High-level secretion of tissue factor-rich extracellular vesicles from ovarian cancer cells mediated by filamin-A and protease-activated receptors

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Summary
Thromboembolic events occur frequently in ovarian cancer patients. Tissue factor (TF) is often overexpressed in tumours, including ovarian clear-cell carcinoma (CCC), a subtype with a generally poor prognosis. TF-coagulation factor VII (fVII) complexes on the cell surface activate downstream coagulation mechanisms. Moreover, cancer cells secrete extracellular vesicles (EVs), which act as vehicles for TF. We therefore examined the characteristics of EVs produced by ovarian cancer cells of various histological subtypes. CCC cells secreted high levels of TF within EVs, while the high-TF expressing breast cancer cell line MDA-MB-231 shed fewer TF-positive EVs. We also found that CCC tumours with hypoxic tissue areas synthesised TF and fVII in vivo, rendering the blood of xenograft mice bearing these tumours hypercoagulable compared with mice bearing MDA-MB-231 tumours. Incorporation of TF into EVs and secretion of EVs from CCC cells exposed to hypoxia were both dependent on the actin-binding protein, filamin-A (filA). Furthermore, production of these EVs was dependent on different protease-activated receptors (PARs) on the cell surface. These results show that CCC cells could produce large numbers of TF-positive EVs dependent upon filA and PARs. This phenomenon may be the mechanism underlying the increased incidence of venous thromboembolism in ovarian cancer patients.

Keywords
Thrombosis, ovarian cancer, tissue factor, microvesicles, coagulation factor VII

Introduction
Venous thromboembolism (VTE) is a common complication in cancer patients and is closely associated with morbidity and mortality (1–4). In particular, ovarian cancer is associated with an increased risk of VTE events, such as deep-vein thrombosis and pulmonary embolism (5). VTE risk can be predicted by detection of several biomarkers, with the level of expression of tissue factor (TF) considered important. Indeed, elevated TF expression in plasma or tissue has been associated with the frequency of VTE in patients with various types of cancer (1–4). Among ovarian cancers, clear cell carcinomas (CCC) express high levels of TF and are associated with a high risk of VTE (5–7).

TF is a transmembrane protein expressed in various tissues that is essential for initiation of the extrinsic coagulation mechanism (8). TF binds to a circulating serine protease, coagulation factor VII (fVII), resulting in activation of the coagulation cascade (8). We have previously shown that both TF and fVII levels are increased in CCC cell lines in response to hypoxia, and that an adaptive response to tissue hypoxia is an important component of cancer progression (6, 9–12).

Cancer cells, including those of ovarian cancer, shed extracellular vesicles (EVs), including exosomes (up to hundreds of nanometers in size); membrane-derived microvesicles (MV; often referred to as microparticles and ectosomes; ranging from hundreds of nanometers to a few μm in size); and larger apoptotic bodies...
Materials and methods

Cell lines and cell culture

Ten ovarian cancer cell lines were used in this study, including four CCC (OVSAVOY, OVISE, OVMANA, and OVTOKO), two serous adenocarcinoma (OVSAHO and OVKATE), one mucinous adenocarcinoma (MCAS), one endometrioid adenocarcinoma (TOV112D), and two undifferentiated carcinoma (TRAMOCH, KURAMOCHI, and TYK-nu cell lines). Glioma (U87) and breast cancer (MDA-MB-231) cells were used as control cell lines. TOV112D was obtained from the American Type Culture Collection (Manassas, VA, USA). The other cell lines have been described previously (10, 24). All cell lines were cultured in RPMI-1640 medium as previously described (10). Hypoxic cell culture was performed under normoxic or hypoxic conditions. During this time, cells were treated with monoclonal mouse IgG (10), monoclonal anti-TF antibody (BD-Pharmingen, #550312), PE-conjugated mouse IgG (BD-Pharmingen, #555749), monoclonal mouse IgG, monoclonal anti-PAR1 (ATAP2 and WEDE15 [10]), and/or rabbit polyclonal anti-PAR2 (10) as required. The medium was filtered through a 5-µm pore membrane (Acrodisc, 25-mm Syringe Filter, PN4199, Pall Corporation, Ann Arbor, MI, USA) and centrifuged at 100,000 g for 90 minutes (min) at 10 °C as described (6). The precipitates were resuspended in PBS and used for further experiments.

