THE ROLE OF TUMOR MICROPARTICLES IN CANCER-ASSOCIATED THROMBOSIS

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ABSTRACT

Julia E. Geddings: The Role of Tumor Microparticles in Cancer-Associated Thrombosis
(Under the Direction of Nigel Mackman)

Cancer patients have a ~4-fold increased risk of venous thromboembolism (VTE) compared with the general population, and cancer patients with VTE have reduced survival compared with non-VTE patients. Microvesicles (MVs), also known as microparticles (MPs), are small membrane vesicles that are constitutively released from tumors, and tumor cell-derived, tissue factor (TF)-positive (TF+) MVs may contribute to thrombosis in cancer patients. I tested the hypothesis that tumor cell-derived, TF+ TMVs activate platelets in vitro and in vivo, and this contributes to cancer-associated VTE.

For this study I first had to establish a reproducible model of cancer-associated thrombosis. The in vivo model chosen for this purpose was the inferior vena cava (IVC) stenosis model in mice. This model was chosen because it mimics certain features of human deep vein thrombosis, including the triggering of thrombosis with minimal endothelial damage, maintenance of blood flow at the site of thrombosis, and chronic thrombus development that can be followed over time in vivo. The strengths and weaknesses of this model are summarized in chapter 2 of this dissertation.

The IVC stenosis model of thrombosis was used to evaluate the role of TF+ TMVs in the enhancement of thrombosis. In the study included as chapter 3 in this dissertation, I found that TF+ TMVs isolated from two human pancreatic cell carcinoma cells lines activate isolated human platelets in a TF- and thrombin-dependent manner in vitro. TF+ TMV-mediated platelet activation was also inhibited by the ADP scavenger apyrase and the P2Y12 receptor inhibitor 2MesAMP. Further, TF+ TMVs enhanced thrombosis in two mouse models of venous thrombosis in a TF-dependent manner.
TF+ TMV-enhanced thrombosis was reduced in Par4 deficient mice and in wild-type mice treated with the platelet inhibitor clopidogrel, suggesting that TF+ TMV-enhanced thrombosis in mice was, in part, dependent on platelets. These studies identify TF+ TMV-induced platelet activation as a possible mechanism of thrombosis in cancer and suggest that platelet inhibitors, such as aspirin and clopidogrel, may be considered for the prevention of VTE in high risk cancer patients.
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<tr>
<td>α</td>
<td>alpha</td>
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<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>µm</td>
<td>micromolar</td>
</tr>
<tr>
<td>ACD</td>
<td>acid citrate dextrose</td>
</tr>
<tr>
<td>aPC/PS</td>
<td>activated protein C/ protein S complex</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ASCO</td>
<td>America Society of Clinical Oncology</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>CATS</td>
<td>cancer and thrombosis study</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
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<tr>
<td>conc</td>
<td>concentration</td>
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<tr>
<td>CFSE</td>
<td>5(6)-Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>DVT</td>
<td>deep vein thrombosis</td>
</tr>
<tr>
<td>EPCR</td>
<td>endothelial cell protein C receptor</td>
</tr>
<tr>
<td>FII</td>
<td>prothrombin</td>
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<tr>
<td>FIIa</td>
<td>thrombin</td>
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<tr>
<td>FIX</td>
<td>factor IX</td>
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<tr>
<td>FX</td>
<td>factor X</td>
</tr>
<tr>
<td>FXa</td>
<td>activated factor X</td>
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FXII  factor XII
FXIIIa  activated factor XIII
FV  factor V
FVa  activated factor V
FVII  factor VII
FVIIa  activated factor VII
FVIIai  active-site inhibited factor VIIa
fsc  forward scatter
gen  generation
IP₆  inositol hexakisphosphate 6
IVC  inferior vena cava
LMWH  low molecular weight heparin
MFI  mean fluorescence intensity
mg  milligram
mm  millimeter
MP  microparticle*
MUC-1  mucin-1
MV  microvesicle*
NET  neutrophil extracellular trap
ng  nanogram
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>NOAC</td>
<td>novel oral anticoagulant drug</td>
</tr>
<tr>
<td>P-sel</td>
<td>P-selectin</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor type-1</td>
</tr>
<tr>
<td>PAMAM</td>
<td>poly (amido amine)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PE</td>
<td>pulmonary embolism</td>
</tr>
<tr>
<td>PGE1</td>
<td>prostaglandin E1</td>
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<td>plt</td>
<td>platelet</td>
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<tr>
<td>PolyP</td>
<td>polyphosphates</td>
</tr>
<tr>
<td>PRP</td>
<td>platelet rich plasma</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidyl serine</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>TAFI</td>
<td>thrombin-activatable fibrinolysis inhibitor</td>
</tr>
<tr>
<td>TAT</td>
<td>thrombin-antithrombin</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TF</td>
<td>tissue factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>tissue factor pathway inhibitor</td>
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TMP  tumor-derived MP
TMV  tumor-derived MV
tPA  tissue plasminogen activator
U    units
uPA  urokinase plasminogen activator
VKA  vitamin K antagonist
VTE  venous thromboembolism
wt   wild-type
(wt/vol)  weight/volume

*The terms MV and MP are used interchangeably throughout this dissertation. During the completion of these studies there was a shift in the field towards the preferential use of the term MV. Some of the text included in this manuscript was published prior to this shift, and thus uses the term MP (Chapters 1.2, 1.4, and 2).
CHAPTER 1: INTRODUCTION

1.1 Tissue Factor and the Coagulation Cascade

Hemostasis is the process by which the body maintains vascular integrity by arresting bleeding. This process can be separated into primary and secondary hemostasis. During primary hemostasis the rapid recruitment of platelets to the site of vascular injury results in the formation of a platelet plug. This platelet recruitment occurs due to the interactions between platelets and the exposed vascular subendothelium. Platelet glycoprotein VI binds to collagen, platelet integrin $\alpha_2\beta_1$ binds to collagen and fibrinogen, and platelet integrin $\alpha_{IIb}\beta_3$ binds to von-Willebrand factor or fibrinogen. The platelet integrin $\alpha_{IIb}\beta_3$ is then involved in platelet aggregation by binding fibrinogen.

Exposure of the vascular subendothelium to blood also initiates secondary hemostasis, referred to as coagulation, when subendothelial TF initiates the extrinsic pathway of the coagulation cascade. The coagulation cascade is made up of a series of serine proteases whose serial activation results in the generation of a cross-linked fibrin network that stabilizes the platelet clot. TF is a transmembrane protein that is constitutively expressed by several cell types in the subendothelium, including vascular smooth muscle cells, pericytes, and adventitial fibroblasts. Upon exposure to the blood, TF serves as a receptor for Factor VII (FVII) resulting in its proteolytic conversion to activated Factor VII (FVIIa). The TF/FVIIa complex then activates both factor IX (FIX) and factor X (FX). FX can also be activated through the intrinsic pathway of the coagulation cascade initiated by the activation of FXII due to exposure to a negatively charged surface. Activated FX (FXa) is assembled into a complex with its cofactor activated factor V (FVa) on a negatively charged phospholipid surface, and this activates prothrombin (FII) to thrombin (FIIa).
Thrombin then cleaves fibrinogen to fibrin, which is cross-linked by activated factor XIII (FXIIIa).\textsuperscript{10,11} Thrombin also performs a multitude of other functions in the enhancement of thrombosis including the activation of FV, FVIII, FXI, and platelets.\textsuperscript{12-18}

Activation of coagulation is counter-balanced by several inhibitors and negative feedback pathways. The coagulation cascade is primarily downregulated by 3 major anticoagulant pathways (Figure 1.1).\textsuperscript{10,19-23} Tissue factor pathway inhibitor (TFPI) is the primary regulator of TF-initiated coagulation.\textsuperscript{19} There are two different isoforms of TFPI. TFPlα is released by activated platelets and TFPlβ is expressed on endothelial cells.\textsuperscript{19,24} TFPI inhibits FXa and the TF/FVIIa complex in a FXa-dependent manner.\textsuperscript{19} The inhibition of FXa by TFPlα activity is also enhanced by protein S.\textsuperscript{25} The protein C pathway is initiated by the interaction of thrombin with the endothelial cell transmembrane receptor thrombomodulin. This interaction changes the substrate specificity of thrombin from fibrin to protein C, resulting in the generation of activated protein C.\textsuperscript{21} Activated protein C in combination with its cofactor protein S inactivates the cofactors FVIIIa and FVa, resulting in the downregulation of the coagulation cascade.\textsuperscript{20} The activation of protein C by thrombomodulin is further enhanced by endothelial cell protein C receptor (EPCR).\textsuperscript{20} Antithrombin inhibits the activity of many of the coagulation proteases in the coagulation cascade, particularly FXa and thrombin. Antithrombin activity is increased by the anticoagulant drug heparin.\textsuperscript{22,23}

Once a fibrin clot is formed it is then degraded by the fibrinolytic system.\textsuperscript{26,27} This process is initiated by the conversion of plasminogen to plasmin. Plasmin then cleaves cross-linked fibrin into soluble breakdown products allowing its removal from the circulation and maintaining vascular patency. Plasminogen is converted to plasmin by two plasminogen activators known as tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). tPA is found in circulation and is synthesized by vascular endothelial cells and macrophages, with increased release during cell stress. Activation of plasmin by tPA is accelerated in the presence of fibrin because plasminogen binds to fibrin.\textsuperscript{27} uPA plays a
more significant role in cell signaling processes than in the fibrinolytic pathway but has still been shown to cleave and activate plasminogen.\textsuperscript{26} The primary inhibitor of tPA activity is plasminogen activator inhibitor type-1 (PAI-1).\textsuperscript{26} Fibrin clots are also stabilized by thrombin-activatable fibrinolysis inhibitor (TAFI) which decreases the affinity of plasmin for fibrin.\textsuperscript{28}

More recent studies suggest that FVII is prebound to subendothelial TF even before being exposed to blood due to the low molecular weight of FVII enabling it to pass through intact endothelium.\textsuperscript{29,30} This prebound TF/FVIIa contributes to a phenomenon in healthy organisms known as “idling” which is a baseline level of activation of coagulation in the absence of endothelial injury.\textsuperscript{31-33} Idling allows a very rapid response to vascular endothelial injury without triggering overt thrombosis.\textsuperscript{29,34} The progression of this baseline level of coagulation to thrombosis in healthy individuals is prevented by the limited availability of FVIII and FV in the subendothelial space due to their high molecular weights and the lack of phosphatidyl serine (PS) rich platelet surfaces for the assembly of coagulation factor complexes.\textsuperscript{29} Low TF mice, which have a transgene expressing low levels of human TF in a murine TF null background, exhibit spontaneous bleeding in many organs, particularly the brain, uterus, heart, and lungs, indicating that low level activation of the extrinsic pathway of the coagulation cascade is required to maintain baseline hemostasis in these organs.\textsuperscript{10} Complete TF knockout in mice is embryonic lethal.\textsuperscript{10}

Hemostasis is a complicated and highly regulated process that allows for the maintenance of blood flow by stopping bleeding from damaged vascular endothelium without inducing excessive clotting. TF is the primary physiological initiator of the coagulation cascade but pathological expression has been shown to contribute to thrombosis in multiple disease states.\textsuperscript{35-39}
Figure 1.1: The coagulation cascade and major regulators

The coagulation cascade consists of a series of serine proteases whose serial activation results in the formation of a fibrin network that stabilizes a platelet thrombus. Each step of the cascade is highly regulated to maintain hemostatic balance. The major targets for anticoagulant regulators (red) tissue factor protease inhibitor (TFPI), activated protein C/ protein S complex (aPC/PS), and antithrombin are shown. Brackets indicate cofactors.
1.2 New Players in Hemostasis and Thrombosis

This review will discuss recent advances in our understanding of the role of inorganic polyphosphates (PolyP) in hemostasis and thrombosis and factors that contribute to thrombosis but not hemostasis. We will summarize recent studies on three new players: factor XII (FXII), tissue factor (TF)-positive microparticles (MPs), and neutrophil extracellular traps (NETs).

The coagulation protease cascade

The coagulation cascade is essential for hemostasis and has been studied for many years. However, we still have a limited number of drugs that are used clinically to prevent and treat thrombosis. Importantly, all of these drugs are associated with bleeding side effects because they target key proteases in the coagulation cascade. This includes the novel oral anticoagulant (NOAC) drugs, which target either factor Xa or thrombin.

The coagulation cascade can be divided into the extrinsic, intrinsic, and common pathways. The extrinsic pathway produces small amounts of thrombin that activates a variety of components in the cascade that allows amplification of the cascade via the intrinsic pathway to produce large amounts of thrombin (Figure 1.2). Thrombin cleaves fibrinogen to fibrin resulting in clot formation (Figure 1.2). The extrinsic pathway of the coagulation cascade is initiated upon the exposure of extravascular TF to blood. Formation of the TF/FVIIa complex triggers the coagulation cascade by activating both FX and FXI. This pathway is “extrinsic” to blood since significant levels of TF are not present in blood in healthy individuals. The extrinsic pathway is essential for hemostasis. The intrinsic pathway of the coagulation cascade is comprised of three proteases, FXIIa, FXIa, FIIa and the cofactor FVIIIa. Under physiologic conditions, this pathway is activated by thrombin cleavage of FXI. Deficiencies in FIX and VIII lead to mild to severe bleeding in humans (hemophilia B and A, respectively) while FXI deficiency results in only a minor increase in bleeding with injury (hemophilia C). The intrinsic pathway can be activated ex vivo by negatively-
charged compounds, such as kaolin, that activates FXII. FXII activation has been showed to directly modify fibrin clot structure by increasing fibrin fiber density. The common pathway consists of the proteases FXa, thrombin, and the cofactor FVa. Proteases in the common pathway are the major targets of current anticoagulant therapy (Figure 1.2).

Renne and colleagues were the first to show that FXII deficient mice exhibit reduced thrombosis in several different arterial thrombosis models without any increase in tail vein bleeding time. This observation was important because it suggested that thrombosis could be separated from hemostasis and suggested that FX could be a new target for the development of safe anticoagulant drugs. FXI deficient mice have no apparent hemostatic defects, and humans with FXI deficiency have a small increase in bleeding after injury. These observations suggest that inhibition of FXIa might also reduce thrombosis with minimal effects on hemostasis.

Despite the knowledge that FXII is activated by negatively-charged substances it was unclear what was activating FXII in vivo. Several candidates have been proposed as FXII activators over the years. Preissner and colleagues reported that extracellular RNA bound to both FXII and FXI and suggested that this was the “long sought after natural foreign surface” for the activation of the intrinsic pathway. There is also evidence that DNA-rich NETS and PolyP activate FXII and initiate the intrinsic pathway of the coagulation cascade.

