# Physical Parameters for the Procoagulant Phenotype of Circulating Cancer Cells

By

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CERTIFICATE OF APPROVAL

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Dedicated to my mom, the smartest person I ever knew.

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# List of Abbreviations

| | |
|---|---|
| Ab | antibody |
| ANOVA | analysis of variance |
| APC | activated protein C |
| ATIII | antithrombin III |
| BSA | bovine serum albumin |
| CTC | circulating tumor cell |
| CTI | corn trypsin inhibitor |
| CVD | cardiovascular disease |
| DIC | differential interference contrast |
| DMEM | Dulbecco's Modified Eagle Medium |
| EC | endothelial cell |
| ECM | extracellular matrix |
| FBS | fetal bovine serum |
| FII | coagulation factor II, prothrombin |
| FIIa | activated coagulation factor II, thrombin |
| FITC | fluorescein isothiocyanate |
| FIX(a) | (activated) coagulation factor IX |
| FV(a) | (activated) coagulation factor V |
| FVII(a) | (activated) coagulation factor VII |
| FVIII(a) | (activated) coagulation factor VIII |
| FX(a) | (activated) coagulation factor X |
| FXI(a) | (activated) coagulation factor XI |
| FXII(a) | (activated) coagulation factor XII |

| | |
|---|---|
| FXIII(a) | (activated) coagulation factor XIII |
| Gla | gamma-carboxyglutamic acid |
| GP | glycoprotein |
| GPCR | G-protein-coupled receptor |
| GPIb | glycoprotein Ib or CD42b |
| GPVI | glycoprotein VI |
| GPRP | H-Gly-Pro-Arg-Pro-OH |
| HBSS | Hank’s Balanced Salt Suspension |
| mAb | monoclonal antibody |
| PAR | protease-activated receptor |
| PBS | phosphate buffered saline |
| PPACK | D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone |
| PPP | platelet poor plasma |
| PRP | platelet rich plasma |
| PS | phosphatidylserine |
| RPMI | Roswell Park Memorial Institute |
| RT | room temperature |
| SEM | standard error of the mean |
| TF | tissue factor |
| TFPI | tissue factor pathway inhibitor |
| TXA2 | thromboxane A2 |
| v/v | volume/volume |
| VTE | venous thromboembolism |

| | |
|---|---|
| vWF | von Willebrand factor |
| w/v | weight/volume |

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# Abstract

## Physical Parameters for the Procoagulant Phenotype of Circulating Cancer Cells

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Cancer is a hypercoagulable state. Thrombosis is the second leading cause of death in cancer, and development of thrombosis confers a worse prognosis for patients with cancer. Conventional anticoagulants are effective at preventing thrombosis (thromboprophylaxis) in patients with cancer, yet thromboprophylaxis is not safe for routine administration in cancer care. A biomarker to identify patients with cancer at risk to develop thrombosis would allow personalized thromboprophylaxis for those in need, while sparing the risks of anticoagulation for those not at elevated risk to develop thrombosis. An increased incidence of thrombosis has been observed in patients with metastatic cancer and acute leukemia. Undifferentiated myeloid leukemia cells are present in the peripheral blood of patients with acute myelogenous leukemia (AML). Thrombosis in AML has been associated with elevated counts of leukemia cells in the peripheral blood. In metastatic cancer, tumor cells are shed into the vasculature, circulate in the blood, and form secondary metastases at distant sites. An elevated count of circulating tumor cells (CTCs) confers a poor prognosis in metastatic cancer. My central hypothesis is that the procoagulant phenotype of circulating cancer cells is regulated by physical parameters.

Blood coagulation occurs on the membranes of procoagulant cells. Flow regulates blood coagulation by controlling the mass transport of coagulation factors to the procoagulant cell surface. Cancer cells circulate as a suspension in the blood, and exhibit a spatial distribution that is unique from cells in a primary tumor, in the bone marrow, or that have become immobilized on vessel walls. In the absence of flow, diffusion predominates as the driver of mass transport. Results in this thesis indicate that the procoagulant phenotype of circulating cancer cells is dependent upon the following physical parameters: local hemodynamic parameters, spatial separation of procoagulant cells (cell concentration), and the variation of coagulation factor concentrations within the physiological range.

Blood coagulation is triggered following contact with cells that express tissue factor (TF) and phosphatidylserine (PS) at their surface. We found that the promotion of coagulation by both malignant epithelial and AML cell lines in blood and plasma was dependent on cell concentration, TF and PS. A fluorescent biomarker to assess circulating cancer cell procoagulant activity may help facilitate clinical studies to establish the association between procoagulant circulating cancer cells and thrombosis. In order to characterize the procoagulant phenotype of circulating tumor cells (CTCs), we developed a panel of novel fluorescent probes that relies on the mechanism of surface assembly of coagulation factors as a potential fluorescent label to identify procoagulant CTCs. We propose a PS index, which combines PS exposure and cell count, as a fluorescent biomarker for AML cell procoagulant activity.

Collectively, this dissertation provides novel insight into the role of physical parameters in the procoagulant phenotype of circulating cancer cells and highlights potential biomarkers for assessment of the risk for patients with cancer to develop thrombosis.

# Chapter 1: Introduction

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## 1.1. Physiology of Blood

Human blood is composed of a proteinaceous liquid phase called plasma, and cellular components that include platelets, leukocytes, and erythrocytes. Blood has several physiological functions that include the transport of nutrients, hormones, and cellular waste; immunological surveillance; and coagulation, which initiates wound healing in the event of an injury (Staindl 1979). Although dysregulated blood coagulation is also critically important to diseases such as myocardial infarctions, strokes, and hemophilia, regulated blood coagulation is critically important to maintain health during acute events such as surgery or trauma (Mann 2003).

### *1.1.1 Overview of blood coagulation*

Blood coagulation is a process by which inactive, precursor zymogens in plasma are converted to active enzymes through proteolysis. In health, initiation of coagulation occurs on the surfaces of immobilized extramural cells or on activated platelets that contain tissue factor (TF) (Nemerson 1968; Mann et al. 2003; Furie et al. 2008). TF is not surface expressed by cells in the bloodstream in health, but is constitutively expressed by

extravascular cells. The initiation of coagulation in health is in response to an injury that disrupts blood vessel architecture so that flowing blood contacts extramural TF-expressing cells at the site of vessel injury. TF serves as the membrane receptor and cofactor for the serine protease activated coagulation factor VIIa (FVIIa). TF, in complex with FVIIa initiate the extrinsic pathway of blood coagulation. The products of blood coagulation form a thrombus that seals the blood vessel injury in order to prevent further blood loss. Propagation of coagulation requires coagulation factors to transport from bulk plasma to the surface-bound coagulation enzyme complexes. In the physiological scenario of a vessel injury, convective mass transport supplies coagulation factors from the blood to the TF-exposing cell membrane (Gemmell et al. 1988; Andree et al. 1994; Hathcock 2006).

TF is essential for hemostasis, as TF knock-out mice are embryonic lethal, and no TF deficiencies are found in humans (Mackman 2009). FVII is also essential for hemostasis. FVII deficiency is rare and is accompanied by spontaneous hemorrhage in the mucosa, skin and joints (Mackman 2009). TF•FVIIa activates coagulation factor X (FX) to activated coagulation factor X (FXa) and coagulation factor IX to activated coagulation factor IXa (FIXa) (Osterud et al. 1977; Lawson et al. 1991). FIXa in complex with activated coagulation factor VIII (FVIIIa), in the presence of calcium and a phospholipid surface, can generate FXa independent of TF (Kane et al. 1988; Ahmad et al. 2003). FVIII and FIX deficiency are known as Hemophilia A and B, respectively. Patients with Hemophilia A and Hemophilia B develop spontaneous hemorrhage, often into the joints (hemarthroses), highlighting the roles for FVIII and FIX in generating sufficient thrombin to maintain hemostasis (Ratnoff et al. 1973). FXa, in complex with activated coagulation

factor V (FVa), forms the prothrombinase complex in the presence of $Ca^{2+}$ and anionic phospholipids, and converts soluble fibrinogen to insoluble fibrin (Blomback et al. 1978; Tracy et al. 1985). Fibrin monomers undergo self-polymerization to form fibrin fibrils, which supply mechanical integrity to the formed thrombus (Ferry et al. 1952; Furie et al. 2008). In addition to forming fibrin, thrombin can activate coagulation factors V (FV), VIII (FVIII), and XI (FXI) (Gailani et al. 1991). In turn, activated FXI (FXIa) can activate FIX, allowing thrombin to propagate its own generation independent of TF. FXI deficiency, also known as Hemophilia C, is associated with a mild bleeding disorder and a lower incidence of stroke, suggesting a role for FXI in hemostasis and thrombosis (Salomon et al. 2008). Thrombin also activates coagulation factor XIII (FXIII), which cross-links formed fibrin polymer strands to form a mesh (Siebenlist et al. 2001). Thrombin spatially limits its own propagation by binding to thrombomodulin and endothelial protein C receptor on the surface of endothelial cells where it converts protein C to activated protein C (APC) (Esmon et al. 1981). APC, in turn, can inactivate FVa and FVIIIa, limiting the generation of thrombin to the site of initiation (Kisiel et al. 1977; Walker et al. 1979; Fulcher et al. 1984). The steps involved in TF-initiated coagulation are shown schematically in Figure 1.1.

#### *1.1.1.2 Endogenous regulation of blood coagulation*

TF-initiated blood coagulation exhibits a threshold behavior, and proceeds when procoagulant mechanisms overcome anticoagulant mechanisms (Mann et al. 2003). In the event of an injury, a robust burst of thrombin can lead to fibrin formation in order to maintain hemostasis. However, an equally robust reaction must occur to counteract the propagation of thrombin generation in order to localize the hemostatic response to the

injury site. APC can spatially limit the spread of a thrombus growth by localizing the generation of thrombin to the site of injury, as described above. Antithrombin is a serine protease inhibitor present in blood that antagonizes many of the activated coagulation factors including FXa and FIIa (Mann et al. 2003). Tissue factor pathway inhibitor (TFPI) inhibits the extrinsic tenase complex preventing it from activating FIX or FX (Crawley et al. 2008). Moreover, blood flow regulates thrombus formation, as the flux of thrombin from the vessel wall needed to form fibrin increases for increasing flow rates (Neeves et al. 2010).

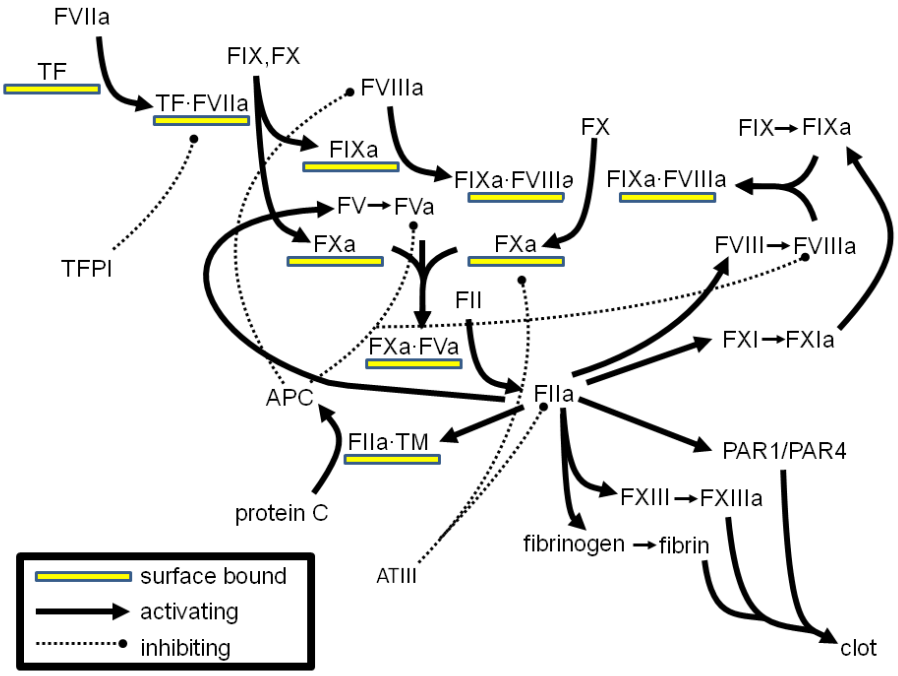


**Figure 1.1. A schematic representation of the events triggered by exposure of tissue factor that lead to the formation of a blood clot.**

*1.1.2 Overview of platelet function*

In addition to the exposure of TF and subsequent activation of blood coagulation, vessel injuries expose extracellular matrix proteins (ECM) such as collagen to the bloodstream, which initiates blood platelet adhesion to the site of injury. Platelets are small (2-3 μm), anucleate blood cells that arrest at sites of ECM exposure following vessel injury.

Arrested platelets undergo homotypic aggregation to form plugs at vessel injury sites. The requirement that cells bind to subendothelial matrix proteins while they are entrained in the bloodstream places stringent physical demands on the receptors involved in the initial adhesion and arrest of platelets, and specifically, on the platelet receptors involved in the adhesion and arrest of platelets. In humans, blood circulates at different flow velocities depending on anatomical location and presence in the venous versus the arterial circulation. Flow exerts shear stress and determines shear rates close to the vessel wall, which directly influences the residence time of a platelet above a subendothelial matrix protein. In humans, shear rates span two orders of magnitude, from 50-60 $s^{-1}$ in the vena cava to 1,000-5,000 $s^{-1}$ in the arterioles (Whitmore 1968; Lipowsky et al. 1977). At shear rates above 500 $s^{-1}$, the vWF/GPIb receptor-ligand interaction facilitates platelet adhesion (Savage et al. 1996). The adhesion of bound platelets in the presence of blood flow exerts a hydrodynamic shear stress on the receptor-ligand bond, which often limits the duration of time the ligand-receptor bond persists (off-rate). Subsequently, native vWF binds GPIb only when subjected to high fluid shear rates or when vWF is first bound to collagen (Dong et al. 2001). Therefore, at lower shear rates, or for arrested platelets, other receptor-ligand interactions must occur for sustained arrest of platelets. Stable adhesion to collagen involves contributions from collagen receptors $\alpha_2\beta_1$ and GPVI (Auger et al. 2005). In addition, fibrin(ogen) deposited at sites of vessel disruption may bind platelets via $aII_bB_3$, which contributes to homotypic aggregation of platelets, a requirement for forming a plug to stem blood loss following an injury (Wagner et al. 1996).

Platelets contain numerous receptors for and are activated by collagen, thrombin, adenosine diphosphate (ADP), thromboxane (TXA2), serotonin, histamine, and

epinephrine (Offermanns 2006). Activated platelets undergo a calcium and ATP-dependent transformation upon activation, whereby platelet granules are released into blood and phospholipid asymmetry in the platelet membrane is lost and external exposure of anionic phospholipids, most notably phosphatidylserine (PS), occurs (Bevers et al. 1982; Bevers et al. 1983; Dachary-Prigent et al. 1995). Exposed PS serves as a surface for coagulation factors to bind to, and thereby provides a catalytic surface localized to the sites of platelet activation for coagulation to proceed (Shearer 1990; Sunnerhagen et al. 1995). The steps that lead to platelet arrest, aggregation and facilitation of blood coagulation are illustrated in Figure 1.2.

*1.1.2.2 Endogenous regulation of platelet activation*

Endogenous inhibitors of platelet activation include prostacyclin ($PGI_2$) and nitric oxide (NO) produced by the endothelium (Hoyer et al. 1994). $PGI_2$ binds to a platelet G-protein-coupled receptor (GPCR) to activate $G_s$ and increases platelet cAMP levels via adenylyl cyclase, while NO increases platelet cGMP levels by activating cytosolic guanylyl cyclase, both of which (cAMP and cGMP) inhibit platelet activation (Marcus et al. 1965; Radomski et al. 1987).

*1.1.3 Pharmacological regulation of platelet function and blood coagulation*

Pharmacological inhibition of platelet activation may be achieved via inhibition of cyclooxygenase (COX) with non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin or ibuprofen, or antagonism of $P2Y_{12}$ signaling (Awtry et al. 2000; Hollopeter et al. 2001). Pharmacological inhibition of platelet aggregation may be achieved by targeting $\alpha_{IIb}\beta_3$, which when blocked, prevents platelet aggregation (EPIC 1994).

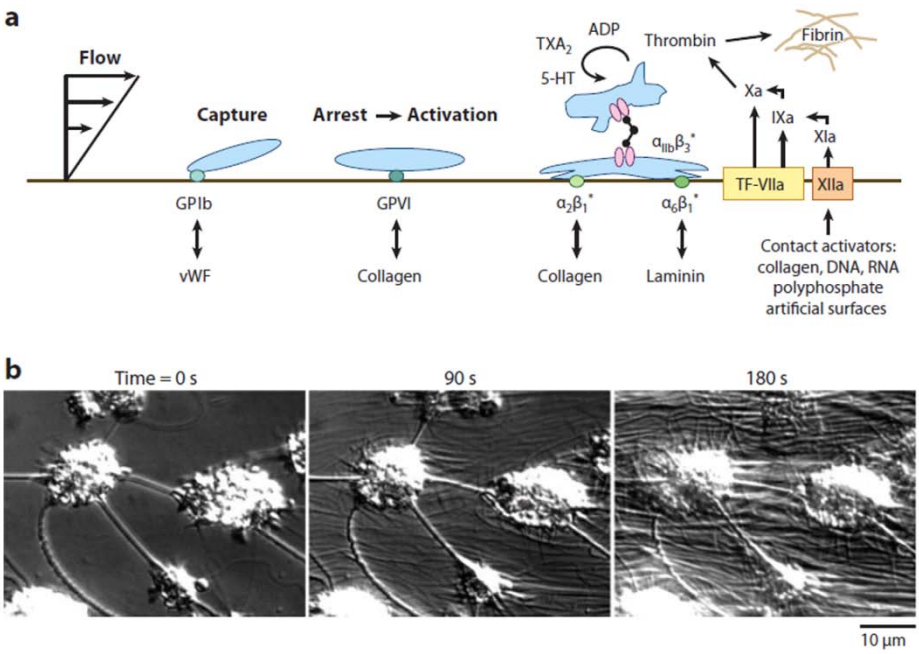


**Figure 1.2. Initial arrest of platelets to a site of injury is facilitated by GPIb/vWF. Stable adhesion is achieved through GPVI/collagen, which also activates platelets to release platelet activators that include thromboxane ($TXA_2$), serotonin (5-HT), and adenosine diphosphate (ADP) to recruit more platelets to form an aggregate. (a) Activated platelets then facilitate coagulation reactions upon their surface following exposure of phosphatidylserine. (b) This process can be imaged in real time utilizing differential interference microscopy (DIC) and flow chambers to recreate the physiological environment within which coagulation occurs.**

Coagulation is pharmacologically inhibited with several drugs. Coumarins antagonize the vitamin K-dependent hepatic enzyme epoxide reductase which catalyzes the formation of gamma carboxyglutamic acid residues (Gla) on FX, FIX, FVII, FII, protein C and protein S (Ansell et al. 2008). Heparins bind and activate antithrombin III (ATIII) to inhibit FXa, FIXa and FIIa (Chuang et al. 2001). Direct FXa inhibitors (rivaroxaban, epixaban) and thrombin inhibitors (dabigatran) have demonstrated anticoagulant effects as well (Roehrig et al. 2005; Wallentin et al. 2010).

### *1.1.4 Overview of thrombosis*

Thrombosis results from a blood clot that grows to occlude the blood vessel lumen and interrupt blood supply. In general, thrombosis occurs when procoagulant reactions

overwhelm anticoagulant reactions. Thromboembolism occurs when a formed thrombus dislodges and is carried with the blood to a distant site where it occludes blood flow. Together, thrombosis and thromboembolism significantly contribute to the mortality of cardiovascular disease, the leading cause of death in the United States (Furie et al. 2008; Lloyd-Jones et al. 2010).

Specific mechanisms that underlie thrombosis are a topic of active research. It is evident that mechanisms of thrombosis depend on where the thrombosis occurs. Arterial thrombi, which form in the presence of high shear rates, are mainly composed of platelets, while venous thrombi are mainly composed of fibrin and erythrocytes (Baumgartner 1973). The development of thrombosis may be explained by normal coagulation and platelet responses to specific pathologies (i.e. advanced atherosclerosis), or pathological coagulation responses to normal procoagulant stimuli (i.e. minor trauma). Thrombophilia, a tendency towards thrombosis, may be inherited via loss of function mutations in anticoagulant proteins. A deficiency of any of the anticoagulant proteins, protein C, protein S or antithrombin III, is associated with an increased risk to develop thrombosis (Rosendaal et al. 2009). Factor V Leiden refers to a mutation in the gene for FV that conveys a resistance to APC inhibition. Therefore, patients with FV Leiden may develop pathological thrombi that continue to propagate away from the initiating site (Svensson et al. 1994). Prothrombin G20210A is a mutation in the prothrombin gene and is associated with a 2- to 3-fold increased risk to develop thrombosis (Rosendaal et al. 2009).

*1.1.4.2 Thrombosis in cancer*

Thrombophilia is acquired in certain disease states such as cancer. Cancer is associated with increased rates of thrombosis (Baron et al. 1998; Blom et al. 2005; Oehadian et al.

2009; Noble et al. 2010; Tafur et al. 2011). Thrombosis contributes significantly to the morbidity and mortality of cancer, and the mechanisms underlying the hypercoagulability of cancer have remained elusive, frustrating the clinical care of patients (Ambrus et al. 1975; Sorensen et al. 2000; Khorana et al. 2007). Patients with cancer who develop thrombosis experience worse outcomes than patients with cancer who do not develop thrombosis (Sorensen et al. 2000). Existing anticoagulation strategies are effective at preventing recurrent venous thrombosis in patients with cancer (Kuderer et al. 2007; Akl et al. 2011). However, anticoagulation is not safe enough to prophylactically treat the cancer patient population as a whole (Akl et al. 2011). Despite nearly two centuries since the first observation that thrombosis occurs at a higher rate in patients with cancer, treatment of thrombosis, prophylaxis against future thrombosis, and early identification of patients at risk to develop thrombosis as a part of their disease is still a topic of active research (Bouillard et al. 1823; Trousseau 1865; Donati et al. 2012).

The development of thrombosis in cancer is complex and may be multifactorial. Personal and family history of prior thrombosis, fibrinogen and platelet levels, immobility and fatigue, indwelling catheters and certain chemotherapeutics are all associated with an increased thrombotic risk in cancer (Dimopoulos et al. 2004; Khorana et al. 2007; Elice et al. 2009; Khorana et al. 2009; Mandala et al. 2010). Thrombosis can herald the symptoms and diagnosis of cancer (Baron et al. 1998; Sorensen et al. 1998; Iodice et al. 2008). This, in itself, underlines the role for cancer in disrupting the normal physiological balance of hemostasis. The incidence of thrombosis in cancer is closely tied to cancer subtype and origin (i.e. adenocarcinoma versus carcinoma, colon versus breast, acute myelogenous leukemia (AML) subtype M3 versus M2) suggesting that cancer cells directly contribute

to the hypercoagulable state in cancer (Baron et al. 1998; Sorensen et al. 1998; Rickles et al. 2001; Blom et al. 2004). In metastatic cancer, cells from the primary tumor are shed into the circulation. Circulating tumor cells (CTCs) accompany metastatic disease across all types of solid tumors (Allard et al. 2004). Undifferentiated myelogenous blast cells, normally present in the bone marrow, are routinely found in the peripheral blood of patients with acute myelogenous leukemia (AML). Tumors and leukemia cells have been shown to overexpress TF, and TF expression correlates with metastatic potential of tumor cells (Zacharski et al. 1983; Zacharski et al. 1986; Yan et al. 2010; Lee et al. 2011; Liu et al. 2011; Ma et al. 2011; Tian et al. 2011; Xu et al. 2011; Gil-Bernabe et al. 2012). As such, metastatic cancer and AML are accompanied by the presence of TF-expressing cells in peripheral blood of patients, violating the classic view of TF exposure only at sites of vessel injury. Whether circulating cancer cells are involved in the development of thrombosis has not been established.

### 1.2 Thesis Overview

Thrombosis significantly contributes to morbidity and mortality in cancer. In AML, freshly isolated peripheral leukemic cells express TF, while levels of circulating TF are elevated in patients with metastatic cancer. This thesis centers on the coagulation kinetics of cells in suspension and under flow, and identifies unique physical parameters that regulate the procoagulant phenotype of circulating cancer cells.

Flow is a potent mediator of coagulation. Analyses in Chapter 3 define the role of flow in regulating the surface flux of coagulation factors from plasma. Studies in Chapter 3 highlight the relevance of coagulation factor concentrations within the physiological range of variation to regulate the procoagulant phenotype of circulating cancer cells.

TF-initiated coagulation is a surface phenomenon. Procoagulant cells in suspension therefore represent novel spatial distributions of procoagulant foci in blood. Studies in Chapter 4 utilize a model system to describe how the mean spatial separation of particles in suspension (particle concentration) influences coagulation kinetics.

TF-initiated coagulation requires activation of a number of coagulation enzymes. The delivery of coagulation factor in plasma to the surface of a procoagulant cell is directly related to its plasma concentration. Coagulation factor concentrations vary by over 3-fold within the physiological range of variation. Studies in Chapter 5 utilize a model system to determine how concentrations of coagulation factors influence coagulation kinetics of TF particles in suspension.

Leukemia cells express TF and exhibit procoagulant activity. TF procoagulant activity is dependent upon exposure of the anionic phospholipid phosphatidylserine (PS). The procoagulant phenotype of an AML cell may be dependent upon a genetic predisposition to overexpress TF coupled with physiological or pharmacological events that induce exposure of PS. Studies in Chapter 6 describe the relative roles of cell count and surface expression of TF and PS to determine the procoagulant phenotype of AML cells.

The isolation and enumeration of circulating tumor cells in patients with metastatic cancer relies on fluorescent detection of extremely rare cells amongst normal blood cells. Efforts to assess whether CTCs contribute to the development of thrombosis would benefit from a fluorescent marker of procoagulant activity. Studies in Chapter 7 describe the development of a panel of fluorescent coagulation factors to label procoagulant cancer cells.

The studies outlined in Chapters 3-7 provide new insights into the physiology of blood coagulation in the context of procoagulant cells in the circulation. In Chapter 8, the key findings from my thesis research are summarized and opportunities for future work are highlighted.

# Chapter 2: Common Materials and Methods

## 2.1 Ethical Considerations

Studies in this thesis were conducted using human blood. All human donors were healthy and gave full informed consent in accordance with the Declaration of Helsinki. Experiments using human donors were performed with approval of the Oregon Health & Science University Institutional Review Board.

## 2.2 Collection of Human Blood and Preparation of Blood Cells or Plasma

### *2.2.1 Blood collection*

Human venous blood was collected by venipuncture from healthy adult male and female volunteers directly into anticoagulated syringes. The anticoagulant used depended upon the experiment performed.

### *2.2.2 Platelet-poor plasma preparation*

Blood was collected into a one-tenth volume of sodium citrate (3.2% w/v). Blood was transferred to polypropylene tubes and platelet-rich plasma (PRP) was prepared by centrifugation at 230 × *g* for 10 minutes. Platelet poor plasma (PPP) was prepared from PRP by pooling with PRP from a minimum of 3 donors, and subjecting to centrifugation at 2150 × *g* for 10 minutes. PPP was stored frozen at -80° C until use.

## 2.3 Coagulation Assays

### *2.3.1 Plasma coagulation*

Blood coagulation involves a series of proteolytic reactions that result in the generation of thrombin and formation of fibrin. In this thesis, the formation of fibrin was measured optically with a spectrophotometer or mechanically with a coagulometer. Optical detection of fibrin formation relies on a change in turbidity of plasma as the fibrin formation reaction proceeds (Jungi 1990; Pratt et al. 1992). Mechanical clot formation

was measured in a KC4 coagulation analyzer (Trinity Biotech, Wicklow, Bray Co., Ireland), which utilizes the adherence of a steel bead to a magnet inside rotating cuvettes containing plasma under conditions of coagulation. As coagulation proceeds, the plasma transforms into a gel that dislodges the bead from the magnet. The time between the initiation of coagulation and dislodging of the bead is detected by the coagulation analyzer and measured as the clotting time.

*2.2.2 Chromogenic determination of enzyme activity*

The procoagulant activity of cells is dependent upon the ability of that cell to convert inactive zymogen precursor to active enzyme. The formation of active enzyme from inactive zymogen in the presence of cells was measured by the change in absorbance of 405 nm wavelength light in the presence of a chromogenic substrate. The chromogenic substrate used depended on the enzyme to be assayed. In purified systems, specific enzyme activation was measured in a two-stage assay. First, cells were incubated with a precursor enzyme and cofactor (in selected experiments) for 10 minutes at room temperature (RT). Then, the cell suspension was added to a well containing purified zymogen and a chromogenic substrate that is hydrolyzed by the formed enzyme to be measured. In plasma, the generation of enzymes by procoagulant cells was performed in a one-stage assay. Here, plasma containing a chromogenic substrate was mixed with cell suspension for 10 minutes at RT before coagulation was initiated by adding calcium (8.3 mM, final concentration). In both purified systems and in plasma, the change in absorbance of 405 nm wavelength light was measured over time. The rate of change of absorbance measures the ability of a cell to catalyze enzyme activation.

*2.3.1 Whole blood occlusive thrombus formation*

The ability of procoagulant cells to promote experimental thrombus formation was measured in an *ex vivo* model of occlusive thrombus formation (Berny et al. 2010). For this assay, rectangular glass chambers (0.2 mm × 2.0 × mm × 50 mm glass capillaries; VitroCom, Mountain Lakes, NJ) were coated with fibrillar collagen from Chrono-Log (Havertown, PA) for 1 hour at RT. Following washing with phosphate buffered saline (PBS, pH=7.40), capillaries were incubated with denatured and 0.45 μm-filtered bovine serum albumin (BSA, 5 mg $mL^{-1}$ in PBS) for 1 hour at RT to block non-specific interactions between the blood and glass. After a second wash with PBS, the capillary was inserted into a silicone tubing system and mounted onto a syringe. The syringe was mounted vertically, with the distal end of the glass capillary immersed in a reservoir of PBS. Prior to the experiment, perfusion through the capillary was primed by forcing PBS through the capillary. Sodium citrate anticoagulated blood was replenished with the divalent cations calcium (7.5 mM) and magnesium (3.75 mM), and recalcified blood was serially added to the reservoir to maintain a predetermined height. This apparatus allowed a gravity-driven constant pressure system to drive blood through the syringe, and initial wall shear rates, at the predetermined blood reservoir height, were approximately 300 $s^{-1}$. The time to occlusion of the capillary was recorded as the time blood ceased to flow from the capillary.