Isolation of EVs

Cells (2.5 × 10⁶) were seeded in 100-mm dishes and cultured in RPMI-1640 medium containing 10% foetal calf serum for 24 hours (h). The cells were washed once with 5 ml of phosphate-buffered saline (PBS) and twice with 5 ml of serum-free RPMI-1640 medium lacking phenol red dye, and were cultured in the same medium for 4 h. The medium was then replaced with new serum-free medium, and the cells were cultured for an additional 48 h under normoxic or hypoxic conditions. During this time, cells were treated with monoclonal mouse IgG (10), monoclonal anti-PAR1 (ATAP2 and WEDE15 [10]), and/or rabbit polyclonal anti-PAR2 (10) as required. The medium was filtered through a 5-µm pore membrane (Acrodisc, 25-mm Syringe Filter, PN4199, Pall Corporation, Ann Arbor, MI, USA) and centrifuged at 100,000 g for 90 minutes (min) at 10 °C as described (6). The precipitates were resuspended in PBS and used for further experiments.

Flow cytometry

Flow cytometry was performed according to published procedures (6) using a BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA, USA). Antibodies used were PE-conjugated monoclonal anti-TF antibody (BD-Pharmingen, #550312), PE-conjugated mouse IgG (BD-Pharmingen, #555749), monoclonal mouse IgG, monoclonal anti-PAR1 (ATAP2 and WEDE15), and rabbit polyclonal anti-PAR2 (10).

Determination of protein concentrations in EVs

The concentrations of proteins present in the MV fractions were estimated using the Protein UV method program of a NanoPhotometer™ (Implen, Inc. Munich, Germany) or Micro BCA Protein Assay Kit (23235; Thermo Scientific, Rockford, IL, USA).

Evaluation of EVs by flow cytometry

Aliquots (10 µl) of MVs prepared as described above were suspended in 290 µl of PBS, and the total number of MVs in 250 µl of each suspension was counted using a BD Accuri™ C6 flow cytometer.

Nanoparticle tracking analysis

EVs were diluted (1:1000) with PBS and analysed by nanoparticle tracking using a NanoSight LM10HS equipped with blue laser instrument and NTA 2.3 Analytical Software. Particles were tracked for 60 seconds (s) with a camera level of 13 and a detection threshold of 7.

Quantitation of live cells

Live cells were counted using a Countess automated cell counter based on trypan blue exclusion methodology (Life Technologies, Carlsbad, CA, USA). Cell viability was estimated by MTS using the

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Materials and methods

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(13, 14). All of these shed particles can potentially contribute to pathological situations. Functionally active TF circulates in the blood as a component of MVs produced by cancer cells (13, 14) and platelets (15). We have demonstrated that CCC cells can secrete TF-fVII-positive EVs under hypoxic conditions (6). In vivo studies revealed that TF-positive EVs are produced by tumour tissues, leading to the activation of coagulation activity (16–18). Additional studies showed that TF-positive MVs cause prothrombotic activity in vivo (19, 20). These results suggest that secretion of procoagulant EVs may contribute to the VTE events that frequently occur in CCC patients.

It is unclear whether alternatively spliced TF contributes directly to thrombosis in malignancy (21) and how different patterns of TF expression correlate with the frequency of VTE observed in ovarian cancer patients. The risk of VTE is less evident for breast cancer patients (1, 2), despite the relatively high TF production by cancer cells (22). Therefore, understanding precisely how and to what extent TF contributes to hypercoagulability in different cancer types requires further investigation. Additionally, the mechanisms underlying EV production and TF integration into EVs produced by ovarian cancer cells are poorly understood.