There is a large literature on the effect of different FXII and FXI inhibitors on thrombosis in various animal models (Figure 1.2). FXII inhibitors have been shown to reduce both venous and arterial thrombosis in multiple animal models without an associated increase in bleeding. These inhibitors include H-D-Pro-Pro-Phe-Arg-chloromethylketine and infestin 4, which was cloned from the midgut of the blood-sucking insect Triatoma infestans. Inhibitory anti-FXI antibodies and small molecules have been shown to reduce both venous and arterial thrombosis in mice, rats, rabbits, and baboons without an associated increase in bleeding. Antisense oligonucleotides (ASOs) have also been used to reduce levels of different
coagulation factors and determine the effect on thrombosis. ASOs target complementary mRNA resulting in RNA degradation and a reduction in protein expression. They are particularly promising for the targeting of genes expressing coagulation proteins because of the high level of sensitivity of liver tissue to ASOs.57 FXII and FXI ASOs have been shown to reduce arterial and venous thrombosis in various animal models without increasing bleeding.49,51,57,58

Role of PolyP in hemostasis and thrombosis

PolyP is a highly anionic linear polymer that is synthesized from ATP.59 In humans, this molecule is secreted by platelets after activation.9 Morrissey and colleagues were the first to report a role for PolyP in blood coagulation.60 PolyP was shown to affect numerous steps in the coagulation cascade, including activating FXII, enhancing the activation of FV, increasing the activity of thrombin-activated fibrinolysis inhibitor (TAFI), and inhibiting tissue factor pathway inhibitor (TFPI).60 Other studies have extended this early observation and have also demonstrated that PolyP enhances fibrin clot structure stability.47,61,62 These results led to the suggestion that PolyP may be considered for use as a general procoagulant agent in the treatment of patients with hemostatic defects.63

Microorganisms store PolyP in subcellular organelles termed acidocalcisomes. In mammalian platelets PolyP is stored in dense granules. There are important differences between bacterial and platelet PolyP. Bacteria produce long-chain PolyP (up to several thousand phosphate units) whereas platelets contain short-chain PolyP (60-100 phosphates units).59 PolyP of different size have different activities in the coagulation cascade.62 Long chain PolyP polymers (>250-500mers) have been shown to be a strong activator of FXII and affect fibrin clot structure.62 On the other hand, shorter polymers (<100mers) increase FV activation and inhibit TFPI.64 Polyphosphate in both size categories also serve as a cofactor for thrombin activation of FXI.64 Renne and colleagues showed that human platelet PolyP induced pulmonary embolism and increased vascular permeability in mice in a FXII-dependent manner.47 The study concluded that PolyP
links platelet plug formation (primary hemostasis) and fibrin generation (secondary hemostasis). If platelet PolyP is playing a central role in blood coagulation by activating FXII then one would expect FXII deficient humans and mice to have a major hemostatic defect. However, FXII deficient humans and mice demonstrate normal hemostasis. Importantly, a second group could not reproduce the in vivo results produced by Renne and colleagues using the mouse pulmonary embolism model and concluded that platelet PolyP is a weak activator of FXII. Drs. Renne and Morrissey provided separate rebuttals to the Faxalv study and demonstrated that the negative in vivo results may be due to the extended storage of PolyP resulting in degradation and loss of activity. The role of platelet PolyP as a weak activator as opposed to a strong activator of FXII is supported by previous studies. Clearly, more studies are needed to resolve this controversy and determine the role of platelet PolyP in regulating the coagulation cascade.

Does platelet PolyP play a role in hemostasis? This question was addressed in a recent study that generated mice deficient in inositol hexakisphosphate 6 (IP₆) kinase, which is an enzyme required for the synthesis of platelet PolyP. Platelets from these mice had a 3-fold reduction in phosphate levels. These IP₆ kinase knockout mice had slower platelet aggregation, increased plasma clotting times, and altered fibrin structure. Furthermore, the mice had prolonged tail vein bleeding times and were resistant to thromboembolism. Since the mice had defects in both platelet aggregation and clotting it is unclear if the in vivo hemostatic defect was due to a primary effect on platelets and/or coagulation. Nevertheless, this genetic approach supports the notion that PolyP is a general procoagulant agent.

The above polyphosphate studies have triggered the development of new anticoagulant drugs that can inactivate these polyphosphates. Sullenger and colleagues screened a variety of nucleic acid binding polymers and identified a cationic poly (amido amine) (PAMAM) dendrimer called PAMAM-G3 that prevented thrombosis without increasing tail vein bleeding in mice. Similarly, Morrissey and colleagues found that the cationic compounds 1.0 dendrimer and polymyxin B reduce both venous and arterial thrombosis in mice.
**Microparticles in hemostasis and thrombosis**

MPs are small (0.1-1.0 µm) membrane vesicles released from activated or apoptotic cells and cells that have undergone oncogenesis. These vesicles were originally described as “platelet dust” that was observed being released from activated platelets by microscopy. MPs are formed by the outward blebbing of the plasma membrane with subsequent release after proteolytic cleavage of the cytoskeleton. The majority of MPs have phosphatidylinerine (PS) exposed on their surface, although PS-negative MPs can be formed after platelet activation. They contain a selective group of cell surface proteins, which includes TF. MPs are procoagulant due to the presence of negatively-charged phospholipids, such as PS, and TF on the surface. TF binds FVII/FVIIa and PS facilitates the assembly of positively-charged coagulation factor complexes. There is indirect evidence for a role of MPs in hemostasis. Injection of soluble P-selectin-Ig into hemophilia A mice resulted in an increase in the number of circulating MPs, including TF-positive MPs, and a correction of the tail-vein bleeding time. Similarly, infusion of red blood cell-derived MPs into thrombocytopenic rabbits corrected ear bleeding time in a dose-dependent manner. However, there is no evidence that circulating MPs contribute to hemostasis in healthy individuals. The majority of MPs in blood in healthy individuals are derived from platelets and it is difficult to separate the role of platelet-derived MPs in hemostasis from the role of the platelets themselves.

MP enhancement of experimental thrombosis is well documented. For instance, injection of MPs isolated from cardiac bypass surgery patient plasma was shown to enhance thrombosis in a TF dependent manner using a rat IVC stenosis model of venous thrombosis. One study examined the docking of tumor-derived MPs to sites of ferric chloride-induced mesenteric vessel injury and laser-induced cremaster arteriole injury in vivo. MPs derived from PANC02 mouse pancreatic adenocarcinoma tumors accumulated at sites of vascular injury and thrombosis. This MP accumulation was reduced by treatment
of the mice with an inhibitory P-selectin antibody.\textsuperscript{87} Mouse pancreatic tumor-derived MPs were also found to enhance ferric chloride-induced thrombosis in mice.\textsuperscript{87} However, cleavage of surface ligands on these tumor MPs by trypsinization or treatment of mice with an inhibitory P-selectin antibody prior to MP infusion attenuated the MP-induced enhancement of thrombosis.\textsuperscript{87} We found that growth of human pancreatic tumors expressing TF was associated with release of TF-positive MPs into the circulation and activation of coagulation. In addition, injection of TF-positive tumor-derived MPs enhances thrombosis in an inferior vena cava (IVC) stenosis model of venous thrombosis.\textsuperscript{88}

Statin treatment is associated with a reduction in venous thrombosis.\textsuperscript{89,90} The Jupiter trial demonstrated a reduction in VTE in patients with high levels of C-reactive protein but normal blood lipids that were prophylactically treated with rosuvastatin.\textsuperscript{89} Our laboratory has shown that hypercholesterolemia can induce monocyte TF expression and release of TF-positive MPs in vivo.\textsuperscript{91} Moreover, we found that simvastatin treatment attenuated this increase in TF-positive MPs in both hypercholesterolemic mice and in monkeys.\textsuperscript{91} Patients with a variety of thrombotic diseases often have elevated levels of TF-positive MPs suggesting that they may contribute to the prothrombotic state and thrombosis.\textsuperscript{35,73,75}

We hypothesize that TF-positive MPs could represent a new target for anticoagulant therapy that would have minimal effect on hemostasis. A concern of this therapy, however, would be that it would only be effective in prothrombotic disease states that are shown to be dependent on MPs. While there is evidence for a role of MPs in thrombosis in vivo in some diseases such as pancreatic cancer associated thrombosis, definitive evidence for a reduction in thrombosis associated with targeting MPs in patients does not yet exist. Potential strategies would include blocking the formation, increasing the clearance and/or preventing the docking of the MPs to the activated endothelium. P-selectin inhibitors are already in development for use as anti-thrombotic agents.\textsuperscript{92-94} However, these inhibitors block platelet aggregation which would affect hemostotic, potentially resulting in bleeding side effects.
NETs and thrombosis

NETs are primarily released by activated neutrophils via a cell death program called NETosis that is distinct from either apoptosis or necrosis. NETs consist of nucleic acids decorated with histones and other proteins that are involved in the entrapment and killing of both Gram-negative and Gram-positive bacteria as well as fungi in innate immunity. NETosis involves chromatin decondensation followed by the fragmentation of the nuclear envelope and neutrophil granules allowing for the mixing of components within the cell before plasma membrane lysis and NET release. Non-suicidal pathways of NETosis have also been identified which involve the release of nuclear or mitochondrial DNA by living cells through the budding of NET-filled vesicles. Of note, simply assaying for free DNA or histones in the plasma is not sufficient evidence for NET release as free DNA can also be released in other forms of lytic cell death.

NETs have been shown to enhance experimental venous and arterial thrombosis through multiple mechanisms. As mentioned above, they provide a negatively-charged surface for the activation of FXII. Histones, a major component of NETs, have also been shown to induce activation and aggregation of platelets. Neutrophils that have been stimulated to release NETs activate FXII in vitro in a histone H2A and H2B dependent manner. NETs enhance activation of the extrinsic pathway of the coagulation cascade through the inactivation of TFPI. They also increase venous thrombosis by binding both platelets and red blood cells. These structures have been imaged within venous thrombi in both mice and primates. Importantly, mice with impaired NET formation have a decreased incidence of thrombosis in the IVC stenosis model. Two studies have demonstrated protection from venous thrombosis in mice that have been treated with DNase 1, which degrades cell free DNA and NETs. The anticoagulant heparin has been shown to displace histones from NETs resulting in their degradation and a reduction in venous thrombosis. Similarly, carotid artery thrombosis is reduced in mice that are deficient in two neutrophil serine proteases that are present on NETs. Infusion of purified histones
into mice has also been shown to enhance venous thrombosis. Polysialic acids have been shown to neutralize histones resulting in a reduction in NET-mediated cytotoxicity, although these agents have not yet been tested in a thrombosis model.

At present, there is little evidence that NETs are required for hemostasis. The tail vein bleeding time was prolonged two-fold in mice that were deficient in two neutrophil serine proteases that are known to be present on NETs. Also, while histones are only one component of NETs, it is of note that infusion of histones into mice causes thrombocytopenia that is associated with a profound increase in tail vein bleeding time.

Recent studies provide evidence for an association between NET release and thrombosis in humans. Patients diagnosed with acute VTE have been shown to have increased plasma nucleosomes and activated neutrophils. Further, both acute VTE and thrombotic microangiopathies are associated with increased plasma DNA and myeloperoxidase after diagnosis. Finally, neutrophils and NETs were present in thrombi isolated from patients with acute myocardial infarction.

Conclusion

There are many people that have contributed to our increased understanding of the pathways involved in hemostasis and thrombosis. The discovery that PolyP affects many aspects of the clotting cascades suggests that it may be a useful hemostatic agent for the treatment of a variety of bleeding disorders. In contrast, several factors have been identified that appear to contribute to pathologic thrombosis but not hemostasis. These include FXII, TF-positive MPs, and NETs. Inhibition of FXII activators with nucleic acid polymers may provide a safe way to reduce thrombosis. However, these drugs may only
be effective in patients in which thrombosis is triggered via the intrinsic pathway and are likely to be less efficacious than current anticoagulant drugs that target the common pathway of coagulation. At present, it is unclear if interfering with NET formation will be a viable approach to reducing arterial and venous thrombosis in patients.
Current anticoagulant therapies target the common pathways and, as a result, have significant bleeding side effects. Targeting thrombotic triggers as opposed to targeting the pathways involved in hemostasis could potentially allow for the creation of novel antithrombotic agents with minimal bleeding side effects.

Figure 1.2: Separating thrombosis and hemostasis in the coagulation cascade.
1.3 Cancer-Associated Thrombosis

Cancer patients are known to have a higher rate of venous thromboembolism (VTE) than non-cancer patients with estimates of 4 to 5-fold increased risk for VTE development for patients with cancer versus the general population. One study found a rate of VTE of 12.6% among cancer patients versus 1.4% in non-cancer patients. Further, 18-20% of first time VTE has been found to be associated with active malignancy. Cancer patients who are diagnosed with VTE are at an increased risk of death due to all causes compared with non-VTE patients. VTE in cancer patients also has a significant impact on disease morbidity due to pain, loss of mobility, and interruption of cancer chemotherapy. The absolute incidence of VTE in cancer patients varies by study and has increased over time, in part due to improved diagnostic procedures. In addition, new cancer treatment strategies leading to improved cancer survival and a more aged population of patients with cancer have increased the rate of VTE. Risk of VTE in cancer patients is highest within the first 3 months of cancer diagnosis, decreases but is still high between 3-12 months, and returns to basal levels 10 years after cancer diagnosis.

Risk of cancer-associated thrombosis is known to vary with cancer type. Pancreatic, hematologic, brain, and ovarian cancer are known to have the highest rate of VTE. The reported incidence of VTE in pancreatic cancer patients varies from 59-102 per 1000 person years or 5.3%-26% depending on the study. Brain cancer patients have a VTE incidence of 48-116 per 1000 person years or 1.6%-26%. VTE incidence in breast cancer patients on the other hand, who are considered low risk for VTE, varies from 5-55 per 1000 person years or 0.4%-8.1%. In general, it has been noted that more aggressive cancers have the highest rates of VTE. Timp and colleagues recently compiled data from 3 different studies which demonstrated a clear negative correlation between cancer type 1-year survival and reported incidence of VTE.