### 2.4 Cell culture

*2.4.1 Leukemia cell lines*

Leukemia cell lines were cultured with RPMI 1640 culture medium supplemented with 10% v/v fetal bovine serum, 1 × penicillin and streptomycin, and supplemented weekly with 2.05 mM L-glutamine. Leukemia cell concentrations were maintained between 2 × $10^5$ and 2 × $10^6$ cells $mL^{-1}$ as counted with a hemocytometer. Cell viability was

maintained above 90% as measured with Trypan Blue staining. Cells utilized in experiments were between passages 3 and 8.

*2.4.2 Epithelial cell lines*

Epithelial cell lines were cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v fetal bovine serum supplemented with 1× penicillin and streptomycin. Cells were grown to confluence and harvested by immersing in TrypLE Express for 20 minutes at $37^{o}$ C. Dissociated cells were collected and washed in complete medium before suspending in serum-free medium prior to experiments.

**2.5 Common Reagents**

Unless otherwise specified, reagents used for blood collection, preparation of blood components, or for flow chamber assays were from Sigma-Aldrich (St. Louis, MO). Recombinant, lipidated tissue factor (thromboplastin, Innovin®) was from Dade Behring (Deerfield, IL). Polymer microspheres were from Bangs Laboratories (Fishers, IN). The serine protease inhibitor D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) was from Calbiochem (San Diego, CA). Antibodies to human FXI were generated as previously described (Tucker et al. 2009). Daunorubicin hydrochloride (daunorubicin, Teva Parenteral Medicines, Inc, Irvine, CA) was from the OHSU Doernboecher Children's Hospital pharmacy. Fluorescein isothiocyanate- (FITC) conjugated bovine lactadherin and plasma-derived FVII, FVIIa, FIX, FX, FXa, FVIII, FV, FVa, FII, FIIa and plasmas immunodepleted of FVII, FIX, FX, FVIII, FV or FII were from Haematologic Technologies, Inc. (Essex Junction, VT). FITC-conjugated anti-TF antibodies were from Lifespan Biosciences (Seattle, WA). Equine fibrillar collagen was from ChronoLog (Havertown, PA). Fetal bovine serum was from American Type Cell

Culture (Manassas, VA). Anti-TF antibodies, TF ELISA kit, Spectrozyme FXa® and Spectrozyme TH® were from American Diagnostica (Stamford, CT). Cell lines were from ATCC (Manassas, VA) or DKFZ (Heidelberg, Germany).

*2.5.1 Fluorescently labeled coagulation factors*

To generate active-site, fluorescently labeled coagulation factors, human coagulation FVIIa, FXa and FIIa were active-site labeled with fluorescein isothiocyanate conjugated D-Phe-Pro-Arg chloromethyl ketone (PPACK). Prior to labeling, loss of protease activity was confirmed (Bock 1992; Panizzi et al. 2006).

## 2.6 Calculation of Spatial Separation

The presence of cells or particles in plasma presents a 3-dimensional distribution unique from cells in the primary tumor, bone marrow or immobilized to an experimental surface or blood vessel-wall. In this thesis, experiments were designed to investigate the role for the distribution of cells in plasma and blood (cell concentration) to control coagulation kinetics. For this, the average spatial separation of cells in suspension was calculated. This calculation normalized the plasma volume by the number of cells added to give the average volume of plasma surrounding each cell (inverse of cell concentration). The cubic root of the volume per cell then provides a measure of the average linear separation between nearest neighbor cells, or spatial separation. A schematic representation of how this calculation measures the distribution of cells in plasma is shown in Figure 2.1. This calculation assumes the cells are uniformly distributed throughout the plasma volume.

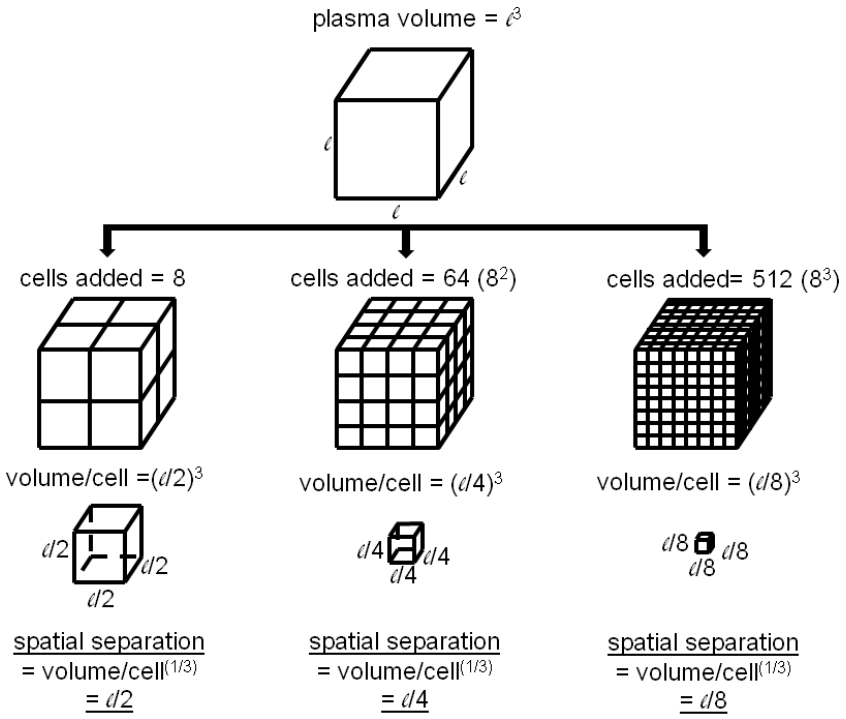


**Figure 2.1. Schematic of method to calculate the spatial separation of cells in plasma based on knowledge of the volume of plasma to be tested, the number of cells added, and an assumption of uniform distribution of cells within the plasma.**

# Chapter 3: Analysis of Coagulation Factor Mass Transport Under Shear

Garth W. Tormoen, Ayesha Khader, András Gruber, Owen J.T. McCarty

## 3.1 Abstract

Blood coagulation initiates when soluble coagulation factors in plasma become activated following contact with enzyme complexes on the surface of a TF-expressing cell. Mass transport of coagulation factors from plasma to a TF-exposing surface is rate-limiting for enzyme activation under shear flow (Forman et al. 1986; Andree et al. 1994; Contino et al. 1994). The aim of this analysis was to derive equations that describe the mass transport of coagulation factors over time in the absence of flow, and contrast with established equations of mass transport under shear. Diffusion kinetics of coagulation factors depend on molecular size and concentrations in plasma. The flux of coagulation factors to a surface determines the number of binding events in a given period. The surface flux of coagulation factors then decreases with time due to the growth of a depletion layer above the extrinsic tenase complex. In the presence of shear, coagulation factors are transported by flow in close proximity to the reactive surface, which diminishes the depth of the plasma depletion zone. The effect of this is to reach a steady-state surface flux, which is proportional to the magnitude of the shear rate. Physiological variations in plasma concentration levels of coagulation factors are predicted to influence coagulation kinetics.

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## 3.2 Introduction

Studies conducted in this thesis were designed to investigate physical parameters that regulate circulating TF coagulation kinetics. As coagulation occurs on the surface of TF-expressing cells, the transport of coagulation factors in the blood to the cell may be rate limiting. Model systems have shown that coagulation kinetics are dependent upon flow rates in a manner explained by hydrodynamic theory of diffusion mass transport across a depleted zone (Forman et al. 1986; Andree et al. 1994; Contino et al. 1994; Hathcock 2006). Shear rates in the vasculature are highest at the vessel wall, and decrease proportionally away from the wall. As such, circulating TF experiences less shear rates than vessel wall immobilized TF, therefore the transport of coagulation factors by diffusion may control coagulation kinetics of circulating TF. We performed an analysis based upon fundamentals of mass transport theory to calculate how the flux of coagulation factors to a TF-expressing cell changes over time and as a function of shear rate. Our analysis predicts that the surface flux is proportional to shear rate. Surface flux is a function of plasma levels of coagulation factors, time and coefficients of diffusion. As levels of coagulation factors vary by over three-fold in the normal population, our analysis predicts a significant difference in procoagulant enzyme generation in the presence of circulating TF.

## 3.3 Background

The initiation of coagulation by TF is a surface phenomenon. TF in complex with FVIIa on the surface of a cell forms the extrinsic tenase complex, which can generate FIXa and FXa (Lawson et al. 1991). However, prior to activation, FIX and FX need to contact the extrinsic tenase complex. Coagulation factors are soluble in blood and entrained in the bloodstream, and therefore must contact the surface-bound extrinsic tenase for

coagulation to proceed. Under shear flow, convective (flow mediated) forces dominate the movement of coagulation factors along the vessel length, while radial movement is governed by diffusion. The coagulation factors nearest the extrinsic tenase complex are activated first, which leaves a depleted region of these coagulation factors. Continued generation of enzyme by the extrinsic tenase complex requires soluble coagulation factors to diffuse across the depleted zone in order to reach the extrinsic tenase complex. For surface reactions with reaction rates that exceed the mass transport rates of substrate (coagulation factors), the mass transport rates can be rate limiting to the overall coagulation reaction. Model systems have shown that the activation of factor X under shear flow increases with the one-third power of shear rate, in agreement with a transport rate-limited reaction (Kobayashi et al. 1974; Gemmell et al. 1988; Hathcock 2006). Flow supplies upstream coagulation factors to the vicinity of the extrinsic tenase complex. Therefore, flow effectively diminishes the depth of the depleted layer. Shear rates increase with flow rates for Newtonian fluids under fully developed, laminar flow, and therefore, the dimensions of the depleted layer are inversely proportional to the shear rate. As such, the surface flux (J) per unit length of coagulation factors under shear flow is a function of coagulation factor concentration ($C_{bulk}$), wall shear rate ($\gamma_w$), the coagulation factor coefficient of diffusion (Hathcock 2006):

[3.1]

A circulating tumor cell is entrained in the bloodstream and removed from the vessel wall. The circulating cell moves along the length of the blood vessel due to the same viscous forces that transport coagulation factors. Therefore, shear rate at the surface of a circulating cancer cell is reduced relative to the vessel wall. For reduced shear flow

conditions, the depleted layer can grow outward from the cell, and a time-dependent decrease in activation of coagulation factors IX and X is expected. To model this, we rely on fundamental principles of diffusion, and assume the cell membrane is a flat surface where the depletion zone grows perpendicular from the surface.

### 3.5 Results

The diffusive flux (J) of a substrate down a concentration gradient in direction (—) is described by Fick's first law of diffusion:

— [3.2]

As coagulation proceeds with time, the plasma closest to the procoagulant surface becomes relatively depleted of coagulation factors, and a depletion zone grows above the procoagulant surface in the direction . To supply the procoagulant surface with new coagulation factors from the bulk, we need to know how the depth of the depletion zone changes with time, which is described by Fick's second law of diffusion:

— — [3.3]

To continue with our analysis, we need to set initial and boundary conditions. Andree et al. demonstrated that the surface flux of soluble FX was rate limiting for its activation, rather than reaction rate limited (Andree et al. 1994). As such, we will assume that once coagulation factors reach the procoagulant surface they become activated, and the surface concentration ($y = 0$) of the coagulation factor is always 0. Moreover, we assume that before the reaction begins, the concentration of coagulation factors in plasma is uniform ($C = C_{bulk}$), and that at infinite distances from the procoagulant surface ($y=\infty$), the plasma concentration is unchanged ($C=C_{bulk}$) for all time points.

Initial condition: $t = 0$: $C = C_{bulk}$ for all y

Boundary conditions: $0 < t < \infty$: $y = 0$, $C = 0$

$y=\infty$, $C=C_{bulk}$

Boltzmann solved equation 3.3 by defining a new variable, z (Boltzmann 1894):

[3.4]

then:

[3.5]

and:

[3.6]

then, we rewrite equation 3.3 as:

[3.7]

which simplifies to:

[3.8]

next, we restate the boundary conditions:

Boundary conditions: $z = \infty$: $C = C_{bulk}$

$z = 0$: $C = 0$

we can simplify our calculations by letting — = b, and rewrite equation 3.8 as:

[3.9]

or:

[3.10]

After calculating the integral of equation 3.10, and reverting z back to y, we get the concentration profile over distance y away from the extrinsic tenase complex.

— [3.11]

Next, we calculate the exponent of each side:

— [3.12]

and revert b back to —:

— — [3.13]

and:

[3.14]

Calculate the Gaussian integral, and utilize our boundary conditions to solve, —= . Then, equation 3.13 becomes:

— — — [3.15]

we then rewrite equation 3.2:

—— [3.16]

and:

— — — [3.17]

Equation 3.17 describes the flux of coagulation factor to a surface (y = 0) as a function of bulk concentration ( , coefficient of diffusion ( and over time ( . This allows us to compare surface flux in the presence (equation 3.1) and absence (equation 3.17) of

shear flow. Using this approach, we compare surface flux for a number of shear rates over time in Figure 3.1.

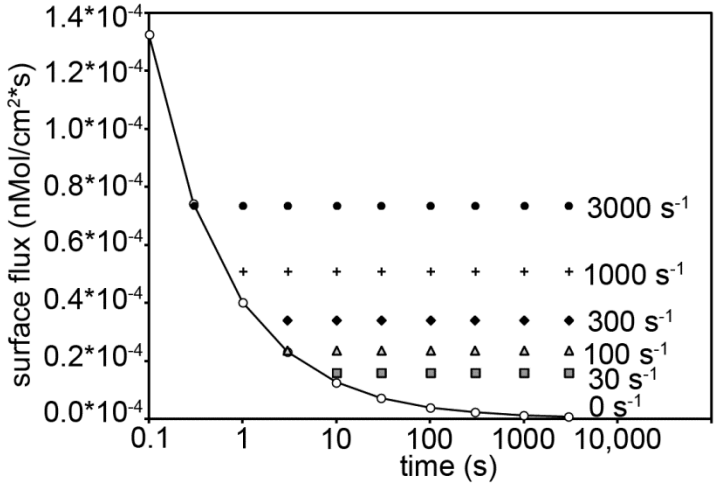


**Figure 3.1. Surface flux for coagulation factors to the extrinsic tenase complex as a function of shear rate.**

Figure 3.1 shows that in the absence of flow, surface flux decreases over time. To quantify the difference, the integral of surface flux with time was calculated and the cumulative differences in surface flux as a function of shear rate over time is shown in Figure 3.2. Figure 3.2 indicates that even for moderate shear rates seen in the venous vasculature ($\gamma_w$ = 300 $s^{-1}$), after 300 seconds approximately 15-fold more coagulation

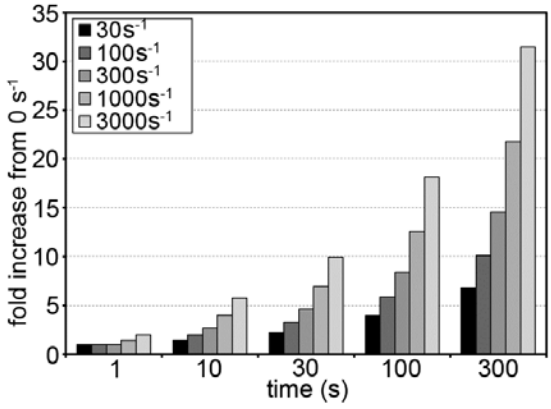


**Figure 3.2. Ratio of surface flux in the presence of shear flow compared to surface flux in the absence of shear flow.**

enzyme would have been generated than in the absence of flow. Coagulation exhibits threshold behavior in that coagulation proceeds once procoagulant stimuli overwhelm anticoagulant inhibition (Mann 2003). Therefore, a 15-fold difference in generation of procoagulant enzymes may result in a cell that exhibits a procoagulant phenotype in the presence of flow that is not procoagulant in the absence of flow. The surface flux of coagulation factors is directly determined by $C_{bulk}$, or in the case of coagulation factors, plasma levels of coagulation factors. Levels of coagulation factors vary by over three-fold in the normal population. Therefore, in the case of diffusion-limited coagulation of circulating cancer cells, we would expect at least a three-fold difference in enzyme generation rates induced by procoagulant circulating cancer cells in a patient population. Physiological variations in coagulation factor levels are not typically of concern in wound healing, as flow, which increases the supply of coagulation factors to an injury site, compensates for differences in activation rates of coagulation factors due to differences in coagulation factor levels (Gemmell et al. 1988).

Surface flux depends upon the coefficient of diffusion (D) in addition to $C_{bulk}$. While D is dependent upon the viscosity of plasma that may vary between patients, it is largely due to the size of the coagulation factors. Masses of coagulation factors vary roughly 7-fold, from ~50 kDa for FIX and FX, to 330 kDa for FV. Therefore, the diffusive fluxes of coagulation factors are predicted to vary due to differences in the coefficients of diffusion (D). Coefficient's of diffusion for coagulation factors in plasma have not been measured, but can be approximated according to the Stokes-Einstein equation:

[3.18]

Where:

$k_B$ = Boltzmann's constant

T = temperature

$\mu$ = viscosity of plasma

R = Stokes radius

We utilize a plasma viscosity of 1.2 mPa s, T = 293 K, and reported R of the different coagulation factors form the literature to calculate D ($cm^2\ s^{-1}$) for coagulation factors of the extrinsic pathway of coagulation in Table 3.1. As TF•FVIIa initiates coagulation, we normalized the calculated flux to that of FVII after t = 300 seconds, and report the range of relative fluxes from normal population coagulation factor levels. The Stokes' radius for FX has not been reported, so we used the commonly assumed coefficient of diffusion ($5 \times 10^{-7}\ cm^2\ s^{-1}$).

**Table 3.1. Table of calculated coefficient's of diffusion and surface flux ratios**

| Factor | Stokes Radius (nm) | C (nM) | $D^{20,plasma}(10^{-7}\ cm^2\ s^{-1})$ | $J/J^{VII}$ |
|---|---|---|---|---|
| VII | 3.5 (Gladhaug et al. 1970) | 7-13 (Fair 1983) | 5.1 | 1 |
| IX | 4.1 (Suomela 1976) | 40-142 (Yang 1978)* | 4.4 | 3-22 |
| VIII | 8.8 (Hoyer et al. 1981) | 0.4-1 (Hoyer 1994)* | 2 | <<1 |
| X | | 101-162 (Epstein 1984) | 5 | 8-23 |
| V | 9.5 (Esmon 1979) | 12-42 (Kamphuisen 2000) | 1.9 | 2-10 |
| II | 4.1 (Stenflo 1972) | 1200-1730 (Legnani 2003) | 4.4 | 100-266 |

* calculated (FIX: 100% = 4.5 $\mu$g $mL^{-1}$; FVIII: 100% = 0.2 $\mu$g $mL^{-1}$)

### 3.6 Discussion

Calculated values from Table 3.1 suggests large differences in surface fluxes for coagulation factors, and that the ratios of coagulation factor fluxes may vary by over 7-fold in the normal population. As such, this analysis predicts significant person-to-person differences in sensitivity to circulating cancer cell induced coagulation. As initiation of coagulation exhibits threshold characteristics when procoagulant properties overcome

anticoagulant properties, the role for coagulation factor levels to dictate coagulation initiated by circulating cancer cells demands attention. In addition, competition for binding sites of coagulation factors (i.e. FII versus FX) may influence a cell's procoagulant phenotype, where membrane-binding events are regulated by surface flux. Moreover, as transport fundamentally occurs over distances, reducing the distance between coagulation factors in plasma and a procoagulant (active TF expressing) cell may have significant impacts on the generation of procoagulant enzymes. Therefore, as circulating cancer cells are distributed in the bloodstream, and may come in close proximity to each other at high cell concentrations; the distribution (i.e. spatial separation) of circulating cancer cells in suspension may regulate their procoagulant phenotype.

# Chapter 4: The Role of Carrier Number in the Procoagulant Activity of Tissue Factor in Blood and Plasma

Garth W. Tormoen, Sandra Rugonyi, András Gruber, Owen J.T. McCarty

## 4.1 Abstract

TF is a transmembrane glycoprotein cofactor of activated blood coagulation factor VII (FVIIa) that is required for hemostatic thrombin generation at sites of blood vessel injury. Membrane-associated TF detected in circulated blood of healthy subjects, referred to as intravascular or circulating TF has been shown to contribute to experimental thrombus propagation at sites of localized vessel injury. Certain disease states, such as metastatic cancer, are associated with increased levels of intravascular TF and an elevated risk of venous thromboembolism. However, the physiological relevance to circulating TF to hemostasis or thrombosis is ill-defined. This study was designed to assess whether the spatial separation of intravascular TF carriers in blood, demonstrated with TF-inducible human monocytic cell line U937 or TF-coated polymer microspheres, affected procoagulant activity and hence thrombogenic potential. Experiments were performed to characterize the effects of TF-carrier number on the kinetics of clot formation in both open and closed systems. The procoagulant activity of TF carriers was found to correlate with spatial separation in both closed, well-mixed systems and open, flowing systems. TF carriers enhanced the amidolytic activity of FVIIa toward the chromogenic substrate, S-2366, as a function of carrier count. These results suggest that TF-initiated coagulation by circulating TF is kinetically limited by mass transport of TF-dependent coagulation factors to the TF-bearing surface, a constraint that may be unique to circulating TF.

Spatial separation of circulating TF carriers is therefore a critical determinant of the procoagulant activity of circulating TF.

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## 4.2 Introduction

The analysis performed in Chapter 3 describes a critical role for mass transport to drive TF initiated coagulation kinetics. Intrinsic to this analysis is the requirement for coagulation factors in the bulk plasma to traffic to a TF-bearing surface in order to drive coagulation. A physical parameter unique to circulating TF is the three-dimensional distribution of these surfaces in blood. To provide further insight into physical determinants of circulating TF, experiments in Chapter 4 were designed to determine the role that spatial separation, a measure of the average separation distance of TF surfaces in suspension, plays in determining coagulation kinetics. Data presented here suggest a critical role for spatial separation to contribute to procoagulant activity of circulating TF.

## 4.3 Background

The increased incidence of venous thromboembolism for metastatic cancer patients is a well-known phenomenon first described nearly two centuries ago and embodies the second leading cause of death in patients with cancer (Bouillard et al. 1823; Trousseau 1865). The association between metastatic cancer and activation of coagulation suggests that metastasizing cancer cells are not inert to the blood environment.

Patients with cancer who suffer thromboses have demonstrated elevated levels of circulating TF in the form of TF-bearing microparticles, while cancer cell lines in culture

have been shown to express TF (Mackman 2009; Berny-Lang et al. 2011). TF, the physiological initiator of coagulation, complexes with FVIIa to activate FIX and FX. The surface-bound nature of TF, its requirement for phospholipids in order to initiate coagulation and the need for FVIIa to form a complex with TF to achieve physiologically relevant reaction rates imply that the initiation of coagulation by TF is a surface phenomenon (Nemerson 1968; Maynard et al. 1975). Kinetics of TF-initiated enzyme activation have been found to depend upon physicochemical characteristics of the TF-bearing surface and its interface with blood (Gemmell et al. 1988; Pickering et al. 2004; Okorie et al. 2008). The discovery of intravascular, circulating TF has brought anew the importance of understanding kinetic limitations on TF-initiated thrombin generation, as the proximity and mixing of circulating TF to blood is different in many aspects compared to TF locally exposed on the vascular wall at sites of disease or injury (Lerner et al. 1971; Giesen et al. 1999; Aras et al. 2004; Ritis et al. 2006; Moosbauer et al. 2007; Panes et al. 2007). *In vivo* and *in vitro*, circulating TF has been shown to be active in initiating coagulation and indeed contribute to experimental thrombosis at sites of vessel injury (Giesen et al. 1999; Falati et al. 2003; Chou et al. 2004; Pawlinski et al. 2010). However, healthy subjects have been shown to have a basal level of circulating TF without evidence for pathological initiation of coagulation (Giesen et al. 1999; Cvirn et al. 2007). Conversely, induction of TF-bearing microparticles restored hemostasis in a mouse model of hemophilia (Hrachovinova et al. 2003). Therefore, the circumstances that dictate whether circulating TF contributes to thrombotic or hemorrhagic coagulopathy versus normal hemostasis have not been reconciled.

Studies on the ability of wall-bound TF to initiate coagulation have shown that the critical

TF surface density to result in surface deposition of fibrin is largely dependent upon flow parameters (Okorie et al. 2008). Generation of FXa was shown to depend upon the presence of phospholipids and FVII levels in an *in vitro* system of TF surface-catalyzed coagulation (Gemmell et al. 1988). Studies with lymphoid cell lines support the notion that the membrane environment, rather than TF exposure, is the dominating factor in determining procoagulant activity of TF (Pickering et al. 2004). How TF influences cancer cell procoagulant activity for vessel-wall adherent versus circulating cancer cells has not been determined. The presence of convective mass transport from blood flowing past an adherent TF-expressing cancer cell presents the potential for different procoagulant activities as compared to a circulating cell, which experiences significantly less relative flow of blood across its surface. Therefore, it is critical to determine the TF procoagulant activity for circulating cells or microparticles under physiological conditions of flow.

This study was designed to characterize the role for spatial distribution in the procoagulant phenotype of intravascular TF. We hypothesize that increasing the proximity of TF carriers to bulk plasma, through increasing the distribution of TF-bearing surfaces throughout blood, will increase the procoagulant activity of circulating TF by reducing the average distance between coagulation factors and a TF surface. We characterized the procoagulant and prothrombotic phenotype of TF carriers, consisting of the TF-inducible monocytic cell line U937 and TF-coated polymer microspheres, in both open and closed systems, and in both static and flow conditions. Our results demonstrate that for circulating TF, spatial distribution plays a role in determining coagulation initiation and propagation kinetics, and suggest that circulating TF procoagulant and

prothrombotic activity may be independent of whole blood TF concentration.

## 4.4 Materials and Methods

### *4.4.1 Reagents*

All reagents were from Sigma-Aldrich (St. Louis, MO) or previously described sources unless otherwise specified (White-Adams et al. 2010). Monoclonal antibodies (mAbs) to factor XI were generated as previously described (Tucker et al. 2009). Neutralizing anti-TF antibodies were from Genentech (South San Francisco, CA) and fluorescein isothiocyanate (FITC)-conjugated anti-TF antibodies were from Lifespan Biosciences (Seattle, WA). Recombinant, lipidated TF (Dade® Innovin®) was from Siemens Healthcare Diagnostics (Deerfield, IL). Fibrillar equine collagen was from Chrono-Log (Havertown, PA). Biowhittaker® Premium FBS was from Lonza (Basel, Switzerland).

### *4.4.2 Blood donations*

All blood donations were from healthy human subjects and obtained in accordance with Oregon Health and Science University IRB approval. Blood was collected by antecubital venipuncture in to a one-tenth volume 3.8% sodium citrate for occlusive thrombus assays or 3.2% sodium citrate for coagulation and enzyme generation assays. Platelet-poor plasma (PPP) for coagulation studies was prepared by centrifugation of citrated blood at 2150 × *g* for 10 minutes. PRP was decanted from the spun blood, pooled with PRP from two other donors and subjected to centrifugation at 2150 × *g* for 10 additional minutes. PPP was collected by decanting the supernatant. Aliquots of PPP were frozen at -80$^{o}$ C until use.

### *4.4.3 Human monocytic U937 cell culture*

U937 cells were from ATCC (Manassas, VA). Cells were cultured in non-treated T75 flasks in a medium consisting of RPMI 1640 containing 10% FBS and 1× penicillin and streptomycin and kept in an incubator at 37$^{o}$ C maintained at 5% $CO_2$. The culture medium was supplemented weekly with L-glutamine. Flasks were seeded at a concentration of $2 \times 10^5$ $mL^{-1}$ and kept below $2 \times 10^6$ $mL^{-1}$ with viability maintained above 90% as measured with Trypan Blue staining. All experiments were with cells in passages 4 through 7.

*4.4.4 U937 induction of TF*

U937 cells were incubated with 50 μg $mL^{-1}$ anti-TF antibody to minimize the influence of constitutively expressed TF. Cells were then split and suspended in the normal culture media or media containing 10 μg $mL^{-1}$ lipopolysaccharide (LPS) and placed in an incubator for 24 hours. Then, cells were subjected to centrifugation at 130 × *g* for 10 minutes and suspended in Hank's Balanced Salt Suspension (HBSS). Cell suspensions were diluted from a concentration of $1\times10^7$ to $1\times10^2$ $mL^{-1}$ with HBSS as determined by a hemocytometer.

*4.4.5 Microsphere coating*

Polymeric microspheres (diameter = 9.86 μm) that approximated the size of human monocytes (diameter ~ 7 -10 μm) were purchased from Bangs Laboratories (Fishers, IN). Stock solution microsphere concentrations were creating by diluting in $H_2O$ (Resistivity = 18.2 MΩ $cm^{-1}$) and counted with a hemocytometer. Microspheres were then dispensed into a 1.7 mL vial ($10^6$, final count). TF coating solutions were prepared by diluting TF stock to 1 nM in $H_20$. Coating solution was added to the microspheres (1 mL, final volume) and allowed to coat for 60 minutes at room temperature (RT). Coated

microspheres were subjected to centrifugation at 16,100 × *g* for 10 minutes and surface blocked with 0.5% denatured-filtered BSA in PBS. Coated and blocked microspheres were suspended in HBSS.

*4.4.6 Flow cytometry analysis*

LPS-stimulated U937 cells or TF-coated microspheres ($10^6$, final count) were washed with HBSS prior to incubation with a FITC-conjugated anti-TF antibody (50 μg $mL^{-1}$) for 30 minutes at RT. Following labeling, TF carriers were washed with HBSS and next analyzed on a FACS Calibur flow cytometer with CellQuest acquisition and analysis software (Becton Dickinson, Franklin Lakes, NJ). Unlabeled carriers served as negative controls.

*4.4.7 Clotting time determination*

TF carriers were suspended in HBSS and counted with a hemocytometer. Suspension concentrations were then diluted from $1 \times 10^6$ to $1 \times 10^2$ $mL^{-1}$. TF carrier suspension was added 1:1 v/v to PPP and allowed to mix for 3 minutes at $37^o$ C, after which 50 μL of 25 mM $CaCl_2$ in $H_2O$ was added and the clotting time recorded on a KC4 coagulation analyzer (Trinity Biotech, Bray Co., Wicklow, Ireland). To determine the procoagulant mechanism of microspheres and U937 cells, anti-FXI mAbs were added to PPP and allowed to mix for 5 minutes at $37^o$ C prior to the addition of microsphere or cell suspension and the clotting time measured. Anti-TF antibodies were mixed with the carrier suspensions for 5 minutes at $37^o$ C before mixing with the PPP, and the resultant clotting time measured. Reported values represent that average value for a minimum of 3 independent experiments.