The first aim of this study was to test whether TF-positive EVs released from CCC cells could contribute to hypercoagulability in CCC patients. Thus, this study examined the EVs produced by ovarian cancer cells of various histologic origins, and tested whether xenograft CCC-derived tumours could influence blood coagulability. The second aim of this study was to examine the mechanisms of TF-positive EV production in CCC cells specifically exposed to a tumour-associated stress condition, hypoxia. The mechanisms underlying TF-positive MV production by CCC cells exposed to hypoxic conditions were investigated, as was the involvement of the actin-binding protein filamin-A (filA) and protease-activated receptors (PARs), as these proteins reportedly influence the incorporation of TF into MVs and the subsequent shedding of MVs (23).
Western blotting and quantitation

Immunoblotting was performed using EVs and whole cell lysates (WCLs) prepared from cultured cancer cells and xenograft tumour tissue. The antibodies used were monoclonal anti-TF antibody (TT9 10H10 and TF 9C3 [21]), anti-fVII (R0882 [24]), anti-follitelin-2 (sc-28320; Santa Cruz Biotechnology), anti-filamin 1 (sc-28284; Santa Cruz Biotechnology), anti-HIF-1α (sc-10790; Santa Cruz Biotechnology), anti-HIF-2α (NB100–122; Novus Biologicals, Littleton, CO, USA), anti-PAR1 (ATAP2 [10]), anti-PAR-2 (sc-13504; Santa Cruz Biotechnology), anti-factor X (BS6600; Bioworld Technology, Inc. St. Louis Park, MN, USA), anti-vinculin (V9131; Sigma, St. Louis, MO, USA), anti-β-actin (A5441; Sigma), and anti-caspase-3 (#9662; Cell Signaling Technology, Danvers, MA, USA). Antibody binding was detected using ECL-prime, ECL-select detection reagents (GE Healthcare, Buckinghamshire, UK) or ImmunoStar® LD (Wako Pure Chemical Industries, Osaka, Japan). Mouse plasma was processed using the ProteoPrep Blue Albumin & IgG Detection Kit (Sigma) prior to SDS-PAGE. Expression levels of TF were densitometrically determined using ImageJ software (http://rsb.info.nih.gov/ij/).

Xenograft experiments

The Institutional Review Board at Kanagawa Cancer Center Research Institute approved this study. OVISE (2 × 10^6 cells) or MDA-MB-231 (5 × 10^6 cells) were subcutaneously injected into NOD-SCID mice (Charles River Laboratories Japan, Inc. Yokohama, Japan). Tumour growth was monitored by calculating tumour volume according to the equation: long diameter × (short diameter)² × 1/2. Mice were sacrificed when tumour volume reached approximately 500 mm³, and tumours were isolated for further experiments. Blood was collected into a one-tenth volume of 0.1 M sodium citrate buffer directly from the heart under general anaesthesia with isoflurane. Plasma was prepared from the blood supernatant after centrifugation (3000 ×g for 10 min.).

Tilt tube plasma clotting assay

The tube plasma clotting assay was performed as previously described (6), with slight modifications because of the limited amount of plasma samples. Citrated mouse plasma (2 µl) was mixed with 100 µl of reaction buffer (10 mM Hepes [pH 7.5], 150 mM NaCl, 4 mM KCl, 11 mM glucose, 5 mM Ca²⁺, and 0.5 mg/ml bovine serum albumin) and incubated at 37°C for 30 min. Plasma samples were judged as coagulation positive if the reaction mixture formed a semisolid gel and did not flow when the tube was tilted. Samples were judged as coagulation negative if the reaction mixture remained in a fluid state under the same experimental conditions. To test whether the observed coagulation activity was a downstream event of TF activation, assays were also performed in the presence of 50 µg/ml of anti-TF (5G9) antibody (10) or 2 µM of the fXa inhibitor, NAP-5 (10, 25).

fXa generation assay

The fXa generation assay was performed as previously described (10). Briefly, EVs were incubated in reaction buffer (10 mM HEPES [pH 7.5], 150 mM NaCl, 4 mM KCl, 11 mM glucose, 5 mM Ca²⁺, and 1 mg/ml bovine serum albumin) containing 175 nM fX at 37°C for 30 min. A 20-µl aliquot was removed and mixed with stopping solution. A total of 100 µl of the above reaction mixture was mixed with the same volume of 5 mM Spectrozyme FXa and the development of optical absorbance was monitored. The fXa concentration in the samples was determined by comparing the data of the standard samples.