Cancer treatment has been shown to impact rates of VTE. Surgery is a well-known risk factor for VTE even in non-cancer patients, so it is no surprise that patients with cancer are also at an increased risk
of VTE after surgery. Cancer-patients are twice as likely to develop post-operative VTE as non-cancer patients undergoing the same procedure.\textsuperscript{127} Multiple clinical studies confirm the benefit of VTE prophylaxis in cancer patients following surgery, including laproscopic procedures.\textsuperscript{128-130} Chemotherapy is associated with an increased risk of VTE in cancer patients as well.\textsuperscript{113,131,132} Breast cancer patients treated with a combination of tamoxifen plus cyclophosphamide/ methotrexate/ fluorouracil therapy had increased thromboembolic events versus patients treated with tamoxifen alone (13.6% versus 2.6%, p<0.0001).\textsuperscript{131} While cancer patients were found to have a 4-fold increased risk of VTE versus non-cancer patients, cancer patients treated with chemotoxic chemotherapy were found to have a 6-fold increased risk of VTE versus non-cancer patients in a population based case-controlled study.\textsuperscript{113} In a record-linkage study, chemotherapy was associated with an increased risk of VTE within one year of cancer diagnosis in patients with and without metastasis, but radiotherapy was not.\textsuperscript{132} Further, cisplatin-containing chemotherapy regimens were associated with an increased rate of VTE in a randomized controlled trial of patients with gastroesophageal cancer versus patients treated with non-cisplatin containing chemotherapy regimens (12.6% versus 7.6%, p=0.0003).\textsuperscript{133} Erythropoiesis stimulating agents, red blood cell transfusions, and angiogenesis inhibitors also increase the risk of thrombosis in cancer patients.\textsuperscript{134,135}

Patients with cancer who are diagnosed with a VTE are treated with low molecular weight heparin (LMWH) for a minimum of 6 months after the event.\textsuperscript{130} Like non-cancer patients, the clinical decision to maintain a cancer patient on anticoagulant therapy beyond the 6 month period is based on their risk of VTE recurrence.\textsuperscript{136} Non-cancer patients, on the other hand, are treated acutely with LMWH and then bridged to a vitamin K antagonist for secondary thromboprophylaxis.\textsuperscript{137,138} Two clinical trials have shown that vitamin K antagonists are less effective in the treatment of cancer-induced VTE than LMWHs.\textsuperscript{139,140} In the largest clinical trial 676 cancer patients who had suffered an acute symptomatic VTE were randomized to either continue LMWH (dalteparin) therapy after acute LMWH treatment or be bridged to a vitamin K antagonist (warfarin) as per the current standard of VTE care. Recurrence of VTE in these patients was
monitored for 6 months and was 8% higher in patients receiving warfarin therapy versus dalteparin therapy (17% versus 9% VTE recurrence, p=0.002) with no significant difference in bleeding events. These findings were confirmed by another smaller clinical trial. While thromboprophylaxis is recommended for inpatients with active malignancy, current guidelines do not recommend the use of available anticoagulant therapies for the primary prevention of VTE in the outpatient setting for ambulatory cancer patients. These recommendations were made based on the results of 9 randomized controlled trials in the outpatient setting, and three in the inpatient setting. These studies showed that the risk of first-time VTE in non-selected ambulatory outpatients was not sufficiently high to justify the increased risk of bleeding events seen with LMWH therapy. For example, in a randomized placebo-controlled clinical trial of nadoparin in ambulatory cancer patients undergoing chemotherapy, anticoagulated patients experienced a VTE rate of 2.0% versus 3.9% in the control group (p=0.02) with no difference in major bleeding events (0.7% vs. 0.0%, p=0.18). The CONKO 004 trial is a randomized open-label clinical study of enoxaparin treatment for the prevention of VTE in pancreatic cancer patients who are being treated with palliative chemotherapy. This study included 312 patients and found that VTE risk was reduced from 10% to 1% by enoxaparin, however there was no statistically significant effect on patient survival (p=0.054).

The ability to identify cancer patients who are at the greatest risk of VTE would enable clinicians to better direct primary thromboprophylaxis towards those patients who were most likely to benefit. A number of VTE risk scoring models have been developed in cancer patient populations for precisely this purpose. The most extensively studied risk-scoring model to date is the Khorana score. This model uses common clinical readouts (site of cancer, platelet count, hemoglobin level, leukocyte count, body mass index) to predict future VTE in cancer patients undergoing chemotherapy. The Khorana score has been clinically validated multiple times. Use of this predictive model to guide VTE prophylaxis in cancer patients has been recommended by multiple clinical consensus publications including the most
recent guidelines published by the American Society of Clinical Oncology (ASCO).\textsuperscript{130,137} This model has also been expanded to include brain cancer as a very high risk cancer, as well as the biomarkers D-dimer and soluble P-selectin. These additions improve the predictive value of the model.\textsuperscript{154} However, one criticism of this extended model is that soluble P-selectin is not a commonly available laboratory test in the clinical setting.\textsuperscript{156}

Further study is needed in order to determine if thromboprophylaxis is beneficial in ambulatory cancer patients who are identified as high risk for the development of VTE. Two such studies are already in progress.\textsuperscript{156,157} Preliminary results of the MicroTec study indicate that advanced cancer patients with high levels of TF+ TMVs would benefit from LMWH thromboprophylaxis.\textsuperscript{157} In this study, TF+ TMVs are counted by impedance-based flow cytometry, which is a technique for MV TF measurement that has not been validated outside of the laboratory that developed it. A second ongoing clinical trial is evaluating the efficacy of LMWH therapy in reducing VTE risk in high-risk patients identified by the Khorana score.\textsuperscript{156} Preliminary results indicate a reduction in the rate of VTE with dalteparin thromboprophylaxis in high-risk ambulatory patients with cancer (10% vs. 23% VTE) with no major bleeding events reported during the intervention phase of the study.
1.4 Tumor-derived Tissue Factor-positive Microparticles and Venous Thrombosis in Cancer Patients

Introduction

Cancer and its treatment are frequently complicated by the development of venous thromboembolism (VTE) which is a collective term for deep vein thrombosis (DVT) and pulmonary embolism (PE). The development of VTE in cancer patients is one condition included in the spectrum of prothrombotic conditions in cancer encompassed by Trousseau’s syndrome. Thrombosis increases the morbidity and mortality of cancer patients. Pancreatic and brain cancer have the highest rates of VTE among all cancer types with cumulative incidences reported at 5.3-26% and 1.6-26% respectively. Hematologic malignancies are also known to have a high rate of VTE. Reported incidence values for VTE in certain types of lymphoma are as high as 59.5% and for multiple myeloma are as high as 58% with highly prothrombotic chemotherapy regimens. In comparison, lung and colorectal cancer have lower rates of VTE (1.6-13.6% and 3.1-10.2% respectively) while breast and prostate cancer have the lowest rate (0.4-8.1% and 0.5-1.3% respectively). In fact, cancer type was used in the development of a risk model to predict the development of VTE in cancer patients. The epidemiology and treatment of cancer-induced thrombosis will be discussed in detail in two other reviews in this series by Cannegieter and colleagues and Lee and colleagues. Tissue factor (TF) is a transmembrane receptor that binds plasma factor VII/VIIa and triggers blood coagulation after vascular injury and in various diseases. TF is expressed by many cancer cells, particularly in cancers of epithelial origin. Cancer cells also spontaneously release small membrane microparticles (MPs) bearing TF. An early study proposed that these MPs may explain the prothrombotic state associated with malignancy. In this review, we will summarize studies analyzing the role of TF-positive, tumor-derived MPs (TMPs) in the activation of coagulation and thrombosis in mouse models, and the association between TF-positive MPs and the development of VTE in cancer patients.
**Development of a Venous Thrombus**

Venous clots form on the surface of a largely intact vascular endothelium due to a combination of changes in the vessel wall, blood flow disturbances, and thrombogenic factors in the blood itself (reviewed in1,78,174-177). The most common site for the development of venous thrombi is valve pockets.175,176,178-181 A number of different mouse models have been used to study thrombosis.182 Generally, animal models of thrombosis that do not produce extensive damage to the vessel wall are better models of venous thrombosis than models that expose vessel wall TF. Studies with healthy mice have shown that leukocyte TF contributes to thrombosis in the inferior vena cava (IVC) stenosis model, whereas vessel wall TF drives thrombosis in the IVC ligation and electrolytic injury models.101,183-186 In small vessels, thrombosis has been shown to be dependent on TF expression by neutrophils, hematopoietic cell-derived TF-positive MPs and vessel wall TF.184,185

**MPs**

Cells release 3 types of membrane vesicles: exosomes (50-100 nM), MPs (0.1-1 µm), and apoptotic bodies (1 to 3 µm).71 This review will focus on TF-positive MPs. Cells undergoing apoptosis release apoptotic bodies.72 Exosomes are released from the cell by the fusion of intracellular multivesicular bodies with the plasma membrane.72 A recent study reported that cancer cells undergoing epithelial-to-mesenchymal transition release TF positive exosomes.187 MPs are released from most cell types upon activation, and platelets are the major cellular source of MPs in blood. Indeed, MPs were originally described as “platelet dust”.73,75,78 Importantly, tumor cells spontaneously release MPs.170,171 MPs are formed by the outward blebbing of the plasma membrane and are subsequently released as small phospholipid vesicles after proteolytic cleavage of the cytoskeleton.72,75 There is selective packaging of surface proteins into MPs72 including TF1,170,188 and adhesion molecules.77,81 MPs are procoagulant due to the presence of TF1,78 and the exposure of negatively-charged phospholipids, such as phosphatidylserine
The negatively-charged surface facilitates the assembly of positively-charged coagulation protein complexes. 

Methods for the Quantification of MPs

Several methods have been used for the quantification of TF-positive MPs in human plasma that include antigen- and activity-based assays. We believe that functional assays are the best way to measure TF in MPs because they are the most sensitive methods for detecting the very low levels of active TF in plasma. In addition, the specificity of some TF antigen-based assays has been questioned. One study failed to observe a correlation between MP TF activity and two antigen-based assays (flow cytometry and a commercial TF ELISA). In contrast, our laboratory found a strong correlation between MP TF activity and plasma TF protein levels using an “in-house” human TF ELISA. Several functional assays have been developed that measure TF activity in isolated MPs using either a single-stage clotting assay or a two-stage factor Xa generation assay in the presence or absence of an inhibitory anti-TF antibody. Thaler and colleagues measured levels of MP TF activity in cancer patients using two different activity assays and found a good correlation between the assays. We observed comparable levels of TF activity in MPs isolated from LPS stimulated human whole blood using two centrifugation speeds, either 20,000 xg for 20 minutes (primarily MPs) or 100,000 xg for 1 hour (MPs and exosomes) suggesting that the majority of vesicle TF is on MPs.

Light scatter flow cytometry is the most commonly used method for the quantification of MPs in clinical samples. Several studies have used flow cytometry to detect TF-positive MPs in cancer patients. However, the low sensitivity of flow cytometry makes it difficult to reliably detect the low levels of TF-positive MPs in clinical samples. A recent publication reported that the level of TF-positive MPs measured using a functional MP TF activity assay correlated with the development of VTE in cancer patients, whereas no correlation was found using flow cytometry to measure TF-positive MPs.
Interestingly, we failed to detect TF-positive MPs by flow cytometry in samples from LPS treated whole blood that had high levels of MP TF activity.\textsuperscript{197} Impedance-based flow cytometry is an alternative method for measuring levels of TF-positive MPs\textsuperscript{203} and has been used to detect TF-positive MPs in plasma from cancer patients.\textsuperscript{157,204}

**TF-Positive MPs in Animal Studies**

Animal studies have found that TMPs are released from a variety of tumors in vivo.\textsuperscript{88,172,205} An early study found that guinea pigs bearing hepatocarcinoma tumors had increased procoagulant activity in cell-free ascites fluid that could be pelleted by ultracentrifugation. The presence of membrane vesicles less than 1 µm in size in this pellet was confirmed by electron microscopy.\textsuperscript{172} The procoagulant activity of the MPs released from these tumor cells in vitro was later determined to be TF dependent.\textsuperscript{173} A more recent study found that severe combined immunodeficiency (SCID) mice bearing TF expressing human colorectal tumors had increased levels of tumor-derived human TF protein in plasma.\textsuperscript{205} Further, circulating TF levels correlated with the size of the tumor in these mice.\textsuperscript{205} Circulating TF-positive TMPs have also been found to be associated with the activation of coagulation in mice.\textsuperscript{88,170,187} Specifically, increased circulating levels of human TF protein in nude mice bearing orthotopic human pancreatic tumors was associated with activation of coagulation as monitored by plasma levels of thrombin-antithrombin (TAT) complex.\textsuperscript{170} In vitro studies showed that the plasma from tumor bearing nude mice generated increased thrombin compared to control plasma and this increase could be inhibited by an anti-human TF monoclonal antibody.\textsuperscript{170} A similar elevation in plasma TAT complex levels was seen in SCID mice bearing TF-positive tumors formed by the human squamous cell carcinoma cell line A431.\textsuperscript{187} We found that inhibition of human TF with a species-specific monoclonal antibody reduced TAT levels in nude mice bearing an orthotopic tumor derived from human pancreatic adenocarcinoma cell line HPAF-II.\textsuperscript{88}
However, the relative contribution of TF expression by the TMPs and the tumor cells themselves to the activation of coagulation could not be determined.\textsuperscript{88}

Several studies have also shown that TMPs enhance the development of thrombosis in mice in vivo. Thomas and colleagues found that both endogenously generated and exogenously injected PANC02 TMPs but not tumor cells accumulated at the site of ferric chloride-induced mesenteric vessel injury and laser-induced cremaster arteriole injury.\textsuperscript{87} In addition, accumulation of endogenous TF-positive TMPs in these orthotopic PANC02 pancreatic tumor bearing mice was shown to be P-selectin-dependent. Interestingly, this study found that both human and mouse pancreatic cell lines expressed P-selectin glycoprotein ligand-1 (PSGL-1).\textsuperscript{87} In contrast, we did not detect PSGL-1 expression by 4 different human pancreatic cell lines but found that they bound to an immobilized P-selectin-IgG chimera, which indicated that they expressed a P-selectin ligand (J-G Wang and N. Mackman, unpublished data 2012). Injected exogenous TMPs enhanced thrombosis and this enhancement was abolished with an anti-P-selectin antibody.\textsuperscript{87} We found that mice bearing human pancreatic tumors had increased thrombosis compared to control mice in a ferric chloride saphenous vein model.\textsuperscript{88} However, as discussed below this is not the best model for studying venous thrombosis.

The IVC stenosis model of venous thrombosis is particularly suited for the evaluation of the mechanisms of VTE initiation because thrombosis is triggered by endothelial cell activation and changes in blood flow rather than denudation of the endothelium and vessel wall damage.\textsuperscript{101} It should be noted, however, that there is significant variability in thrombosis observed in the IVC stenosis model.\textsuperscript{88,101,106,206,207} One study reported increased incidence of thrombosis in this model in C57BL/6J mice bearing subcutaneous PANC02 tumors.\textsuperscript{208} In contrast, we did not observe an increase in IVC thrombosis in nude mice bearing TF-positive human HPAF-II pancreatic tumors.\textsuperscript{88} We did find that injection of exogenous TF-positive HPAF-II MPs enhanced thrombosis, although the amount of injected TMPs required to increase thrombosis was 40 times higher than those present in mice with HPAF-II tumors.\textsuperscript{88} At present, it is unclear
the reasons for these different results but it may be due to differences in experimental conditions, the use of different mouse strains, tumor size, and the species of the tumors (mouse versus human). Clearly, further studies are needed to understand how TF-positive TMPs contribute to thrombosis in different mouse models.