*4.4.8 Occlusive thrombus assay*

*Ex vivo* occlusive thrombus assay was performed as previously described (Berny et al. 2010). Briefly, glass capillary tubes (2.0 mm × 0.2mm × 50 mm; VitroCom, Mountain Lakes, NJ) were coated with 100 μg $mL^{-1}$ equine fibrillar collagen in 10 mM acetic acid for 60 minutes at RT while rotating on a carousel. Next, tubes were washed with PBS and surface-blocked with 0.5% denatured-filtered BSA in PBS for 60 minutes at RT while rotating on a carousel. Blocked tubes were then washed and one end fitted with a 1 cm length of 0.40" internal diameter silicone tubing and attached to a suspended 3 mL syringe while submerging the distal end in PBS. TF carrier suspensions were mixed with citrated blood and recalcified immediately before subjecting them to flow. The time for the blood solutions to occlude flow in the collagen-coated capillary was measured as the time to occlusion.

*4.4.9 Enzyme generation assay*

The chromogenic substrate, S-2366 (15 μL at 4 mM; Chromogenix, Milan, Italy) was combined with 50 μL of PPP and dispensed into individual wells of a 96-well plate. Next, 50 μL of TF-coated microsphere suspensions ranging from a concentration of $1 \times 10^6$ to $1 \times 10^3$ $mL^{-1}$ were added to the wells and allowed to mix for 15 minutes at $37^o$ C. Then, 50 μL of 25 mM $CaCl_2$ in $H_20$ was added and immediately followed by measurement of absorbance of 405 nm wavelength light at 1 minute intervals for 1 hour in a spectrophotometer (Tecan, Mannedorf, Switzerland). Absorbance data exhibited a sigmoid relationship with time. The data were analyzed by first normalizing by the maximum and subtracting the baseline absorbance. Next, the initiation lag was recorded as the time corresponding to the first measurable increase in absorbance. Then, the molar equivalent of S-2366 added to the plasma (600 pMoles) was divided by the time

difference between 0% and 100% absorbance through extrapolation of the slope at 50% absorbance.

*4.4.10 Spatial separation calculation*

The average distance between TF carriers in suspension, referred to as spatial separation, was obtained by calculating the cubic root of the volume of the liquid divided by the number of TF carriers added. This approach assumes that TF carriers are uniformly distributed in suspension.

*4.4.11 Statistical analysis*

Data are presented as mean and standard error of the mean (SEM). Statistical significance between means was determined with a student's t-test. Significance for all tests required $p<0.05$.

## 4.5 Results

*4.5.1 Monocytic cell-line U937 and TF-coated polymer microspheres portray surface-bound TF*

To determine if surface expression of TF on TF-coated microspheres and U937 cells, flow cytometry in the presence and absence of a FITC-conjugated anti-TF antibody was performed. As shown in Figure 4.1, flow cytometry analysis confirmed that both TF carriers portrayed TF on their surface. Unlabeled U937 cells resulted in a mean fluorescence of 3.1, while antibody labeled cells yielded a mean fluorescence of ~130.1. Non-stimulated U937 cells yielded a mean fluorescence of ~109.3 (data not shown). Unlabeled TF-coated microspheres yielded a mean fluorescence of ~2.8, while FITC-conjugated antibody labeled TF-coated microspheres yielded a mean fluorescence of ~21.9. Antibody labeled BSA-coated negative control microspheres yielded a mean fluorescence of ~5.9.

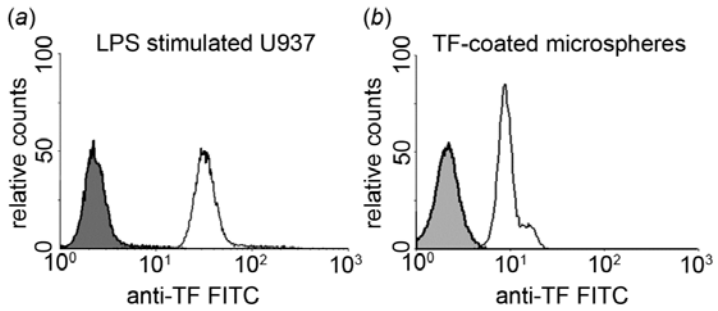


**Figure 4.1. Characterization of TF expression on LPS-stimulated U937 cells and TF-coated polymer microspheres. Cultured U937 cells that were stimulated with 10 μg mL$^{-1}$ LPS for 24 hours or TF-coated microspheres were labeled with a FITC-conjugated anti-TF antibody (50 μg mL$^{-1}$) and analyzed by flow cytometry. The shaded histograms represent background fluorescence of cells or beads; white histograms represent a shift in fluorescence in the presence of an anti-TF antibody.**

*4.5.2 Monocytic cell-line derived TF is procoagulant in a carrier number- and TF-dependent manner*

To verify that our assays were sensitive to hematopoietic TF, the ability of TF-expressing U937 cell suspensions to coagulation plasma was investigated. As shown in Figure 4.2(a), our results indicate that LPS-stimulated cells had higher procoagulant activity than non-stimulated U937 cells. The clotting times decreased with increasing counts of U937 cells from $1 \times 10^4$ to $1 \times 10^6$ mL$^{-1}$ (Figure 4.2(b)), and their ability to clot plasma was abrogated by the addition of an anti-TF antibody (Figure 4.2(a)). The clotting time correlated linearly with the spatial separation of U937 cells (Figure 4.2(c)), $R^2 = 0.9949$). No significant effect on clotting time was observed for U937 cell counts below $1 \times 10^5$ mL$^{-1}$.

*4.5.3 Synthetic TF carriers are procoagulant in a carrier number- and TF-dependent manner*

We next investigated the ability of TF-coated microspheres to promote coagulation. Clotting times in the presence of $1 \times 10^6$ mL$^{-1}$ TF-coated microspheres were dramatically reduced in a TF-dependent manner (Figure 4.3(a)). Clotting times were unaffected by the blockade of the contact pathway with the anti-FXI mAbs, 14E11 and 1A6, unless the TF

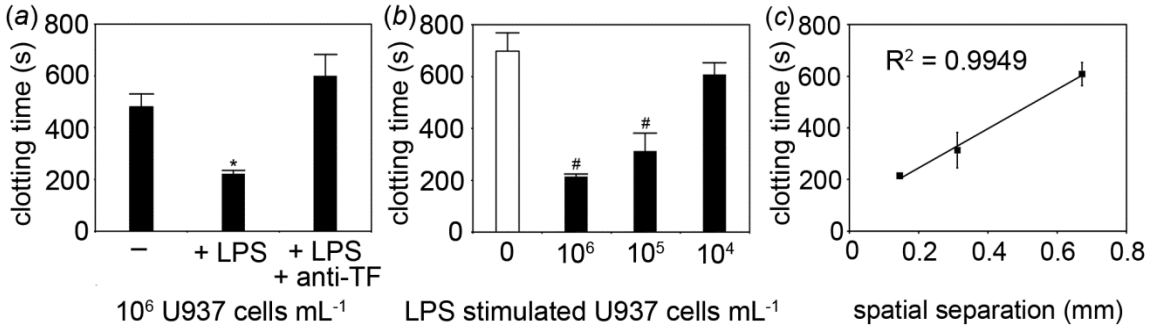


**Figure 4.2. Characterization of the procoagulant activity of monocytic cells in a closed system. U937 cells were procoagulant in a TF-dependent (a) and cell concentration-dependent manner (b) that correlated with the spatial separation of TF surfaces (c). Human sodium citrate anticoagulated plasma was pretreated with vehicle (-) or a neutralizing antibody to TF (anti-TF, 20 μg mL$^{-1}$) prior to addition of cultured U937 cells ($10^4$ - $10^6$ mL$^{-1}$) for 3 minutes at 37$^o$ C. U937 cells were stimulated with LPS (10 μg mL$^{-1}$) for 24 hours. Coagulation of plasma was initiated by recalcification using 8.3 mM $CaCl_2$ (final concentration) and clotting times were recorded on a KC4 coagulation analyzer (Trinity Biotech, Wicklow, Bray Co, Ireland). *P < 0.05 versus non-LPS stimulated cells. #p < 0.05 versus vehicle.**

pathway was blocked concomitantly (Figure 4.3(a)). TF carrier suspensions from $1 \times 10^2$ to $1 \times 10^6$ mL$^{-1}$ yielded clotting times that varied with carrier count (Figure 4.3(b)). Clotting time correlated with the spatial separation of TF-coated microspheres (Figure 4.3(c), $R^2 = 0.9992$).

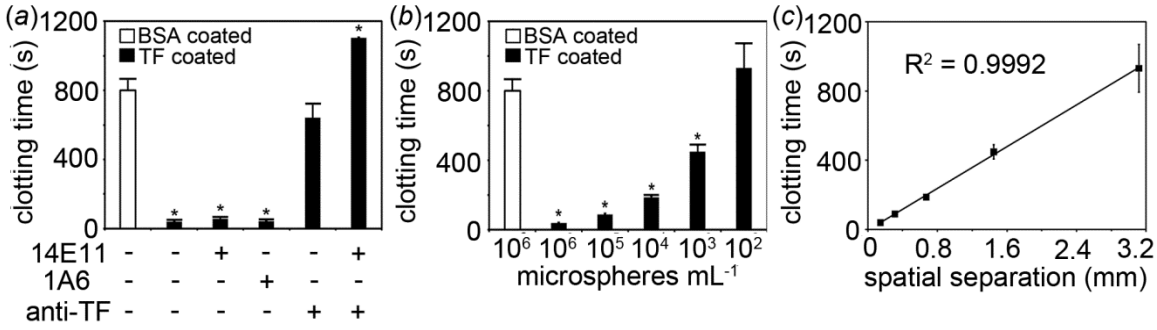


**Figure 4.3. Characterization of the procoagulant activity of TF microspheres in a closed system. TF-coated polymeric microspheres were procoagulant in a TF- (a) and carrier concentration-dependent manner (b) that correlated with spatial separation of TF surfaces (c). Human sodium citrate-anticoagulated plasma was pretreated with neutralizing antibodies to TF (anti-TF, 50 μg mL$^{-1}$) and FXI (14E11 or 1A6, 10 μg mL$^{-1}$) prior to addition of BSA-coated (white bars) or TF-coated (black bars) microspheres ($10^2$ – $10^6$ mL$^{-1}$) for 3 minutes at 37$^o$ C. Data in (a) were obtained with microsphere additions of $10^6$ mL$^{-1}$. Coagulation of plasma was initiated by recalcification using 8.3 mM $CaCl_2$ (final concentration) and clotting times were recorded on a coagulometer. *p < 0.05 versus BSA-coated microspheres.**

*4.5.4 TF carriers promote occlusive thrombus formation in a TF-and carrier number-dependent manner*

Experiments were designed to determine the role of carrier count on the prothrombotic activity of TF carriers in a flowing, open, whole blood assay by measuring the time required for the occlusion of flow. Addition of $1 \times 10^6$ TF-coated microspheres $mL^{-1}$ significantly reduced the time to occlusion in a TF-dependent manner (Figure 4.4(a)). The prothrombotic activity of TF-coated microspheres was abrogated by the addition of an anti-TF antibody (Figure 4.4(a)). Blockade of the contact pathway with 14E11 did not influence time to occlusion unless an anti-TF antibody was also included. TF-coated microspheres reduced the time to occlusion in a carrier number-dependent manner (Figure 4.4(b)). Time to occlusion correlated with spatial separation of TF-coated microspheres (Figure 4.4(c), $R^2 = 0.9909$).

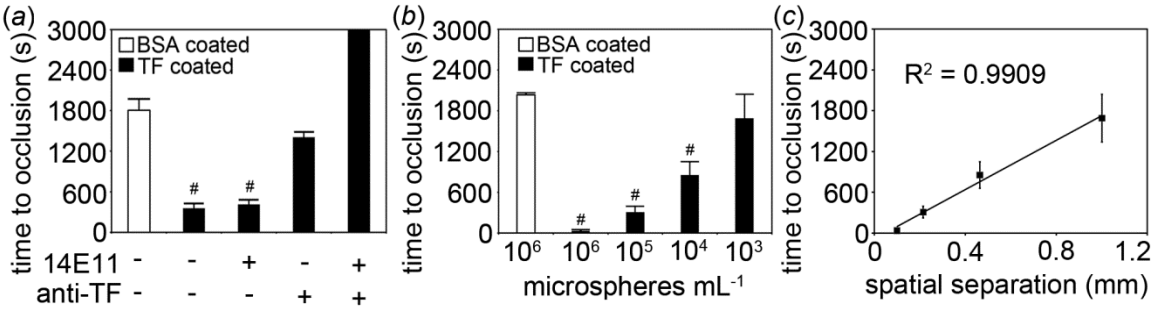


**Figure 4.4. Characterization of prothrombotic activity of TF microspheres in an open system. TF-coated polymeric microspheres were prothrombotic in a TF-(a) and carrier concentration-dependent manner (b) that correlated with spatial separation (c) when added to recalcified whole blood in an *ex vivo* occlusive thrombus assay. The procoagulant activity of $10^5$ $mL^{-1}$ TF-coated microspheres was abrogated with an anti-TF antibody. Blockade of the contact pathway of coagulation with 14E11 (anti-FXI) had no effect on time to occlusion for TF microsphere suspensions in the absence of anti-TF antibodies (c). TF-coated microspheres ($10^3$ - $10^6$ $mL^{-1}$ final concentration) were added to human sodium citrate-anticoagulated blood prior to recalcification using 8.3 mM $CaCl_2$ (final concentration). Occlusive thrombus formation was initiated by flowing the pretreated blood at a constant pressure through a collagen-coated capillary tube and the time to occlusion was recorded when flow ceased. Data are reported as mean and standard error. *p < 0.05 versus non-LPS stimulated cells. #p < 0.05 versus BSA-coated microspheres.**

*4.5.5 Enzymatic initiation time and reaction rate are carrier number dependent*

Experiments were designed to investigate whether the time required for assembly of active enzyme complexes on the TF carriers or the reaction rates of assembled complexes were dictated by carrier number. Cleavage of the chromogenic substrate S-2366 was performed to measure initiation time lag (initiation time) for activated enzyme to form as well as the rate at which formed active enzymes cleaved the substrate (rate). The initiation time decreased as the TF-coated microsphere carrier count increased (Figure 4.5(a)). The rate of substrate cleavage, on the other hand, increased with increases in carrier count (Figure 4.5(b)). The initiation time correlated with spatial separation (figure 5(c), $R^2 = 0.9643$), whereas enzyme reaction rates correlated with inverse spatial separation (Figure 4.5(d), $R^2 = 0.9927$).

**4.6 Discussion**

This and other studies have shown that the addition of TF-expressing cells to plasma results in shortened clotting times in a cell concentration-dependent manner. The ability of TF-expressing cells to initiation coagulation has been shown to depend on the physicochemical properties of the cell-plasma interface. In other words, the presence of TF does not dictate its ability to promote coagulation, even though the mechanism of coagulation initiation is TF dependent. In this study, we implemented a model system utilizing a single, fully active form of TF (thromboplastin) adsorbed onto the surfaces of polymer microspheres to determine if the spatial separation of TF carriers regulated coagulation in well-mixed, closed system plasma coagulation assays (Figures 4.2(c) and 4.3(c), $R^2 = 0.9949$ and 0.9992, respectively). Additionally, the time to occlusion linearly correlated with the spatial separation of TF carriers in a flowing, open-system whole

blood occlusive thrombus assay (Figure 4.4(c), $R^2 = 0.9909$). In a purified system, the initiation time

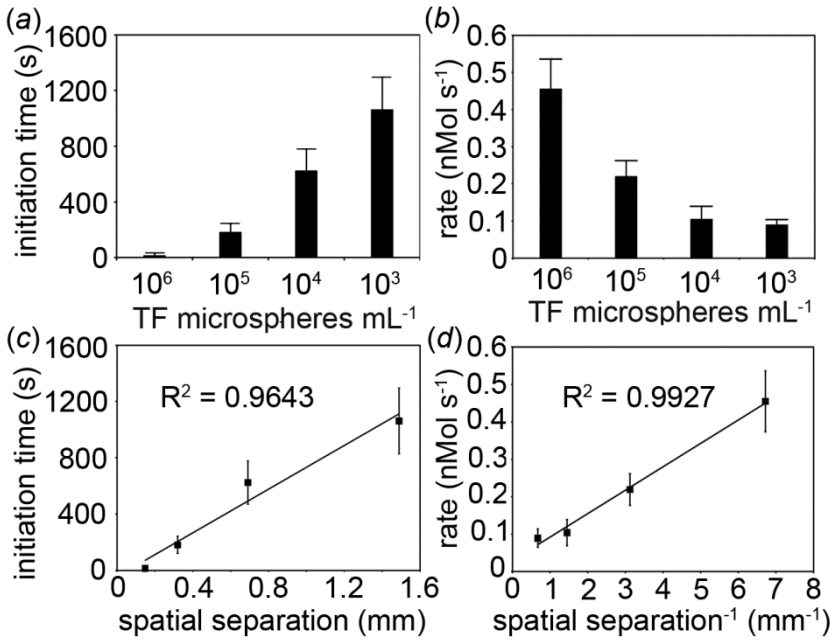


**Figure 4.5. Characterization of enzyme activation kinetics initiated with TF microspheres. TF-coated polymeric microspheres promoted assembly of active enzyme complex in a carrier concentration-dependent manner (a). The rate of substrate cleavage was dependent upon carrier count (b). Enzyme initiation time yielded a direct relationship with spatial separation (c). Enzyme reaction rate yielded an inverse relationship with spatial separation for TF microspheres (d). human sodium citrate-anticoagulated plasma was pretreated with 15 μL of S-2366 before addition of 50 μL of TF microspheres ($10^3$ - $10^6$ $mL^{-1}$) and recalcification using 7.6 mM $CaCl_2$ (final concentration). Absorbance of 405 nm wavelength light was recorded with a spectrophotometer at 1 minute intervals for 60 minutes.**

correlated with spatial separation (Figure 4.5(c), $R^2 = 0.9643$), while the enzyme reaction rate showed an inverse correlation with spatial separation (Figure 4.5(d), $R^2 = 0.9927$).

Results from this study demonstrate that the spatial separation of TF surfaces, as calculated from carrier count, strongly correlates with procoagulant and prothrombotic activity. These results suggest that mass transport may be a prominent kinetic limitation for intravascular TF under steady-state conditions and supports the notion that procoagulant activity of circulating TF is kinetically constrained by enzyme complex

formation on the TF-bearing surface (Giesen et al. 1991; McGee et al. 1992). Immobilized TF has been shown to transition from diffusion-limited to convection-assisted reaction kinetics above wall shear rates of 120 $s^{-1}$ (Gemmell et al. 1988). A transition from diffusion-limited reaction kinetics for circulating TF (lower procoagulant activity) to convection assisted kinetics for immobilized TF (higher procoagulant activity) may explain how TF can circulate without initiation of clinically evident coagulation, yet contribute to thrombus propagation when incorporated into the growing thrombus. In essence, the procoagulant activity of TF may be regulated independent of whole blood TF concentration, but rather by the distribution of TF within the whole blood as well as the relative flow of blood past the TF-bearing surface. Thus, data presented here suggest the potential for circulating TF carriers to increase procoagulant activity based solely on changes in spatial separation. Further, our results suggest that a TF-expressing cell, such as a metastatic cancer cell, may yield different procoagulant activities based on its relative mobility within the vasculature. Specifically, a vessel wall adherent cell, as may be encountered for an intravasating or extravasating cell, would yield a procoagulant activity that is dictated by convection-mediated transport, while a circulating cell's procoagulant activity is dictated by diffusion-mediated transport. Additionally, these results also support the idea that turbulent flow would increase circulating TF procoagulant activity through enhanced substrate mass transport to the TF carrier surface through convection-assisted mixing. Anecdotally, regions of turbulent flow due to branching vessels in a baboon sepsis model were shown to undergo increases in TF-dependent coagulation (Lupu et al. 2005).

In suspension, increasing the TF carrier count, and thus the proximity of reacting TF surface to soluble substrate, increases the rate of formation of active enzyme complexes per unit volume. Increases in TF carrier count in blood, such as due to an increase in the number of circulating TF-bearing microparticles, could promote thrombosis. Here, in its simplest form, a parent cell sheds microparticles. These microparticles, being composed largely of the parent cell membrane, would not alter the concentration of circulating TF within the blood. However, the TF carrier count per unit volume of blood would be dramatically increased, resulting in plasma coagulation factors having greater access to TF surfaces, hastening their conversion to active enzyme. This may elevate the procoagulant activity of circulating TF in the blood. For instance, the induction of microparticles by monocytes in models of endotoxemia and in patients with paroxysmal nocturnal hemoglobinuria has been reported, both of which are associated with thrombotic complications (Liebman et al. 2003; Aras et al. 2004). Similarly, a link between intravascular TF and cancer cell-derived microparticles has been observed, perhaps explaining why patients with cancer experience elevated rates of venous thromboembolism (Langer et al. 2008). Our results suggest a novel TF procoagulant activity-regulating mechanism, independent of the concentration of TF, which may contribute to understanding the physiological significance and clinical relevance of intravascular TF.

The case for spatial separation dictating the procoagulant activity of intravascular TF may highlight a novel therapeutic strategy for coagulopathy associated with intravascular TF. While reducing expression of TF could reduce the thrombogenic activity of a microparticle, such as by inhibiting TF expression on LPS-induced monocytic cell lines

by activated protein C, it is possible that inhibiting the formation of microparticles from the TF-expressing parent cell could also be effective at preventing thrombosis (Shu et al. 2000). For instance, IL-10 administration was found to reduce thrombin generation of LPS-stimulated monocytes, which resulted in concomitant reductions in microparticle formation as well as TF expression (Poitevin et al. 2007). Moreover, blockade of the leukocyte receptor, PSGL-1, has been shown to reduce leukocyte-derived microparticles, while PSGL-$1^{-/-}$ mice were unable to incorporate circulating TF into thrombi at sites of vessel injury (Falati et al. 2003; Hrachovinova et al. 2003). Therefore, whether reduction in circulating TF activity is due to inhibition of TF expression or microparticle generation is difficult to know, as inhibition of one is often linked with inhibition of the other.

This study aimed to determine the impact of TF carrier count on the procoagulant activity of TF. A direct relationship of clotting time, time to occlusion and enzyme initiation was seen with spatial separation, while enzyme reaction rates yielded an inverse correlation with spatial separation, a direct function of carrier count and measure of the distribution of particles in suspension. These findings were evaluated in light of mass transport limitations, which suggest that determinants of prothrombotic intravascular TF activity, include the TF carrier number. The paucity of thrombotic events in the presence of intravascular events may be explained by kinetically constrained mass transport of coagulation factors to the TF carrier. Such a limitation may be overcome to achieve clinically relevant coagulation activation when the carrier number reaches a critical level or when a transition from diffusion-limited to convection-assisted reaction kinetics occurs when circulating TF incorporates into a growing thrombus or a circulating cell adheres to a vessel wall. It is interesting to note that these are the physical parameters, independent

of the changes in a patient's coagulation profile and whole blood TF concentration, which may influence the procoagulant activity and clinical relevance of circulating TF.

# Chapter 5: Physiological Levels of Blood Coagulation Factors IX and X Control Coagulation Kinetics in an In Vitro Model of Circulating TF

Garth W. Tormoen, Ayesha Khader, András Gruber, Owen J. T. McCarty

## 5.1 Abstract

Thrombosis significantly contributes to cancer morbidity and mortality. The mechanism behind thrombosis in cancer may be circulating tissue factor (TF), as levels of circulating TF are associated with thrombosis. However, circulating TF antigen level alone has failed to predict thrombosis in patients with cancer. We hypothesize that coagulation factor levels regulate circulating TF-induced blood coagulation. Coagulation was measured as a function of individual coagulation factor levels and TF particle concentration. Clotting times increased when pooled plasma was mixed at or above a ratio of 4:6 with PBS. Plasma clotting times in the presence of TF particles increased when pooled plasma was mixed at or above a ratio of 8:2 with factor VII-depleted plasma, 7:3 with factor IX- or factor X-depleted plasmas, or 2:8 with factor II-, V- or VIII-depleted plasmas. Addition of coagulation factors VII, X, IX, V and II to depleted plasmas shortened clotting and enzyme initiation times, and increased enzyme generation rates in a concentration-dependent manner for a single TF particle concentration. Only additions of factors IX and X from low-normal to high-normal levels shortened clotting times and increased enzyme generation rates. Our results demonstrate that coagulation kinetics for TF particles are controlled by factor IX and X levels within the normal physiological range. We hypothesize that individual patient factor IX and X levels may be prognostic for susceptibility to circulating TF-induced thrombosis.

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## 5.2 Introduction

In Chapter 3, an analysis of mass transport elucidated the role for coagulation factor levels to determine surface flux of coagulation factors, and thus coagulation kinetics for transport-limited coagulation reactions. However, coagulation is a multi-step reaction involving a number of different coagulation factors that exhibit unique diffusion properties and are present in humans at varying concentrations, and it is not clear which factor or factor(s) may be rate limiting for coagulation. In Chapter 4, the role of spatial separation and the implications for mass transport to control coagulation kinetics was highlighted for TF carriers in pooled plasma and whole blood. Pooling plasma from multiple donors equilibrates donor differences in factor levels to give pooled plasma ~ 100% factor levels. However, individual levels of coagulation factor levels vary, and the effects that these individual variances have on susceptibility to circulating TF induced coagulation is not known. In Chapter 5, plasmas with varying levels of individual coagulation factors are generated and the resultant effects on coagulation kinetics measured for TF particle counts of $10^3$, $10^4$, and $10^5$ $mL^{-1}$.

## 5.3 Background

Cancer is a hypercoagulable state. Patients with cancer are at a 4-7 fold increased risk of developing venous thrombosis (Iodice et al. 2008). The development of thrombosis confers worse outcomes for patients with cancer and contributes significantly to patient morbidity; however, the mechanisms underlying thrombosis in cancer remain ill-defined (Khorana et al. 2007; Khorana et al. 2008; Elice et al. 2009; Mandala et al. 2010). The

association between having cancer and developing thrombosis has been observed for centuries, yet we are unable to accurately predict if an individual patient with cancer will develop thrombosis over the course of their disease (Bouillard et al. 1823; Trousseau 1865). Further, only a relative minority of patients with cancer will develop thrombosis; thus, anticoagulation prophylaxis is not part of routine care for patients with cancer.

Efforts to correlate cancer-specific properties with incidence of thrombosis have found that cancer site of origin, histology and stage contribute to an increased risk for developing thrombosis (Chew et al. 2006; Chew et al. 2008; Iodice et al. 2008). The observation that specific tumor characteristics influence the risk of thrombosis suggests that cancer cells are a procoagulant stimulus. Procoagulant mechanisms of cancer cells include surface expression of tissue factor (TF) (Tanaka et al. 1993; Iodice et al. 2008; Berny-Lang et al. 2011; Welsh et al. 2012). TF is a 47 kDa transmembrane protein, which serves as the physiological initiator of coagulation during blood vessel injury. TF is not expressed on the surface of normal cells within the blood, and therefore control of its procoagulant activity is largely due to exposure only at sites of vessel injury. Unless a bleeding diathesis is present, virtually every vessel injury results in a hemostatic response to stem blood loss. Some pathological states, such as metastatic cancer and acute myelogenous leukemia (AML), introduce TF-expressing cells or TF-expressing cell derived microvesicles into the blood circulation (Lopez-Pedrera et al. 2006; Zwicker et al. 2009). Elevated levels of circulating TF are associated with thrombosis in cancer, yet presence of circulating TF by itself is not prognostic for development of thrombosis in patients with cancer (Khorana et al. 2008; Zwicker et al. 2009; Auwerda et al. 2011; Thaler et al. 2011). Therefore, the impact of circulating TF on coagulation remains

unclear. For instance, low levels of circulating TF have been detected in healthy subjects with no indication of coagulation initiation (Giesen et al. 1999; Falati et al. 2003; Chou et al. 2004). Therefore, the procoagulant activity of circulating TF appears to be inherently different than TF exposed locally at a vessel injury.

Circulating TF experiences less relative blood flow than wall-bound TF exposed at the site of a vessel injury. The transmembrane nature of TF suggests that TF-dependent coagulation reactions occur on the surface of TF-expressing cells or cell-derived microvesicles. As such, coagulation factors entrained in the bloodstream must diffuse to the cell or cell-derived microvesicle surface in order for coagulation to proceed. For TF exposed at the site of an injury to a vessel wall, blood flowing past the injury supplies coagulation factors to propagate the coagulation reaction. Conversely, for a particle of TF in the bloodstream, coagulation factors must diffuse to the TF particle, as there is very little relative flow (blood flowing past the particle) with which to supply coagulation factors. The rate at which coagulation factors diffuse to the cell or microvesicle surface can be rate-limiting for coagulation reaction kinetics (McGee et al. 1992). Diffusion transport of coagulation factors can be quantified by a diffusive flux, which depends upon the plasma concentration of the coagulation factor. Concentrations of individual coagulation factors are known to vary by over 3-fold in the normal population, implying that transport of coagulation factors vary by over 3-fold in the normal population. Moreover, the rate of thrombin generation from an identical procoagulant stimulus has been shown to vary by over 3-fold in healthy populations (Brummel-Ziedins et al. 2004; Dielis et al. 2008).

The risk to develop thrombosis in cancer is increased for patients with a prior history of thrombosis. Moreover, patients whose peak thrombin generation values (using a TF-dependent assay) rank in the upper quartile are associated with an increased incidence of thrombosis (Mandala et al. 2010; Ay et al. 2011). Thus, patients whose blood responds more robustly to procoagulant stimuli *in vitro* are more susceptible to thrombosis induced by cancer. Taken together, the development of thrombosis in patients with cancer may be the culmination of the procoagulant stimulus introduced by the patient's cancer cells and a heightened blood coagulation response to this stimulus. As such, the procoagulant stimulus of cancer itself may not be sufficient to induce thrombosis in all patients. Instead, thrombosis may occur in patients whose blood is most sensitive to the cancer induced procoagulant stimulus. Moreover, attempts to predict thrombosis based solely on quantification of the procoagulant stimulus (i.e. circulating TF) may lack specificity to predict thrombosis. We hypothesize that susceptibility to circulating TF-induced thrombosis lies in levels of a patient's coagulation factors, with patients whose coagulation factor levels reside in the high-normal range to be more susceptible to cancer-derived circulating TF than patients whose coagulation factor levels reside in the low-normal range. Further, quantification of coagulation factor levels may provide added specificity for predicting thrombosis in cancer.