Immunohistochemistry

Routinely processed formalin-fixed paraffin-embedded specimens were sectioned and stained with antibodies to TF (TF 9C3; 7.6 µg/ml), CD31 (Dianova GmbH, Hamburg, Germany; 6.7 µg/ml), and HIF-1α (BD Biosciences; 2.5 µg/ml). Immunoreactivity was visualised by the peroxidase-labelled amino acid polymer method using a Histofine simple stain MAX-PO’ (Nichirei Co. Tokyo, Japan) and the avidin-biotin-peroxidase complex method (LSAB⁺; DakoCytomation Co. Tokyo, Japan) according to the manufacturer’s instructions. Sections were counterstained with haematoxylin and eosin. Positive areas for CD31 and TF staining were quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

Statistics

Means were compared using two-sided Student’s t-tests. P-values <0.05 were considered statistically significant.

Results

Measurement of EV levels secreted by ovarian cancer cells

EVs produced by various ovarian cancer cell lines of different histological origin were assayed. As positive controls for EV production, we used cell lines U87 (glioma) and MDA-MB-231 (breast cancer, hereafter 231) (26). EV fractions were prepared by high-speed centrifugation of conditioned medium (CM) from these cell lines. Flow cytometry analysis (FCA) of EV suspensions revealed that secretion levels were dependent on cell type (Figure 1A).
Notably, EV production by the two CCC cell lines OVISE and OV-SAYO was high. EVs were further examined by nanoparticle tracking analysis (NTA) and protein levels. We found that relative particle numbers estimated by both NTA (Figure 1B) and protein levels (Suppl. Figure 1A, available online at www.thrombosis-online.com) were mostly consistent with those estimated by FCA. NTA revealed that most EVs released from OVISE cells fall within 50–400 nm in size (Figure 1C and D), and thus these EVs may contain both exosomes and MVs (27). Sizes of EVs secreted from other cell lines also fall within this range, as observed in 231 cells (Suppl. Figure 1B and C, available online at www.thrombosis-online.com).

**Ovarian CCC cancer cells robustly shed TF-rich EVs**

TF is a known marker of MVs (27). Thus, we next examined the levels of TF in WCLs and EVs prepared from cancer cells. Western blotting revealed that TF was present in WCL from multiple CCC cell lines (Figure 2A). Consistent with previous findings, TF expression in WCL from 231 cells was relatively high (28). Western blotting with equal volumes of EV suspensions, which reflect the level of EVs secreted from each cell line, revealed high TF levels in EVs from OVISE and OV-SAYO cells. TF was also detected in EV suspensions from OV-TOKO cells, although the total number of EVs from this cell line was low. Furthermore, while high levels of TF were present in WCL from 231 cells, TF levels in the EV fraction of 231 cells were lower than in ovarian cancer cells.

TF can be secreted into the culture media as an alternatively spliced soluble form in addition to the full-length (fl) form (21). We therefore tested whether these splice variants are expressed by OVISE and 231 cells. Western blotting with an anti-TF antibody that can recognise both fl-TF and truncated forms of TF showed that fl-TF was the predominant form in both cell lines (Figure 2B). We also confirmed that TF levels were low in 231 cell-CM
Expression of TF and fVII in xenograft tumours

Using a xenograft tumour model, we tested whether CCC cells could secrete high levels of TF in vivo. The CCC cell line OVISE was selected for its favourable tumorigenicity. Additionally, we previously showed that fVII transcription can be ectopically induced in this cell line under hypoxic conditions (10, 11). We also used 231 cells, as this cell line is devoid of ectopic fVII (24). OVISE and 231 cells were subcutaneously injected into NOD-SCID mice and tumour growth was monitored (Figure 3 A). Tumours were isolated upon reaching approximately 500 mm$^3$ in volume. Western blotting analysis of TF expression revealed that tumours predominantly produce fi-TF, with similar levels of expression in OVISE and 231 tumour samples after normalisation to β-actin levels (Figure 3 B and C). Only OVISE tumour tissue expressed high levels of fVII. Conversely, western blotting of plasma samples showed that tumour-specific TF was present in plasma samples obtained from OVISE tumour-bearing mice (Figure 3 D). FCA showed that the amount of EVs tends to be high in OVISE tumour plasma compared with that of 231 tumour plasma (Figure 3 E).