**TF Expression in Cancer**

Cancer cells are well known to express TF and release TF-positive MPs. In addition, TF expression increases with histologic grade in different cancer types, including pancreatic cancer. Two studies reported a correlation between the level of TF in pancreatic and brain tumors and VTE. In addition to its proposed role in cancer-associated thrombosis, TF has been shown to be involved in tumor growth and metastasis (reviewed in 36, 211).

Similarly, TF-positive MPs released from cancer cells have been implicated in non-VTE-related cancer processes. Tesselaar and colleagues found that advanced breast cancer patients but not early stage patients had elevated levels of MP TF activity compared with healthy controls. Similarly, we did not detect elevated levels of MP TF activity in a group of 26 early stage breast cancer patients. Thaler and colleagues recently identified a correlation between increased MP TF activity and worsened cancer stage, grade, and survival in patients with metastatic non-resectable pancreatic cancer.

**Circulating TF-positive TMPs and Activation of Coagulation in Cancer Patients**

Numerous studies have analyzed levels of circulating TF-positive MPs in cancer patients. Hron and colleagues used flow cytometry to detect increased levels of TF-positive MPs in advanced colorectal cancer patients (n=20) as compared to age matched healthy controls. Levels of TF-positive MPs in the cancer patients correlated with activation of coagulation as determined by D-dimer levels. A study of early stage prostate cancer patients (n=69) also observed increased MP TF activity in cancer patients with
a modest correlation between MP TF and D-dimer levels.\textsuperscript{193} In glioblastoma patients, plasma levels of TF-positive MPs but not MP TF activity correlated with D-dimer levels.\textsuperscript{214,215} An observational study of one giant cell lung carcinoma patient with a high rate of thromboembolic events reported vesicle-associated plasma TF antigen levels that were 41 times higher than 16 healthy controls.\textsuperscript{188}

Hron and colleagues found that most of the circulating TF-positive MPs in their advanced colorectal cancer patients co-expressed platelet antigen CD41a, indicating that were derived from platelets.\textsuperscript{200} Platelets do not appear to synthesize TF but have been shown to bind monocyte-derived, TF-positive MPs.\textsuperscript{216-218} Recently, we have found that TMPs bind to platelets (Geddings, Bergmeier, Mackman, unpublished data). Therefore, TF present on platelet-derived MPs may be due to the binding of TF-positive TMPs to platelets and re-processing to form platelet-derived MPs. We found an increase in TF activity in combined platelet and MP samples from cancer patients compared with healthy controls.\textsuperscript{219} Therefore, levels of TF activity in a combined platelet and MP sample may be a more accurate measure of thrombotic risk in cancer patients compared to TF activity of MPs alone.

**Cancer Chemotherapy and TF-positive MPs**

Cancer chemotherapy is known to be associated with increased thrombosis.\textsuperscript{125,161} We found that treatment of the human monocytic cell line THP-1 with cytotoxic chemotherapy agents enhanced cellular TF activity without increasing TF expression by increasing cellular PS exposure.\textsuperscript{220} Further, these cells demonstrated increased release of TF-positive MPs.\textsuperscript{220} Increased PS expression or release of PS-positive MPs has also been observed with cytotoxic chemotherapy treatment of other cell-types including endothelial cells, red blood cells, and acute promyelocytic leukemia cells.\textsuperscript{221-224}

Tesselaar and colleagues found that cancer patients receiving chemotherapy do not have elevated plasma MP TF activity in comparison to untreated cancer patients.\textsuperscript{225} We evaluated the effect of chemotherapy on MP TF activity in 26 early stage breast cancer patients.\textsuperscript{213} Plasma samples were analyzed
at days 0, 2, and 8 after the start of chemotherapy during the first 2 cycles of chemotherapy but no increase in MP TF activity was observed.\textsuperscript{213} A recent study suggested that the activation of coagulation in early stage breast cancer patients undergoing chemotherapy may be mediated by the release of free DNA.\textsuperscript{226}

The findings above suggest that mechanisms other than increased levels of circulating TF-positive MPs are responsible for thrombosis during cancer chemotherapy, such as release of nucleic acids and increased cellular PS exposure.

**TF-positive TMPs and VTE in Cancer Patients**

(i) Retrospective studies

The earliest retrospective study that analyzed the link between TF-positive MPs and VTE in cancer patients was performed by Tesselaar and colleagues in 2007 (Table 1.1).\textsuperscript{196} This study included 23 patients with non-resectable pancreatic adenocarcinoma, 27 patients with breast ductal adenocarcinoma (10 early and 17 late stage breast cancer patients), 7 patients with idiopathic VTE, and 37 healthy controls. The mean MP TF activity was significantly higher in pancreatic and metastatic breast cancer patients compared with either healthy controls or idiopathic VTE patients. Interestingly, pancreatic cancer patients had higher MP TF activity than breast cancer patients. Cancer patients with VTE had higher plasma MP TF activity than cancer patients without VTE.\textsuperscript{196} Approximately 50% of MPs in the cancer patients expressed the tumor antigen mucin-1 (MUC-1) on their surface, which suggested that they were derived from the tumor. Further, no MUC-1-positive TMPs were detected by light-scatter flow cytometry after breast tumor resection.\textsuperscript{196}

Zwicker and colleagues observed elevated levels of TF-positive MPs in the plasma of pancreatic, breast, colorectal, ovarian, and non-small cell lung cancer patients using impedance-based flow cytometry.\textsuperscript{204} This study included 30 cancer patients with VTE at study entry and 60 case matched cancer
patient controls without VTE. TF-positive MPs were detected in the plasma of 60% of the cancer patients
with VTE compared to 27% of cancer patients without VTE. Again, 50% of the circulating TF-positive MPs
in 3 pancreatic cancer patients expressed MUC-1. Similar to the study by Tesselaar and colleagues, plasma
levels of TF and MUC-1 double-positive MPs in these 3 patients was reduced after surgical
resection of the primary tumor.

A second study by Tesselaar and colleagues measured plasma MP TF activity levels in 51
unselected cancer patients who presented with VTE in comparison to case matched cancer patients
without VTE. The cancer types in this study included gastrointestinal tract (n=27), genitourinary tract
(n=12), and a variety of other cancer types (n=13). MP TF activity was higher in the VTE patients compared
with the non-VTE patients. Interestingly, pancreatic cancer patients with VTE had the highest levels of MP
TF activity. Increased MP TF activity was found to be associated with decreased cancer patient survival.
Cancer patients with VTE also had increased plasma TAT levels, and there was a modest correlation
between MP TF activity and TAT levels. Similar to Tesselaar and colleagues, we found increased levels
of MP TF activity in cancer patients with VTE (n=53) in comparison to non-VTE patients (n=13) with the
highest levels being in pancreatic cancer patients. A more recent retrospective study also found
increased levels of circulating TF-positive MPs identified by flow cytometry in cancer patients with VTE as
compared to cancer patients without VTE.

(ii) Prospective studies

The earliest prospective study was performed by Khorana and colleagues in 2008. MP TF activity
was measured in the plasma of 10 pancreatic cancer patients receiving chemotherapy. Blood samples
were drawn prior to starting chemotherapy and every four weeks over a 20 week period. MP TF activity
was increased in 2 out of the 10 patients and these 2 patients developed VTE during the study. This is the
first study to provide evidence to support the hypothesis that elevated levels of MP TF activity precede thrombosis and may be predictive of VTE in pancreatic cancer patients.\textsuperscript{194}

Since this initial study, several other studies have evaluated the association between plasma levels of TF-positive MPs and development of VTE in cancer patients. In the second portion of the study by Zwicker and colleagues, they measured plasma levels of TF-positive MPs in cancer patients and then followed these patients for the development of VTE.\textsuperscript{204} This study found a one year VTE rate estimate in patients with detectable TF-positive MPs of 34.8\% (4 out of 16) as compared to 0\% (0 of 44) in patients without detectable TF-positive MPs.\textsuperscript{204} Another study measured MP TF activity in 122 newly diagnosed multiple myeloma patients who were eligible for high dose chemotherapy.\textsuperscript{228} MP TF activity levels were elevated in multiple myeloma patients but were not predictive of future VTE events.\textsuperscript{228} However, the rationale for this study is not clear since neoplastic plasma cells have not been reported to express TF.\textsuperscript{229} Van Doormaal and colleagues followed 43 cancer patients (13 pancreatic) for a period of 6 months for the development of VTE.\textsuperscript{202} 5 patients developed VTE during the study 3 of whom were pancreatic cancer patients. Levels of TF antigen were measured by flow cytometry and ELISA, and TF and TF-factor VIIa activity of MPs were measured using the MP TF activity assay and a fibrin generation test with or without an anti-factor VIIa blocking antibody, respectively.\textsuperscript{202} In addition, levels of PS-positive MPs were measured using flow cytometry and a functional assay. This study found that there was an association between both MP TF activity and MP TF-factor VIIa-dependent fibrin generation and the development of VTE. In contrast, VTE did not correlate with either of the TF antigen-based assays or the level of PS-positive MPs.\textsuperscript{202} These results indicate that it is important to measure levels of MP TF activity and not simply levels of PS-positive MPs.\textsuperscript{230}

The most extensive study to date measured MP TF activity in plasma samples from the Vienna cancer and thrombosis study (CATS).\textsuperscript{198} Four different types of cancer patients (60 pancreatic, 43 gastric, 126 colorectal, and 119 brain) were chosen because these have a relatively high rate of VTE. These
patients were followed for up to two years for the development of VTE. For many samples plasma MP TF activity was measured using both the end point\textsuperscript{195} and kinetic\textsuperscript{196} assays. Pancreatic and gastric cancer patients expressed higher levels of MP TF activity compared with brain and colorectal cancer patients. MP TF activity was associated with decreased survival in pancreatic and gastric cancer patients. Pancreatic cancer patients demonstrated borderline significance for an association between MP TF activity and VTE in the end point assay.\textsuperscript{198}

It is somewhat surprising that level of circulating MP TF activity was not increased in brain cancer patients in the above study.\textsuperscript{198} It is possible that the blood-brain barrier may limit the release of TMPs into the circulation in these patients. Sartori and colleagues found that MP TF activity and TF-positive MPs were increased in 61 preoperative glioblastoma multiforme patients.\textsuperscript{214,215} Levels of TF-positive MPs were also increased over baseline at 7 days and at 1 month following tumor resection.\textsuperscript{215} This increase is most likely due to disruption of the blood-brain barrier during surgery.\textsuperscript{215} Furthermore, the 11 patients that developed VTE had significantly higher levels of non-tumor-derived TF-positive MPs at baseline than the non-VTE patients.\textsuperscript{215} Levels of MP TF activity were not elevated (Bradford and Key, unpublished data).

We have also analyzed MP TF activity in 117 patients with newly diagnosed pancreaticobiliary cancer at all stages. These patients are part of the Roswell Park Cancer Institute Data Bank and Biorepository.\textsuperscript{231} Blood was collected from each patient after cancer diagnosis. In this study, elevated plasma MP TF activity was associated with the future development of VTE and decreased survival.\textsuperscript{231}

\textbf{Timing of Sample Collection for the Prediction of Future VTE in Cancer Patients}

A general issue with prospective studies is that the collection of blood samples may occur many months before the thrombotic event. Most studies rely on a single sample to predict future events. We found that in two pancreatic cancer patients MP TF activity serially increased in the months before the
development of thrombosis. Further, MP TF activity was found to correlate with VTE in a study with a 6 month follow-up but only weakly with a study with a 2 year follow-up. Clearly, it would be better to collect multiple blood samples from each patient which would allow monitoring of levels of TF-positive MPs over time. We have an ongoing study in which we collect 8 blood samples from patients with either advanced pancreatic cancer or advanced colorectal cancer before and during 4 cycles of chemotherapy and monitor for the development of symptomatic and asymptomatic VTE. Using this study design we will further examine the hypothesis that elevated levels of MP TF activity are predictive of symptomatic and asymptomatic VTE in these patients.

Conclusions

In summary, TF is expressed by tumor cells and expression is increased in advanced cancer. Tumor cells spontaneously release high levels of TF-positive MPs that are associated with a prothrombotic state and enhanced thrombosis in animal models. In human cancer patient studies, elevated levels of MP TF activity are predictive of VTE in the majority of studies suggesting that MP TF likely contributes to thrombosis in cancer patients, particularly in pancreatic cancer. However, the current laboratory-based assays need to be improved before they can be used for clinical diagnosis. The clinical utility for the use of plasma MP-TF in the guiding of thromboprophylaxis in cancer patients also remains to be determined. The first study using plasma MP-TF to guide this type of treatment decision has produced promising results. The MicroTEC study found that use of the low molecular weight heparin enoxaparin for thromboprophylaxis in cancer patients with high plasma MP-TF reduced VTE and improved patient survival.