The goal of our study was to characterize the role of individual coagulation factor levels on coagulation kinetics for a constant burden of TF using TF and phospholipid-coated microspheres (TF particles) suspended in plasma as a model for circulating TF. This model of circulating TF allows the precise control of TF burden by controlling the amount of TF particles added ($10^5$, $10^4$ or $10^3$ $mL^{-1}$) which presented TF levels of 3.5

pM, 350 fM and 35 fM, respectively. By varying individual coagulation factor levels below, within and above normal physiological ranges, we aimed to find the coagulation factor or factors whose physiological levels may be limiting to circulating TF-induced coagulation kinetics. The levels of coagulation factors VII, IX, X, VIII, V and II were independently controlled by mixing pooled plasma with coagulation factor-depleted plasma, by adding purified coagulation factors to coagulation factor-depleted plasma, or by mixing pooled plasma with PBS. Clotting times and enzyme generation assays were utilized to characterize the effect of coagulation factor levels on initiating coagulation reactions (initiation time), propagating coagulation reactions (rate), and functional clotting of plasma (clotting time) in the presence of TF particles.

## 5.4 Materials and Methods

### *5.4.1 Materials*

Anti-factor XI antibodies were generated as previously described (Tucker et al. 2009). Purified human coagulation factors and immunodepleted plasmas were from Haematologic Technologies, Inc (Essex Junction, VT). Thromboplastin (recombinant TF and phospholipids (Dade® Innovin®)) was from Siemens Healthcare Diagnostics (Deerfield, IL). Polymeric microspheres (9.86 μm diameter) were from Bangs Laboratories (Fishers, IN). All other reagents were from Sigma-Aldrich (St. Louis, MO).

### *5.4.2. Generation of human plasmas with controlled levels of coagulation factors*

Globally diluted plasmas were created by mixing pooled plasma with phosphate buffered saline (PBS, pH = 7.40) in stepwise ratios from (10:0, 9:1, 4:1, 7:3, 3:2, 1:1, 2:3, 3:7, 1:4, or 1:9). In a similar manner, pooled plasma was mixed with plasma depleted of a single coagulation factor (<0.1% as reported by supplier). As the depleted plasma contains

normal levels of all other coagulation factors, mixing pooled plasma with depleted plasma results in plasmas varying in levels of a single coagulation factor. Plasmas with varied coagulation factor concentrations were generated by adding purified coagulation factors to depleted plasmas.

## 5.5 Results

### *5.5.1. Clotting times for pooled plasma diluted with phosphate-buffered saline*

Pooled plasma was mixed with TF particle suspensions containing $10^3$, $10^4$, or $10^5$ TF particles $mL^{-1}$ (TF particle burden) to determine the effect of TF particle burden on clotting time. Higher TF particle burden resulted in shorter clotting times (clotting times = 60, 110, and 210 seconds for TF burdens $10^5$, $10^4$, and $10^3$, respectively). Pooled plasma was mixed with PBS to determine the effects of plasma dilution on clotting times (Figure 5.1A). Clotting times from diluted plasmas were normalized to the 100% plasma value and reported as the percent increase in clotting time (Figure 5.1B). Diluting plasma with PBS did not significantly increase clotting times until the dilution reached a 4:6 ratio of pooled plasma to PBS, at which point the clotting times increased by nearly 50% (clotting time increase at a TF particle burden of $10^5$ $mL^{-1}$ (95% C.I = (15.2, 68.6)). Stepwise increases in the dilution of pooled plasma resulted in stepwise increases in clotting times (dilution ratios of 3:7, 2:8 resulted in clotting time increases of 61 and 114%, respectively, at a constant TF particle burden of $10^5$ $ml^{-1}$). Clotting times were not measurable (>1200 seconds) when plasma was diluted at 2:8 with PBS for a TF burden of $10^3$ $mL^{-1}$, or when plasma was diluted at 1:9 with PBS for TF particle burdens of $10^4$ $mL^{-1}$ and $10^5$ $mL^{-1}$ (Figure 5.1B).

### *5.5.2. Clotting times for pooled plasma mixed with plasma depleted of specific coagulation factors*

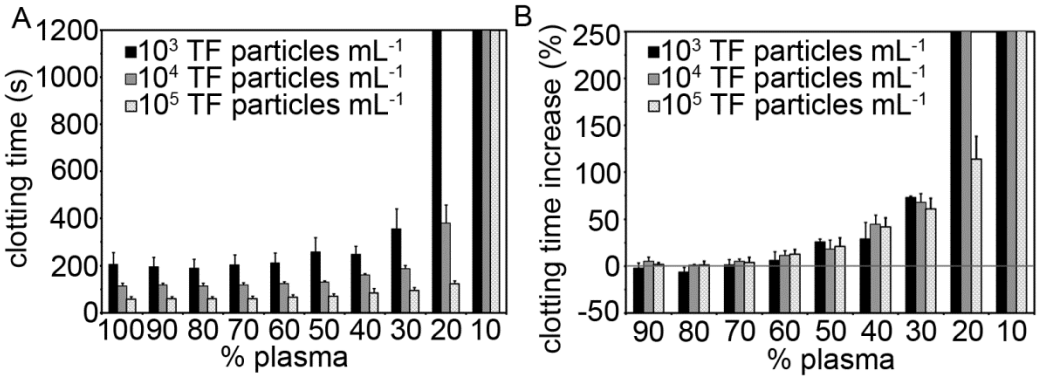


**Figure 5.1. Clotting times for plasma mixed with PBS. Citrate anticoagulated pooled plasma was mixed with PBS and incubated with TF-coated polymer microspheres (TF particles) at a count of $10^3$, $10^4$, or $10^5$ $mL^{-1}$ for 3 minutes at 37° C prior to recalcification (8.3 mM, final $Ca^{2+}$ concentration). The time for plasma to clot was recorded on a KC4 coagulation analyzer (A). Clotting time elongation was calculated for diluted plasmas by normalizing to clotting times obtained with 100% plasma (B). Data are mean and standard error (n=3).**

Pooled plasma was mixed with plasma depleted of coagulation factors VII, X, IX, VIII, V, or II at specific ratios to determine the effect of depleting individual coagulation factors on clotting time as a function of TF particle burden (Figure 5.2). Plasma depleted of factors VII, X, or II did not clot (clotting time >1200 seconds at a TF particle burden of $10^4$ $mL^{-1}$) in our assay. Mixing pooled plasma with factor VII-depleted plasma at a ratio of 8:2 significantly increased the clotting time for TF particle burdens of $10^4$ $mL^{-1}$ and $10^5$ $mL^{-1}$ (Figure 5.2A; 95% C.I. = (1.9, 13.7) and (7.1, 9.5), respectively). Stepwise increases in the ratio of factor VII-depleted plasma to pooled plasma resulted in stepwise increases in clotting times. Mixing pooled plasma with factor VII-depleted plasma significantly increased clotting times at a TF particle burden of $10^3$ $mL^{-1}$ by 37% (95% C.I. = (1.8, 72.6)) at a ratio of 4:6. Mixing pooled plasma with factor X-depleted plasma significantly increased clotting times at a pooled plasma to factor X-depleted plasma ratio of 7:3 (Figure 5.2B; clotting time increase at TF particle burdens of $10^4$ $mL^{-1}$ and $10^5$ $mL^{-1}$ (95% C.I. = (4.3, 12.7) and (2.1, 6.5), respectively). Stepwise increases in the ratio of factor X-depleted plasma to pooled plasma resulted in stepwise increases in clotting

times. Mixing pooled plasma with factor II-depleted plasma did not significantly increase clotting times until a pooled plasma to factor II-depleted plasma ratio of 1:9 was obtained (clotting time increase at TF particle burdens of $10^4$ $mL^{-1}$ and $10^5$ $mL^{-1}$ (Figure 5.2F; 95% C.I. = (5.3, 18.2) and (8.8, 38.5), respectively).

Plasmas depleted of factors VIII, V and IX clotted in our assay (clotting times = 180, 890 and 500 seconds, respectively, at a constant TF burden of $10^4$ $mL^{-1}$). Mixing pooled plasma with factor VIII-depleted plasma at a ratio of 9:1 increased clotting times by 23% as compared to pooled plasma alone for a TF particle burden of $10^5$ $mL^{-1}$ (95% C.I. = (16.5, 30.3)). Increasing the ratio of factor VIII-depleted plasma to pooled plasma did not cause further increases in clotting times. Mixing pooled plasma with factor VIII-depleted plasma did not increase clotting times at any mixing ratio for TF particle burdens of $10^4$

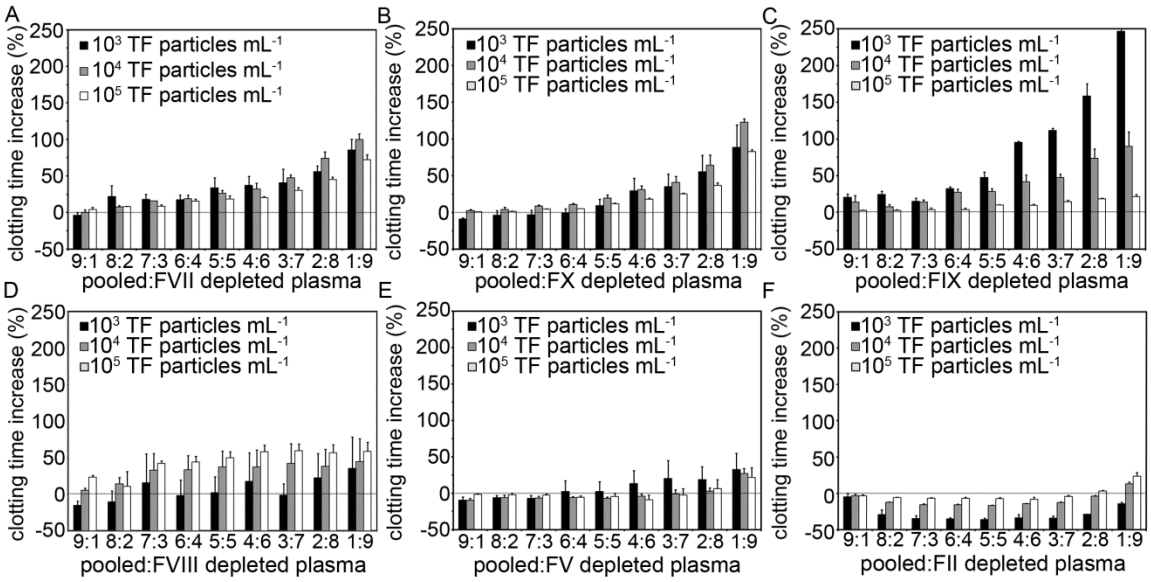


**Figure 5.2. Clotting times for plasma mixed with plasma immunodepleted of specific coagulation factors. Citrate anticoagulated pooled plasma was mixed with plasma immunodepleted of either FVII (A), FX (B), FIX (C), FVIII(D), FV(E), or FII (F) and incubated with thromboplastin-coated microspheres at a count of $10^3$, $10^4$, or $10^5$ $mL^{-1}$ for 3 minutes at 37° C prior to recalcification (8.3 mM, final $Ca^{2+}$ concentration). The time for plasma to clot was recorded on a KC4 coagulation analyzer. Clotting time elongation was calculated for mixed plasmas by normalizing to 100% plasma clotting times. Data are mean and standard error (n=3).**

$mL^{-1}$ or $10^3$ $mL^{-1}$ (Figure 5.2D). Mixing plasma with factor V-depleted plasma significantly increased clotting times by 27% at a 1:9 ratio of pooled plasma to factor V-depleted plasma for a TF particle burden of $10^4$ $mL^{-1}$ (Figure 5.2E; 95% C.I. = (6.9, 47.3)). Mixing pooled plasma with factor IX-depleted plasma at a 9:1 ratio increased clotting times by 20% at a TF particle burden of $10^3$ $mL^{-1}$ (Figure 5.2C; 95% C.I. = (8.1,32.7)), by 14% for a 7:3 ratio of factor IX-depleted plasma:plasma for a TF particle burden of $10^4$ $mL^{-1}$ (95% C.I. = (5.4, 22.2), and by 10% for a 5:5 ratio of factor IX-depleted plasma:pooled plasma for a TF particle burden of $10^5$ $mL^{-1}$ (95% C.I. = (8.6, 11.6)).

*5.5.3. Clotting times as function of coagulation factor concentration*

Plasmas containing specific concentrations of coagulation factors were generated by adding purified coagulation factors to depleted plasma. Clotting times were determined as a function of coagulation factor concentration at a TF particle burden of $10^4$ $mL^{-1}$ (Figure 5.3). Gray regions in the graph represent the range of coagulation factor concentrations reported for normal populations.

Adding purified factor VII to factor VII-depleted plasma decreased clotting times in a concentration-dependent manner (Figure 5.3A; plasma factor VII concentrations of 0.1, 0.3, 1.0, and 3.0 $\mu g$ $mL^{-1}$ resulted in clotting times of 5600, 440, 400, and 360 seconds, respectively). Adding purified factor X to factor X-depleted plasma did not result in a measurable clotting time until a concentration of 6 $\mu g$ $mL^{-1}$ was achieved (clotting time of 1160 seconds), after which further increasing the concentration of factor X shortened clotting times (Figure 5.3B; clotting times = 640 and 430 seconds at factor X concentrations of 10 and 30 $\mu g$ $mL^{-1}$, respectively). Adding factor II to factor II-depleted

plasma shortened clotting times in a concentration-dependent manner (Figure 5.3F; clotting times = 850 and 370 seconds for factor II concentrations 10 and 30 μg $mL^{-1}$, respectively). Further increasing factor II concentration above 30 μg $mL^{-1}$ had no effect on clotting times. Adding factor IX to factor IX-depleted plasma shortened clotting times in a concentration-dependent manner (Figure 5.3C; clotting times of 380, 350, 300, and 280 seconds for plasma factor IX concentrations of 1, 3, 10, and 50 μg $mL^{-1}$, respectively). Adding factor V to factor V-depleted plasma shortened clotting times in a concentration-dependent manner (Figure 5.3E; plasma factor V concentrations of 1, 3, 10, and 30 μg $mL^{-1}$ resulting in clotting times of 520, 310, 260, and 200 seconds, respectively). Adding factor VIII to factor VIII-depleted plasma at concentrations up to 1 μg $mL^{-1}$ had no effect on clotting times in our study (Figure 5.3D; clotting times of 260 +/- 2.5 seconds at all factor VIII concentrations tested).

*5.5.4. Enzyme initiation times as a function of coagulation factor concentration*

The time before the generation of proteolytic enzymes as a function of coagulation factor concentration was analyzed by measuring 405 nm light absorbance over time from plasmas containing the chromogenic substrate S-2366 at a constant TF particle burden of $10^4$ $mL^{-1}$ (Figure 5.4). Adding factor VII to factor VII-depleted plasma shortened the initiation time from 2520 to 930 and 600 seconds for factor VII concentrations of <0.01, 0.1, and 0.3 μg $mL^{-1}$, respectively. Increasing FVII concentration from 0.3 to 3 μg $mL^{-1}$ had no effect on initiation times (Figure 5.4A). Adding factor IX to factor IX-depleted plasma shortened the initiation time from 630 to 520, 500, and 380 seconds at factor IX concentrations of 1, 3, and 10 μg $mL^{-1}$, respectively. Increasing factor IX concentration from 10 to 30 μg $mL^{-1}$ had no effect on the initiation time (Figure 5.4C). Adding factor X

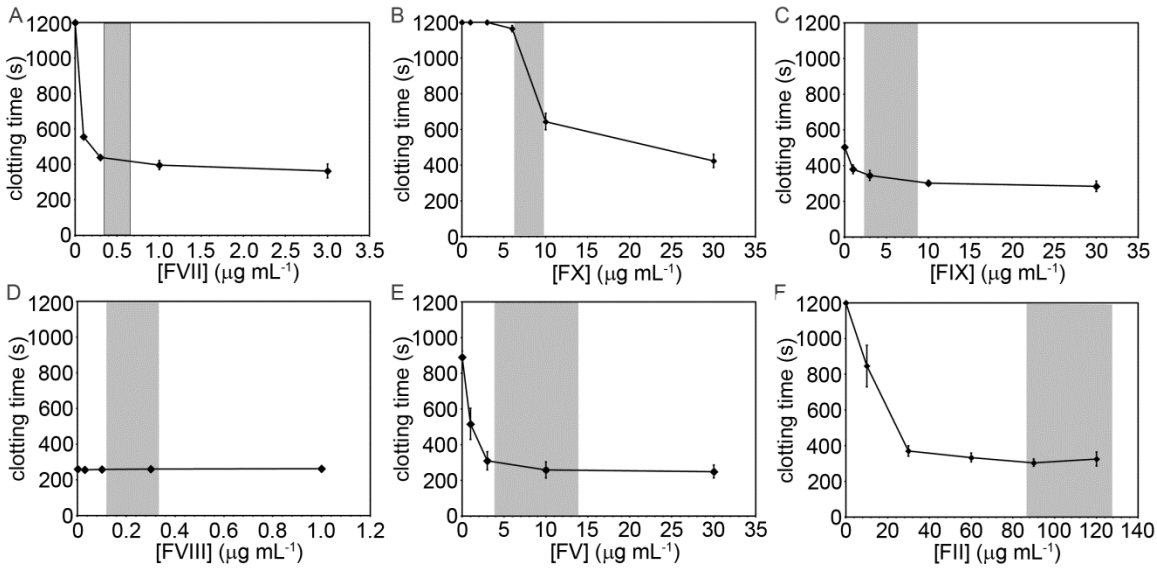


**Figure 5.3. Clotting times for plasma as a function of coagulation factor concentration. Immunodepleted human plasma was reconstituted at indicated coagulation factor levels and incubated with TF-coated microspheres at a count of $10^4$ $mL^{-1}$ for 3 minutes at 37° C prior to recalcification (8.3 mM, final $Ca^{2+}$ concentration). Data are mean and standard error (n=3).**

to factor X-depleted plasma shortened initiation times from >7200 to 3480, 2000, 2020, 1020, and 830 seconds at factor X concentrations of 1, 3, 6, 10, and 30 μg $mL^{-1}$, respectively (Figure 5.4B). Adding factor II to factor II-depleted plasma shortened initiation times from >7200 to 650 and 470 seconds for factor II concentrations of 10 and 30 μg $mL^{-1}$, respectively. Increasing factor II concentrations from 30 to 90 μg $mL^{-1}$ had no effect on the initiation time, while increasing factor II from 90 to 120 μg $mL^{-1}$ increased initiation times from 430 to 540 seconds (Figure 5.4F). Adding factor V to factor V-depleted plasma resulted in shortening initiation times from 3100 to 2670, 670, 330, and 240 seconds at factor V concentrations of 1, 3, 10, and 30 μg $mL^{-1}$, respectively (Figure 5.4E). Adding factor VIII to factor VIII-depleted plasma had no effect on initiation times for factor VIII concentrations up to 1 μg $mL^{-1}$ (Figure 5.4D).

*5.5.5. Enzyme generation rates as a function of coagulation factor concentration*

The rate at which proteolytic enzymes were generated as a function of coagulation factor concentration was analyzed by measuring the change in 405 nm light absorbance over time from plasmas containing the chromogenic substrate S-2366 at a TF particle burden of $10^4$ $mL^{-1}$ (Figure 5.5).

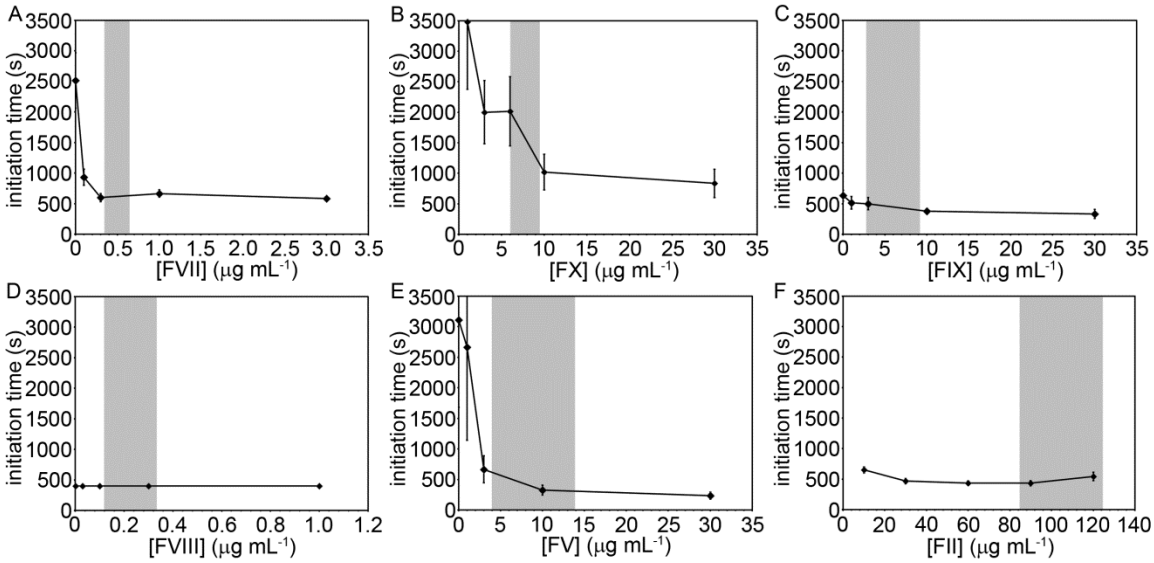


**Figure 5.4. The time to initiate enzyme activity (initiation time) for plasma as a function of coagulation factor concentration. Immunodepleted human plasma was reconstituted at indicated coagulation factor levels and incubated with the chromogenic substrate S-2366 and TF-coated microspheres at a count of $10^4$ $mL^{-1}$ for 3 minutes at 37° C prior to recalcification (8.3 mM, final $Ca^{2+}$ concentration). Absorbance of light (405 nm) was recorded with a spectrophotometer at 1 minute intervals. Samples yielded a sigmoid relationship between absorbance and time, and the initiation time was recorded as the time when absorbance increased above baseline. The gray regions represent the reported range of normal coagulation factor levels. Data are mean and standard error (n=3).**

Adding factor X to factor X-depleted plasma increased enzyme generation rates in a concentration dependent manner from <0.01 $sec^{-1}$ for factor X-depleted plasma to 0.017, 0.027, 0.031, 0.044, and 0.063 $sec^{-1}$ at factor X concentrations of 1, 3, 6, 10, and 30 μg $mL^{-1}$, respectively (Figure 5.5B). Adding factor II to factor II-depleted plasma increased enzyme generation rates in a concentration dependent manner from <0.01 $s^{-1}$ for factor II-depleted plasma to 0.11 and 0.19 $s^{-1}$ at factor II concentrations of 10 and 30 μg $mL^{-1}$,

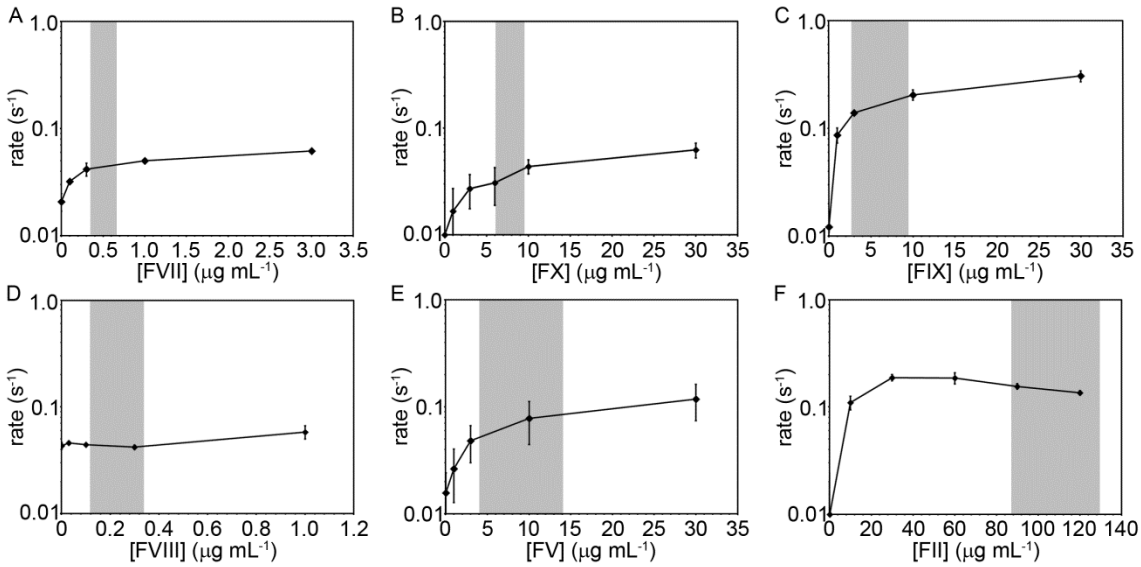


**Figure 5.5. The rate of enzyme generation (rate) for plasma as a function of coagulation factor concentration. Immunodepleted human plasma was reconstituted at indicated coagulation factor levels and incubated with the chromogenic substrate S-2366 and thromboplastin-coated microspheres at a count of $10^4$ $mL^{-1}$ for 3 minutes at 37° C prior to recalcification. The absorbance of light (405 nm) was measured at 1 minute intervals. Results yielded a sigmoid relationship between absorbance and time, and the rate was recorded as the slope of the curve at which 50% of the total change in absorbance had occurred. The gray regions represent the reported range of normal coagulation factor levels. Data are mean and standard error (n=3).**

respectively. Further increasing factor II concentration to 60, 90, and 120 μg $mL^{-1}$ slowed enzyme generation rates in a concentration-dependent manner to 0.187, 0.156, and 0.136 $s^{-1}$, respectively (Figure 5.5F). Adding factor VII to factor VII-depleted plasma increased enzyme generation rates from 0.02 $s^{-1}$ for factor VII-depleted plasma to 0.03, 0.04, 0.05, and 0.06 $s^{-1}$ at factor VII concentrations of 0.1, 0.3, 1 and 3 μg $mL^{-1}$, respectively (Figure 5.5A). Adding factor IX to factor IX-depleted plasma increased enzyme generation rates in a concentration-dependent manner from 0.01 $s^{-1}$ for factor IX-depleted plasma to 0.09, 0.14, 0.2 and 0.31 $s^{-1}$ at factor IX concentrations of 1, 3, 10, and 30 μg $mL^{-1}$, respectively (Figure 5.5C). Adding factor V to factor V-depleted plasma increased enzyme generation rates in a concentration dependent manner from 0.02 $s^{-1}$ for factor V-depleted plasma to 0.03 to 0.05, 0.08, and 0.12 $s^{-1}$ at factor V concentrations of 1, 3, 10, and 30 μg $mL^{-1}$,

respectively (Figure 5.5E). Adding factor VIII to factor VIII-depleted plasma had no effect on enzyme generation rates for factor VIII additions up to 0.3 μg $mL^{-1}$ (rate = 0.04 $s^{-1}$). Increasing factor VIII from 0.3 to 1 μg $mL^{-1}$ increased the enzyme generation rate to 0.06 $s^{-1}$ (Figure 5.5D).

**5.6 Discussion**

Thrombosis significantly contributes to morbidity and mortality in cancer. In metastatic cancer, where TF-expressing cells or cell-derived microvesicles enter the bloodstream, a minority of patients develop a thrombosis. It is unclear if the development of thrombosis in patients with cancer is due to an increased procoagulant stimulus, such as an increased level of TF-bearing microvesicles or procoagulant circulating tumor cells, or if patients that develop thrombosis are more sensitive to the procoagulant stimulus induced by cancer. A significant obstacle to implementing anticoagulant prophylaxis to patients with cancer is identifying the relative minority of patients who go on to develop a thrombosis. Moreover, risks of routine anticoagulant prophylaxis of the entire cancer patient population outweigh the benefits gained by sparing a minority from thrombosis. A method to predict individual patient risk of developing thrombosis would allow personalized indications for anticoagulation prophylaxis.

Circulating tissue factor is elevated in the blood of patients with cancer (Tesselaar et al. 2007; Khorana et al. 2008; Zwicker et al. 2009; Manly et al. 2010). Concentration of circulating tissue factor is associated with thrombosis, yet this alone lacks sufficient specificity to predict thrombosis (Khorana et al. 2008; Zwicker et al. 2009; Thaler et al. 2011). Patients with cancer who also have a past history of venous thrombosis, or whose peak thrombin generation values (a TF-dependent clinical assay) score in the upper

quartile have an increased risk to develop thrombosis in cancer (Mandala et al. 2010; Ay et al. 2011).

We hypothesize that patients with cancer who develop thrombosis are more susceptible to the procoagulant stimulus provided by circulating TF than patients with cancer who do not develop thrombosis. Transport of coagulation factors from the blood to the TF-bearing cell or cell-derived microvesicle is a rate-limiting step in the generation of coagulation enzymes (Gemmell et al. 1988; McGee et al. 1992). The rate of generation of coagulation enzymes has been attributed to flux of zymogen from bulk solution to the surface-bound enzyme complexes (Forman et al. 1986; Gemmell et al. 1988; Andree et al. 1994; Giesen et al.). The flux of substrate from bulk solution to a procoagulant surface is directly dependent upon the bulk concentration. The concentrations of individual coagulation factors differ by 3-fold in normal populations (Yang 1978; Epstein et al. 1984; Howard et al. 1994; Hoyer et al. 1994; Kamphuisen et al. 2000; von Ahsen et al. 2000; Ceelie et al. 2001; Legnani et al. 2003). Therefore, the flux of coagulation factors to and subsequent generation of procoagulant enzymes by circulating TF carriers would be expected to vary by up to 3-fold for normal populations. Further, the generation of thrombin in response to an identical TF-dependent procoagulant stimulus has been shown to vary by over 3-fold in healthy populations (Brummel-Ziedins et al. 2004; Dielis et al. 2008). Along these lines, we hypothesize that patient-specific levels of coagulation factors may be predictive of susceptibility to develop thrombosis in the presence of circulating TF.

Individually diluting coagulation factors by mixing pooled plasma with coagulation factor-depleted plasma was more effective at increasing clotting times than

simultaneously diluting all coagulation factors by mixing pooled plasma with PBS. For example, mixing pooled plasma with PBS at a ratio of 7:3 had no effect on clotting times as compared to pooled plasma alone, yet mixing pooled plasma with factor VII-depleted plasma at a 7:3 ratio increased clotting times by 17%. In other words, lowering factor VII levels to ~70% of normal values did not influence coagulation times if other coagulation factor levels were also lowered to 70% of normal levels. Conversely, if factor VII levels were lowered to 70% independently from other coagulation factor levels, a significant increase in clotting time was observed. Therefore, our data suggests that the ratio of coagulation factors to each other regulates coagulation kinetics more than overall coagulation factor levels.