TF is highly expressed in hypoxic tissues within xenograft tumours

We next examined whether TF was differentially induced in OVISE and 231 tumours in response to tissue hypoxia. Immuno-histochemistry confirmed that TF was expressed in hypoxic tumour regions associated with both poor vasculature and nuclear accumulation of the hypoxia marker HIF-1α (HIF1; Figure 4 A and B for OVISE and Suppl. Figure 2 for 231, available online at www.thrombosis-online.com). Further FCA revealed that the TF level in EVs released by OVISE cells was lower under normoxic than hypoxic conditions (Suppl. Figure 3, available online at www.thrombosis-online.com). However, most EVs under hypoxic conditions were TF positive. In 231 cells, more EVs were TF positive than TF negative under normoxic conditions. However, the increase in TF-positive EVs in response to hypoxia was lower than for OVISE-EVs (Suppl. Figure 3, available online at www.thrombosis-online.com).

Plasma coagulation activity of tumour bearing mice

Clotting analysis was performed to determine whether CCC tumours could activate blood coagulation. Using tilt tube plasma clotting assays, we found that plasma from OVISE-tumour bearing mice could solidify over 30 min in reaction buffer containing calcium chloride (Table 1). In contrast, plasma prepared from 231 tumour-bearing mice remained in a fluid state under the same experimental conditions. As expected, fXa generation assay with EVs prepared from cells cultured under hypoxia revealed that higher procoagulant activity was precipitated in OVISE cells than in 231 cells (Figure 4 C). Furthermore, plasma clotting was inhibited in the presence of NAP-5, a specific inhibitor of factor X, or anti-TF (5G9) antibody (Table 1).
EV production by CCC cells is not enhanced in response to hypoxia

Tumour tissues are considerably hypoxic, suggesting that production of EVs by CCC cells may be affected by hypoxia. Moreover, the level of EVs released by 231 cells is reportedly increased under hypoxic conditions (29). We therefore examined the effect of hypoxia on the shedding of EVs by CCC cells by comparing the viability of cancer cells cultured under normoxic (20 % O₂) or hypoxic (1 % O₂) serum-free conditions. The viability of OVISE cells, evaluated by the number of live cells and caspase-3 cleavage, an apoptosis marker (Suppl. Figure 4A and B, available online at www.thrombosis-online.com), was not affected by hypoxic conditions, but the viability of OVSAYO cells decreased (Suppl. Figure 4C, available online at www.thrombosis-online.com). FCA revealed that EV release by either cell line was not increased in response to hypoxia (Suppl. Figure 4D, available online at www.thrombosis-online.com). However, the TF level in EVs secreted by OVISE (Suppl. Figure 3, available online at www.thrombosis-online.com, and [6]) and OVSAYO (6) cells increased in response to hypoxia.

FilA is responsible for integration of TF into CCC derived EVs

The C-terminal domain of TF binds to the actin-binding protein FilA (30), and previous studies showed that the integration of TF into MVs released by endothelial and 231 cells occurs in a FilA-dependent manner (23). Calpain-dependent hydrolysis of FilA is also required for the vesicle formation process (31) and incorporation of TF into MVs (32), and this hydrolysis is enhanced in melanoma cells subjected to hypoxia (33). We therefore assessed whether FilA is involved in the loading of TF into MVs produced by CCC cell lines under hypoxic conditions.

We examined FilA expression in OVISE cells cultured under normoxic and hypoxic conditions, with 231 cells serving as a control. We confirmed that HIF1 expression was higher in both cell types cultured in serum-free media under hypoxic than normoxic conditions (Figure 5A). Calpain-dependent hydrolysis of FilA is also required for the vesicle formation process (31) and incorporation of TF into MVs (32), and this hydrolysis is enhanced in melanoma cells subjected to hypoxia (33). We therefore assessed whether FilA is involved in the loading of TF into MVs produced by CCC cell lines under hypoxic conditions.