Most studies of cancer patients focus on measurement of MP TF activity rather than TF activity in other cells. However, levels of MP TF activity may represent the tip of the iceberg in terms of the MP-associated procoagulant activity present in the blood of cancer patients as TMPs may be binding to other
vascular cell types. Indeed, a recent study analyzed circulating TF activity in the blood of patients undergoing total knee arthroplasty and found that the majority of circulating TF was associated with monocytes with only ~5% of the TF activity present in the free MP fraction. It is possible that measurement of TF activity in vascular cell populations will be more predictive of thrombosis than MP TF activity. More studies are needed to determine the distribution of TF-positive TMPs in the circulation in animal models and cancer patients and to characterize the tumor markers and adhesion proteins that are present on the surface of the TMPs (Figure 1.3). One study found that P-selectin was required for the TMP-induced enhancement of microvascular thrombosis. However, it seems likely that other receptors contribute to the delivery of TMPs to thrombosis sites and that these receptors may represent good targets for the development of cancer-specific anti-thrombotic drugs.
Figure 1.3: TMP surface proteins and their functions
TMPs are constitutively released from tumors into the circulation. The procoagulant activity of TMPs is mediated by the expression of TF and the exposure of PS on the MP surface. The tumor markers such as MUC-1 can allow for the identification of TMPs in the circulation. Adhesion proteins including P-selectin ligand CD24 and E-selectin ligand CD43 have been proposed to be involved in the binding of TMPs to endothelium and thrombosis sites.\cite{78,87} Delivery of TMP TF to the site of thrombosis can then initiate thrombosis. This diagram is an example of proteins that can be expressed on the surface of TMPs. Protein expression on the surface of TMPs varies with each tumor. Abbreviations: TMP, tumor microparticle; TF, tissue factor; FVIIa, factor VIIa; PS, phosphatidylserine; MUC-1, mucin-1
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**Table 1.1: Studies evaluating the association between TF-positive MPs and VTE in cancer patients**

Abbreviations: PRP - platelet rich plasma; MP – microparticle; TF – tissue factor; VTE – venous thromboembolism; n/a – not applicable
1.5 Focus of This Dissertation

Despite the development of many novel anticoagulant drugs over the past few years, VTE is still a significant public health burden. The studies comprised in this dissertation work to identify mechanisms through which TF+ tumor microvesicles enhance venous thrombosis in order to identify new potential targets for the prevention and treatment of cancer-associated thrombosis. The first study evaluates the usefulness of a relatively novel mouse model of large vein thrombosis by identifying the strengths and weaknesses of the model along with summarizing the technical variations between each site where this surgical model is performed as a first step towards model standardization. The next study then uses this model in combination with another mouse model of large vein thrombosis and studies in washed human platelets to identify a potential mechanism of cancer-associated thrombosis.
1.6 REFERENCES


CHAPTER 2: STRENGTHS AND WEAKNESSES OF THE INFERIOR VENA CAVA STENOSIS MOUSE MODEL OF VENOUS THROMBOSIS

2.1 Introduction

Deep vein thrombosis (DVT) is a significant cause of morbidity and mortality throughout the world. Risk factors for DVT include major surgery, immobilization, trauma, and cancer among others. Many mouse models of thrombosis have been used to study mechanisms of thrombosis, including those involving small and large veins. In this report from the Animal Models Subcommittee of the International Society of Thrombosis and Hemostasis, we summarize the strengths and weaknesses of the inferior vena cava (IVC) stenosis mouse model of venous thrombosis.

2.2 History

In this model, the lumen volume of the IVC is reduced by ~90%. This model was adapted from a comparable rat model called the “St. Thomas Model” involving IVC stenosis followed by endothelial damage with vascular clamps upstream of the stenosis site. Many variants of the St. Thomas model have been described. The IVC stenosis mouse model described in this article was first reported in 2011 and does not involve a vascular damage step. Since then several groups have used the model with slight variations. Thrombosis in this new model is thought to be initiated by the combination of endothelial activation, a reduction in blood flow velocity, and disturbed blood flow upstream of the stenosis site.
2.3 Method

To perform this model, a midline laparotomy is performed on anesthetized male mice that are at least 8 weeks old and 22 grams in weight.\textsuperscript{3,4,7} The IVC is exposed by atraumatic blunt dissection and then carefully separated from the abdominal aorta. A spacer is placed on top of the exposed IVC, and a non-reactive permanent narrowing ligature (7.0 or 8.0 monofil polypropylene) is secured around both the IVC and spacer directly below the renal veins. The spacer is then removed resulting in a ~90% reduction in IVC lumen size at the stenosis site.\textsuperscript{3} Injury to the IVC wall is avoided during the procedure. Mice with any bleeding from the IVC are excluded. The surgical site is then closed and mice are allowed to recover under the influence of appropriate analgesia.

2.4 Sources of Variability

The spacers used for this model range from 0.26 to 0.36 mm in diameter and consist of guide wires, sutures, or blunted needles.\textsuperscript{3,4,6-10} The most common spacers used to date are a 0.36mm guide wire\textsuperscript{4,10} and a blunted 30-gauge needle.\textsuperscript{3,7,8} The effect of spacer length on thrombosis in this model has not been evaluated. The method of surgical anesthesia also varies, with an isoflurane/oxygen mixture being the most common.\textsuperscript{3,4,6-10} There is some debate as to whether side branches should be ligated to control for IVC anatomy variation. A concern with side branch ligation is that it may result in endothelial damage and increase the duration of surgery. One group compared the size of thrombi in mice with and without side branch ligation and found no difference between the two groups.\textsuperscript{10} This same study, however, found that side branches that were less than 1.5 mm from the stenosis site resulted in smaller thrombi.\textsuperscript{10}
2.5 Quantification of Thrombus Size

Thrombus size in this model is typically quantified by measuring thrombus mass or size at a given time point after the induction of stenosis. In order to obtain these measurements mice are sacrificed and the IVC harvested. We and others have extended the utility of this model by using high frequency ultrasonography to image blood flow and quantify IVC thrombus growth in vivo (Figure 2.1 A, B, C).\textsuperscript{10}

2.6 Strengths and Weaknesses of the New Model

It has been well documented that human DVT initiates most often within the valve sinus of large veins on an intact endothelium.\textsuperscript{2} A strength of this model is that thrombosis is initiated in a large vein in the mouse in the absence of major vessel damage. Further, while there are no valves in the IVC, the disturbed blood flow occurring around the stenosis site may mimic changes in blood flow that occur over valves and within valve pockets in large veins in humans (Figure 2.1 D). The thrombi that develop in this model are also histologically similar to human venous thrombi.\textsuperscript{3}

The major concern with this model is that thrombus development is variable. The reported incidence of thrombosis at 48 hours in wild type mice ranges from 44% to 100%.\textsuperscript{3,6,9,10} We have a lot of experience with this model in our lab and followed the development and growth of thrombi in fifteen 14-18 week old C57Bl/6J mice over 48 hours. High-frequency ultrasonography was used to measure thrombus size in each mouse at 3, 6, 9, and 48 hours after the initiation of thrombosis (Table 2.1). As expected, we found that thrombus size at each timepoint along with time to thrombus initiation was variable with thrombi appearing in the IVC anywhere from 3 to 48 hours after stenosis (thrombus area, incidence: 3 hours 0 – 8.5 mm\textsuperscript{2}, 13%; 6 hours 0 – 11.1 mm\textsuperscript{2}, 33%; 9 hours 0 – 10.1 mm\textsuperscript{2}, 40%; 48 hours 0 – 11.1 mm\textsuperscript{2}, 53%). Another limitation of this model is that it is performed in a vein that does not have valves.
2.7 Conclusions

As with any model, this mouse model of venous thrombosis does not accurately reflect all aspects of human DVT. However, as discussed above, it does have certain strengths over other available models. Primarily, the initiation of thrombosis occurs without inducing major vascular damage which more closely mimics human DVT than models triggered by chemical or physical vessel damage. Nevertheless, the high variability in thrombus development is a significant concern that limits its utility to detect small differences between groups.
Figure 2.1: In vivo imaging of the IVC stenosis model

IVC stenosis was performed in C57Bl/6J mice with ultrasound imaging of the stenosis site (A) before and (B) after thrombus formation. (C) Longitudinal thrombus area was measured in mice at 24 hours by ultrasound. Thrombi were then immediately harvested and weighed. Linear regression analysis for longitudinal thrombus area versus thrombus mass identified a strong correlation ($r^2 = 0.96$). (D) Color Doppler imaging reveals a pattern of disturbed blood flow around the stenosis site prior to thrombus development.
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Table 2.1: Size and Incidence of thrombosis in the IVC stenosis model
2.8 REFERENCES


CHAPTER 3: TUMOR MICROVESICLES ENHANCE THROMBOSIS IN A PLATELET-DEPENDENT MANNER

3.1 Introduction

Cancer patients have a 4-fold increased risk of venous thromboembolism (VTE) compared with the general population.\(^2\,^5\) VTE in cancer patients has a significant impact on disease morbidity due to pain, loss of mobility, and interruption of cancer chemotherapy.\(^6\,^7\) Only 12% of cancer patients who develop VTE survive past one year versus 36% of patients who do not develop VTE.\(^8\) Pancreatic cancer patients have been shown to have a particularly high rate of VTE development in comparison to other cancer types.\(^9\,\,^{11}\) Many mechanisms have been proposed to explain the enhancement of thrombosis in cancer patients including increased circulating procoagulant molecules, leukocyte activation, platelet activation, and thrombocytosis.\(^12\) Since cancer is a heterogeneous disease, in all likelihood the mechanisms of cancer-associated VTE are also heterogeneous.

Microvesicles (MVs) are small membrane vesicles released by most cell types, with increased release by activated and apoptotic cells.\(^13\,\,^{14}\) MVs are constitutively released by tumor cells, and we have designated them as tumor-MVs (TMVs).\(^15\,\,^{16}\) They are distinct from other membrane vesicles including apoptotic bodies and exosomes due to their size, mechanism of release, and presence of characteristic surface proteins.\(^17\) Importantly, tissue factor (TF) makes MVs highly procoagulant.\(^18\,\,^{19}\) TF is the primary initiator of the extrinsic pathway of the coagulation cascade that culminates in thrombin generation and fibrin formation.\(^20\,\,^{25}\) Thrombin is also a potent platelet activator, mediating the activation of platelets via Par1 and Par4 in humans and Par3 and Par4 in mice.\(^26\) Several studies have reported increased plasma TF+ MVs in patients with multiple cancer types.\(^27\,\,^{29}\) Further, plasma
levels of TF+ MVs and MV TF activity have been shown to increase prior to VTE in pancreatic cancer patients, which suggests a role for TF+ MVs in cancer-associated thrombosis.27,28,30,31

Tumor cell TF expression has been shown to increase with histologic grade in many cancer types, including pancreatic cancer.32-34 The MVs released from these tumors into the blood via leaky tumor microvasculature also express TF.15,35,36 TMVs are released into the circulation by tumor cells at high levels even before tumor metastasis.29,37,38

While venous clots have less platelets and more red blood cells than arterial clots, there is clinical evidence that platelets play a role in recurrent VTE. For instance, a meta-analysis of two clinical trials of the platelet inhibitor aspirin showed a reduction in VTE.39-42 In cancer patients, thrombocytosis is a risk factor for the development of VTE and decreased survival.43-46 In addition, cancer patients have increased plasma markers of platelet activation.46-52 Platelets from patients with metastatic cancer also exhibit hyperreactivity to low dose agonists.46,53 Platelets have also emerged as a key mediator of tumor growth, angiogenesis, and metastasis, making an understanding of their role in cancer important.42

Previous studies have suggested a role for TF+ TMV-initiated platelet activation in cancer-associated thrombosis.36,54,55 However, the role of TF+ TMVs in platelet activation and thrombosis has not been studied directly. The goal of this study was to determine if TF+ TMVs activate platelets in vitro and enhance thrombosis in mice, in part, via platelet activation.
3.2 Materials and Methods

Reagents and antibodies

Clopidogrel was obtained from Bristol-Myers Squib (New York, NY, USA). Prostaglandin E1 (PGE1) was obtained from Cayman Chem (Ann Arbon, MI, USA). HTF-1 was a generous gift from Dr. Ronald Bach (University of Minnesota, Minneapolis, MN, USA). The substrate benzyloxycarbonyl-Gly-Gly-Arg-7-amido-4methylcoumarin• HCl (Z-GGR-AMC) was obtained from Bachem (Torrance, CA, USA). AnnV- Pac Blue was obtained from Life Technologies (Grand Island, NY, USA). Megamix beads were obtained from Biocytex (Marseille, France). Anti-CD41a-PE (clone HIP8), anti-CD62P-APC (clone AK-4), Pac-1-FITC, PE-IgG isotype control, and anti-CD142-PE (HTF-1) were obtained from BD Biosciences (San Jose, CA, USA). Citrated mouse plasma, mouse IgG, apyrase, 2 MesAMP, and acid citrate dextrose (ACD) were obtained from Sigma (Saint Louis, MO, USA). Sodium citrate was obtained from Ricca Chemical Co (Pocomoke City, MD, USA). Hirudin was obtained from Accurate Chem and Scientific (Westbury, NY, USA). Active-site inhibited FVIIa (FVIIai) was obtained from American Diagnostica (Stamford, CT, USA). Human α-thrombin was obtained from Thermo Scientific (Waltham, MA, USA). Recombinant relipidated human TF (Innovin™) was obtained from Dade Behring (Liederbach, Germany). 5(6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) was obtained from eBioscience (San Diego, CA, USA).

Cell Lines

The human pancreatic adenocarcinoma cell lines BxPc-3 and L3.6pl were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in RPMI (Gibco, Grand Island, NY, USA) plus 10% fetal bovine serum (Omega Scientific, Tarzana, CA, USA) and 1% penicillin streptomycin (Gibco, Grand Island, NY, USA). TMVs were isolated from 72 hour serum free cell culture supernatant. Apoptotic bodies and cell debris were precleared from culture supernatant by
centrifugation at 3000 x g for 5 minutes. TMVs were then pelleted at 20,000 x g for 20 minutes at 4°C, resuspended in PBS, and stored at -80°C until use. MV concentration was determined by protein content using a BCA protein assay (Thermo Scientific, Rockford, IL, USA).

**TF Activity Assays**

Cellular TF activity was determined by one stage clotting assay using a Start4 clotting machine (Diagnostica Stago, Parsippany, NJ, USA) as described. MV TF activity was determined by two-stage chromogenic assay in the presence and absence of inhibitory anti-human TF antibody HTF-1 as described and normalized to protein content after TF activity was determined.

**Flow Cytometry**

Washed human platelets were resuspended to a concentration of 5 x 10^8 plt/mL in Tyrode’s buffer (137 mM NaCl, 2.7 mM KCl, 2 mM MgCl_2, 0.42 mM NaH_2PO_4, 5 mM glucose, 10 mM Hepes, pH 7.3-7.4) containing 1 mM CaCl_2 with or without the addition of human plasma as indicated. Platelets were preincubated with indicated inhibitors for 5 minutes at room temperature in the presence of 2 µL anti-CD41a-PE, anti-P-selectin-APC, and Pac-1-FITC. TMVs were incubated with platelets for 15 minutes at 37°C in a final reaction volume of 30 µL. Platelet reactions were then diluted to 500 µL with Tyrode’s buffer containing 0.5% formalin. For MV ligand studies, 0.1 µg of MVs were stained with 2 µL CD142-PE and AnnV-Pac Blue in 100 µL of staining buffer (140 mM NaCl, 10 mM Hepes, 5 mM CaCl_2) for 30 minutes on ice. Samples were diluted to 500 µL before running. Megamix beads were used to set an upper size limit of 1 µm for MVs. Negative controls for each staining antibody used for MV studies included using the same volume of antibody in MV-free plasma (total MVs removed by ultracentrifugation at 100,000 x g for 4 hours). Platelet and MV samples were analyzed using a
Stratedigm Ex1000 flow cytometer (Stratedigm, San Diego, CA). Data were analyzed using Flowjo version X.0.7 software.