In mixing studies, lowering factor VIII, V and II showed no effect on clotting times until levels reached approximately 20% of normal, well below the physiologically normal range. Therefore, normal variations in factor VIII, V and II would not influence TF-induced coagulation in our assay. Conversely, lowering factor VII, IX and X levels to only 80% of normal levels, well within the physiological range of these coagulation factors, increased clotting times. Lowering factor IX levels resulted in the biggest increase in clotting times, despite factor IX not being essential to clot plasma in our assay. The magnitude by which lowering factor IX levels increased clotting times was diminished at higher TF burdens. Conversely, factors VII and X were essential to clotting in our assay and factor VII and X levels controlled coagulation kinetics equally for all TF burdens tested.

In studies where purified coagulation factor was added back to depleted plasma, levels of all coagulation factors except factor VIII influenced clotting times, initiation time and

enzyme generation rates. For clotting time assays, the concentration at which a saturation effect was reached was different for each coagulation factor. Additions of factors VII, IX and V shortened clotting times in a concentration dependent manner up to low-normal physiological levels, after which further additions, even at supraphysiological levels did not significantly decrease clotting times. Factor X additions continued to shorten clotting times in a concentration dependent manner, even when added at supraphysiological levels. Adding factor II beyond 30 $\mu g\ mL^{-1}$ (normal level ~100 $\mu g\ mL^{-1}$) had no effect on clotting time. Taken together, only physiological variations of factor X levels influenced clotting times for TF particles in suspension.

Enzyme initiation times emulated the concentration-dependent shortening seen with clotting times, in that additions of factors VII and V shortened initiation times up to low-normal physiological levels, after which further increases did not significantly shorten clotting times. Additions of factor IX had minimal influence on shortening initiation times. Adding factor X to factor X-depleted plasma shortened initiation times up to a concentration of 10 $\mu g\ mL^{-1}$, which is the high end of physiological levels, suggesting that physiological variations of factor X would influence initiation times in our assay.

Enzyme generation rates increased with coagulation factor levels in a concentration-dependent manner for all coagulation factors tested except factor VIII; however, the increases in enzyme generation rate leveled off as coagulation factor levels approached normal levels. Only factors IX and X showed significant variation in enzyme generation rates within the physiological ranges of these coagulation factors. However, supraphysiological additions of factors VII, IX and V showed continued increases in

enzyme generation rate as coagulation factor levels crossed normal levels into supraphysiological levels. Adding factor II to factor II-depleted plasma above 30 μg $mL^{-1}$ had no effect on enzyme generation rates, again suggesting that physiological variations in factor II levels would not influence enzyme generation rates. Factor IX levels had the biggest impact on enzyme generation rates.

All measures of coagulation kinetics demonstrated in this study suggest that factor II, when present at normal levels, is well in excess of levels that may influence coagulation kinetics. Factors V and VII did not significantly influence coagulation kinetics when added above low-normal physiological levels. Factor X shortened clotting and initiation times, and increased enzyme generation rates in a concentration dependent manner across the physiological range of factor X levels. Factor IX increased enzyme generation rates across the physiological range of factor IX levels. Our data suggests that levels of factors X and IX, within the physiological range of variation, are sufficient to influence circulating TF-induced coagulation kinetics.

Thrombosis in cancer is different from spontaneous venous thrombosis in a number of ways that suggest that the pathogenesis of cancer-associated thrombosis is unique. Along these lines, an association between circulating TF and thrombosis in cancer exists, but circulating TF lacks specificity for identifying which patients will develop thrombosis. This suggests that patients who do develop thrombosis in the presence of circulating TF have a predisposed susceptibility to circulating TF-induced coagulation. We demonstrate that moderate perturbations of factors X and IX levels within physiological ranges can significantly influence *in vitro* coagulation kinetics for a model of circulating TF. The mechanism by which coagulation factor levels are maintained *in vivo* are ill-defined;

however, it has been shown that these levels vary widely between individuals. Similar variation has been demonstrated in individual coagulation responses to a single procoagulant stimulus (Karnicki et al. 2002; Brummel-Ziedins et al. 2004; Dielis et al. 2008). Studies linking clinical coagulation assays and risk to develop thrombosis have shown that patients who mount the largest coagulation response to a set procoagulant stimulus are at increased risk to develop thrombosis in cancer (Ay et al. 2011). Moreover, therapeutic lowering of an individual coagulation factor with antisense oligonucleotides has been demonstrated *in vivo* to lower risk of thrombosis in a dose-dependent manner without increasing the risk of bleeding (Zhang et al. 2010). Along these lines, therapeutically lowering of individual levels of factors X or IX may be a rational approach to preventing thrombosis in cancer without inducing a risk of bleeding.

In this study, we characterize the role for individual coagulation factors to control coagulation induced by TF particles in suspension. Varying individual coagulation factors altered functional clotting more than globally lowering all coagulation factor levels. Adding factor IX to factor IX-depleted plasma resulted in the largest increase in enzyme generation rates. At low TF particle burdens, factor IX levels had the biggest impact on clotting times. Within normal physiological ranges, factor X levels had the biggest impact on clotting times and enzyme initiation times, while supraphysiological levels of factor X shortened clotting times compared to normal physiological levels. We provide evidence to support therapeutic manipulation of factor IX or X levels as a rational approach to inhibiting circulating TF induced coagulation. Moreover, quantification of patient factor IX and X levels may assist efforts to predict thrombosis in patients with cancer.

# Chapter 6: Phosphatidylserine Index as a Marker of the Procoagulant Phenotype of Acute Myelogenous Leukemia Cells

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Owen J. T. McCarty

## 6.1 Abstract

Patients with Acute Myelogenous Leukemia (AML) are at increased risk for thrombotic complications. Risk to develop thrombosis is closely tied to leukemia subtype, and studies have shown an association between leukemia cell count and an increased risk of thrombosis. We investigated the relative roles of cell count and the surface expression of tissue factor (TF) and phosphatidylserine (PS) in the procoagulant phenotype of AML cells. The TF-positive AML M3 cell lines, NB4 and HL60, and AML M2 cell line, AML14, exhibited both extrinsic tenase and prothrombinase activity in a purified system and promoted experimental thrombus formation. In contrast, the TF-negative AML cell line, HEL, exhibited only prothrombinase activity and did not affect the rate of occlusive thrombus formation. In plasma, NB4, HL60 and AML14 shortened clotting times in a cell-count, PS- and TF-dependent manner. Exposure of cultured NB4, HL60, and AML14 cells to the chemotherapeutic agent daunorubicin increased their extrinsic tenase activity and PS expression. Clot initiation time inversely correlated with logarithm of PS index, defined as the product of multiplying leukocyte count with cell surface phosphatidylserine exposure. Cultured AML cell lines promote coagulation in a cell count-, TF- and PS-dependent manner. We propose that leukemia cell PS index may serve as a biomarker for procoagulant activity and help identify patients with AML that may benefit from thromboprophylaxis.

## 6.2 Introduction

Studies in Chapters 3-5 have utilized a model system of circulating TF to determine how physical parameters of spatial separation and mass transport regulate blood coagulation. Cellular TF activity is dependent upon a number of factors that include presence of disulfide bonds, colocalization with anionic phospholipids, and the presence of anticoagulant proteins on the cell surfaces. In Chapter 6, we extend our findings from Chapters 3-5 to a panel of AML cell lines. After characterizing AML cell procoagulant mechanisms, we investigate a fluorescent-labeling strategy to serve as a marker of AML cell procoagulant activity. We propose a phosphatidylserine index as a potential biomarker to help identify patients with AML who may benefit from thromboprophylaxis.

## 6.3 Background

Thromboses significantly contribute to the morbidity and mortality of patients with acute myelogenous leukemia (AML) (De Stefano et al. 2005; Ziegler et al. 2005; Mohren et al. 2006; Falanga et al. 2009; Oehadian et al. 2009). The cumulative incidence of thrombosis in AML has been reported from 3-12%, with variation in thrombosis rates between AML subtypes (De Stefano et al. 2005; Mohren et al. 2006; Ku et al. 2009). Anticoagulation is effective at preventing thrombosis in AML; however, it is not safe enough to be part of routine patient care due to thrombocytopenia during induction therapy (De Stefano et al. 2005; Melillo et al. 2007). A better understanding of the mechanism of thrombophilia in AML may help identify patients at high risk to develop thrombosis who may benefit from prophylactic anticoagulation.

Benign, differentiated leukocytes do not typically express TF on their surface, but TF can be translocated to the cell surface following cell activation (Conkling et al. 1988; Moosbauer et al. 2007). Surface expression of tissue factor (TF) has been identified on leukemic cells from some, but not all, patients with different subtypes of AML (Carson et al. 1990; Lopez-Pedrera et al. 2006). TF expression has been shown to contribute to leukemic cell procoagulant activity (Tanaka et al. 1993; Falanga et al. 1995; Barrowcliffe et al. 2002; Pickering et al. 2004; Pickering et al. 2008; Marchetti et al. 2012). Co-localization of fibrin with leukemia blast cells in marrow and peripheral vasculature suggests a causal relationship between the presence of AML cells and aberrant, intravascular blood coagulation (Meyer et al. 1973; Takemori et al. 1993). Several leukemia-specific oncogenic alleles, including the pathognomonic t(15;17) translocation in AML M3, have been shown to induce overexpression of TF (Falanga et al. 2007; Yan et al. 2010). TF is an integral membrane protein and serves as the essential hemostatic cell receptor and cofactor for coagulation factor VIIa (FVIIa). Normally, extravascular TF in complex with FVIIa catalyzes the conversion of factor X (FX) to activated factor X (FXa), as well as factor IX (FIX) to activated factor IX (Lawson et al. 1991). FXa, in complex with its non-enzyme cofactor FVa, as the prothrombinase complex, activates prothrombin to thrombin, which, in turn clots fibrinogen and activates platelets to form a hemostatic plug and stop bleeding after injuries (Mackman 2009). Pathological intravascular activation of the hemostatic response may lead to thrombosis, however, not all patients whose leukemic cells express TF develop clinically evident signs of DIC or thrombosis (Tanaka et al. 1993; Engelmann et al. 2013).

TF procoagulant activity is closely tied with phosphatidylserine (PS) exposure on the outer cell membrane of TF-expressing cells (Kunzelmann-Marche et al. 2000; Shaw et al. 2007; Boles et al. 2012). PS is a charged constituent of a cell's lipid membrane and not exposed on the cell membrane outer leaflet under normal conditions. Surface exposed PS on activated platelets supports the assembly of vitamin K-dependent blood coagulation enzyme complexes (Berny et al. 2010). As such, PS enhances the formation of coagulation products but does not initiate coagulation on its own. Cellular events in benign cells, such as apoptosis and infection, have been shown to translocate PS to the outer membrane leaflet of leukocytes (Bevers et al. 1982; Van Dam-Mieras et al. 1987; Thiagarajan et al. 1991; Martin et al. 1995; Goth et al. 2001; Elliott et al. 2005). Undifferentiated leukemic cells have been shown to express PS and exhibit procoagulant activity, which may be due to increased rates of apoptosis of peripheral AML blasts (Connor et al. 1989; Utsugi et al. 1991; Pickering et al. 2004; Zhou et al. 2010). Thrombin formation was directly related to the extent of apoptosis *in vitro*, suggesting a role for PS exposed upon apoptosis to hasten leukemia cell procoagulant activity (Wang et al. 2001). Moreover, procoagulant activity of the TF-expressing, AML M3 derived cell line, HL60, has been shown to directly correlate with the extent of experimentally induced PS exposure (Langer et al. 2004). Taken together, separate genetic and physiologic events result in the expression of TF and PS, respectively. As both contribute to procoagulant activity, the procoagulant phenotype of leukemia cells *in vivo* may be a result of genetic predisposition of AML cells to express TF, combined with physiological events that induce the exposure of PS on TF-expressing AML cells.

During AML, undifferentiated malignant blast cells enter the peripheral blood circulation. The peripheral blood leukemia cell count varies between patients, and an elevated white blood cell count is associated with an increased risk of thrombosis (Breccia et al. 2007; Stoffel et al. 2010). In a series of patients with AML M3, peripheral blast count directly related to markers of thrombin activation (Scharf et al. 1990). Whether elevated peripheral leukemia cell counts, below that which causes leukostasis, could directly contribute to the development of thrombosis is not known.

In this study, we characterize the prothrombotic and procoagulant phenotypes of three TF-expressing AML cell lines, NB4, HL60 and AML14, and one non-TF-expressing AML cell line, HEL as a function of cell count. The NB4 and HL60 cell lines were derived from different patients each with a diagnosis of AML M3 (Gallagher et al. 1979; Lanotte et al. 1991). The AML14 cell line was derived from a patient with AML M2 and have some properties of eosinophils (Paul et al. 1993; Moosbauer et al. 2007). HEL cells have erythroleukemic characteristics and do not express TF (Marchetti et al. 2012).

The goal of our study was to characterize the relative roles for TF and PS to contribute to AML cell procoagulant activity as a function of cell count. We measured plasma clotting times, clot initiation times, clot growth rates, and whole blood occlusive thrombus formation in the presence of NB4, HL60, AML14 and HEL cells. In plasma, NB4, HL60 and AML14 shortened clotting times in a cell-count, PS- and TF-dependent manner. Extrinsic tenase activity increased, clot initiation times shortened and clot growth rates increased for all cells following exposure to the chemotherapeutic agent daunorubicin. Our results demonstrate that extrinsic tenase activity corresponds with the ability of AML cells to drive coagulation and promote occlusive thrombus formation. Clot initiation time

inversely correlated with PS index, which is the logarithmic value of the product of PS fluorescent intensity and labeled cell count. We propose a phosphatidylserine index as a potential biomarker to help identify patients with AML who may benefit from thromboprophylaxis.

## 6.4 Materials and Methods

### *6.4.1 Materials and reagents*

Daunorubicin hydrochloride (daunorubicin, Teva Parenteral Medicines, Inc, Irvine, CA) was obtained from the OHSU Doernboecher Children's Hospital pharmacy. Anti-FXI antibodies were generated as previously described (Tucker et al. 2009). Fluorescein isothiocyanate (FITC)-conjugated bovine lactadherin and plasma derived FVII, FVIIa, FIX, FX, FXa, FVa, FII, and plasmas immunodepleted of FVII, FIX or FX were from Haematologic Technologies, Inc. (Essex Junction, VT). FITC-conjugated anti-TF antibodies were from Lifespan Biosciences (Seattle, WA). Anti-TF antibodies, Spectrozyme FXa® and Spectrozyme TH® were from American Diagnostica (Stamford, CT). All other reagents were purchased from Sigma or obtained from previously mentioned sources (White-Adams et al. 2010).

### *6.4.2 Blood collection*

All blood donations for coagulation studies were collected from healthy volunteers in accordance with Oregon Health and Science University Institutional Review Board Policy. Blood was collected by venipuncture directly into sodium citrate (3.2% w/v) at a ratio of 9:1 v/v. To prepare plasma for clotting analysis, blood was subjected to centrifugation at 230 × *g* for 10 minutes. Platelet rich plasma was collected and mixed with the platelet rich plasma of three other donors. Pooled plasma was then subjected to

centrifugation at 2150 × *g* for 10 minutes, and platelet poor plasma (PPP) dispensed into 1 mL aliquots and stored at -80$^{o}$ C.

*6.4.3 Preparation of plasma with variable concentrations of coagulation factors*

Plasmas immunodepleted of FVII, FIX or FX (<1%) but containing normal levels of all other coagulation factors were replenished with FVII, FIX or FX at 30% to 300% of normal concentrations, respectively. Normal (100%) levels of FVII, FIX and FX were set at 0.5, 4.5 and 10 μg mL$^{-1}$, respectively.

*6.4.4 AML cell lines, cell culture, and exposure to daunorubicin*

All cell lines were from ATCC (Manassas, VA). AML cell lines were cultured in RPMI-1640 media containing 2 mM L-Glutamine, 10% fetal bovine serum, and 1 × penicillin and streptomycin. Cells in suspension were seeded into non-treated tissue culture flasks and kept in an incubator at 37$^{o}$ C and 5% $CO_2$. Cell density was maintained between 2 × $10^5$ and 2 × $10^6$ mL$^{-1}$.

Exposure to daunorubicin was carried out by first splitting the cells and suspending in the normal culture media or media containing daunorubicin (0.2 μg mL$^{-1}$) for 2 days prior to harvesting cells. Cells were harvested by washing and suspending in Hank's Balanced Salt Suspension (HBSS) from $10^7$ to 3 × $10^2$ mL$^{-1}$ as counted by a hemocytometer.

*6.4.5 Plasma clotting times*

Citrate-anticoagulated platelet-poor plasma (PPP) was pretreated with buffer or anti-FXI antibodies (12.5 μg mL$^{-1}$) for 10 minutes at RT. Cells were pretreated with an anti-TF antibody (50 μg mL$^{-1}$), the PS blocking protein bovine lactadherin (200 nM) or buffer for 10 minutes at RT. Plasma was then mixed 1:1 with vehicle or cell suspensions ($10^2$ to $10^6$

$mL^{-1}$, final count) for 3 minutes at 37° C on a KC4 Coagulation Analyzer (Trinity Biotech, Wicklow, Bray Co., Ireland) before recalcification (8.3 mM, final $Ca^{2+}$ concentration). To determine the effect of levels of coagulation factor levels on clotting times, 65 μL of PPP was mixed with 30 μL of cell suspension ($10^6$ $mL^{-1}$) for 3 minutes at 37° C prior to adding 5 μL of 160 mM $CaCl_2$ in $H_2O$. Clotting experiments were carried out for 25 minutes. If clotting had not occurred in that time, the experiment was stopped and a time of 25 minutes recorded. The time for the plasma to clot was recorded in duplicate and repeated 3 independent times.

*6.4.6 Ex vivo occlusive thrombus formation assay*

Glass capillary tubes (2.0 × 0.2 × 50 mm, VitroCom, Mountain Lakes, NJ) were coated with 100 μg $mL^{-1}$ fibrillar collagen in 10 mM acetic acid, washed in phosphate buffered saline (PBS, pH=7.4) and blocked with 5 mg $mL^{-1}$ bovine serum albumin (BSA) in PBS. Collagen-coated and BSA-blocked capillary tubes were flushed and primed with PBS prior to addition of blood. Citrate-anticoagulated whole blood was mixed 9:1 v/v with cell suspension ($10^6$ cells $mL^{-1}$, final cell count) and calcium and magnesium (7.5 mM/ 3.75 mM, respectively) immediately before adding to the glass capillary. A constant gravity-driven pressure gradient was maintained throughout the experiment that resulted in initial wall shear rates of approximately 300 $s^{-1}$. The time for blood to cease flowing through the tube was measured as the time to occlusion. The time to occlusion was measured and repeated 3 independent times.

*6.4.7 Chromogenic measurement of enzyme activity*

Activation of FX (tenase activity) and prothrombin (prothrombinase activity) in the presence or absence of leukemia cells at 37° C was measured by using the initial-rate

method of chromogenic substrate hydrolysis. Washed cells ($10^6$, final count) or buffer (0.1 M Tris, 0.1 M NaCl, 5 mM $CaCl_2$, 0.1% BSA, pH=8.40) were incubated with factor VIIa (10 nM), or factor Va (10 nM) and factor Xa (15 nM) for 10 minutes prior to mixing with factor X (150 nM) and Spectrozyme FXa (400 nM), or prothrombin (200 nM) and Spectrozyme TH® (500 nM), respectively. Absorbance of 405 nm light was recorded at 1 minute intervals for up to 2 hours, and the slope of absorbance versus time measured and reported as activity ($s^{-1}$). Enzyme activities were measured and repeated 3 independent times.

*6.4.8 Clot initiation and growth assay*

Measurements of the initiation of fibrin clot formation and growth rate were performed by pretreating plasma with an anti-FXI antibody (12.5 μg $mL^{-1}$) for 10 minutes at RT prior to mixing with cells ($10^6$ to $10^2$ $mL^{-1}$). Next, the plasma mixture was recalcified (8.3 mM, final $Ca^{2+}$ concentration) and the absorbance of 405 nm light at $37^o$ C was recorded at 1 minute intervals for 2 hours. The initial departure from baseline absorbance was recorded as the initiation time, and the slope measured and reported as the growth rate ($s^{-1}$). Clot initiation times and growth rates were measured and repeated 3 independent times.

*6.4.9 Flow cytometry*

Washed AML cells were incubated with vehicle, FITC-conjugated anti-TF antibody (30 μg $mL^{-1}$) or FITC-conjugated bovine lactadherin (8.3 μg $mL^{-1}$) for 30 minutes at RT. Cells were then washed twice in HBSS before measuring fluorescence with a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

*6.4.10 Quantification of TF antigen*

One million cells were lysed (50 mm Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate pH=7.5) in the presence of protease inhibitors, for 2 hours followed by centrifugation at 16,000 × *g* for 10 minutes. TF antigen levels of the supernatant were determined with a TF ELISA (IMUBIND, American Diagnostica) according to manufacturer's specifications.

*6.4.11 Data analysis*

Data represents mean and standard error for a minimum of three independent experiments. Flow cytometry results are presented as representative histograms from experiments performed in duplicate and repeated. Statistical significance between means was calculated using an ANOVA followed by a Tukey's post hoc analysis. Linear regression analysis was performed using the method of least squares to calculate correlations. Significance required $p<0.05$.

## 6.5 Results

*6.5.1 NB4, HL60 and AML14 cells express active TF and promote experimental thrombus formation*

We first measured the TF antigen levels and extrinsic tenase and prothrombinase activity for the AML cell lines NB4, HL60, AML14 and HEL. Our results confirm that NB4, HL60 and AML14 had detectable levels of TF antigen, while TF was not detected in HEL cells by ELISA (Figure 6.1A). While all cell lines demonstrated significant degrees of prothrombinase activity, only NB4, HL60 and AML14 showed significant extrinsic tenase activity relative to buffer (Figure 6.1B, C). We next measured the ability of AML cells to promote occlusive thrombus formation in whole blood. NB4, HL60 and AML14

cells significantly shortened the time to occlusion as compared to buffer, while HEL cells had no effect on time to occlusion (Figure 6.1D).

### *6.5.2 Characterization of the roles of TF, PS, FXI and FXII, and cell count in the procoagulant phenotype of NB4, HL60 and AML14 cells*

Previous studies have shown that leukemia cells are procoagulant in a coagulation factor XII, TF- and PS- manner. Our next experiments were designed to investigate the relative roles of TF, PS and factor XII in the ability of NB4, HL60 and AML14 cells to induce clotting of plasma. In our assay, adding AML cells to plasma shortened clotting times

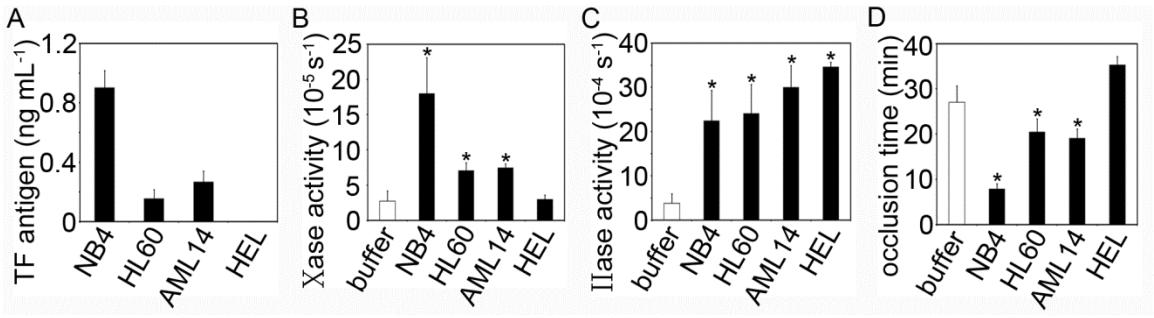


**Figure 6.1. NB4, HL60, AML14 and HEL cells were lysed and TF antigen levels detected with an ELISA (A). Cells were incubated with factor VIIa (200 nM) and the activation of factor X (30 μM) measured by tracking 405 nm light absorbance in the presence of Spectrozyme Xa® (B) Cells were incubated with factor Xa (500 nM) and factor Va (1.72 μM) and the activation of prothrombin measured by tracking 405 nm light absorbance in the presence of Spectrozyme TH® (C) Hank's Balanced Salt Suspension (buffer) or AML cells were spiked into whole blood prior to insertion in an *ex vivo* occlusive thrombus assay and the time to form an occlusive thrombus measured (D). * p<0.05 vs. buffer.**

from over 25 minutes to less than 6 minutes (Figure 6.2A). Clotting times were shortest for NB4 cells (~1.3 minutes), followed by HL60 cells (~2.7 minutes) and AML14 cells (~5.5 minutes). Pretreating the plasma with an anti-FXI antibody that blocks the activation of the contact pathway by FXII, failed to block the procoagulant effect of NB4, HL60 or AML14 cells. In contrast, pretreating the cells with the PS-blocking protein, bovine lactadherin (anti-PS), prolonged clotting times of NB4 cells to ~5.6 minutes, HL60 cells to ~9.9 minutes, and AML14 cells to ~25 minutes, although the increase of

NB4 cells clotting time did not reach statistical significance ($p<0.07$). Pretreating the cells with a TF-blocking antibody prolonged the clotting times of NB4 cells to ~21 minutes, HL60 cells to ~25 minutes, and AML14 cells to ~22 minutes. Taken together, our results suggest NB4, HL60 and AML14 cells are procoagulant in a TF- and PS-dependent manner, independent of the FXII/FXI pathway.

Previous studies have shown an association between elevated leukocytosis and thrombosis (Breccia et al. 2007; Stoffel et al. 2010). Our next experiments were designed to investigate the role of cell count in the ability of NB4, HL60 and AML14 cells to shorten clotting times. In our assay, clotting times were inversely dependent upon cell

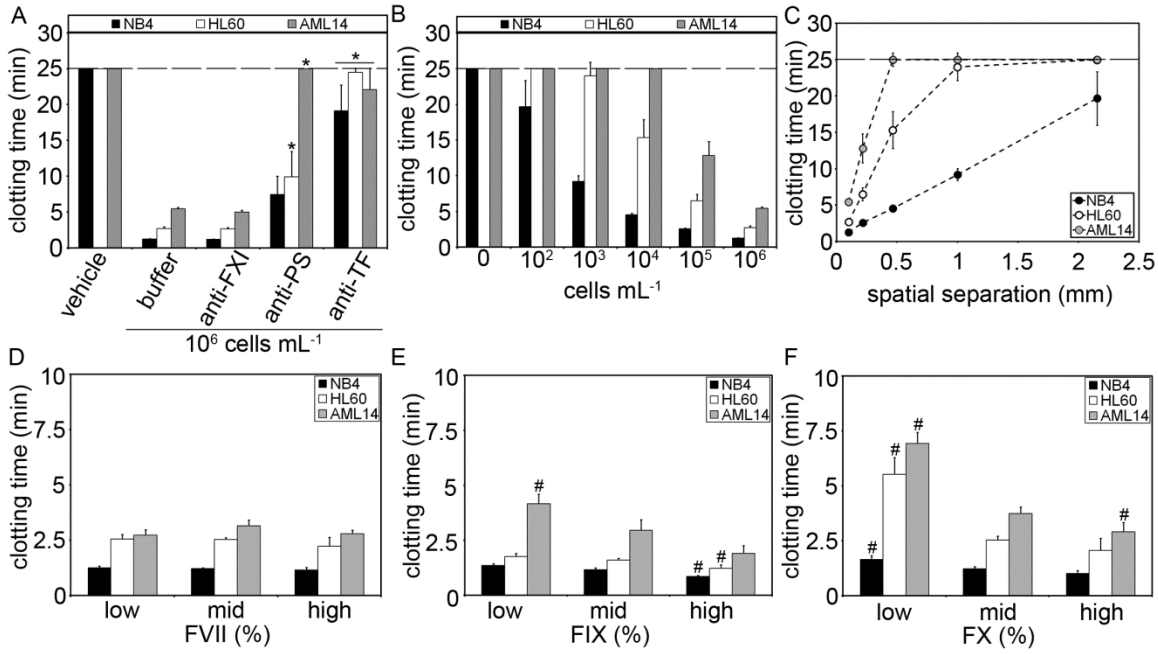


**Figure 6.2. Cells were incubated with buffer, anti-TF antibodies (50 μg $mL^{-1}$) or bovine lactaderin (anti-PS, 200 nM) for 10 minutes prior to mixing with human pooled plasma (+/- anti-FXI antibody (12.5 μg $mL^{-1}$) at 37° C for 3 minutes prior to recalcification (8.3 mM $Ca^{2+}$, final concentration) and the clotting times measured on a KC4 coagulation analyzer (A). Final cell count added to plasma was varied ($10^2$ to $10^6$ $mL^{-1}$, final count) and clotting times measured (B). Clotting times correlated with the calculated separation distance (spatial separation) of cells in plasma (C). Human plasmas immunodepleted of either factor VII, factor IX or factor X were replenished with factor from 30% to 300% of normal levels prior to mixing with AML cells and measuring clotting times (D-F). * $p<0.05$ versus buffer treated cells. # $p<0.05$ versus mid factor levels.**

count (Figure 6.2B). The cell count at which clotting times of plasma failed to occur within 25 minutes (the maximum time allotted) was $10^4$ $mL^{-1}$ for AML14 cells and $10^3$ $mL^{-1}$ for HL60 cells, while NB4 cells promoted clotting at all cell counts tested. Clotting times were plotted against the calculated spatial separation of cells in suspension (spatial separation, cube root of plasma volume/cell, Figure 6.2C).

Previous studies have shown that levels of soluble coagulation factors determine extrinsic tenase activity, and that levels of coagulation factors vary by over 3-fold in the normal population. Our next experiments were designed to investigate whether varying the plasma levels of coagulation factors VII, IX and X from 30% to 300% of normal levels had an effect on plasma clotting times of NB4, HL60 and AML14 cells. Varying coagulation factor VII levels had no effect on clotting times (Figure 6.2D). Increasing factor IX and factor X levels from 30-50% (low-normal) to 150-300% (high-normal) significantly shortened clotting times by AML cells (Figure 6.2E,F). Our results show that the procoagulant phenotypes of NB4, HL60 and AML14 cells were sensitive to the levels of plasma coagulation factor IX and X within the physiological range of variation.