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In contrast, both fl- and cleaved (cv) forms of filA were detected in 231 cells, with the expression pattern and cell viability unaffected by hypoxia (Figure 5B and Suppl. Figure 4B, available online at www.thrombosis-online.com).

We next tested whether silencing of filA expression affected the integration of TF into EVs secreted by OVISE cells exposed to hypoxia primarily using the filA siRNA reagent sc-35374 (see Materials and methods). Western blotting analysis of WCL from OVISE cells, in which more than 80% of filA was silenced by 40 nM of siRNA (Suppl. Figure 5A, available online at www.thrombosis-online.com), revealed that cellular TF levels were considerably increased by filA knockdown (Figure 5C). Only fl-filA was present in the EV fraction (Figure 5D), consistent with an earlier report using 231 cells (23). In contrast, TF levels in the EV fraction were largely diminished by filA knockdown (Figure 5D), suggesting that incorporation of TF into EVs under hypoxic conditions was blocked by filA silencing, leading to accumulation of TF in the cell body. Similar results were observed using a second filA siRNA (Silencer® Select s5277) (Suppl. Figure 5B, available online at www.thrombosis-online.com).

FilA is essential for EV shedding from CCC cells

Large numbers of TF-bearing EVs are from OVISE cells in the absence of cv-filA, suggesting that filA hydrolysis is not required for vesicle formation. Furthermore, filA negatively regulates the release of EVs from endothelial and cancer cells (23). We therefore tested whether filA contributed to shedding of EVs from CCC cells cultured under hypoxic conditions. OVISE cells were transfected with nonsense (NS) or filA siRNA and cultured for 24 h, followed by further culture under 1% O2 in serum-free media for 48 h. FCA and NTA revealed that the number of EVs decreased following filA knockdown (Figure 5E and F), but the distribution of vesicle size remained the same (Figure 5F). The same conclusion was deduced when EVs were estimated by protein level (Figure 5G). Similar results were observed when cells were cultured under normoxia for the same time period (Figure 5H and I). We confirmed that the number of live cells post-hypoxic cell culture was not diminished between NS siRNA treated and filA siRNA treated cells (Figure 5J), suggesting that filA contributed to both the incorporation of TF into EVs and the shedding of EVs from CCC cells.
cells. Similar results were obtained using a second filA siRNA reagent (Suppl. Figure 5C, available online at www.thrombosis-online.com) and by experiments with an additional CCC cell line (OVSAYO; Suppl. Figure 6, available online at www.thrombosis-online.com).

**PARs contribute to shedding of MVs**

PARs can be activated through TF-fVII complex formation on the cell surface, with activation of PAR2 facilitating the integration of TF into EVs released by multiple cells through phosphorylation of its cytoplasmic domain (23, 34). A complex of TF and ectopically synthesised fVII can activate PAR1, but not PAR2, thereby enhancing CCC cell motility and invasiveness (10). Additionally, PAR2 is upregulated in response to hypoxia, promoting tumour angiogenesis in endothelial cells (35). We therefore tested whether PARs could influence the incorporation of TF into EVs and/or the release of EVs produced by OVISE cells cultured under hypoxic conditions. Cells were cultured under hypoxia for 48 h in the presence of negative control IgG or cleavage-blocking PAR1 and PAR2 antibodies. TF levels in WCL and EV fractions were not affected by the presence of anti-PAR antibodies (Figure 6G). Moreover, as the TF-fVIIa-fXa ternary complex can cleave PAR1 (36), we also examined expression of fX. Western blotting analysis with concentrated CM revealed that fX is synthesised and secreted from OVISE and OVSAYO cells as in the case of HepG2 cells (Figure 6H). The expression is higher in OVSAYO cells than in OVISE cells (Figure 6H). Notably, fX secretion increased in response to hypoxia (Figure 6H).

