the non-metastatic MCF-10A cell line. Taken together, our data demonstrate that the presence of both metastatic and non-metastatic cells of epithelial origin, in suspension, strongly promotes coagulation of recalcified plasma.

3.2. Mechanisms of MDA-MB-231 and MCF-10A cell procoagulant activity

A number of recent reports have suggested a role for TF in metastasis and the development of cancer-associated thrombosis. TF has been reported to be expressed on the

surface of a number of native and cultured cells, including breast cancers, and in general, its surface expression level has been shown to increase with advanced disease [23]. To first determine if MDA-MB-231 and MCF-10A cells express TF, cells were labeled with a FITC-conjugated anti-TF antibody and analyzed by flow cytometry. Results indicate that TF is expressed on the surface of both MCF-10A and MDA-MB-231 cells (figure 2(*a*)).

To investigate how TF expression on MDA-MB-231 and MCF-10A cells contributes to their procoagulant activity, we examined the role of the TF pathway in the plasma recalcification assay. When the TF pathway was inhibited by an excess molar concentration of a competitive TF pathway inhibitor, inactivated FVIIa (FVIIai), or an anti-TF antibody, clotting times dramatically increased (figure 2(*b*)). An exogenous addition of TF to plasma samples containing MDA-MB-231 or MCF-10A cells caused a further decrease in clotting times. These results indicate that the TF pathway plays an important role in the procoagulant activity of both MDA-MB-231 and MCF-10 cells.

In order to determine the role of the members of the tenase and prothrombinase complexes in the procoagulant activity of breast epithelial cells, additional plasma recalcification experiments were performed in the presence of inhibitors of the coagulation enzymes FXa and thrombin. Our data demonstrate that clotting times were prolonged more than tenfold in the presence of either the FXa inhibitor, rivaroxaban, or thrombin inhibitor, hirudin (figure 2(*c*)), indicating that the accelerated coagulation of recalcified plasma, in the presence of suspended epithelial cells, was mediated by thrombin. Inhibition of negatively charged phosphatidylserine on cell surfaces by the addition of a high concentration of annexin A5 (∼10 000 times the physiological plasma concentration [24]) dramatically prolonged clotting times (>20 min), suggesting a role for exposure of negatively charged lipids during epithelial cell-induced coagulation. In contrast, pretreating the plasma with the FXIIa inhibitor, corn trypsin inhibitor (CTI, 4 μM), or the anti-FXI monoclonal antibodies, 1A6 or 14E11 (20 μg ml^{-1}), had no effect on clotting times in the presence of either MDA-MB-231 or MCF-10A cells (data not shown), providing evidence against the primary involvement of contact activation and the intrinsic coagulation cascade in the procoagulant activity of these cell lines. Taken together, these results demonstrate that the procoagulant activity of MDA-MB-231 and MCF-10 cells is primarily dependent upon activation of the extrinsic TF pathway of blood coagulation on the surface of cells.