### *6.5.3 Characterization of the role for cell count in clot initiation times and growth rates for NB4, HL60 and AML14 cells*

The kinetics of fibrin formation triggered by procoagulant cells is known to proceed by a sigmoid growth rate following a significant time-lag (Jungi 1990). Our next experiments were designed to investigate whether AML cell count affected the initiation time and fibrin growth rates in plasma. A representative fibrin formation curve following the addition of HL60 cells to plasma is shown in Figure 6.3A, while a reference curve for exogenously added thrombin-induced fibrin formation is shown in Figure 6.3B. Our

results show that the initiation time was inversely dependent upon cell count for NB4, HL60 and AML14 cells. Clot growth rates were independent of cell count for HL60 and AML14 cells, while clot growth rates increased for NB4 cells above $10^4$ cells $mL^{-1}$ (Figure 6.3C).

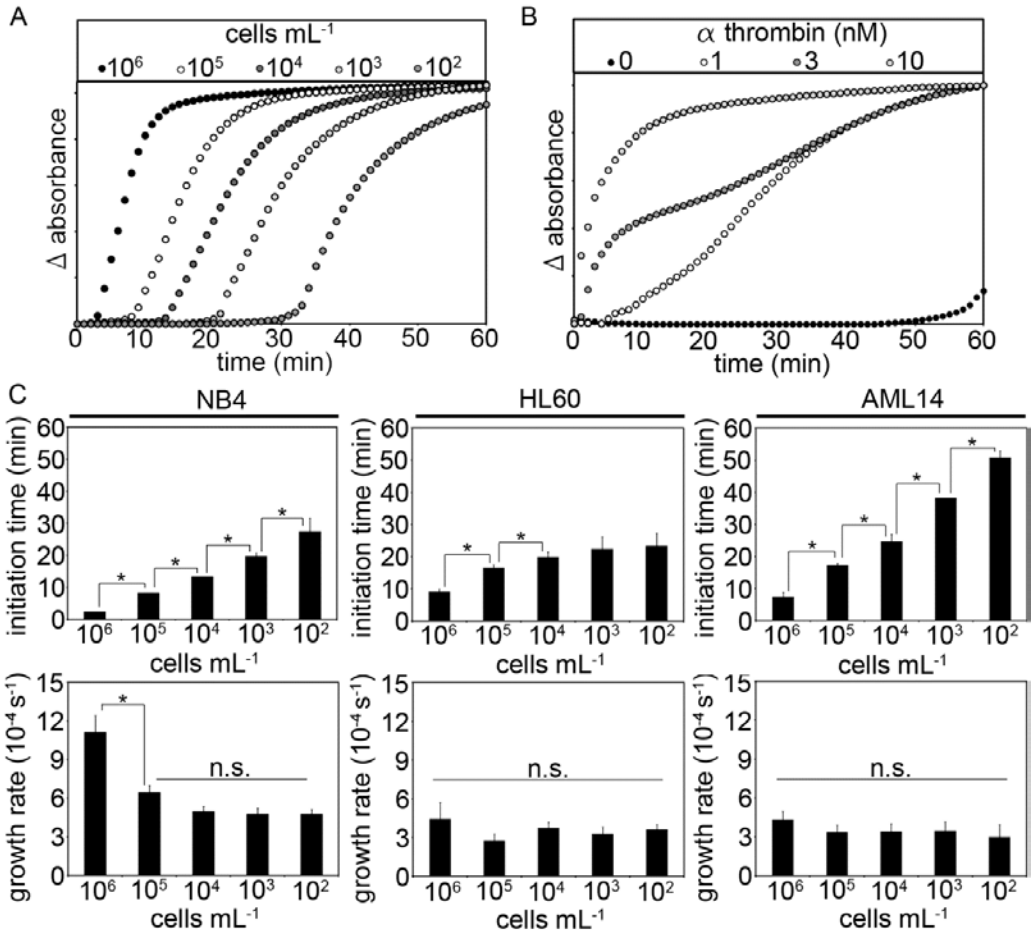


**Figure 6.3. AML cells were mixed with human pooled plasma ($10^2$ to $10^6$ $mL^{-1}$) for 10 minutes at RT prior to recalcification (8.3 mM, final $Ca^{2+}$ concentration) and the absorbance of 405 nm light measured as a function of time on a microplate reader. A representative clot formation result for adding AML cells is shown (A) next to a representative clot formation assay result for adding thrombin directly to plasma (B). Initiation times were recorded as the time at which the absorbance of 405 nM light was increased from baseline, and the growth rate measured as the slope of the absorbance versus time result. (C) Clot initiation times and clot growth rates were measured for NB4, HL60 and AML14 cells at $10^2$ to $10^6$ $mL^{-1}$ final cell counts.**

*6.5.4 Characterization of the effects of daunorubicin exposure on procoagulant activity of NB4, HL60 and AML14 cells*

Previous studies have shown that AML-related activation of coagulation correlates temporally with exposure to systemic chemotherapeutics (Carson et al. 1990; Ku et al. 2009). Daunorubicin is a component of induction therapy in AML and is known to induce apoptosis (Bose et al. 1995; Boland et al. 1997). *In vitro*, exposure of AML cells to the chemotherapeutic agent daunorubicin has been shown to increase the procoagulant activity of cultured cells (Langer et al. 2004; Boles et al. 2012). Our next experiments

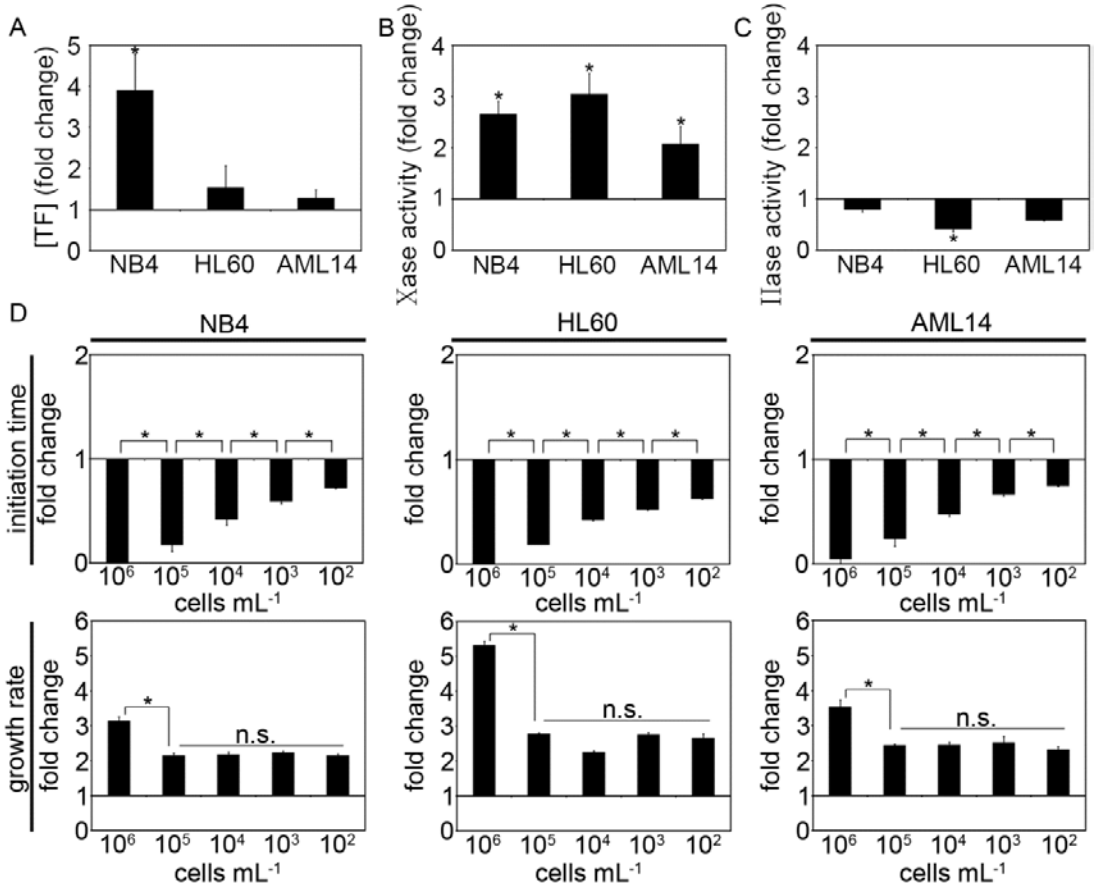


**Figure 6.4. NB4, HL60 and AML14 cells were treated with daunorubicin (0.2 μg mL$^{-1}$) for 2 days. TF antigen levels of treated cells were measured with an ELISA (IMUBIND, American Diagnostica) (A). Treated cells were incubated with factor VIIa (10 nM) for 10 minutes at RT and the activation of factor X measured with a chromogenic assay in the presence of Spectrozyme Xa® (B). Treated cells were incubated with factor Va and factor Xa for 10 minutes at RT prior to measuring the activation of prothrombin in a chromogenic assay in the presence of Spectrozyme TH® (C). Treated cells were mixed with human pooled plasma and clot formation was measured as a change in 405 nm light transmittance. The time before clot initiation began (initiation time) and the rate of change of light transmittance (growth rate) were measured and normalized to untreated cells to determine the net change due to treatment (D).**

were designed to investigate whether treatment of AML cells with daunorubicin changed their procoagulant phenotype. Our results show that TF antigen levels for NB4 cells significantly increased by approximately 4-fold following exposure to daunorubicin for 2 days, while changes in TF antigen levels of HL60 or AML14 cells remained unchanged (Figure 6.4A). The tenase activity of NB4 and HL60 cell increased approximately 3-fold following exposure to daunorubicin, while tenase activity of AML14 cells doubled (Figure 6.4B). Prothrombinase activity remained unchanged for NB4 and AML14 cells, and

significantly decreased for HL60 cells following exposure to daunorubicin (Figure 6.4C). Clot initiation times for NB4, HL60 and AML14 cells shortened in a cell count-dependent manner, and clot growth rates increased for NB4, HL60 and AML14 cells following exposure to daunorubicin (Figure 6.4D). Daunorubicin treatment caused clot growth rates for NB4 cells to increase 3-fold at cell counts of $10^6$ $mL^{-1}$, and double for cell counts from $10^5$ to $10^2$ $mL^{-1}$. Clot growth rates for HL60 cells increased approximately 5.5- fold over untreated cells at $10^6$ cells $mL^{-1}$, and approximately 3-fold at cell counts from $10^5$ to $10^2$ $mL^{-1}$. Clot growth rates for AML14 cells increased approximately 3.5-fold over untreated cells at $10^6$ cells $mL^{-1}$, and 2.5-fold for cell counts from $10^5$ to $10^2$ $mL^{-1}$ following exposure to daunorubicin. We next characterized the effect of daunorubicin on PS expression as measured by flow cytometry. Untreated AML cells were all weakly positive for TF and PS. Daunorubicin treatment resulted in a moderate increases in TF labeling (Figure 6.5A), yet dramatic increases in PS labeling (Figure 6.5B) for NB4, HL60 and AML14 cells. Taken together, we observed that exposure to daunorubicin caused an increase in TF and PS exposure, increased extrinsic

tenase activity, shortened clot initiation times in a cell count-dependent manner, and increased clot growth rates for NB4, HL60 and AML14 cells.

*6.5.6 PS Index correlates with clot initiation times for NB4, HL60 and AML14 cells across cell counts and cell treatments*

We next analyzed whether a fluorescent index (mean fluorescence intensity × fluorescent cell count) could account for differences between NB4, HL60 and AML14 procoagulant activities. Clot initiation times for NB4, HL60 and AML14 cells in both untreated and daunorubicin exposed conditions were plotted for all clotting experiments performed in the plate reader. By itself, the logarithm of cell count alone did not correlate with clot

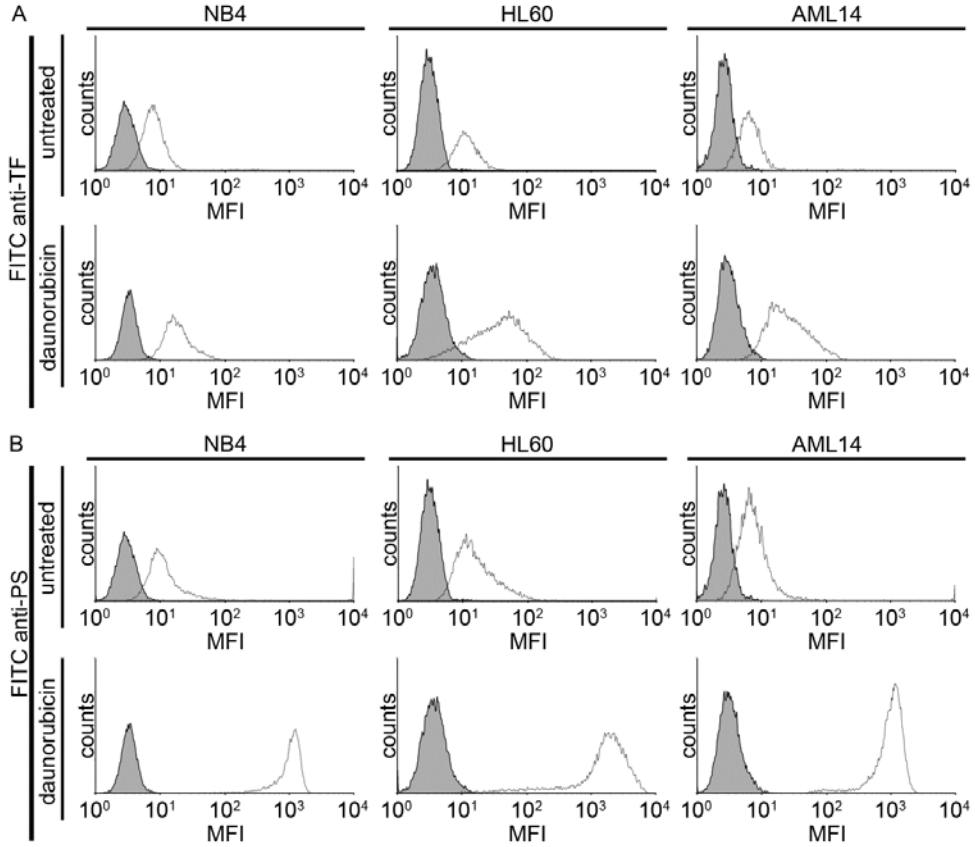


**Figure 6.5. NB4, HL60 and AML14 cells were treated with vehicle (untreated), daunorubicin (dauno, 0.2 μg mL$^{-1}$) for 2 days prior to incubation with buffer, FITC conjugated anti-TF (30 μg mL$^{-1}$) or FITC conjugated bovine lactadherin (8.3 μg mL$^{-1}$) for 30 minutes at RT, washed and fluorescence measured with flow cytometry. Shaded histograms represent unstained (buffer incubated) cells.**

initiation times ($R^2$=0.49, p = 4.4 × $10^{-5}$, Figure 6.6A). The logarithm of TF index, the product of TF mean fluorescent intensity and cell count, resulted in an improved, but still weak correlation with clot initiation times ($R^2$=0.61, p =1.0 × $10^{-6}$, Figure 6.6B). The logarithm of PS index, the product of PS mean fluorescent intensity and cell count, correlated strongly with clot initiation times for NB4, HL60 and AML14 cells in untreated and daunorubicin exposed conditions ($R^2$=0.81, p = 2.4 × $10^{-11}$, Figure 6.6C). Taken together, our results suggest that PS index may provide a fluorescent labeling strategy to account for cell-type differences in procoagulant activity of TF-expressing AML cells.

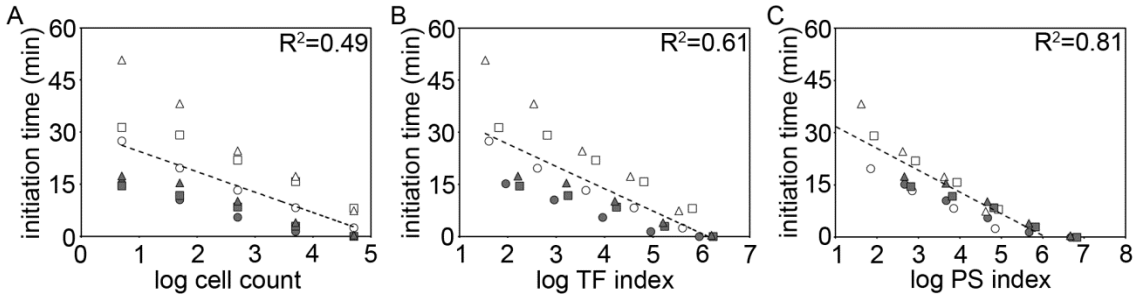


**Figure 6.6. Mean clot initiation times are plotted against cell count, PS and TF index (FITC conjugated bovine lactadherin or anti-TF MFI × cell count) for plate reader clotting experiments with untreated and daunorubicin exposed (0.2 μg $mL^{-1}$ for two days). Regression analysis was performed using the method of least squares and represented by $R^2$ values.**

## 6.6 Discussion

The aim of our study was to characterize the procoagulant phenotypes of AML cell lines under specific conditions known to alter procoagulant activity, and correlate changes in procoagulant activities with independent measures of PS and TF exposure. We measured TF antigen level and fluorescent labeling for TF and PS as a function of cell type and treatment as compared to measures of procoagulant activity for the AML cell lines NB4, HL60, AML14. The NB4 and HL60 cell lines, which were both isolated from patients with a diagnosis of AML M3, and are known to express TF-dependent procoagulant

activity (Gallagher et al. 1979; Lanotte et al. 1991; Falanga et al. 1994; Bach et al. 1997; Langer et al. 2004; Zhou et al. 2010). We included HEL cells in our study as a non-TF expressing cell. We also included the AML14 cell line, an AML M2 subtype derived cell line whose procoagulant phenotype, to our knowledge, has not yet been described in the literature. Our results show that NB4, HL60 and AML14 cells all express significant levels of TF activity on their surface and are procoagulant in a TF- and PS-dependent manner. We did not detect any TF antigen nor did we observe any extrinsic tenase activity or promotion of experimental thrombus formation from HEL cells. NB4, HL60 and AML14 cells shortened occlusion times, providing evidence for prothrombotic activity of NB4, HL60 and AML14 cells in whole blood under shear. NB4 cells had the highest extrinsic tenase activity of the cells tested. NB4 cells also shortened experimental thrombus formation and plasma clotting times more than HL60 and AML14 cells. Tenase activity increased, clot initiation times shortened, and growth rates increased for all cells following treatment with daunorubicin. Prothrombinase activity was not different between cell lines, and decreased following exposure to daunorubicin. As functional clotting assays did not correspond with changes in prothrombinase activity between cell types and following exposure to daunorubicin, our results suggest that prothrombinase activity does not account for differences in the coagulation kinetics of NB4, HL60 or AML14 cells. Conversely, extrinsic tenase activities were different between cells and corresponded with differences in coagulation kinetics of untreated cell types. Therefore, our results support a role for extrinsic tenase activity, but not prothrombinase activity, as a driver of the procoagulant phenotype of AML cell lines. Moreover, as in the case of

HEL cells, facilitation of the prothrombinase complex was not sufficient to promote experimental thrombus formation in the absence of extrinsic tenase activity.

Extrinsic tenase activity is a measure of the TF•FVIIa activation kinetics of coagulation factor X. However, TF•FVIIa is capable of activating FIX as well. Levels of coagulation factors, including coagulation factors involved in the extrinsic tenase complex, are known to vary over three-fold in the normal population and increase with age (Sweeney et al. 1993). We demonstrated that increasing factor IX and factor X levels plasma shortened clotting times for NB4, HL60 and AML14 cells. Our results suggest that the kinetics of leukemia cell-induced coagulation kinetics may be sensitive to the physiological variation of levels of coagulation factors IX and X. As thrombosis occurs in a minority of patients, it is enticing to speculate that the few patients with high-normal levels of coagulation factors IX or X are more susceptible to AML cell procoagulant activity and therefore at higher risk to develop thrombosis than patients with low-normal or normal levels. A clinical cohort study is required to test this hypothesis, and is beyond the scope of this study.

Patients whose leukemic cells express TF and show procoagulant activity do not always present with thrombotic complications. Several disease parameters, including leukemic cell count, vary between patients and are associated with an increased risk for thrombosis (Scharf et al. 1990; Breccia et al. 2007; Stoffel et al. 2010). We investigated the clotting time, clot initiation time and clot growth rate for AML cells as a function of cell count. *In vitro* studies, performed in plasma in closed systems, have established that clotting times decrease with increasing concentrations of procoagulant cells (Berny-Lang et al. 2011; Welsh et al. 2012). The current study suggests that the procoagulant phenotype of AML

cells is a function of spatial separation, which is a measure of the distribution of cells in suspension. The approximately 2-fold increase in clot growth rates observed for NB4 cells from $10^5$ to $10^6$ cells $mL^{-1}$ was equal to the increase in clot growth rate for following exposure to daunorubicin. We propose the following mechanism: thrombin is generated at the surface of a procoagulant AML cell. As thrombin generation proceeds over time, a concentration gradient builds at the cell membrane and extends radially outward from the cell. Activated protein C (APC) limits thrombin generation, and protease inhibitors may continually inhibit the generated thrombin, keeping the local concentration low and limiting the spatial dissipation of thrombin. Fibrin formation and platelet activation occurs, but are negated by fibrinolytic mechanisms, leading to low-level chronic disseminated intravascular coagulation. Therefore, thrombin generation from a single procoagulant cell is unable to overcome local inhibitory or fibrinolytic effects to cause fibrin formation to spread out form the cell. However, if dissipation of thrombin from the cell surface is inhibited, as occurs in a closed-system assay, local thrombin concentrations passively increase, and the cell gains a prothrombotic phenotype. For instance, physical confinement of thrombin generated by a single thromboplastin bead in a closed system resulted in fibrin formation, which was not observed from a similar bead in an open system (Shen et al. 2009). Physical confinement of leukemia cells does not likely occur *in vivo*, however, confinement could also occur chemically. As dissipation of thrombin is driven by concentration gradients, thrombin generated from neighboring leukemia cells may oppose dissipation of thrombin from the cell surfaces, which would result in passive thrombin accumulation near the leukemia cells. Therefore, AML cells lacking sufficient

prothrombotic activity to overwhelm local anticoagulant mechanisms, may do so in synergy with neighboring leukemic cells if in close enough proximity (Lee et al. 2012).

Alterations in antithrombotic mechanisms, which may also affect thrombin generation and thrombus formation, have not been directly evaluated in this study. An approach to glean cellular prothrombotic activity by measuring an imbalance of procoagulant surface features (i.e. TF) to anticoagulant features (i.e. thrombomodulin or urokinase plasminogen activator receptor) showed potential for prognosticating thrombosis in AML (Nadir et al. 2005). Along these lines, quantification of the tissue type plasminogen activator receptor Annexin II in AML M3 cells correlated with generation of plasmin, a potent fibrinolytic enzyme which may contribute to the bleeding diathesis seen in AML M3 (Menell et al. 1999). In a purified system, TF-dependent fibrin formation was limited by adding thrombomodulin (Panteleev et al. 2006). Thrombomodulin, in complex with thrombin, converts protein C to APC, which has several anticoagulant properties. As such, loss of thrombomodulin expression could result in increased cellular prothrombotic by failing to limit the spread of thrombin from the surface of a leukemia cell surface. Homocysteine inhibits the ability of thrombomodulin to APC, and hyperhomocysteinemia has been associated with the development of thrombosis in AML (Hayashi et al. 1992; Melillo et al. 2007). However, NB4 and HL60 cells have been shown to constitutively express thrombomodulin, and therefore, the procoagulant activities reported in this study are not dependent upon a loss of thrombomodulin expression (Saito et al. 1996; Nishiguchi et al. 1999). Whether incorporation of cell count, or enlisting PS labeling as a procoagulant feature for exiting strategies, would result in improved prognostication of thrombosis has not been determined.

The ability of NB4, HL60 and AML14 cells to drive coagulation activation was cell count-, TF- and PS-dependent. Despite a common procoagulant mechanism, NB4 cells shortened plasma clotting times and experimental thrombus formation times more than HL60 and AML14 cells. A prior study with HL60 cells observed that PS labeling correlated with experimentally increased procoagulant activity at a single cell count (Langer et al. 2004). Further, the majority of procoagulant activity of AML cells isolated from different patients with AML M3 was due to PS exposure (Zhou et al. 2010). Through calculation of a PS index, we extend the potential for PS labeling to correlate with procoagulant activity across a number of cell types, in untreated and daunorubicin exposed conditions, and across different cell counts. As the cell lines utilized in this study were isolated from different patients with different AML subtypes, our results suggest a broader role for TF- and PS- expression to contribute to the coagulopathy in AML beyond AML M3. TF and PS have been shown to contribute to procoagulant activity of freshly isolated AML cells from virtually all AML subtypes, while marked leukocytosis has been associated with increased risk of thrombosis in AML. We demonstrate that the procoagulant phenotype of cultured AML cells is dependent upon the cell count. Thus, we hypothesize that the number of TF- and PS-expressing cells in the peripheral blood of patients with AML may predict risk to develop thrombosis. As such, we propose that the thrombotic phenotype of a patient with AML is a function of both the procoagulant activity of that patient's AML cells and AML cell count. Moreover, physiological events or pharmacological treatments that increase AML cell PS exposure without increasing cell counts, may increase thrombin formation kinetics at the cell surface sufficiently to overwhelm local anticoagulant mechanisms to form a fibrin clot and/or activate platelets.

Anecdotal support for the former scenario is supported by the association of elevated leukocyte counts and thrombosis in AML, and the latter, by increased risk of thrombosis following administration of cytotoxic therapies.

Our study demonstrates that the logarithm of PS index (lactadherin labeling intensity × number of labeled cells) correlated with procoagulant activity across cell types and cell counts, supporting a role for PS-labeling in determining TF-positive AML cell procoagulant activity. PS is not sufficient to initiate coagulation, therefore the role for PS-labeling to correlate with procoagulant activity of isolated cells would likely require a determination of TF antigen as well. Similarly, labeling of TF and PS together may increase specificity of a PS index. Moreover, including physical parameters of cell size into PS index did not yield a better correlation than PS index alone. A clinical trial is needed to test whether procoagulant leukemia cell count could predict thrombosis, and would likely require a fluorescent marker that identified procoagulant cells (i.e. TF and PS expressing) to allow for enumeration of freshly isolated procoagulant leukemia cells via flow cytometry.

We provide evidence to support a role for cell count and the extrinsic tenase activity to determine the prothrombotic phenotype of AML cells. The procoagulant phenotype of NB4, HL60 and AML14 cells varied as a function of plasma concentrations of FIX and FX. Treatment of cultured NB4, HL60 and AML14 cells with daunorubicin was followed by an increase in PS exposure, which was associated with an increase in specific procoagulant activity. The logarithm of PS index, the product of PS labeling intensity and cell count, correlated strongly with clot initiation times. We propose that PS index has potential as a biomarker for thrombophilia in AML.

# Chapter 7: Development of Coagulation Factor Probes for the Identification of Procoagulant Circulating Tumor Cells

Garth W. Tormoen, Flor A. Cianchetti, Paul E. Bock, Owen J. T. McCarty

## 7.1 Abstract

Metastatic cancer is associated with a hypercoagulable state, and pathological venous thromboembolic disease is a significant source of morbidity and the second leading cause of death in patients with cancer. Here we aimed to develop a novel labeling strategy to detect and quantify procoagulant circulating tumor cells (CTCs) from patients with metastatic cancer. We hypothesize that the enumeration of procoagulant CTCs may be prognostic for the development of venous thrombosis in patients with cancer. Our approach is based on the observation that cancer cells are capable of initiating and facilitating cell-mediated coagulation *in vitro*, whereby activated coagulation factor complexes assemble upon cancer cell membranes. Binding of fluorescently-labeled, active site-inhibited coagulation factors VIIa, Xa and IIa to the metastatic breast cancer cell line, MDA-MB-231, nonmetastatic colorectal cell line, SW480, or metastatic colorectal cell line, SW620, was characterized in a purified system, in anticoagulated blood and plasma, and in plasma under conditions of coagulation. We conclude that a CTC labeling strategy that utilizes coagulation factor-based fluorescent probes may provide a functional assessment of the procoagulant potential of CTCs, and that this strategy is amenable to current CTC detection platforms.

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## 7.2 Introduction

Taken together, the studies carried out in Chapters 3-6 were designed to identify parameters of procoagulant cells that determine procoagulant activity. We hypothesize that circulating cancer cells underlie the hypercoagulable state in cancer, and therefore have potential to serve as a patient-specific biomarker for risk to develop thrombosis. Studies in Chapter 4 and 6, when taken together, suggest that the procoagulant phenotype of groups of cells should be considered (cell count or spatial separation), in addition to individual cell parameters. However, in Chapter 6, significant differences in coagulation kinetics between cells was observed between cell types and following exposure to daunorubicin, despite all cells exhibiting TF- and PS-dependent procoagulant mechanisms. PS index (Chapter 6) showed potential to serve as a fluorescent biomarker of procoagulant activity for TF-positive AML cells. Therefore, PS index showed potential to account for cell-type and treatment-dependent differences in procoagulant activities procoagulant activities of AML cells. TF expression in solid tumor CTCs has not been studied, as the cytological detection of these cells has only recently become feasible. Studies in Chapter 7 are focused on the development of novel coagulation factor-based fluorescent probes to identify procoagulant CTCs, enabling patient-specific identification and enumeration of procoagulant CTCs in future attempts to correlate CTCs with thrombosis in cancer.

## 7.3 Background

Cancer is a hypercoagulable state. Patients with cancer have a 4-7 fold increased risk of developing thrombosis, which is a significant source of morbidity and mortality for patients with cancer (Trousseau 1865; Baron et al. 1998; Sorensen et al. 2000; Rickles et al. 2001; Falanga et al. 2009). Recurrent thrombosis can be clinically managed with

anticoagulant therapy; however, the risk of bleeding complications associated with the use of anticoagulants has prevented routine prophylactic anticoagulation for patients with cancer who have not yet developed thrombosis (Akl et al. 2011). Therefore, a method to identify which cancer patients are at imminent risk to develop thrombosis would allow for an objective means by which to administer personalized anticoagulant prophylaxis, reducing the morbidity and mortality for patients with cancer. There is currently a lack of laboratory assays capable of identifying which patients with cancer are at risk of developing thrombosis.