Human Subjects

Healthy non-pregnant adults were recruited into this study under IRB approval from the office of Human Research Ethics at UNC and informed consent obtained. Blood was collected from the antecubital vein into ACD and 1 μg/mL PGE1. The first 3 mL of blood was discarded. Platelet rich plasma (PRP) was isolated by centrifugation at 230 x g for 15 minutes at room temperature. PRP was then spun at 920 x g for 10 minutes with one Tyrode’s buffer wash step to isolate platelets. Platelets were resuspended in Tyrode’s buffer without calcium. Platelets rested at 37°C for at least 20 minutes prior to experimentation.

Electron Microscopy

Isolated MVs were adsorbed onto copper grids covered by thin glow-charged carbon foils and stained for 5 minutes with 2% (wt/vol) uranyl acetate. Samples were examined in a Tecnai 12 transmission electron microscopy (TEM) (FEI, Hillsboro, OR) at 80 kV, and images were captured on a Gatan Orius charge-coupled device (CCD) camera programmed with Digital Micrograph software (Gatan, Warrendale, PA).

Thrombin Generation Assay

The thrombin generation assay was adapted from a previous protocol. Platelets were resuspended to 5X10⁸/μL in Tyrode’s with plasma at the indicated dilutions and combined with a thrombin substrate solution (416 μM z-GGR-AMC, 15 mM CaCl₂, 1.5 M NaCl, 200 mM HEPES, pH 7.4). Thrombin generation was initiated by addition of BxPc-3 or L3.6pl MVs (50 μg/mL) as indicated, and
monitored by SpectraMax M5 fluorescent plate reader (Molecular Devices, Sunnyvale, CA, USA) over 30 minutes. Thrombin generation curves were generated as a function of the rate of change of sample fluorescence.

**Platelet Aggregometry**

Human or mouse washed platelets were resuspended to a concentration of $6 \times 10^8$ plt/mL in Tyrode’s buffer containing 1 mM CaCl$_2$, 0.35% BSA, and 1:100 mouse or human plasma. Inhibitors were preincubated as indicated and aggregation was triggered by the addition of BxPc-3 and L3.6pl MVs at a final concentration of 50 µg/mL. Reactions were performed under stirring conditions (1200 RPM) at 37°C in a Chrono-log 4-channel optical aggregation system (Chrono-log, Havertown, PA). Data are presented as mean absorbance.

**Mice**

Studies were approved by the Institutional Animal Care and Use Committee at the University of North Carolina and comply with NIH guidelines. C57Bl6/J mice were obtained from Jackson laboratories (Bar Harbor, Maine, USA). Par4 knockout mice (-/-) were a generous gift from Dr. Mark Zogg (Blood Center of Wisconsin, Milwaukee, WI) and crossed with C57Bl6/J mice to obtain WT (+/-) littermate controls. Par4+/mice were identified by PCR (Forward 5’ CAGATGTTCCTGGCTGTTG 3’, Reverse WT 5’ ATTGTGGGCTCAGTGCTCCC 3’, Reverse KO 5’ CAGGGTTTTCCCAGTCAGCGC 3’). Wild-type mice were treated with clopidogrel (75 mg/kg) by oral gavage 24 hours and 1 hour before experimentation. Efficacy of this treatment was confirmed by the inability of platelets in clopidogrel treated mice to respond to 2.5 µm ADP (MFI JonA-PE binding saline no ADP 20.7 +/- 2.8; saline + ADP 38.9 +/- 3.2; clopidogrel no ADP 20.7 +/- 1.4; clopidogrel + ADP 13.4 +/- 0.94). All experiments used 8-
22 week old male mice. For inferior vena cava (IVC) stenosis experiments, all mice weighed at least 30 grams.

To obtain mouse platelets, blood was drawn from the IVC under isoflurane anesthesia into syringes containing a final concentration of 0.32% sodium citrate and 1 µg/mL PGE1. PRP was isolated by sequential centrifugation at 130 x g and then 100 x g for 5 minutes at room temperature. Platelets were then isolated by centrifugation at 700 x g for 5 minutes at room temperature with 1 Tyrode’s buffer wash step.

**Mouse Thrombosis Models**

The electrolytic injury model of the femoral vein was performed as described.\(^{59,60}\) The IVC stenosis mouse model of venous thrombosis was performed as described.\(^{61}\) Briefly, stenosis of the IVC was performed around a 30G needle using 8.0 monofilament polypropylene sutures without side branch ligation. Thrombus growth was quantified over 24 hours in vivo by high-frequency ultrasonography on a Visualsonics Vevo® 2100 (Toronto, Ontario, Canada) ultrasound machine. MV dose was titrated to enhance thrombosis without inducing high mortality.

**Statistical Analysis**

Data are presented as mean +/- SEM. TF activity and TF antigen expression data are analyzed by unpaired student’s t-test. Flow cytometry, thrombin generation, and thrombosis model data are analyzed by one or two-way ANOVA as indicated with Bonferroni posttests comparing indicated pairs of data. Survival data are analyzed using Log-rank (Mantel-Cox) Test. Statistical significance is defined on each figure. Statistical analysis were performed on GraphPad Prism software version 5.01.
3.3 Results

TMV characterization

We studied 2 TF-expressing human pancreatic adenocarcinoma cell lines that expressed high (BxPC-3) and low (L3.6pl) levels of TF (Figure 3.1A). BxPc-3 and L3.6pl cells are moderately and poorly differentiated pancreatic adenocarcinoma cell lines, respectively. The BxPc-3 cell line was established from a primary human tumor whereas the L3.6pl cell line was established from a more metastatic isolate of the human pancreatic adenocarcinoma cell line COLO 357. MVs isolated from the cell culture supernatant of each of these cell lines showed a similar pattern of TF expression as the parental cell line with BxPc-3 MVs having a high MV TF activity and L3.6pl MVs having a low MV TF activity (Figure 3.1B). TF antigen expression on MVs was evaluated by MV flow cytometry (BxPc-3, mean fluorescence intensity (MFI) of HTF-1-PE binding 31.7 +/- 3.12 and L3.6pl, MFI of HTF-1-PE binding 3.6 +/- 0.04) (Figure 3.1C). TMVs isolated from BxPc-3 and L3.6pl cells were also imaged using electron microscopy to confirm that isolated MVs were of the expected size and shape and essentially free of cells and apoptotic body contamination (>95% vesicles <0.9 µm in size) (Figure 3.1D).

Binding of TF+ TMVs to platelets

There are at least two potential mechanisms by which TF+ TMVs could initiate the activation of platelets. TMVs could either carry a platelet activating protein on their surface, such as podoplanin, resulting in direct platelet activation, or they could trigger the generation of a platelet activator, such as thrombin. In order to differentiate these potential pathways we first evaluated the ability of BxPc-3 and L3.6pl TF+ TMVs to bind to washed human platelets in vitro. TMVs were stained with CFSE. This dye is only fluorescent after being activated by intracellular esterases, meaning a cell (or vesicle in this case) must have an intact plasma membrane in order to become fluorescent. The binding of CFSE-
labeled BxPc-3 and L3.6pl MVs to washed human platelets was quantified by flow cytometry (Figure 3.1E). Both MV types bound to platelets in a concentration-dependent manner (Figure 3.1E).

**TF+ TMV-initiated thrombin generation in the presence of platelets**

We examined the ability of BxPc-3 and L3.6pl TF+ MVs bound to platelets resuspended in Tyrode’s buffer containing diluted citrated human plasma to generate thrombin. Experiments were performed in the presence of diluted human plasma rather than heparinized or citrated PRP in order to avoid inhibition of the coagulation cascade and to prevent the changes in platelet signaling associated with recalcifying undiluted citrated PRP. TF+ MVs from both cell lines triggered thrombin generation in a plasma concentration-dependent manner (Figure 3.2A). As expected, washed platelets alone did not support thrombin generation. We observed more thrombin generation in the presence of platelets (Figure 3.2A). TF+ MV-dependent thrombin generation was reduced by an anti-TF antibody (Figure 3.2B).
Figure 3.1: TF expression by BxPc-3 and L3.6pl pancreatic adenocarcinoma cells and MVs.
(A) Cellular TF activity was measured using a one stage clotting assay. *p<0.001, n=6. (B) MV TF activity was measured using a two-stage clotting assay. *p<0.005, n=4. TF activity was normalized to total protein. Data were analyzed by unpaired student’s t-test on Graphpad Prism software v5.01. (C) TF antigen expression on MVs was analyzed by MV flow cytometry. Representative histograms are shown for TF expression on MV-sized, PS-positive events for anti-TF-PE (black) and PE isotype control (grey). (D) BxPc-3 and L3.6pl MVs were imaged by transmission electron microscopy. (E) BxPc-3 and L3.6pl TMVs were stained with 4 µm CFSE fluorescent dye followed by 2 wash steps at 20,000 x g for 20 minutes. The indicated concentrations of stained TMVs or wash supernatant were incubated with human platelets in the presence of 1:100 diluted human plasma for 15 minutes at 37°C in the presence of anti-human CD41a-PE. TMV-platelet binding was quantified by measuring the percentage of CFSE-positive platelet events by flow cytometry. A representative experiment of 3 independent experiments is shown.
A

BxPc-3 MVs  L3.6pl MVs

Lag Time (min)

BxPc-3 MVs  L3.6pl MVs

Peak Thrombin Gen (nM)

B

BxPc-3  L3.6pl  BxPc-3  L3.6pl

Lag Time (min)

Peak Thrombin Gen (nM)
Figure 3.2: L3.6pl and BxPc-3 TMVs trigger the generation of thrombin.

(A) BxPc-3 and L3.6pl TMVs (50 µg protein/mL) were incubated with indicated dilutions of human plasma in the presence and absence of human washed platelets. Thrombin generation was monitored over 30 minutes by monitoring the consumption of a fluorogenic thrombin substrate (z-GGR-AMC).

(B) BxPc-3 and L3.6pl TMVs (50 µg protein/mL) were incubated with washed human platelets in the presence of 1:100 diluted human plasma in the presence of IgG (50 µg/mL), HTF-1 (50 µg/mL), Hirudin (1 U/mL), or vehicle control. Thrombin generation was monitored over 30 minutes by monitoring the consumption of a fluorogenic thrombin substrate (z-GGR-AMC). Thrombin generation curves were generated as a function of the rate of change of sample fluorescence and lag time and peak thrombin generation calculated for each sample. *p<0.001, n=3. Data were analyzed by one-way ANOVA with Bonferroni posttests comparing indicated pairs of data on Graphpad Prism software v5.01.
TF+ TMV-initiated platelet activation

In order to differentiate the direct versus indirect activation of platelets by TF+ TMVs, platelet activation was performed in the presence and absence of plasma. TF+ TMV-induced platelet activation was dependent on the presence of plasma for MVs from both cell lines (Figure 3.3A). The ability of BxPc-3 and L3.6pl TF+ TMVs to activate platelets in the presence of 1:100 human plasma was inhibited by the anti-TF blocking antibody HTF-1 (Figure 3.3B). The percent inhibition of platelet activation by HTF-1 for BxPc-3 MVs was 94.1% and for L3.6pl MVs was 100.0%. The involvement of the extrinsic pathway of the coagulation cascade was also confirmed using FVIIai. The percent inhibition for FVIIai of platelet activation by BxPc-3 TMVs was 93.2% and for L3.6pl TMVs was 100% (Figure 3.3C).

As demonstrated above, TF+ TMVs trigger the generation of thrombin in the presence of plasma, making thrombin the most likely candidate for the indirect mediator of TF+ TMV-induced platelet activation. To test this notion, BxPc-3 and L3.6pl TF+ TMVs were incubated with platelets and diluted plasma in the presence of the direct thrombin inhibitor hirudin, along with inhibitors of secondary mediators of thrombin-initiated platelet activation. Platelet activation was quantified by measuring platelet P-selectin expression by flow cytometry. Hirudin inhibited both BxPc-3 (percent inhibition 93.6%) and L3.6pl (percent inhibition 99.7%) TF+ TMV-induced platelet activation (Figure 3.3C). TMV-induced platelet activation was also inhibited by ADP scavenger apyrase or P2Y12 receptor inhibitor 2MesAMP (Figure 3.3C).
Figure 3.3: TMVs induce platelet activation in a thrombin and TF-dependent manner.
(A) BxPc-3 and L3.6pl TMVs (50 µg protein/mL) were incubated with washed human platelets in the presence and absence of diluted human plasma (1:100). Platelet activation was monitored by flow cytometry for PAC-1 binding and P-selectin expression. Data are presented as the mean fluorescence intensity (MFI) of total CD41a positive events (+/- SEM). Data are representative of 3 independent experiments. *p<0.01, n=3. Data were analyzed by one-way ANOVA with Bonferroni posttests comparing indicated pairs of data on Graphpad Prism software v5.01. (B) BxPc-3 and L3.6pl TMVs (50 µg protein/mL) were preincubated with anti-TF blocking antibody HTF-1 (50 µg/ml) or IgG control (50 µg/ml) for 30 minutes at 4°C. TMVs were then incubated with human platelets in the presence of diluted human plasma (1:100). Data are presented as the MFI of total CD41a positive events (+/- SEM). Representative scatter plots are also shown. Data are representative of 3 independent experiments. *p<0.0001, n=3. (C) Platelets were preincubated with inhibitors as indicated for 5 minutes at room temperature. BxPc-3 and L3.6pl TMVs (50 µg/mL) were then incubated with human platelets in the presence of diluted plasma for 15 minutes at 37°C. Recombinant TF (InnovinTM) (0.5 pg) and alpha-thrombin (5 nm) were used as controls. After the activation reaction was complete, the reactions were further diluted in Tyrode’s buffer + 1 mM CaCl₂ + 0.5% formalin and P-selectin expression evaluated by flow cytometry. Data are presented as the mean fluorescence intensity (MFI) of total CD41a positive events (+/- SEM). Representative scatter plots are also shown. Data are representative of 3 independent experiments. *p<0.0001, n=3. Data were analyzed by one-way ANOVA with Bonferroni posttests comparing all data to the no MV control on Graphpad Prism software v5.01.
TF+ TMVs induce platelet aggregation

BxPc-3 and L3.6pl TF+ TMVs were both found to induce platelet aggregation in the presence of 1:100 diluted human plasma (Figure 3.4A, B). Consistent with our previous results, BxPc-3 and L3.6pl TF+ TMV-induced platelet aggregation was dependent on the presence of both MV TF (Figure 3.4A) and thrombin (Figure 3.4B). BxPc-3 TF+ TMVs have a high level of TF activity and triggered platelet aggregation more quickly than L3.6pl TMVs in the presence of control antibody or vehicle control (Figure 3.4A and B). In addition, BxPc-3 TF TMV-induced platelet aggregation was faster and was only partially inhibited by HTF-1, whereas L3.6pl TMV induced platelet aggregation was completely inhibited (Figure 3.4A).