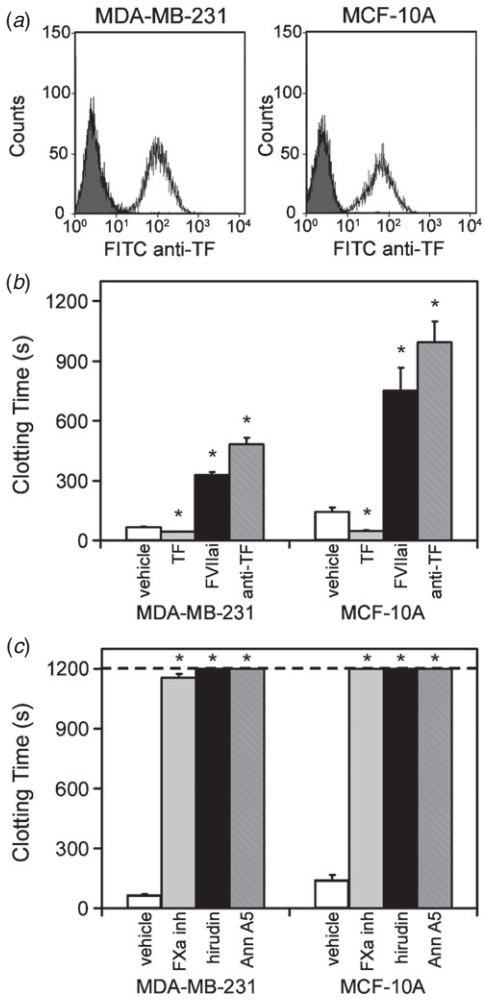

Figure 2. Characterization of the procoagulant activity of breast epithelial cells. (*a*) Cultured MDA-MB-231 or MCF-10A cells (1×10^6 mL^{-1}) were labeled with a FITC-conjugated anti-TF antibody (1 μg mL^{-1}) and analyzed by flow cytometry. Shaded curves represent background fluorescence of unlabeled cells; white curves represent a shift in fluorescence in the presence the anti-TF antibody. Representative curves from two or more independent experiments are shown. (*b*) and (*c*) Human sodium citrate-anticoagulated plasma was pretreated with (*b*) vehicle; TF (TF,10 pM); the TF pathway inhibitor, FVIIai (20 μg mL^{-1}); or a neutralizing antibody to TF (anti-TF, 20 μg mL^{-1}) or (*c*) vehicle; the FXa inhibitor, rivaroxaban (FXa inh, 10 μM); the thrombin inhibitor, hirudin (20 μg mL^{-1}); or the phosphatidylserine binding protein, annexin A5 (Ann A5, 10 μg mL^{-1}). Cultured MDA-MB-231 or MCF-10A cells were added to treated plasma at 1×10^4 mL^{-1}. After 3 min of incubation at 37 °C, coagulation was initiated by the addition of 16.7 mM $CaCl_2$ and clotting times were recorded. Data are reported as mean ± SEM, from four to eight experiments. If clotting did not occur during 20 min of observation, experiments were terminated and a clotting time of 20 min was recorded. $^*P < 0.05$ versus vehicle treatment.

3.3. MDA-MB-231 and MCF-10A cells support the binding of OG-488 thrombin

We next aimed to determine the ability of breast epithelial cells to directly support coagulation factor binding and localization. We have previously shown that both blood platelets and fibrin-rich thrombi support the binding active site fluorescently labeled thrombin (OG-488 thrombin) under physiologically relevant shear flow conditions [25]. Plasma was incubated with OG-488 thrombin (1 μM) and the fibrin polymerization inhibitor, GPRP (10 mM), before the addition of MDA-MB-231 or MCF-10 cells. Coagulation was triggered with 16.7 mM $CaCl_2$, and plasma samples were taken after 5 min. Our data show specific binding of OG-488 thrombin to both MDA-MB-231 and MCF-10A (figure 3), providing direct evidence of the assembly of coagulation factors on the epithelial cell surface under conditions of coagulation.

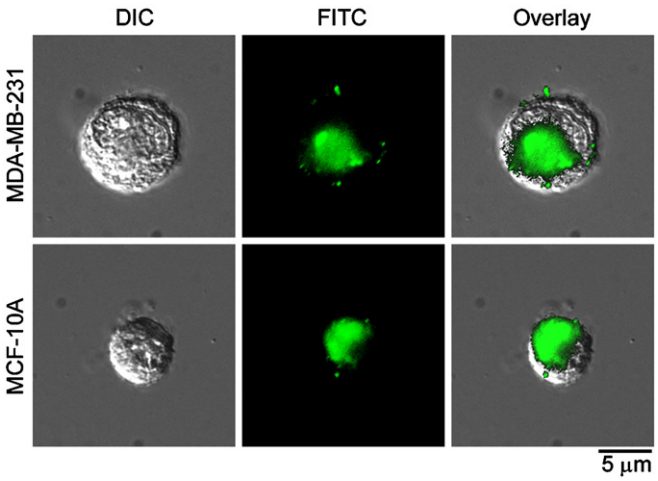

Figure 3. Cultured breast epithelial cells bind thrombin under procoagulant conditions. Human sodium citrate-anticoagulated plasma was incubated with suspended MDA-MB-231 or MCF-10A cells (2×10^5 mL^{-1}) for 3 min at 37 °C in the presence of OG-488 active-site-labeled thrombin (1 μM). Plasma was pretreated with GPRP (10 mM), an inhibitor of fibrin polymerization, to prevent complete gelation. Coagulation was initiated by the addition of 16.7 mM $CaCl_2$ and plasma was sampled 5 min later. Samples were imaged by DIC and fluorescence microscopy, a representative image of an MDA-MB-231 and MCF-10A cell binding thrombin is shown. OG-488 thrombin fluorescence is indicated in green.