Blood coagulation is carried out by a system of serine proteases that are contained within the blood in their inactive, zymogen form. In health, activation of coagulation is restricted to sites of blood vessel injury through the localized exposure of tissue factor (TF), a transmembrane protein constitutively expressed by extravascular tissue not normally exposed to the circulating blood. As blood hemorrhages from an injured vessel, it comes into contact with TF-expressing cells outside of the vasculature. TF serves as the membrane receptor and protein cofactor of coagulation factor VIIa (FVIIa). The TF•FVIIa complex initiates the extrinsic blood coagulation pathway by activating factor X (FX) and factor IX (FIX). FXa and FIXa are initially inhibited by tissue factor pathway inhibitor (TFPI) present in blood at a low concentration (~2.4 nM) by forming a quaternary FXa•TF•FVIIa•TFPI complex (Baugh et al. 1998; Lu et al. 2004). FIXa forms the tenase complex with its protein cofactor FVIIIa on the surface of phosphatidylserine (PS)-containing cell membranes in the presence of calcium ion, which generates additional FXa. FXa produced on PS-containing cell membranes assembles, in a $Ca^{2+}$-dependent manner, the prothrombinase complex with its protein cofactor factor Va

(FVa). The prothrombinase complex converts prothrombin into thrombin (FIIa). FIIa cleaves fibrinogen into self-polymerizing, insoluble fibrin to form a plug at the injury site, effectively stemming blood loss. The localization of the procoagulant stimulus to the injury site, as well as anticoagulant effects of the endothelium downstream of the injury, serve to localize blood coagulation to the site of injury. However, pathologically excessive coagulation, or the initiation of coagulation at sites other than blood vessel injury, can result in thrombosis.

Hematogenous spread of metastatic cancer follows when cells from the primary tumor are shed into the bloodstream. The existence of tumor cells in the blood of patients with cancer has been known for over a century, yet only recently has technology allowed the routine cytological detection of these cells, hereafter referred to as circulating tumor cells (CTCs) (Ashworth 1869). CTCs have been demonstrated to be prognostic for overall patient survival, yet the impact of CTCs on cancer-associated hypercoagulability has not been established (Cristofanilli et al. 2004; Danila et al. 2007; Cohen et al. 2008; de Bono et al. 2008). *In vitro*, cancer cell lines added to plasma are able to induce coagulation. The ability of cancer cell lines to clot plasma is abrogated by incubating with a TF-blocking antibody, or with Annexin V, which blocks the binding of coagulation factors to the PS-containing cancer cell membrane (Berny-Lang et al. 2011). Further, the clotting kinetics for plasma spiked with cancer cells is strongly dependent upon the number of cells added (Berny-Lang et al. 2011; Tormoen et al. 2011; Yates et al. 2011; Welsh et al. 2012). Therefore, it appears that cancer cells are wholly capable of cell-mediated coagulation *in vitro*, whereby they can initiate coagulation through surface expression of TF and

facilitate the propagation of coagulation by binding and assembling coagulation factor complexes upon their cell membranes.

The ability for CTCs to facilitate coagulation in human disease has not been investigated. Technological advancements have allowed the reliable detection of CTCs in patients with cancer through immunofluorescent labeling; specifically, cells that are cytokeratin positive, CD45 negative, and nucleated as apparent with DAPI staining are currently utilized to identify CTCs. On this basis, we sought to develop a functional probe that is amenable to fluorescence microscopic techniques in order to supplement CTC identification with the ability to characterize the procoagulant nature of CTCs. In this study, we characterized the binding of fluorescently-labeled, active site-inhibited coagulation factors VIIa, Xa and IIa to the metastatic breast cancer cell line, MDA-MB-231, nonmetastatic colorectal cell line, SW480, or metastatic colorectal cell line, SW620, in a purified system and in blood plasma. We focused on coagulation factors in the TF-pathway of coagulation based upon the *in vitro* results demonstrating the TF- and phosphatidylserine (PS)-dependent pathways by which cancer cells mediate coagulation. We hypothesize that the identification and enumeration of procoagulant CTCs will be prognostic for venous thrombosis in patients with cancer.

## 7.4 Materials and Methods

### *7.4.1 Reagents*

All reagents were purchased from Sigma or previously described sources (Berny-Lang et al. 2011). H-Gly-Pro-Arg-Pro-OH (GPRP) was purchased from Calbiochem (San Diego, CA). Fluorescein isothiocyante (FITC)-conjugated tissue factor monoclonal antibody was purchased from LifeSpan Bioscences (Seattle, WA). Human coagulation factors VIIa,

Xa, IIa and fluorescein-conjugated D-Phe-Pro-Arg-chloromethyl ketone (PPACK) were purchased from Haematologic Technologies (Essex Junction, VT).

*7.4.2 Blood collection*

Blood samples were obtained and managed in accordance with Oregon Health and Science University Review Board approval. Human whole blood was collected from healthy volunteers by venipuncture into 1:9 v/v 3.2% sodium citrate. Platelet poor plasma (PPP) was obtained similarly, except that the collected blood was then subjected to centrifugation step at 2150 x *g* for 10 minutes, followed by removing the supernatant and mixing with the supernatant from 2 other donors. The pooled supernatant was then subjected to a second centrifugation step at 2150 x *g* for 10 minutes. The supernatant (PPP) was then removed, divided into 1 mL aliquots and stored at -80 $^{o}$C.

*7.4.3 Generation of fluorescent coagulation factor probes*

Coagulation factors were incubated with the fluorophore-conjugated PPACK as previously specified (Bock 1992; Panizzi et al. 2006). In brief, active site inactivation was verified by comparing PPACK-bound coagulation factor activity towards the chromogenic substrates Spectrozyme FVIIa, Spectrozyme Xa or Spectrozyme TH (American Diagnostica). Following inactivation, excess PPACK was removed by dialysis using a Slide-A-Lyzer® MINI Dialysis Unit (Thermo Scientific) with 5 mM HEPES and 0.15 M NaCl (pH = 7.40).

*7.4.4 Cell culture and harvesting*

The metastatic breast cancer cell line, MDA-MB-231, nonmetastatic colorectal cell line, SW480 and metastatic colorectal cell line, SW620, were obtained from American Type Cell Culture (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle's

Medium (DMEM) containing 10% fetal bovine serum and cultured at 37 °C with 5% $CO_2$. Prior to each experiment, cells were detached from the culture flask by immersing in TrypLE Express for 20 minutes at 37 °C, followed by suspension in complete media and subjecting to centrifugation at 210 x *g* for 5 minutes followed by final suspension in serum-free DMEM. Suspended cell concentrations were measured with a hemocytometer.

*7.4.5 Isolation of peripheral blood cells*

To isolate human neutrophils, blood was collected 1:9 into 3.8% sodium citrate, followed by a 1:7 dilution into citrate-phosphate-dextrose as previously described (Itakura et al. 2011). In brief, 5 ml of blood suspension was layered over 5 ml of Polymorphprep and subjected to centrifugation at 500 x *g* for 45 minutes. The neutrophil band was extracted and diluted in Hank's Balanced Salt Suspension (HBSS) to 50 ml, and subjected to centrifugation at 400 x *g* for 10 minutes. The supernatant was removed and the remaining cell pellet was suspended in sterile water for 30 seconds, followed by diluting to 10 mL with 10X PIPES buffer (250 mM piperazine-*N*,*N*′bis [2-ethanesulfonic acid], 1.1 mM NaCl, 50 mM KCl, pH = 7.40), then to 50 mL with HBSS, and subjected to a final centrifugation step at 400 x *g* for 10 minutes. Cells were counted with a hemocytometer and diluted to a final concentration of $10^6$ $mL^{-1}$.

To isolate human platelets, blood was collected as above but then subjected to centrifugation at 200 x *g* for 20 minutes as previously described (White-Adams et al. 2009). In brief, the supernatant containing plasma and platelets was incubated with 0.10 μg/mL of prostacyclin and subjected to centrifugation at 1000 x *g* for 10 minutes. The platelet pellet was suspended in modified Tyrode's buffer (129 NaCl mM, 0.34 mM

$Na_2HPO_4$, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$; pH = 7.30).

*7.4.6 Clotting times*

MDA-MB-231 or SW620 cells were diluted from $3 \times 10^6$ to $1.5 \times 10^3$ cells $mL^{-1}$ in serum-free DMEM. Next, 50 μL of cell suspension or vehicle (DMEM) was mixed with 50 μL of PPP for 180 seconds at $37^o$ C. Then, 50 μL of 25 mM $CaCl_2$ was added and the time required for the plasma to clot was measured on a KC4 coagulation analyzer (Trinity Biotech, Bray, Co. Wicklow, Ireland). To determine the mechanism of the cancer cell procoagulant activity, 50 μL of $3 \times 10^5$ cells $mL^{-1}$ were pretreated with a function blocking anti-TF mAb (50 μg $mL^{-1}$) or the phosphatidylserine function-blocking ligand Annexin V (20 μg $mL^{-1}$) for 5 minutes at RT prior to mixing with plasma. Further, PPP was pretreated with the FXa inhibitor rivaroxaban (20 μg $mL^{-1}$) for 5 minutes prior to mixing with cells.

*7.4.7 Flow cytometry*

Washed MDA-MB-231 or SW620 cells were suspended in 50 μl of PBS, PPP, or PPP treated with the anti-FXI antibody 1A6 (12.5μg $mL^{-1}$) and the fibrin polymerization inhibitor Gly-Pro-Arg-Pro-OH (GPRP 10 mM), and 8.3 mM $CaCl_2$ (final concentration). Cell suspensions were incubated with fluorescently labeled coagulation factors FVIIa (5-500 nM), FXa (50-5000 nM) or FIIa (50-5000 nM) or FITC-conjugated anti-TF (10-100 μg $mL^{-1}$) for 30 minutes at RT. Then labeled cells were diluted to 500 μL with PBS and characterized using a FACS Calibur flow cytometer with CellQuest Pro acquisition and analysis software (Becton Dickinson, Franklin Lakes, NJ.

*7.4.8 Immobilization of cells onto glass coverslips*

Coverslips (#1.5 12 mm; Fischer Scientific) were etched for 30 seconds in a 70% nitric acid bath, immersed in deionized $H_20$ (Resistivity = 18.2 MΩ cm), rinsed in ethanol, and allowed to air dry. Etched and dried coverslips were then placed in individual wells of a 24-well plate. Next, 500 μL of 4% 3-aminopropyltriexothysilane in ethanol was added to the wells and allowed to coat for 2 minutes. Coverslips were then washed once in ethanol and submerged in $H_20$ prior to performing the experiments.

Then, 300,000 MDA-MB-231, SW480, or SW620 cells in serum-free DMEM were dispensed onto the etched coverslips and allowed to adhere for 60 minutes at 37 °C. Nonadherent cells were removed by washing with PBS. Recalcified plasma containing the anti-FXIa antibody 1A6 (12.5 μg $mL^{-1}$) and 10 mM GPRP was dispensed over immobilized cells and allowed to incubate for 30 minutes at RT. Cells were washed in PBS, and incubated with fluorescently labeled coagulation factors FVIIa (500 nM), FXa (5 μM) or FIIa (10 μM) or FITC-conjugated anti-TF mAB (50 μg $mL^{-1}$) in PBS for 30 minutes at RT. Labeled cells were washed in PBS, fixed with 3.7% paraformaldehyde, washed in triplicate and mounted in Fluoromount G (Southern Biotech, Birmingham, AL) and kept at 4 °C overnight. Labeled, adherent cells were imaged on a Zeiss Axiovert 200M at 40X with a Zeiss apochromat 1.4 NA objective using fluorescence and differential interference contrast (DIC) microscopy. A minimum of 3 images were recorded from each experimental condition, with representative images shown for each condition.

Binding of coagulation factors to purified populations of peripheral blood cells was performed by dispensing 300 μL of isolated cells onto silanized coverslips and allowing

them to adhere for 60 minutes at 37$^{o}$ C. Cells were then washed and incubated with PBS (vehicle) or PBS containing active site-inhibited, fluorescent coagulation factor probes for 30 minutes at RT. Cells were then washed in PBS, fixed and mounted as described above.

*7.4.9 Data analysis*

Experiments were performed in duplicate and the average clotting time reported. Clotting time experiments were repeated 3-4 times and plotted as mean ± the standard error of the mean. Statistically significant differences were evaluated using a student's t test (⌘ for p-value<0.05 versus untreated cells,* for p-value<0.05 versus vehicle).

## 7.5 Results

*7.5.1 Clotting times of MDA-MB-231, SW480 and SW620 cells are TF-, PS- and cell count dependent*

To investigate whether the metastatic breast cancer cell line, MDA-MB-231, nonmetastatic colorectal cell line, SW480, or metastatic colorectal cell line, SW620, were sufficient to initiate and propagate blood coagulation, washed cells were suspended in serum-free DMEM and added to recalcified human plasma. The subsequent time required for the plasma to clot (i.e. clotting times) was measured as a function of cell count in a coagulometer (Figure 7.1A-C). All three cell lines hastened the time for plasma to clot as compared to vehicle (DMEM) and the clotting times depended upon the number of cancer cells added to the plasma. To determine the role for cancer cell-expressed TF in clot initiation, a function-blocking anti-TF mAb (50 μg mL$^{-1}$) was added to the cells prior to mixing with plasma. Our results show that the anti-TF mAb abrogated the ability for SW480 and SW620 cells to clot plasma, and prolonged the clotting times for MDA-MB-231 cells (290 seconds *vs.* 39 seconds, respectively). We next designed experiments to

determine whether cancer cell-surface exposed acidic phospholipids were required for clotting. Cancer cells were pretreated with Annexin V (20 μg $mL^{-1}$), which binds to and functionally blocks the ability of PS to bind clotting factors and assemble enzyme complexes on a cell surface. Pretreating SW480 and SW620 cells with Annexin V abrogated the ability of these cells to clot plasma. Pretreatment of MDA-MB-231 cells with Annexin V prolonged clotting times in a concentration-dependent manner (130 seconds at 20 μg $mL^{-1}$ *vs*. 230 seconds at 40 μg $mL^{-1}$). Finally, pretreating the plasma with the FXa inhibitor, rivaroxaban, prior to mixing with the cancer cells completely abrogated the ability of all 3 cell lines to clot plasma. Taken together, our data demonstrate that MDA-MB-231, SW480 and SW620 cells are procoagulant in a TF, PS, FXa, and cell count-dependent manner (Figure 7.1D-F).

*7.5.2 Flow cytometry of labeled cells*

*7.5.2.1 Labeling of MDA-MB-231 and SW620 cells in a purified system*

We next investigated whether fluorescently-labeled, active site-inhibited coagulation factors could be used to label procoagulant cancer cells in a purified system. For this, we utilized the MDA-MB-231 and SW620 cancer cell lines, as they were shown to have the highest and lowest procoagulant activities of the cancer cell lines we tested, respectively. MDA-MB-231 and SW620 cells were suspended in serum-free DMEM and incubated with either vehicle or active site-blocked, fluorescently labeled FVIIa (50 nM), FXa (0.5-5 μM) or FIIa (0.5-10 μM) for 30 minutes at RT. Samples were diluted ten-fold in phosphate buffered saline (PBS) and fluorescence recorded with flow cytometry. Figure 7.2 shows the fluorescence intensity histogram for labeled cells versus vehicle treated controls. The surface expression of TF was verified by staining of the cell lines

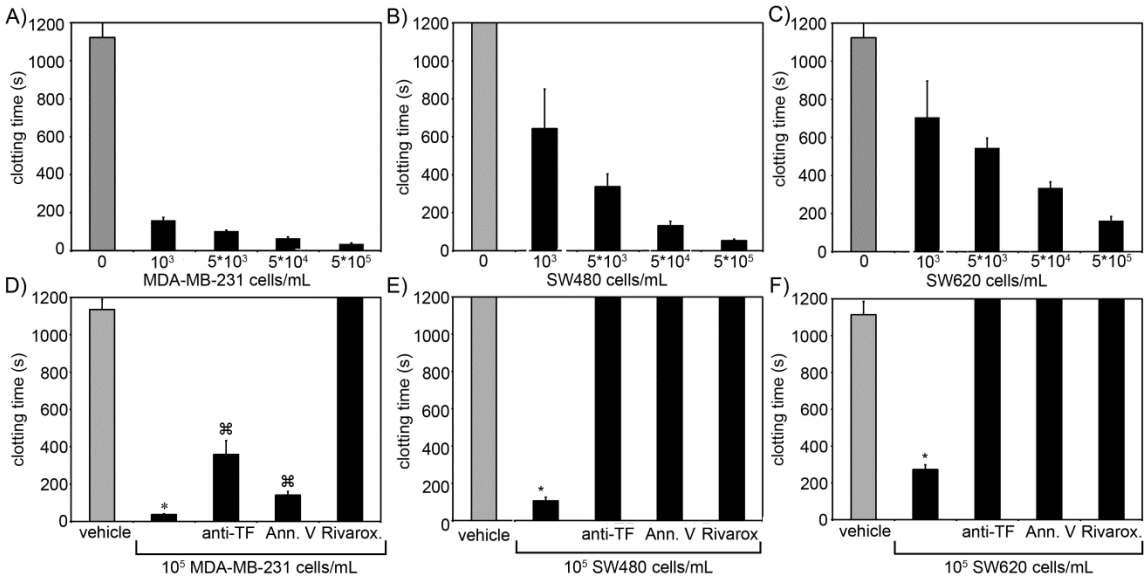


**Figure 7.1. Clotting times for human plasma containing MDA-MB-231, SW480 or SW620 cells. The addition of MDA-MB-231, SW480, and SW620 cells shortened clotting times of plasma in a cell-count dependent manner. (A-C) The ability for these cells to coagulate plasma was inhibited with a function-blocking antibody to tissue-factor (TF) or Annexin V, and completely abrogated with a Factor Xa inhibitor. (D-F) Human sodium citrate-anticoagulated plasma was pretreated with vehicle or the FXa inhibitor rivaroxaban (Rivarox) for 5 minutes at RT. MDA-MB-231, SW480 or SW620 cells were pretreated with vehicle, a neutralizing antibody to TF (anti-TF, 50 μg mL$^{-1}$) or the phosphatidylserine binding protein Annexin V (20 μg mL$^{-1}$) for 5 minutes at RT. Cells and plasma were then mixed together for 3 minutes on a KC4 coagulation analyzer prior to recalcification to 8.3 mM (final concentration). Clotting time experiments were performed in duplicate for each condition and reported as the average. Each experiment was independently repeated 3-9 times. *P<0.05 *vs.* the absence of cells. ⌘P<0.05 *vs.* vehicle pretreated cells.**

with a FITC-conjugated anti-TF mAb (data not shown). Factor VIIa-labeling of MDA-MB-231 cells was clearly evident at 50 nM (mean fluorescence intensity (MFI) = 67 *vs.* 2.6 for unstained cells) and did not change at 100 nM (MFI = 72; Figure 7.2A). Factor VIIa-labeling of SW620 cells was evident at 50 nM (MFI = 10 *vs.* 2.86 for unlabeled cells), with further MFI increases observed at 100 and 500 nM (MFI = 16 and 88, respectively; Figure 7.2B). FXa-labeling of MDA-MB-231 and SW620 cells was evident at 500 nM (MFI = 21.05 for MDA-MB-231 and MFI = 33.31 for SW620), with further increases in fluorescence intensity for labeling at 1 and 5 μM (MFI = 49 and 324 for MDA-MB-231, and MFI = 67 and 281 for SW620, respectively). FIIa (thrombin)

labeling of MDA-MB-231 cells was not apparent at 100 nM (MFI = 6.17) while SW620 cells (MFI = 9.91) were labeled at 100 nM. MDA-MB-231 cells showed increases in labeling intensity with FIIa-based probes from 500 nM to 10 μM (MFI = 24, 42, 305 and 531 for 500 nM, 1, 5 and 10 μM, respectively). SW620 cells showed increases in labeling intensity from 500 nM to 1 μM (MFI = 37, 90, 282 for 500 nM, 1 and 5 μM, respectively), with no further increase seen at 10 μM (MFI = 258). Labeling of cancer cells in a purified system showed cell and factor-specific characteristics for labeling efficacy. Our data show that a concentration of 50 nM FVIIa-based probe was sufficient to label both the MDA-MB-231 and SW620 cells, while a concentration of 500 nM of the FXa- or FIIa-based probes was required to label both MDA-MB-231 and SW620 cells.

*7.5.2.2 Labeling of MDA-MB-231 and SW620 cells in human plasma*

We next investigated whether fluorescently-labeled coagulation factors could be used to label procoagulant cancer cells in plasma. For this, MDA-MB-231and SW620 cells were suspended in sodium citrate anticoagulated PPP containing vehicle or fluorescently labeled FVIIa (50-500 nM), FXa (0.5-5 μM) or FIIa (0.5 – 10 μM) for 30 minutes at RT.

Samples were diluted ten-fold in phosphate buffered saline (PBS) and fluorescence measured with flow cytometry. Figure 7.3 shows the fluorescence intensity histogram for labeled cells versus vehicle treated controls. The surface expression of TF was verified by staining of the cell lines with a FITC-conjugated anti-TF mAb (data not shown). Factor VIIa-labeling of MDA-MB-231 cells in plasma was evident at 50 nM (MFI = 13.8

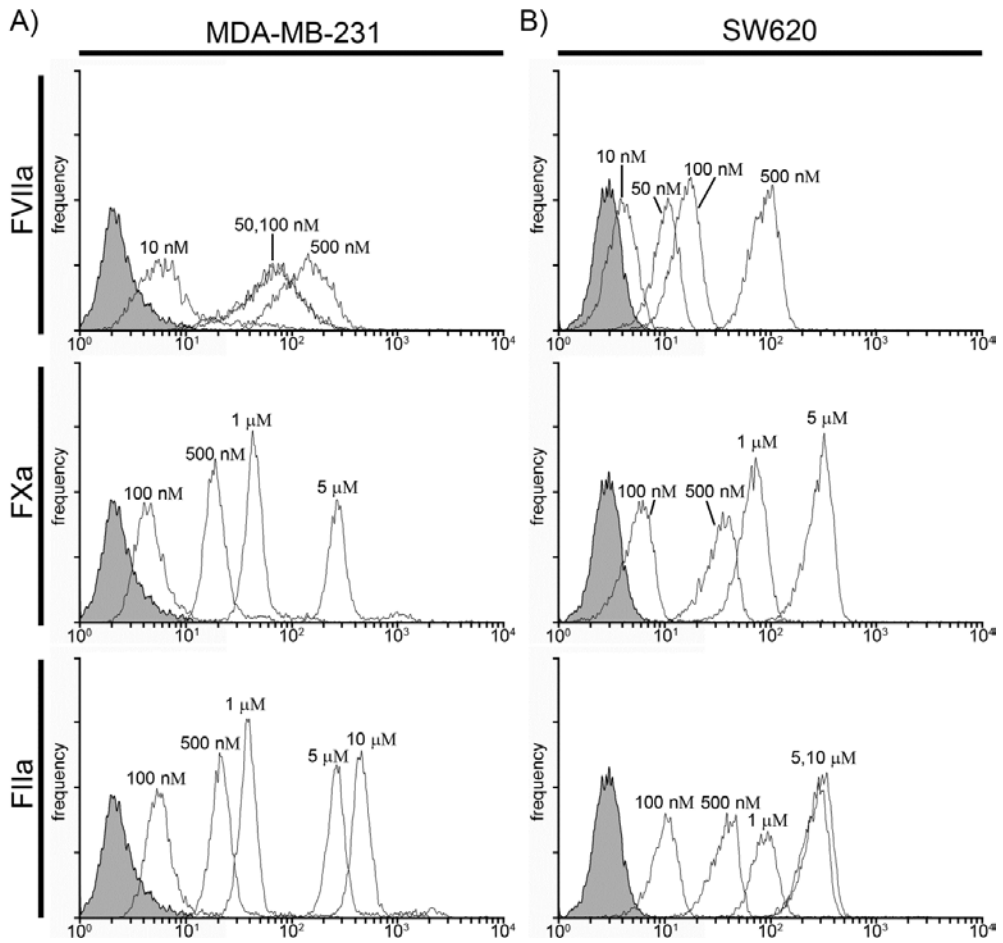


**Figure 7.2. Characterization of fluorescent coagulation factor probe binding to MDA-MB-231 and SW620 cells suspended in DMEM. One-hundred thousand MDA-MB-231 or SW620 cells were incubated with vehicle or fluorescently-modified, active site-inhibited coagulation factors FVIIa (10 – 500 nM), FXa (100 – 5000 nM) or FIIa (100 – 10,000 nM) for 30 minutes at RT. Cells were then diluted 10-fold in sterile-filtered phosphate-buffered saline (PBS, pH = 7.40) and analyzed by flow cytometry. Shaded histograms represent background fluorescence while white histograms represent labeled cells at the fluorescent probe concentrations shown.**

vs. 2.4 for unlabeled cells) and fluorescence labeling increased at probe concentrations of 100 nM (MFI = 18 and 81 for 100 and 500 nM, respectively; Figure 7.3A). FVIIa-based probe labeling of SW620 cells was evident at 50 nM (MFI = 11.7 vs. 2.8 for unlabeled cells) in plasma with a further increases in fluorescence intensity at higher probe

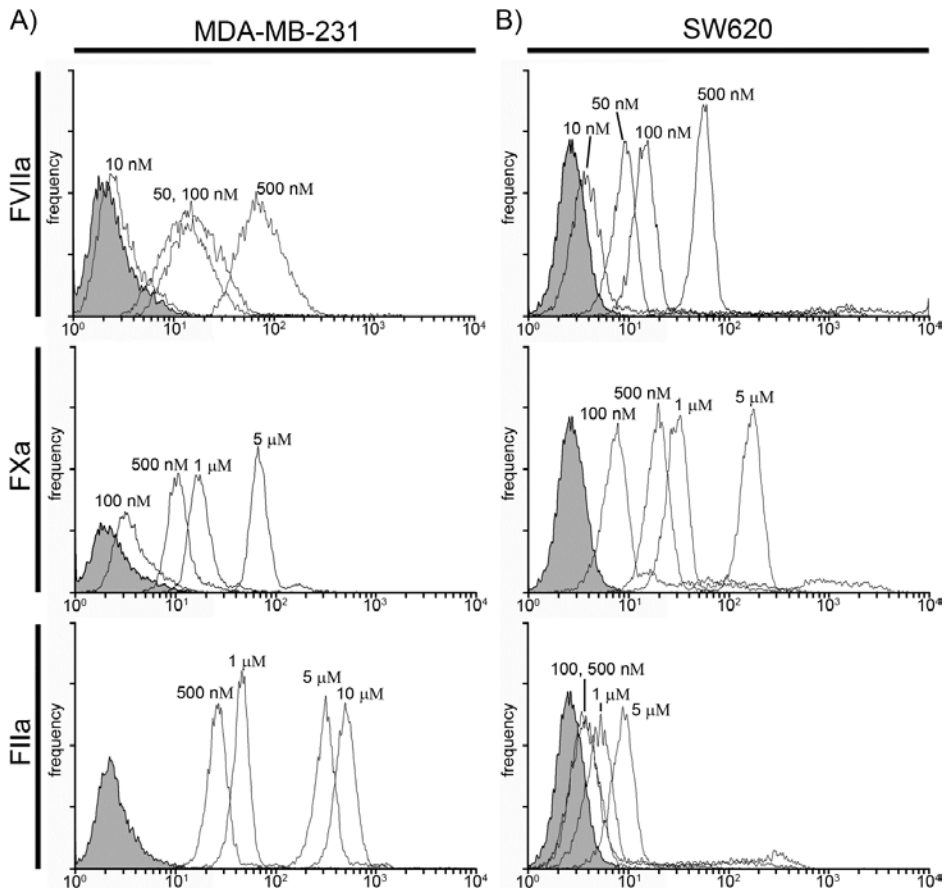


**Figure 7.3. Characterization of fluorescent coagulation factor probe binding to MDA-MB-231 and SW620 cells in human plasma. Washed MDA-MB-231 or SW620 cells were incubated with vehicle or fluorescently-modified, active site-inhibited coagulation factors FVIIa (10 – 500 nM), FXa (100 – 5000 nM) or FIIa (100 – 10,000 nM) in 50 μL of PPP for 30 minutes at RT. Cells were then diluted 10-fold in sterile-filtered phosphate-buffered saline (PBS, pH = 7.40) and analyzed by flow cytometry. Shaded histograms represent background fluorescence while white histograms represent labeled cells at the fluorescent probe concentrations shown.**

concentrations (MFI = 20 and 76 at 100 and 500 nM, respectively) (Figure 7.3B). Labeling of MDA-MB-231 cells with the FXa-based probe in plasma was not evident at 100 nM (MFI = 3.9 vs. 2.4 for unlabeled cells), but could be seen at 500 nM (MFI = 11.3), with further increases in fluorescence labeling at higher probe concentrations (MFI = 18.9 and 74.2 for 1 and 5 μM, respectively). Labeling of SW620 cells with the FXa-based probe in plasma was observed at 100 nM (MFI = 8.05 vs. 2.8 for unlabeled cells) and increases in fluorescence intensity were observed at higher probe concentrations

(MFI = 22, 35 and 210 at 500 nM, 1 and 5 μM, respectively). FIIa-labeling of MDA-MB-231 cells was observed at 500 nM (MFI = 27 vs. 2.6 for unlabeled cells) with further increases in fluorescence intensity seen with probe concentration (MFI = 47, 321 and 515 for 1, 5 and 10 μM, respectively). In contrast, the FIIa-probe did not label SW620 cells at or below 1 μM FIIa-probe concentration (MFI = 3.8, 4.2 and 6.8 for 100, 500 and 1000 nM, respectively).