BxPc-3 and L3.6pl TF+ TMVs also triggered mouse platelet aggregation in the presence of 1:100 diluted mouse plasma (Figure 3.4C). Similar to human platelets, TF+ TMV-induced mouse platelet aggregation was inhibited by hirudin. Hirudin completely inhibited platelet aggregation induced by L3.6pl MVs but only partially inhibited platelet aggregation induced by BxPc-3 MVs.
Figure 3.4: TMVs induce platelet aggregation in a thrombin and TF-dependent manner.
Washed human platelets were resuspended in Tyrode’s buffer containing human plasma at a dilution of 1:100. (A) MVs were pre-incubated with anti-TF blocking antibody HTF-1 (50 µg/ml) or IgG control (50 µg/ml) for 30 minutes prior to being added to platelets. Platelets were then stimulated with either BxPc-3 or L3.6pl TMVs (50 µg protein/mL). (B) The direct thrombin inhibitor hirudin (1 U/ml) or vehicle control were preincubated with platelets for 5 minutes at room temperature prior to the addition of MVs. Platelet aggregation was monitored by light transmittance and reported as percent absorbance. Tracings are representative of 3 independent experiments. (C) Washed mouse platelets were re-suspended in Tyrode’s buffer containing mouse plasma at a dilution of 1:100. Hirudin, or vehicle control were preincubated with platelets for 5 minutes at room temperature. Platelets were then stimulated with BxPc-3 or L3.6pl TMVs (50 µg protein/mL). Platelet aggregation was monitored by light transmittance and is reported as percent absorbance.
**TMV-TF enhances thrombosis in mice**

Due to the fact that TF+ TMVs derived from the human pancreatic adenocarcinoma cell lines BxPc-3 and L3.6pl activate and aggregate mouse platelets in the presence of mouse plasma, we evaluated the effect of TF+ TMVs on venous thrombosis in mice. In vivo studies were performed using TMVs from the TF-high cell line BxPc-3. We examined the effect of TF+ TMVs in the IVC stenosis thrombosis model because it imitates some important features of human DVT.\(^6^1\) Injection of BxPc-3 TF+ TMVs into mice 1 hour following the induction of IVC stenosis resulted in the development of larger thrombi in mice at 3, 6, 9, and 24 hours following stenosis (Figure 3.5A). BxPc-3 TMVs pretreated with HTF-1, however, did not increase thrombus size in this model. BxPc-3 TF+ TMV infusion lead to death of 40% of the mice and this effect was abolished by blocking human TF (Figure 3.5A). TF+ TMV-enhanced platelet and fibrin accumulation at the site of electrolytic injury in the mouse femoral vein was also significantly reduced by HTF-1 (Figure 3.5B).

**Impairment of platelet activation reduces TF+ TMV enhancement of thrombosis in mice**

With the knowledge that TF+ TMV-enhanced thrombosis in mice is dependent on MV TF and MV TF leads to thrombin dependent platelet aggregation and activation in vitro, we evaluated whether platelets were involved in TF+ TMV-enhanced thrombosis in vivo. The thrombus size was significantly smaller in Par4 deficient mice injected with TF+MVs compared to wild-type littermates injected with TF+ MVs (Figure 3.6A). Furthermore, TF+ TMVs increased mortality in wild-type mice but not Par4\(^{-/-}\) mice (Figure 3.6A). Next, we examined the effect of pharmacological inhibition of platelets in wild-type mice using the P2Y12 receptor inhibitor clopidogrel. Wild-type mice that had
been treated with clopidogrel or saline underwent IVC stenosis followed by i.v. injection of BxPc-3 TF+ MVs. Clopidogrel-treated mice injected with TF+ MVs developed significantly smaller thrombi than saline treated controls (Figure 3.6B). In addition, TF+ TMVs induced less mortality in clopidogrel treated mice in comparison to controls (Figure 3.6B).
**A**

**Thrombus incidence**

<table>
<thead>
<tr>
<th></th>
<th>3 hours</th>
<th>6 hours</th>
<th>9 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>14%</td>
<td>42%</td>
<td>57%</td>
<td>57%</td>
</tr>
<tr>
<td>BxPc-3 IgG</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>BxPc-3 HTF-1</td>
<td>57%</td>
<td>57%</td>
<td>57%</td>
<td>71%</td>
</tr>
</tbody>
</table>

**% Survival**

No MVs | BxPc-3 + IgG | BxPc-3 + HTF-1

* indicates a significant difference.
Figure 3.5: TMVs enhance thrombosis in mice in a TF-dependent manner.

(A) Thrombosis in mice was induced by partial ligation (stenosis) of the inferior vena cava (IVC). Following stenosis, mice were injected i.v. with (i) PBS (white bars), (ii) BxPc-3 TMVs pre-incubated with IgG (black bars), or (iii) BxPc-3 TMVs pre-incubated with HTF-1 (1 mg/mL) (grey bars). TMVs were injected at 5 minutes, 30 minutes, and 1 hour after the induction of stenosis (90 ng MVs by protein content in total). Thrombus formation was quantified in mice using high-frequency ultrasonography. Data is present as mean +/- SEM. Percent incidence of thrombosis is reported below each bar. *p<0.01. PBS n=7, BxPc-3 n=8, BxPc-3 + HTF-1 n=7. Data were analyzed by two-way ANOVA with Bonferroni posttests comparing all data to the no MV control at each time point on Graphpad Prism software v5.01. Mouse survival following IVC stenosis is also shown. Mortality was defined as death during the 1 hour following stenosis during which time MVs were being administered. *p<0.05 by log-rank (Mantel-Cox) test.

(B) Thrombosis in mice was induced by electrolytic injury of the femoral vein. Mice were injected i.v. with vehicle (white circles), BxPc-3 TMVs (30 ng) pretreated with IgG (50 µg/mL) (black line), or BxPc-3 TMVs (30 ng) pre-treated with HTF-1 (50 µg/mL) (grey line) immediately prior to injury and 30 minutes following injury. Thrombus size is shown as relative platelet and fibrin intensity over time. *p<0.001, n=8. Data were analyzed by one-way ANOVA with Bonferroni posttests comparing all data sets to the no MV control on Graphpad Prism software v5.01.
Figure 3.6: TMVs enhance thrombosis in mice in a platelet-dependent manner.

(A) Par4 KO mice (−/−) or WT (+/+ ) littermate controls underwent the IVC stenosis model followed by BxPc-3 TMV treatment as above. Thrombus development was quantified by high-frequency ultrasonography. (p<0.001 +/+ n=6, −/− n=5). Data were analyzed by one-way ANOVA on Graphpad Prism software v5.01 and are statistically significant for Par4 +/+ MVs versus Par4 −/− MVs. Mouse survival following IVC stenosis is also shown. p=0.17 by log-rank (Mantel-Cox) test. (B) Clopidogrel or vehicle treated mice were subjected to IVC stenosis followed by BxPc-3 TMV administration as above and thrombus formation was quantified by high-frequency ultrasonography. p<0.001. BxPc-3 TMVs + vehicle n=5, BxPc-3 TMVs + clopidogrel n=6. Data were analyzed by one-way ANOVA on Graphpad Prism software v5.01 and are statistically significant for MVs + clopidogrel versus MVs + vehicle control. Mouse survival following IVC stenosis is also shown. p=0.13 by log-rank (Mantel-Cox) test. (C) Tumor cells spontaneously release TMVs which carry TF. TF initiates the extrinsic pathway of the coagulation cascade, resulting in thrombin generation. Local thrombin generation causes fibrin polymerization and activates platelets. Activated platelets and fibrin can then contribute to the development of cancer-associated thrombosis, including VTE along with other potential roles of platelet activation in cancer patients that are not discussed in this paper.
3.4 Discussion

The current study shows that TF+ TMVs derived from 2 human pancreatic cell lines bind to platelets, leading to an enhancement of thrombin generation in the presence of plasma. This thrombin generation then leads to TF+ TMV-dependent platelet activation and aggregation. Further, we found that the activation of platelets by TF+ TMVs was necessary for TF+ TMVs to enhance thrombosis in a mouse model of large vein thrombosis.

The enhancement of thrombin generation triggered by TF+ TMVs in the presence of platelets can be attributed to the accumulation of MV TF on or near the platelet surface. Our data shows that the binding of TF+ TMVs triggers a low level of thrombin generation that initiates platelet activation. Activated platelets increase their exposure of negatively charged phosphatidylserine (PS), facilitating the assembly of coagulation factor complexes and enhancing further thrombin generation. Once TF+ TMV-initiated thrombin generation reaches a threshold, platelet activation and aggregation occurs. Specifically, thrombin interacts with PARs on the platelet surface, triggering the generation of secondary messengers that then amplify the platelet activation signal. Our results support the involvement of the secondary messenger ADP and its platelet receptor P2Y12 in TF+ TMV-induced platelet activation, which are both known to be downstream of thrombin induced platelet activation. This is not the first time that platelet activation has been shown to occur in the presence of a TF source. LPS activated monocytes, which express TF, trigger platelet activation in the presence of added coagulation factors. The enhancement of thrombin generation in the presence of platelets is consistent with previous studies demonstrating that the presence of platelets changes thrombin generation assay parameters, and platelet agonists increase thrombin generation in PRP.

Previous studies have shown that TF+ MVs from LPS stimulated human monocytes and human monocytic cell lines bind and transfer TF to platelets. We show that BxPc-3 and L3.6pl TMVs both bind to platelets in vitro. Platelets do not appear to express TF themselves and the binding of TF+ MVs
may explain reports of TF+ platelets in different diseases. WT mice with low TF bone marrow have reduced circulating TF+ MVs. These mice have been found to have decreased thrombosis following laser injury of cremaster arterioles. Accumulation of TF+ MVs at the site of thrombosis in this model is dependent on MV PSGL-1 and platelet P-selectin. One study reported that pancreatic tumor lines express PSGL-1 and that inhibition of P-selectin reduced the enhanced thrombosis observed in tumor-bearing mice. However, we did not detect PSGL-1 on BxPc-3 or L3.6pl cells (data not shown). Importantly, we propose that TF+ MVs released from tumors may bind to platelets in a similar manner to monocyte-derived TF+ MVs. This would greatly increase the half-life of the TF+ TMVs in the circulation compared with cell-free TMVs, creating a procoagulant and prothrombotic state in cancer patients.

Non-cancer patients who develop a DVT or PE are treated acutely with low-molecular-weight-heparin (LMWH) and then bridged to a vitamin K antagonist (VKA). However, clinical trials have found that long-term LMWH is more effective at reducing VTE recurrence in cancer patients than VKAs suggesting that the mechanism of action of VTE in cancer patients is different from non-cancer patients. However, 6-9% of cancer patients treated with LMWH still have recurrent VTE despite therapy. Two retrospective studies examined the effect of aspirin on VTE in breast and pancreatic cancer patients. These studies found no effect in breast cancer patients and a marginal effect (p=0.053) in pancreatic cancer patients. In specialized cases such as multiple myeloma patients treated with either thalidomide or lenalidamide aspirin was as effective as LMWH at reducing VTE. Our study shows that TF+ TMVs enhance thrombosis in vivo in a MV TF-dependent manner, identifying a potential new mechanism of thrombosis in cancer patients. Further, we have found that TMV TF-enhanced venous thrombosis is platelet-dependent. These findings are consistent with a previous study which found that clopidogrel reduced microvascular thrombus size in mice bearing mouse pancreatic adenocarcinoma tumors. Of note, previous clinical studies looking at anti-platelet agents
for thromboprophylaxis in ambulatory cancer patients used a general platelet inhibitor as opposed to targeting the thrombin-initiated pathways identified as significant in our study.\textsuperscript{85-88} Also, these clinical trials have not yet targeted patients with pancreatic cancer or patients with high levels of plasma MV TF activity. Our data identifies these specific cancer patient populations as the most likely to benefit from platelet-specific interventions.

The novelty of the current study is that it provides evidence that TF+ TMVs can contribute to the activation of platelets in a TF- and thrombin-dependent manner and that the interaction between TMV TF and platelets contributes to the enhancement of venous thrombosis a mouse model (Figure 3.6C). These findings provide further evidence that an anti-platelet agent that targets thrombin-mediated platelet activation, such as clopidogrel, may provide effective alternative thromboprophylaxis for some cancer patients with high plasma MV TF.

### 3.5 Supplementary Discussion

The following section addresses discussion points that go beyond what was included in the submitted manuscript above. It covers subjects highlighted during the oral defense on this topic and by reviewers of my submitted manuscript.

In order for TMVs to contribute to clot formation they likely need to accumulate at potential sites of thrombosis. In order to accumulate at sites of thrombosis MVs must dock either directly to the thrombosis site or to blood cells that then bind to the thrombosis site. Potential mechanisms for TMV docking in cancer patients are a major topic of discussion in the cancer-associated thrombosis literature and are only briefly mentioned in this manuscript. P-selectin and fibrinogen receptor inhibitors have been shown to reduce the docking of labeled mouse pancreatic adenocarcinoma TMVs to sites of laser-induced microvascular injury in vivo.\textsuperscript{36,55} There is also evidence for ligand-mediated docking by non-tumor MV sources at sites of thrombosis.\textsuperscript{89} Receptors which have been shown to play
a role in MV-platelet docking in vitro include scavenger receptor CD36, CD15, P-selectin, PSGL-1, and the phospholipid phosphatidyl serine.\textsuperscript{70,71,90} TMV docking to blood or endothelial cells could also result in cell activation. Of particular relevance to this study, human umbilical vein endothelial cell-derived MVs, monocyte MVs, platelet MVs, and unselected human blood MVs also enhance ADP-initiated platelet aggregation and activation in a CD36-dependent manner.\textsuperscript{90} During this study, experiments were performed that evaluated the docking of CFSE-labeled BxPc-3 and L3.6pl MVs to unactivated and thrombin-activated washed platelets in vitro (data not shown). Neither PSGL-1 nor P-selectin blocking antibodies reduced MV docking in vitro. Further, neither of these MV types express PSGL-1, although BxPc-3 MVs do express P-selectin ligand CD24 (data not shown). Contrary to this information, static binding assays demonstrated the binding of L3.6pl MVs but not BxPc-3 MVs to human P-selectin-IgG coated wells (data not shown). Since the role of P-selectin in TMV docking to the activated platelets and endothelial cells is of interest, future experiments will evaluate the effect of P-selectin blocking antibodies on TF+-TMV-initiated thrombin generation and platelet aggregation.

This study demonstrates a concentration-dependent relationship between plasma coagulation factors and the TF+ MV-triggered generation of thrombin. We have similarly shown that platelet activation is dependent on the concentration of plasma coagulation factors (data not shown). It would also be of interest to perform MV dose titration experiments to show that the kinetics of thrombin generation and platelet aggregation can be impacted by changing the concentration of MVs used. The effect of changes in TF concentration on thrombin generation kinetics is well known.\textsuperscript{67} As a result, we can predict that changing MV concentration should show similar changes in thrombin generation. Platelet aggregometry studies should also show the same change in time to aggregation, which is already demonstrated in this study using a TF high (BxPc-3) and TF low (L3.6pl) TMV source.