**Table 1: Coagulation activity of plasma samples prepared from tumour-bearing mice.**

<table>
<thead>
<tr>
<th>Coagulation</th>
<th>OVISE plasma No.</th>
<th>231 plasma No.</th>
</tr>
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<tbody>
<tr>
<td>No additive or IgG</td>
<td>1−</td>
<td>1−</td>
</tr>
<tr>
<td>NAP-5 or TF (5G9)</td>
<td>2+</td>
<td>2−</td>
</tr>
<tr>
<td></td>
<td>3+</td>
<td>3−</td>
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<td></td>
<td>4+</td>
<td>4−</td>
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</table>

Tilt tube clotting assays were performed in the presence or absence of 2 µM NAP-5. A tilt tube clotting assay was also performed in the presence of 50 µg/ml IgG or anti-TF (5G9) antibody. Plasma samples were judged as negative if the reaction mixture did not solidify; samples were regarded as negative if the reaction mixture did not solidify. Numbers are indicative of samples prepared from individual mice.

**Discussion**

We found that the level of EVs secreted *in vitro* by various cancer cell lines, including those of ovarian origin, varied widely. We also showed that CCC cells could secrete unusually high levels of EVs enriched with TF, with the TF level in EVs being especially high when cells were exposed to hypoxia, consistent with the higher plasma coagulability of CCC tumour-bearing mice. Furthermore, we found that the integration of TF into EVs in these cells was dependent on filA. However, unlike incorporation of TF into macrophage-derived EVs (32), this process does not associate with filA hydrolysis. Production of EVs was dependent on both filA and PARs.

MDA-MB-231 cells have been used in many studies of EVs (15, 16, 23, 26) and express high levels of TF (28). OVISE and OVSAYO cells produced considerably higher levels of EVs than 231 cells and other cell lines. This increase in EV production is a cause of the high level of TF released by CCC cells.

Hypoxia is a common feature of solid tumours, including CCCs, and TF gene expression is enhanced under hypoxic conditions (37–39). Indeed, TF levels in CCC cells and the EVs they produce are increased in response to hypoxia (6). We found that the levels of TF within both OVISE and 231 tumour tissues were higher in hypoxic than normoxic regions. However, plasma TF levels were higher in OVISE- than in 231-tumour bearing mice, although TF is expressed at similar levels by both tumour tissues. Taken together, these findings suggest that TF-positive EVs from OVISE tumours are responsible for the increased plasma TF levels in these mice. As expected, the coagulability of plasma was higher from OVISE than from 231 tumour-bearing mice, and these results are consistent with clinical findings showing that CCC is associated with a high incidence of VTE and high TF expression (7), while thrombosis is less frequently observed in breast cancer patients and is independent of TF levels (22). Direct demonstration of thrombus formation *in vivo* will further validate the crucial role of EVs and TF in tumour angiogenesis.
contribution of tumour-derived EVs to VTE observed in CCC patients.

We also demonstrated that filA is required for incorporation of TF into MVs released by CCC cells cultured under hypoxic conditions. This result is consistent with a recent finding, showing that incorporation of TF into MVs released from 231 cells cultured under normoxia is mediated by filA (23). FilA is cleaved by calpain, a process enhanced in melanoma cells by hypoxia (32). We also showed that filA cleavage is minimal in OVISE cells compared with 231 cells, and that expression of filA does not change in response to hypoxia. These findings suggest that the elevated TF level in EVs derived from CCC cells subjected to hypoxia may simply reflect increased cellular expression of TF, which is independent of filA.