3.4. MDA-MB-231 and MCF-10A cells decrease the time to occlusion in a ex vivo model of thrombus formation

We next investigated the ability of the cell lines to promote coagulation and occlusive thrombus formation in the presence of shear flow. In our *ex vivo* model of occlusive thrombus formation, recalcified blood was driven by a constant pressure gradient at a physiologically relevant initial wall shear rate of 285 s^{-1} through capillaries coated with fibrillar collagen (figure 4(*a*)). Flow through the capillary was monitored until occlusion. Our data demonstrate that the time to capillary occlusion was significantly decreased in the presence of either MDA-MB-231 or MCF-10A cells (figure 4(*b*)). This reduction in time to occlusion caused by the addition of the cultured tumor cells was erased by the addition of either an anti-tissue antibody or the thrombin inhibitor, hirudin (figure 4(*b*)). These results support the notion that the procoagulant activity of epithelial cells that enter the circulation under pathologic conditions may contribute to thrombus formation in the presence of physiologically relevant shear forces.

4. Discussion

Metastatic cancer has long been linked to coagulopathies such as thromboembolism, a leading cause of death in cancer patients. Here we explore the ability of metastatic and non-metastatic cells of epithelial origin to promote experimental thrombus formation. Using models of coagulation under shear conditions, we show that both non-metastatic MCF-10A cells and aggressively metastatic MDA-MB-231 breast tumor cells can promote coagulation. Metastatic potential, based on cell concentration, correlated with procoagulant activity, as MDA-MB-231 cells were more efficient at forming clots *in vitro* compared to MCF-10A cells.

Previous work has established that TF is present in greater levels in the serum of cancer patients and that tumor cells express high levels of TF [12, 23, 26]. Our work concludes that the prothrombotic potential of circulating tumor cells may be, in part, a consequence of TF expression. Indeed, both