This study uses both a TF high and TF low TMV source for all in vitro experiments. While the TF activity of these two TMV types is vastly different, the resulting platelet activation and aggregation
by each TMV type does not appear to coincide with the level of TF expression. The difference between the level of platelet activation and aggregation triggered by each MV type is statistically significant, however based on how vastly different the TF expression is by each MV type, we would expect these differences to be even larger. One explanation relates to the fact that both thrombosis and platelet activation are threshold events. As a result, any source of TF that has the minimum amount of TF expression required to meet that threshold will trigger platelet activation. Further, the level of platelet activation can be determined by level of P-selectin expression and integrin α_{IIb}β_{3} activation; however there is a limit to how much P-selectin and integrin α_{IIb}β_{3} each platelet can express. Each platelet agonist is known to induce platelet activation to a different amplitude, and thrombin is known as a particularly potent platelet agonist. As a result, any amount of TF expression that is capable of generating enough thrombin to trigger platelet activation will result in strong platelet activation.

In this study, some mice die of apparent pulmonary embolism (PE) during MV injection. For survival analysis during the IVC stenosis model, mortality is defined as death at any time during the 1 hour following stenosis induction. This is the period of time during which MVs are being injected. More specifically, MVs are injected intravenously at a dose of 15 µg protein/mL in a volume of 200 µL at 0, 30, and 60 minutes following stenosis. The mice remain anesthetized during this one hour period. The mice that die start to gasp and then die within minutes of MV infusion, which bears clinical resemblance to the symptoms of pulmonary embolism. Necropsy demonstrates visible clot formation throughout the lungs in mice that die during MV infusion (data not shown). While the mice die due to lung thrombosis, this model cannot be called a PE model since TF+ TMV injection results in spontaneous intravascular lung thrombosis as opposed to mice developing deep vein thrombosis (DVT) which then embolizes to the lungs. Other studies have induced lung thrombosis with many substances, including collagen, epinephrine, polyphosphate, and recombinant TF. Previous studies have found lung thrombosis in this model to be dependent both on platelet aggregation and fibrin
deposition.\textsuperscript{94,97} Future experiments are planned to image platelet deposition in the lung following fatal MV injection in order to confirm the role of TF+ TMV-induced platelet activation and aggregation in TMV-induced lung thrombosis.

In this study, the electrolytic injury model provides the opportunity to image platelet and fibrin deposition at the site of thrombosis over a relatively short period of time. It is interesting to note that while both fibrin and platelet accumulation at the site of thrombosis is increased with the injection of TF+ TMVs, blockade of MV-TF before injection actually reduces fibrin accumulation at the thrombosis site to below baseline levels. HTF-1 treated TMVs cause initial fibrin accumulation that is consistent with thrombus growth in control mice. Once fibrin accumulation reaches a peak, fibrin accumulation in the control mice plateaus whereas fibrin accumulation in HTF-1 treated mice begins to decrease towards baseline. This decrease is not due to the inhibition of subendothelial TF in addition to MV TF because HTF-1 is specific for human TF.\textsuperscript{57} One possibility is that the presence of TMVs in the developing thrombus that are deficient in TF actually serves to compete with platelets as a thrombogenic surface and destabilize the clot. We have not examined these MVs for expression of tissue factor pathway inhibitor (TFPI), however other studies have found TFPI expression on mouse pancreatic adenocarcinoma MVs.\textsuperscript{55}
3.6 REFERENCES


4.1 Summary, Clinical Relevance, Future Directions

Venous thromboembolism (VTE) remains a significant public health concern, with incidence increasing over the last 30 years, in part due to better diagnostic techniques, increasing obesity, and an aging population. In addition to an association with decreased survival in cancer patients, cancer patients who develop VTE also suffer a loss of quality of life with increased pain, disability, and monetary cost. The proposed mechanisms for the hypercoagulable state that contribute to the increased rate of VTE in cancer patients are varied. However, specific therapies designed to target these mechanisms have yet to be developed. This dissertation project elucidated potential mechanisms by which tissue factor (TF)+ tumor cell-derived microvesicles (TMVs) enhance thrombosis and initiate the development of a hypercoagulable state.

In the process of identifying a novel mechanism of cancer associated thrombosis I contributed to a developing pool of literature designed to further standardize the techniques used for the evaluation of thrombosis in vivo and help guide scientists in their choice of the most appropriate thrombosis model to address their particular hypothesis. In chapter 2, I evaluated the strengths and weaknesses of the novel inferior vena cava (IVC) stenosis mouse model for the study of deep vein thrombosis (DVT). In short, the strengths of the model are that thrombosis occurs in the presence of relatively minimal endothelial damage, there is continued blood flow throughout thrombus growth, resulting thrombi contain platelet and red blood cell-rich layers, and thrombus development has a relatively chronic timecourse compared to other models. The weaknesses are that only ~50% of the mice get thrombi, thrombosis occurs in the
IVC which is devoid of valves and not the site of DVT in humans, and thrombus growth occurs upstream of the stenosis site.

A major topic of conversation within the animal models of thrombosis community is whether there is a “best” animal model of thrombosis which should be designated as the gold standard for venous thrombosis research. Ideally, the answer to this question would be those animal models which most accurately and consistently mimic the results of human clinical trials. The majority of animal models of thrombosis have not been around long enough to see a drug taken from animal studies through clinical trials. Further, the varying strengths and weaknesses of each animal model of thrombosis means that there is no one model that will fit every research question. The IVC stenosis model, for example mimics key features of human DVT that arguably could be particularly important in studies looking at the biology of venous thrombosis. However, the low rate of thrombosis and high variability in thrombus formation in this model would make the use of the model cost-limiting for the evaluation of anti-thrombotic agents or genotypes. In the case of anti-thrombotic agent evaluation, a study would benefit from using a mouse model with more consistent thrombus formation such as the electrolytic injury model, or the IVC stenosis model with the addition of a vascular damage step (also known as the St. Thomas Model).\(^5,10\) Both of these models still induce relatively chronic thrombosis in a large vein but with more reproducible thrombus formation. Similarly, the electrolytic injury model or the St. Thomas Model would also be better suited than the IVC stenosis model for the evaluation of thrombolytic agents and for looking at thrombus resolution due to the presence of blood flow at the thrombosis site but, again, in the presence of more reproducible thrombus growth. The IVC stenosis model is particularly useful for the evaluation of modulators which enhance thrombosis as was done in chapter 3 of this dissertation. With such a low rate of thrombus formation at baseline, there is plenty of room for the detection of increased thrombus growth. Along a similar line of reasoning, this model can be used to evaluate the efficacy of anti-thrombotic agents in prothrombotic disease states such as cancer, again as done in chapter 3 of this...
dissertation. With the addition of pro-thrombotic modulators such as TMVs, thrombus growth is enhanced and becomes more reproducible, making it possible to detect an effect with anti-thrombotic interventions.

There is a question as to how worthwhile it is to prevent or treat VTE in cancer patients when the patient’s primary concern is their cancer. Death in cancer patients can be attributed to the progression of cancer in 70.9% of patients.\textsuperscript{11} VTE is the second leading cause of death in this patient population, however it only accounts for 9.2% of patient deaths.\textsuperscript{11} Of note, the development of VTE in cancer patients is associated with an increased risk of death due to all causes.\textsuperscript{12,13} Based on these statistics one would hypothesize that reducing the incidence of VTE in cancer patients would lead to a subsequent increase in survival. Several clinical trials evaluating the efficacy of LMWH therapy in the reduction of VTE have also monitored the impact of anticoagulant therapy on survival in cancer patients. Randomized controlled trials on the use of LMWH versus placebo for primary thromboprophylaxis in non-selected cancer patients or cancer patients with a relatively low risk of thrombosis such as breast cancer have found that LMWH slightly reduces VTE incidence without impacting survival.\textsuperscript{14-16} The largest of these studies included over 1600 patients in each treatment arm with a broad range of solid tumors who were undergoing chemotherapy.\textsuperscript{14} LMWH did reduce the incidence of VTE (placebo 3.4%, LMWH 1.2%, p< 0.001), however there was no change in survival (placebo 44.5%, LMWH 43.4%, p=0.40).\textsuperscript{14}

Studies that focus on pancreatic cancer patients, who are at a high risk for VTE, showed a greater reduction in VTE with LMWH primary thromboprophylaxis, however the studies still failed to demonstrate a survival benefit.\textsuperscript{17-19} The lack impact on survival can likely be attributed to the low numbers of patients included in the studies.\textsuperscript{17-19} For example, a randomized controlled trial of pancreatic cancer patients undergoing gemcitabine therapy found that enoxaparin thromboprophylaxis during chemotherapy reduced the incidence of VTE from 23% to 3.4% (p=0.002).\textsuperscript{19} There was no significant impact on 100 day
patient survival (LMWH 4 of 59, placebo 7 of 62 patients died, p = 0.388). Of note, 3 deaths in the control group can be attributed to VTE in comparison to 0 in the LMWH group.19

While LMWH therapy has been shown to be more effective in the prevention of secondary VTE than vitamin K antagonists (VKAs) such as warfarin, two of three studies have shown a lack of relative impact on patient survival between these two treatments.20-22 A reanalysis of the CLOT trial identified a survival benefit when LMWH as opposed to VKAs were used for the prevention of VTE recurrence in 602 VTE patients with solid tumors.22 LMWH provided a survival benefit over VKAs in cancer patients without metastasis (LMWH 80% survival, VKA 65% survival, p=0.02), but not in cancer patients with metastasis (LMWH 28% survival, VKA 32% survival, p=0.46).22 The two studies which did not find a survival benefit for LMWH versus VKAs for secondary thromboprophylaxis in cancer patients contained a relatively high proportion of patients with metastasis.20,21 A large RCT evaluating the efficacy of LMWH versus VKAs for secondary thromboprophylaxis has met its recruitment goal of 900 patients and will include a secondary endpoint of 6-month survival, however preliminary data indicates a lack of a survival benefit with LMWH versus warfarin.23,24

VTE studies in cancer patients which monitor survival as an endpoint provide important information, however they do not account for the significant impact of VTE on patient quality of life.3,4,25 Deep vein thrombosis (DVT) can lead to post-thrombotic syndrome in 20-40% of patients, symptoms of which includes chronic pain, leg ulcers, and edema which can lead to reduced mobility and the need for long-term nursing care.4,26 Pulmonary embolism (PE), in addition to potentially being fatal, can also result in a long-term reduction in lung function leading to dyspnea and poor physical performance.27,28 Both DVT and PE have variable effects on quality of life scores, ranging from no effect to some patients reporting a post-VTE quality of life that is nearly equivalent to what they would report for death.25 The negative impact of post-thrombotic syndrome on quality of life has also been confirmed in pediatric patients.29,30 As improved anti-cancer therapies increase life-span in cancer patients, therapies which provide a quality
of life benefit become more important. The treatment and prevention of VTE could arguably provide a significant improvement in quality of life by reducing VTE incidence and recurrence, along with reducing the long-term complications.

Chapter 3 of this dissertation elucidated a novel role for the interaction between TF+ TMVs and platelets in cancer-associated thrombosis. TF+ TMVs are found to trigger the local generation of thrombin on platelets in the presence of plasma coagulation factors. This thrombin then triggers platelet activation and aggregation, leading to the platelet dependent enhancement of venous thrombosis in mice. This study shows that TF+ TMVs enhance thrombosis in mice in a TF-dependent manner in two different mouse models of large vein thrombosis, including the IVC stenosis model. TF+ TMV-enhanced thrombosis was reduced in Par4-deficient mice which have impaired thrombin-dependent platelet activation and in wild-type mice treated with the platelet inhibitor clopidogrel. This study shows that TF+ TMVs trigger the activation of platelets in a TF- and thrombin-dependent manner and that platelets play a significant role in the enhancement of thrombosis in a mouse model of cancer-associated venous thrombosis.

Low molecular-weight heparin (LMWH) is the preferred treatment for patients with cancer who develop cancer-associated thrombosis. LMWH has increased efficacy in cancer patients over VKAs, the current standard of care for the treatment of VTE in non-cancer patients. However, VTE recurs in 6-9% of cancer patients despite therapy. Currently, the only evidence-based option for treating recurrent VTE in cancer patients despite secondary thromboprophylaxis is LMWH dose escalation. There are several novel oral anticoagulant drugs that have recently become available, however use of these drugs is not currently recommended in patients with malignancy. This recommendation was made based on the limited clinical trial data available on the use of novel oral anticoagulant agents in this population along with clinical concerns about the combination of unpredictable absorption in the malignant state and lack of available drug level monitoring. Current completed clinical studies examine the efficacy of novel oral anticoagulant agents versus warfarin for secondary VTE prophylaxis. A recent meta-analysis of
these studies found that novel oral anticoagulant agents are at least as effective as warfarin at preventing VTE recurrence.⁴⁹,⁵⁰ These trials were not specifically designed to evaluate VTE treatment in cancer patients, and so they only include small numbers of relatively healthy cancer patients in comparison to the cancer patient populations generally recruited for cancer-associated VTE trials.⁴⁹,⁵⁰ Also LMWH, not Coumadin, is the current standard of care for VTE prophylaxis in cancer patients. There are currently 2 ongoing clinical trials evaluating the novel oral anticoagulant agent rivaroxiban for secondary thromboprophylaxis in patients with cancer.⁴¹ The first is an open-label, randomized study comparing the efficacy of rivaroxiban to the LMWH dalteparin. The second study is a single arm-prospective cohort study.⁴¹

My studies provide evidence that a platelet-targeted approach to thromboprophylaxis may be effective in cancer patients. Chapter 3 of this dissertation identified a platelet-dependent mechanism of cancer-associated thrombosis that could be contributing to the enhancement of thrombosis in patients with increased circulating TF+ TMVs. The efficacy of clopidogrel therapy in the reduction of thrombosis in a mouse model of cancer-associated thrombosis identifies antiplatelet agents as a potential alternative therapy for secondary thromboprophylaxis for some patients with cancer. Two retrospective studies examined the effect of aspirin on VTE risk in patients with breast and pancreatic cancer.⁴²,⁴³ Aspirin had no effect on VTE risk in breast cancer patients and a marginal effect (p=0.053) in pancreatic cancer patients. On the other hand, aspirin reduced VTE risk as effectively as LMWH in multiple myeloma patients.⁴⁴ My studies show that TF+ TMV-dependent platelet activation is dependent on platelet receptor P2Y12, suggesting that clopidogrel should be considered as a candidate agent for thromboprophylaxis in high-risk cancer patients.
4.2 REFERENCES