Figure 5: Filamin-A (filA) is responsible for both TF integration into EVs and shedding of EVs released from OVISE cells. A) Expression of the hypoxia marker HIF1 in cancer cells. N and H indicate cell culture in serum-free medium for 24 h under normoxic and hypoxic (1% O2) conditions, respectively. B) Western blotting analysis of full-length and cleaved forms of filA in WCL from OVISE and 231 cells cultured under normoxia and 1% O2 in serum-free media for 48 h; β-actin levels served as a protein loading control. C) TF levels in WCL prepared from OVISE cells cultured under serum starvation and hypoxic (1% O2) conditions for 48 h following suppression of filA expression by RNAi. TF levels were normalised by β-actin levels and quantified using ImageJ software. Data represent the means ± SD of three independent experiments. D) TF levels in the EV fraction prepared from OVISE cells cultured under serum deprivation and 1% O2 for 48 h following suppression of filA expression by RNAi. Equivalent amounts of EVs (12 µg protein) were loaded into each well. Flotillin-2 levels served as a protein loading control. TF levels were normalised by flotillin-2 levels and quantified using ImageJ software. Data represent the means ± SD of three independent experiments. E) EVs secreted from siRNA treated cells cultured under serum starvation and hypoxia for 48 h were counted by FCA. Data represent the means ± SD of three independent experiments. *P = 0.04 by a two-sided Student's t-test. F) Nanoparticle tracking analysis of EVs secreted from siRNA-treated cells cultured under serum starvation and hypoxia for 48 h. G) EVs shown in E and F were evaluated by protein quantification. H) EVs secreted from siRNA-treated OVISE cells cultured under normoxia for 48 h were counted by FCA. Data are the means ± SD of three independent experiments. I) EVs shown in H were evaluated by protein quantification. J) OVISE cells transfected with nonsense or filA siRNA were cultured for 24 h and live cells were counted (0 h). Cells were further cultured under serum starvation and hypoxia for 48 h and then live cells were counted (48 h).
A previous study found that partial suppression of filA by siRNA did not affect the vesiculation process in 231 cells, while near complete knockdown of filA expression enhanced EV production (23). In contrast, we found that incomplete knockdown of filA was sufficient to impair EV production by CCC cells under both normoxic and hypoxic conditions.

We found that PARs contributed to the production of EVs, but were not involved in the incorporation of TF into EVs under hypoxia. These phenomena may be cell-type dependent events, as TF incorporation into EVs released from 231 cells is mediated by PAR2 (23). Interestingly, unlike OVISE cells, EV secretion from OVISE cells was dependent on PAR1, whereas its secretion by OVISE cells can be facilitated by both PAR1 and PAR2. These observations can be attributed to relatively high expression of PAR1 and Fx in OVISE cells, potentially leading to high expression of active PAR1 via TF-FvIIa-Fx complex formation. This observation is consistent with our earlier report, which found that TF-FvIIa-dependent motility in OVISE cells was mediated by PAR1, but not PAR2 (10). It is feasible that the observed differential PAR dependence of CCC cells is due to the cellular availability of PARs and expression of FX.

Although the mechanisms by which filA acts in conjunction with PARs to promote these effects remain unclear, the actin-regulating protein DRF3 has been shown to inhibit MV release (27) and filA functions in multiple signalling pathways (32, 40, 41). Therefore, while filA can affect blood coagulability by modulating
What is known about this topic?
- Venous thromboembolism is frequent in cancer patients including those with ovarian clear cell carcinoma (CCC).
- Cancer cells shed tissue factor (TF)-positive microvesicles (MVs), potentially leading to thrombosis.
- Filamin-A is required for incorporation of TF into MVs released from some cancer cells.

What does this paper add?
- CCC cells secrete large numbers of TF-rich extracellular vesicles (EVs) particularly under hypoxic conditions.
- The blood of mice bearing CCC tumours is hypercoagulable compared with mice bearing tumours with low secretion of TF-positive EVs.
- Filamin-A contributes to high level production of EVs and incorporation of TF into EVs released by CCC cells.
- Protease-activated receptors differentially participate in shedding of EVs depending on CCC cells.

platelet function (42, 43), it may also contribute to VTE in CCC patients by promoting TF expression.

Here we have demonstrated that CCC cells can robustly shed TF-rich EVs, and that this high level of MV production is positively regulated by both filA and PARs. These results may explain why CCC patients are more prone to thrombosis events compared with breast cancer patients. This knowledge enhances our understanding of why CCC patients who experience VTE events have poor prognosis and could lead to the development of therapeutic strategies that suppress the release of procoagulant EVs from CCC cells.

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Conflicts of interest
None declared.

References