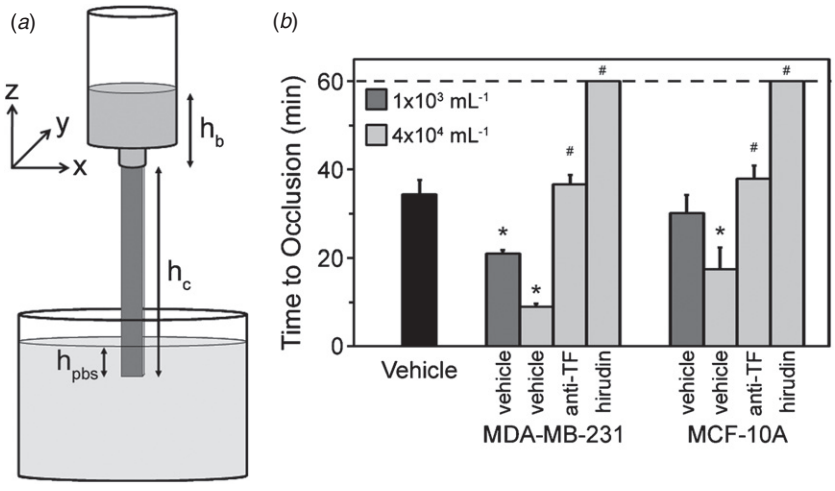

Figure 4. Cultured breast epithelial cells promote TF-dependent occlusive thrombus formation in flowing blood, *ex vivo*. Human sodium citrate-anticoagulated whole blood was mixed with vehicle, MDA-MB-231 or MCF-10A cells (4×10^4 or 1×10^3 mL^{-1}) for 5 min at room temperature. In selected experiments, blood was treated with a neutralizing antibody to TF (anti-TF, 20 μg mL^{-1}) or the thrombin inhibitor, hirudin (20 μg mL^{-1}), in the presence of MDA-MB-231 or MCF-10A cells. (*a*) Treated blood was recalcified with $CaCl_2$ and $MgCl_2$ (final concentration 7.5 and 3.75 mM, respectively), added to a reservoir to a set height (h_b), and allowed to drain through collagen-coated capillaries into a PBS bath as shown. (*b*) The time to thrombotic occlusion (time until blood ceased to flow from the capillary) was recorded. The height of blood in the reservoir was maintained at 1.5 cm, yielding an initial shear rate of 285 s^{-1} in the $0.2 \times 2.0 \times 50$ mm collagen-coated capillary, as described in section 2. Data are mean ± SEM from three or more experiments. $^{*}P < 0.05$ versus vehicle treatment in the absence of cells. $^{\#}P < 0.05$ versus vehicle treatment of the corresponding cell type at 4×10^4 mL^{-1}.

cell lines expressed TF and a neutralizing antibody against TF abrogated the ability of both MDA-MB-231 and MCF-10A breast epithelial cell lines to accelerate blood clotting. We found that epithelial cell-associated TF is an active cofactor for FVIIa and supports the activation of FX, as addition of the FXa inhibitor, rivaroxaban, also blocks the ability of tumor cells to promote coagulation. Interestingly, the addition of annexin A5, which binds specifically to exposed phosphatidylserine, also delayed clotting. This suggests that epithelial cells can expose phosphatidylserine on their surface, possibly upon activation, and this phosphatidylserine exposure has a role in the ability of the cells to promote thrombus formation. While it is known that tumor cells display more phosphatidylserine on their surface in part due to an altered balance of pro- and anti-apoptotic programs [27–29], it remains unclear whether this resultant exposure of phosphatidylserine allows cancer cells to assemble procoagulant complexes on their surface, thus allowing the pirating of the coagulation cascade while in the circulation. Additionally, we show that the surface of MDA-MB-231 and MCF-10A cells support the direct binding of thrombin (figure 3). It has been shown that the MDA-MB-231 cells express PARs for thrombin, but the ability of MCF-10A cells to express PARs is unclear [30, 31]. It is intriguing to speculate that cancer cells express a specific receptor for thrombin, or that perhaps cancer cells can associate with fibrin to establish a platform for thrombin binding and activity. Whether or not the assembly of thrombin on the surface of cancer cells in the fluid phase plays a role in the process of metastasis remains to be determined.

Our study takes advantage of two well-established breast-derived cell lines, MCF-10A and MDA-MB-231. MCF-10A cells were obtained from ductal-like epithelial cells derived from a patient with cystic fibrosis [22]. MDA-MB-231 cells were isolated from the plural effusion from a highly metastatic breast cancer patient [21]. While these cells are at opposite ends of the metastatic spectrum and provide a powerful tool for studying metastasis, we recognize that there are fundamental differences in these cells that could contribute to the observed differences in coagulation response. For instance, the surface expression profile of molecules such as integrins and selectin ligands varies between these two cell types [26, 32–34]. Additionally, individual MDA-MB-231 cells are nearly twice the diameter of MCF-10A cells, resulting in a nearly fourfold increase in the catalytic surface area on a per cell basis. Since the fourfold larger surface area of MDA-MB-231 cells appeared to be associated with an approximately twofold increase in procoagulant potential over MCF-10A cells in the plasma recalcification assay, the underlying relationship between surface area and thrombogenicity remains to be characterized. Future studies that take advantage of circulating tumor cells isolated from patients over the course of varying disease states will overcome these discrepancies and provide more conclusive data linking coagulopathies and metastatic potential.

This study demonstrates that cultured breast-derived epithelial cell lines, MDA-MB-231 and MCF-10A, promote coagulation and the formation of occlusive thrombi under physiological levels of shear. While we show that the coagulation potential of these epithelial cell lines is dependent upon the extrinsic TF pathway, it remains to be determined if circulating tumor cells utilize these mechanisms to promote coagulation during transit within the vasculature and what impact the procoagulant nature of circulating tumor cells has on metastasis.

Acknowledgments

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