*7.5.2.3 Labeling of MDA-MB-231 and SW620 cells in plasma with coagulation*

In the presence of a procoagulant stimulus, such as a procoagulant cancer cell, coagulation factors in plasma undergo limited proteolysis to become activated. In addition, the presence of $Ca^{2+}$ ions may present Ca-dependent binding sites on cells that are inaccessible in the presence of the anticoagulant sodium citrate. To determine if coagulation factor-based probes could be used to label procoagulant cancer cells under conditions of coagulation, cancer cells were suspended in PPP pretreated with an anti-FXIa antibody and GPRP and recalcified to 8 mM $Ca^{2+}$ containing vehicle or fluorescently labeled FVIIa (50-500 nM), FXa (0.5-5 μM) or FIIa (0.5 – 10 μM) for 30 minutes at RT. Figure 7.4 shows the fluorescence intensity histogram for labeled cells versus vehicle treated controls. FVIIa-labeling of both cell types was evident at 50 nM (MDA-MB-231 MFI = 102 *vs.* 2.4 for unlabeled cells (Figure 7.4A), SW620 MFI = 11.4 *vs.* 2.8 for unlabeled cells (Figure 7.4B), and increases in FVIIa-based probe concentrations showed minimal effect on MDA-MB-231 fluorescence intensity (MFI = 122 and 159 for 100 and 500 nM, respectively). SW620 cells exhibited increases in fluorescence intensity with FVIIa-based probe concentrations of 100 and 500 nM (MFI =

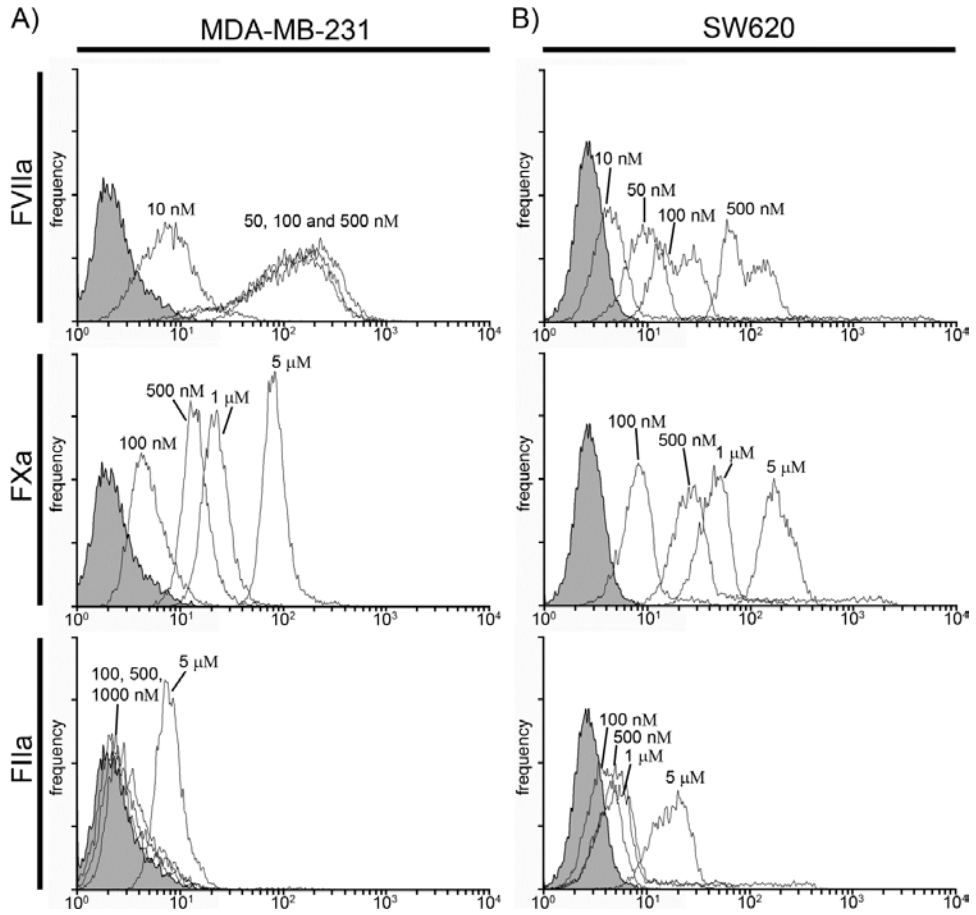


**Figure 7.4. Characterization of fluorescent coagulation factor probe binding to MDA-MB-231 and SW620 cells in human plasma under conditions of coagulation. Washed MDA-MB-231 or SW620 cells were incubated with vehicle or fluorescently-modified, active site-inhibited coagulation factors FVIIa (10 – 500 nM), FXa (100 – 5000 nM) or FIIa (100 – 10,000 nM) in 50 μL of recalcified PPP (8 mM, final $Ca^{2+}$ concentration) containing the fibrin polymerization blocker GPRP (10 mM) and the FXIa-blocking antibody 1A6 (12.5 μg $mL^{-1}$) for 30 minutes at RT. Cells were then diluted 10-fold in sterile-filtered phosphate-buffered saline (PBS, pH = 7.40) and analyzed by flow cytometry. Shaded histograms represent background fluorescence while white histograms represent labeled cells at the fluorescent probe concentrations shown.**

23.6 and 112.3, respectively). FXa- labeling of MDA-MB-231 cells was seen at 500 nM (MFI = 15.5 *vs.* 2.4 for unlabeled cells), and the fluorescence intensity increased with FXa-based probe concentration (MFI = 24 and 90 for 1 and 5 μM, respectively). FXa-based probe labeling of SW620 cells was seen at 500 nM (MFI = 27.3 *vs.* 2.8 for unlabeled cells) and further increases in fluorescence intensity were seen at FXa-based probe concentrations of 1 and 5 μM, respectively. The FIIa-based probe labeled SW620

cells at but not below 5 μM (MFI = 21 *vs.* 2.8 for unlabeled cells), while MDA-MB-231 cells were not clearly labeled at 5 μM under conditions of coagulation (MFI = 8.4 *vs.* 2.4 for unlabeled cells).

### *7.5.3 Fluorescence microscopy of immobilized cells*

#### *7.5.3.1 Labeling of immobilized MDA-MB-231 and SW620 cells in a purified system*

Due to the extreme rarity with which CTCs are present in the blood of patients with cancer, flow cytometry is not routinely utilized to detect CTCs. Rather, various plating or lab-on-chip methods are utilized in combination with fluorescent labels to identify and/or isolate CTCs from a population of cells that consists of both normal blood cell constituents and CTCs (Nagrath et al. 2007; Gleghorn et al. 2010; Marrinucci et al. 2012). We designed a series of experiments to determine whether our labeling strategy was amenable to a cell processing protocol that utilizes cancer cells plated onto glass slides. We immobilized MDA-MB-231, SW480, and SW620 cells on functionalized glass surfaces and exposed them to fluorescently labeled FVIIa (500 nM), FXa (5 μM), FIIa (10 μM). DIC, fluorescence, and merged images are shown in Figure 7.5 for MDA-MB-231, SW480, and SW620 cells. The images showed that the MDA-MB-231 cells were robustly labeled with the FVIIa and FXa probes. The FVIIa and FXa probes weakly labeled the SW480 cells and SW620 cells. The FIIa probe failed to label any of the cell lines.

#### *7.5.3.2 Labeling of immobilized MDA-MB-231 and SW620 cells following exposure to coagulation in a purified system*

Our next set of experiments were designed to determine whether fluorescently labeled coagulation factor probes could label cells that had been exposed to blood plasma under

conditions of coagulation. We immobilized MDA-MB-231, SW480, and SW620 cells on functionalized glass surfaces, exposed the immobilized cells to recalcified plasma, and then incubated the slides with fluorescently labeled FVIIa (500 nM), FXa (5 μM), or FIIa (10 μM). DIC, fluorescence, and merged images are shown in Figure 7.6 for MDA-MB-231, SW480, and SW620 cells. The images showed that all coagulation factor-based probes labeled at least a portion of the adherent cancer cells for all three cancer cell lines.

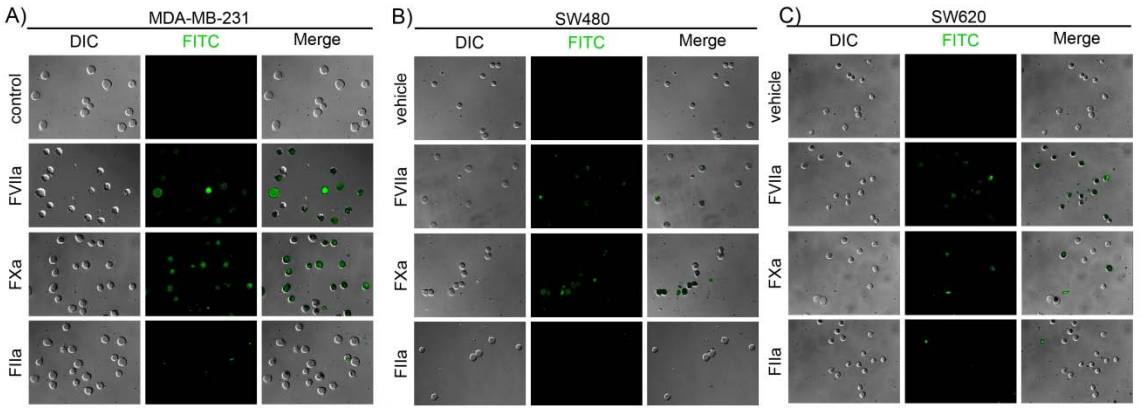


**Figure 7.5. Characterization of fluorescent coagulation factor probe binding to immobilized MDA-MB-231, SW480 and SW620 cells in DMEM. MDA-MB-231, SW480 and SW620 cells were immobilized on silanized glass slides prior to incubation with DMEM (vehicle) or DMEM containing fluorescently labeled coagulation factors FVIIa (500 nM), FXa (5 μM), or FIIa (10 μM) for 30 minutes at RT, washed in DMEM and fixed in 3.7% paraformaldehyde. Immobilized cells were imaged using differential interference contrast (DIC) and fluorescence microscopy.**

The FVIIa probe labeled all the adherent MDA-MB-231 cells. Heterogeneous FVIIa-labeling was observed for both the SW480 and SW620 cell lines, with some of the adherent cells labeling brightly, while other cells on the same slide were not labeled by the FVIIa-probe. The FXa probe showed complete labeling of all cell lines, but pronounced heterogeneity in labeling was observed as some cells were brightly labeled and others showed dim labeling by the FXa probe. The FIIa-probe showed complete labeling of the MDA-MB-231 and SW620 cell lines and heterogeneous labeling of the SW480 cells.

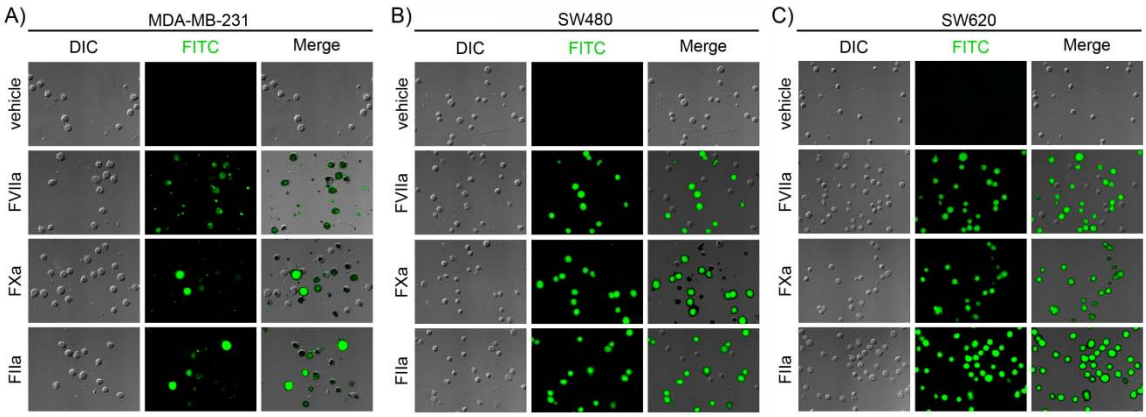


**Figure 7.6. Characterization of fluorescent coagulation factor probe binding to immobilized MDA-MB-231, SW480 and SW620 cells in the presence of coagulation. MDA-MB-231, SW480 and SW620 cells were immobilized on silanized glass slides and incubated with recalcified human plasma containing the anti-FXIa antibody, 1A6, and the fibrin polymerization inhibitor, GPRP, for 30 minutes at RT. Cells were then washed and treated with PBS (vehicle) or PBS containing fluorescently labeled coagulation factors FVIIa (500 nM), FXa (5 μM), or FIIa (10 μM) for 30 minutes at RT, washed in PBS and fixed in 3.7% paraformaldehyde. Immobilized cells were imaged using differential interference contrast (DIC) and fluorescence microscopy.**

*7.5.3.3 Labeling of immobilized MDA-MB-231 and SW620 cells in whole blood*

Following our experiments in cell-free labeling buffers, we next determined whether coagulation factor-based probes could label cancer cells in whole blood. Immobilized MDA-MB-231, SW480 and SW620 cells were incubated with anticoagulated whole blood containing either vehicle, or fluorescent, active site-inhibited FVIIa (500 nM), FXa (5 μM), FIIa (10 μM), or FITC-conjugated anti-TF mAb (50 μg $mL^{-1}$) for 30 minutes at RT. DIC, fluorescence and merged images are shown in Figure 7.7. The FVIIa probe showed heterogeneous labeling of the MDA-MB-231 cells, SW480 and SW620 cells. The labeling of the SW480 cells with the FVIIa probe was greatly diminished as compared to cells that had been exposed to plasma under conditions of coagulation (Figure 7.6). Heterogeneous labeling of all three cell lines with the FXa and FIIa probes was observed, with very few SW480 or SW620 cells labeled. All cell lines were labeled with the anti-TF mAb in whole blood.

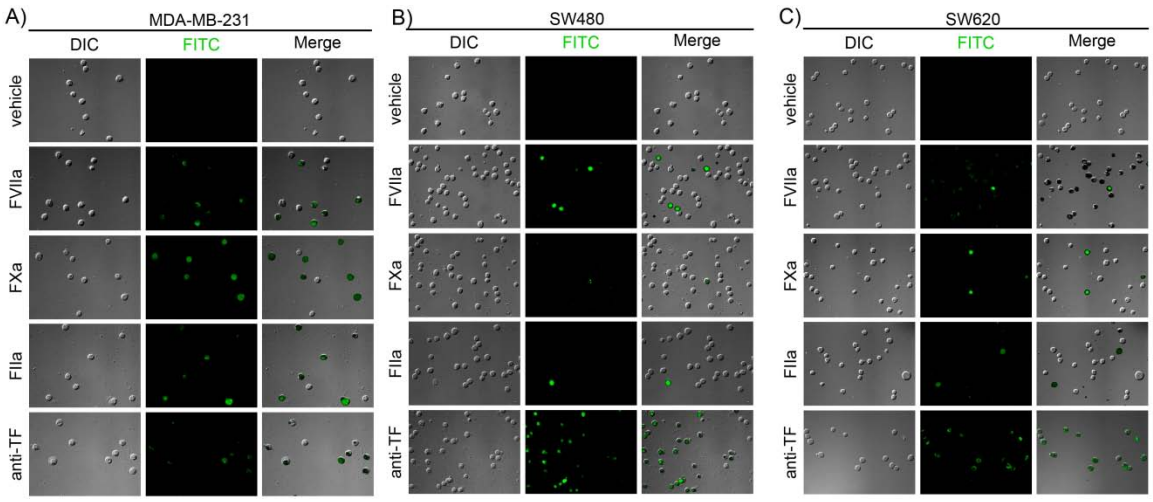


**Figure 7.7. Characterization of fluorescent coagulation factor probe binding to immobilized MDA-MB-231, SW480 and SW620 cells in whole blood. MDA-MB-231, SW480 and SW620 cells were immobilized on silanized glass slides and incubated with sodium citrate anticoagulated whole blood (vehicle) containing fluorescently labeled coagulation factors FVIIa (500 nM), FXa (5 μM), FIIa (10 μM) or FITC-conjugated anti-TF mAb (50 μg mL$^{-1}$) for 30 minutes at RT, washed in PBS and fixed in 3.7% paraformaldehyde. Immobilized cells were imaged using differential interference contrast (DIC) and fluorescence microscopy.**

*7.5.3.4 Labeling of immobilized platelets and neutrophils in a purified system*

Following the reduced labeling of the MDA-MB-231 cells, SW480 and SW620 cells in whole blood as compared to cell-free labeling solutions, we designed a set of experiments to determine if peripheral blood cells might be binding the probe in solution, and thereby causing diminished labeling of immobilized cancer cells. Neutrophils and platelets were purified from peripheral blood draws and immobilized onto silanized glass slides, and incubated with fluorescent FVIIa (500 nM), FXa (5 μM), and FIIa (10 μM). Coagulation factor-based probes failed to bind immobilized peripheral blood cells in a purified system (Figure 7.8). Our data show that neither the FVIIa (500 nM), FXa (5 μM), FIIa (10 μM) probes labeled purified human platelets (Figure 7.8A) or neutrophils (Figure 7.8B). In a complementary experiment, addition of purified human neutrophils to plasma failed to reduce clotting times (605 s *vs.* 670 s for vehicle and $10^5$ mL$^{-1}$ neutrophils, respectively) demonstrating that purified human neutrophils did not exhibit a procoagulant phenotype.

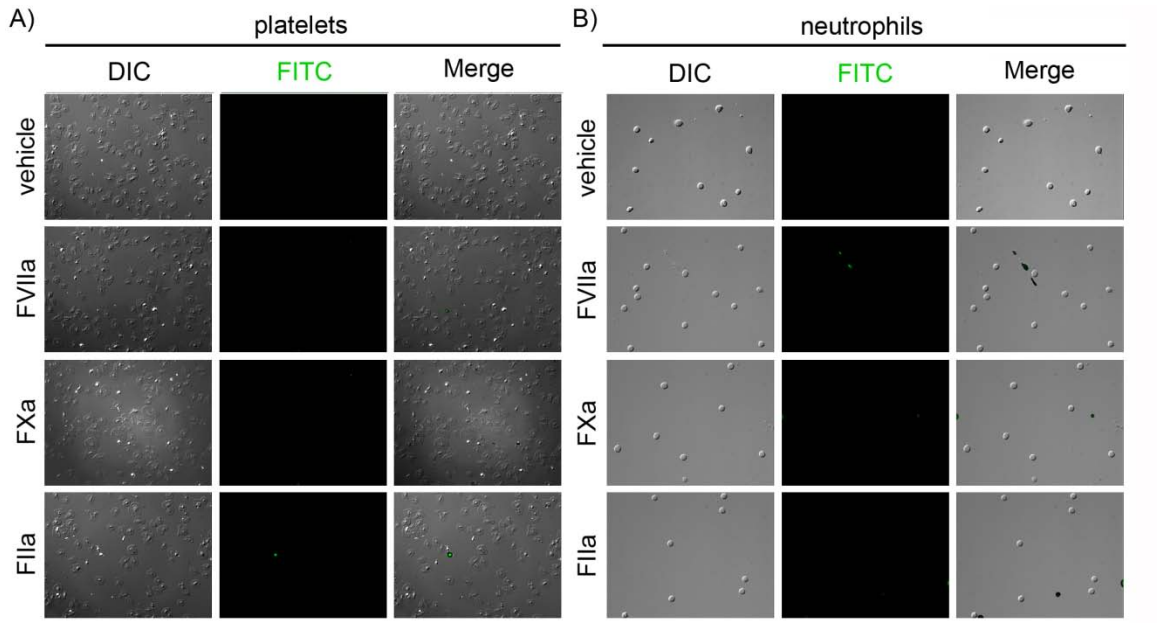


**Figure 7.8. Characterization of fluorescent coagulation factor probe binding to immobilized human neutrophils and platelets. Human platelets (A) and neutrophils (B) were immobilized on silanized glass slides and incubated with PBS (vehicle) containing fluorescently labeled coagulation factors FVIIa (500 nM), FXa (5 μM) or FIIa (10 μM) for 30 minutes at RT, washed in PBS and fixed in 3.7% paraformaldehyde. Immobilized cells were imaged using differential interference contrast (DIC) and fluorescence microscopy.**

### 7.6 Discussion

Metastatic disease accounts for the majority of cancer deaths. Thrombosis significantly contributors to metastatic disease, and accounts for the second leading cause of cancer deaths. No current technology is available to provide a rational basis to predict patient risk for venous thromboembolism. CTC counts have not been evaluated as a potential biomarker for risk to develop cancer associated thrombosis.

In this study, we demonstrate the use of fluorescently-modified, active site-inhibited coagulation factors to label procoagulant cancer cells. The metastatic breast cancer cell line, MDA-MB-231, and metastatic colorectal cell line, SW620, were used due to the fact that these cell lines possess the ability to survive circulation in the blood to establish hematogenous metastases in murine models of cancer metastasis (Zhang et al. 1991;

Sampson-Johannes et al. 1996).The nonmetastatic colorectal cell line, SW480, was derived from the primary tumor from the same individual from which the SW620 was derived. We determined that all three cell lines exhibited a procoagulant phenotype, with the MDA-MB-231 cells resulting in the highest procoagulant activity, while the SW620 cells had the lowest procoagulant activity. The procoagulant activity of the MDA-MB-231, SW480, and SW620 cell lines could be reduced or abrogated by a function-blocking antibody to TF, or by pretreatment with Annexin V. Annexin V blocks the binding of coagulation factors that contain gamma carboxyglutamic acid (Gla) domains to PS on the cell membrane. These results support the notion that TF and PS exposure is a general phenomenon seen for cancer cell-mediated coagulation *in vitro,* and support a role for CTC-mediated coagulation as a potential contributor to the hypercoagulability seen for patients with cancer.

Previous studies have shown that cancer cell procoagulant activity correlates better with PS exposure than with overall TF expression levels, supporting a role for cell membrane effects in regulating procoagulant activity as opposed to surface TF expression (Barrowcliffe et al. 2002; Pickering et al. 2004). Therefore, we aim to develop a function-based CTC labeling strategy to determine whether CTCs are procoagulant, and whether CTC enumeration and procoagulant characterization strategies are clinically useful in predicting thrombosis in patients with cancer. We hypothesized that coagulation factors themselves would serve as functional probes with which to identify procoagulant cells. We focused on the coagulation factors FVIIa, FXa and FIIa, as they are key components of the extrinsic (TF) pathway of coagulation.

Our results with flow cytometry show that the cancer cell lines MDA-MB-231, SW480 and SW620 bind coagulation factor probes. Immobilized cells show that cancer cells exhibited heterogeneity in their ability to bind various fluorescently-modified active site-inhibited coagulation factors. For instance, we observed heterogeneous labeling of SW480 with the FVIIa probe in a both a purified system and in whole blood. Binding heterogeneity was observed over a range of probe concentrations (data not shown), suggesting the heterogeneity was not due to a scarcity of probe concentration. Further, the $K_D$ for FVIIa to TF is in the pM range, 5 orders of magnitude below our labeling concentration. A stronger binding of FVIIa could suggest that these cells are more procoagulant than cells that show weak binding, however, currently no method can be used to determine individual cell procoagulant activity. Moreover, protease activated receptor 2 (PAR2) is another known receptor for FVIIa besides TF. MDA-MB-231 and SW620 cells are known to express PAR2, while SW480 cells have been shown to have little PAR2 surface expression (Morris et al. 2006; Zhou et al. 2008). Whether coagulation factor probe binding levels correlate with procoagulant activity or whether PAR2 expression levels affect binding of FVIIa probe is a focus of future studies.

FIIa-based probes brightly labeled immobilized MDA-MB-231, SW480, and SW620 cells in the presence of coagulation. In contrast, FIIa-probes only weakly labeled these cell lines in whole blood and failed to label any cells in DMEM. One prominent difference between FIIa and the other coagulation factor probes is the absence of the Gla domain for FIIa. The Gla-domain mediates calcium ion-dependent binding of vitamin K-dependent coagulation factors to PS-containing procoagulant cell membranes. Calcium-dependent binding may account for differences in the FVIIa or FXa probe labeling as

compared to FIIa in DMEM, which contains calcium, but fails to account for differences in whole blood in the presence of the calcium-chelator sodium citrate. As FIIa binds fibrinogen and fibrin, it is possible that FIIa labeled cancer cells that were coated in fibrin, a phenomenon that would be expected after exposing a procoagulant cancer cell to plasma under conditions of coagulation. However, using flow cytometry, we observed labeling of cancer cells with a FIIa-based probe in purified systems, suggesting an alternate mechanism for binding of FIIa to the cancer cell surface. Our future work will be focused on identifying the mechanism(s) of FIIa-cancer cell binding.

In this study, we demonstrated the use of fluorescently-labeled, active site-inhibited coagulation factors to label procoagulant cancer cells. We demonstrated that coagulation factor based-probes bound to cancer cell lines in purified systems and in whole blood, yet failed to bind to peripheral blood cells. Labeling of cancer cells was demonstrated via flow cytometry in purified systems, as well as on an immobilized-cell platform similar to what is currently used in some CTC-detection platforms. This work is the first step in the development of a function-based CTC labeling strategy to determine whether CTCs are procoagulant, and whether CTC enumeration and procoagulant characterization strategies are clinically useful in predicting thrombosis in patients with cancer.

# Chapter 8: Conclusions and Future Work

## 8.1 Development of a single cell plasma clotting assay

As shown in this thesis, the procoagulant phenotype of TF-positive cancer cells and TF-coated microspheres correlates with spatial distribution in suspension. TF-initiated coagulation exhibits threshold behavior, and proceeds to form fibrin once procoagulant mechanisms overwhelm anticoagulant mechanisms (Mann 2003). As such, a TF-positive cell with low intrinsic procoagulant activity (i.e. due to low PS-exposure) may not initiate coagulation on its own, but may do so in synergy with a neighboring cell if in close proximity. In an experimental model of TF-initiated coagulation, physical confinement of a TF-coated bead induced fibrin formation whereas no fibrin formation from a similar TF-coated bead in an unconfined environment occurred (Shen et al. 2009). In this experiment, physical confinement inhibited the dissipation of thrombin away from the surface where it was generated, causing local thrombin concentrations to increase to the point where coagulation is initiated. In suspension and under flow, chemical confinement of thrombin dissipation, by juxtaposing neighboring TF-positive cells such that the generation of thrombin at the surface of one cell opposes the dissipation of thrombin from a neighboring cell, could account for a prothrombotic phenotype of a group of circulating cancer cells that is not observed from individual circulating cancer cells, or cells in a less dense population. As such, chemical confinement of thrombin generation may help to explain the role for spatial separation to determine blood coagulation of TF-positive cells in suspension and in flow. To test this hypothesis, a method to control the spacing of cells is needed, in combination with a method to determine the procoagulant phenotype of individual cells. Micropatterned surfaces, which are coated with adhesive

proteins, provide a method to control the spacing of cells on a surface. A fluorescence microscopy image of a micropatterned surface that has been coated with the fluorescently labeled coagulation factor VIIa is shown in Figure 8.1.

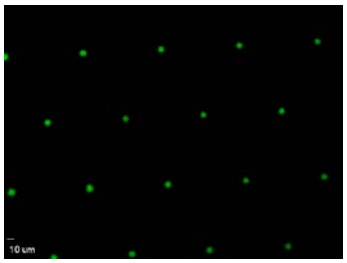

**Figure 8.1. Micropatterned glass substrate that has been coated with fluorescent FVIIa. Cells may be immobilized onto the pattern allowing direct control of spatial separation.**

To test whether neighboring cells synergistically operate to promote coagulation, a patterned surface could precisely control the spatial separation of immobilized cells. Under controlled spatial constraint, live cell imaging can be used to observe the formation of fibrin in real time, allowing direct measurement of initiation times and growth rates from a single cell. Preliminary experiments were performed by immobilizing malignant epithelial cells onto silanized glass surfaces and immersing in plasma under conditions of coagulation. As shown in Figure 8.2, the real-time measurement of fibrin formation was observed at 40× magnification with time-lapse DIC microscopy.

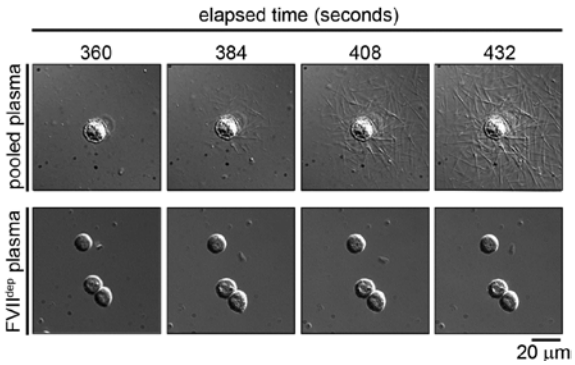


**Figure 8.2. Time lapse microscopy of fibrin formation by SW480 colon adenocarcinoma cells immobilized onto a silanized glass surface, immersed in plasma under conditions of coagulation for (top row) pooled plasma and (bottom row) coagulation factor VII-depleted plasma.**

### 8.2 Assess the correlation between circulating cancer cells and thrombosis

The experiments carried out in this thesis investigated the physical determinants of the procoagulant phenotype of circulating cancer cells. Our results highlight several unique physiological features of a cell in circulation, and provide a framework for future efforts to determine the association between circulating cancer cells and thrombosis in patients with cancer. The majority of patients with cancer have clinical evidence of low-grade disseminated intravascular coagulation (DIC), however, a relative minority of patients with cancer develop thrombosis (Rickles et al. 1983). Thrombosis, and DIC leading to hemorrhage, are thought to be distinct points along the same spectrum of coagulation activation in patients with cancer, and can occur concomitantly (Falanga et al. 2007). In cancer, clinical evidence for activation of coagulation (elevated thrombin-antithrombin complexes) and marked fibrinolysis (elevated fibrin degradation products and thrombocytopenia) fits a picture of slow, intravascular fibrin formation that is matched by fibrinolytic mechanisms. In other words, the rate of fibrin formation is comparable to fibrin degradation, resulting in a futile consumption of coagulation factors (i.e. fibrinogen) which can leave the patient vulnerable to hemorrhage if production of coagulation factors cannot keep up with consumption. Conversely, if fibrin formation overwhelms fibrinolysis, intravascular fibrin formation may lead to thrombosis.

Results in this thesis demonstrate that the rate of fibrin formation by TF-positive cancer cells is dependent upon the cell count as well as PS exposure. In AML M3, where procoagulant activity from circulating cancer cells is both expected due to the oncogenic alleles pathognomonic for the development of AML M3, and verified from freshly isolated AML cells, an elevated peripheral cell count is associated with increased

development of thrombosis. Moreover, administration of cytotoxic chemotherapeutics may not only worsen clinical signs of DIC, but may also be associated with the development of thrombosis. Along these lines, we hypothesize that the procoagulant phenotype of circulating cancer cells underlies the coagulopathy experienced by patients with cancer. We provide evidence to suggest that the rate of fibrin formation by cancer cells in blood is dependent upon the procoagulant phenotype of cancer cells, which includes cell count, TF and PS exposure. Along these lines, we hypothesize that a measure of the procoagulant phenotype of individual patient's cancer cells would serve as a marker for thrombophilia in cancer. Enumeration of procoagulant circulating cancer cell enables prospective studies to determine the association between circulating cancer cells and thrombosis. As such, we developed PS index as a marker for TF-positive AML cell procoagulant activity, and proposed fluorescent coagulation factors as markers of procoagulant circulating tumor cells. Taken together, Figure 8.3 schematically illustrates our hypothesis on the formation of blood clots by circulating cancer cells over time and as a function of cell count, PS exposure, and concentration of F IX and FX.

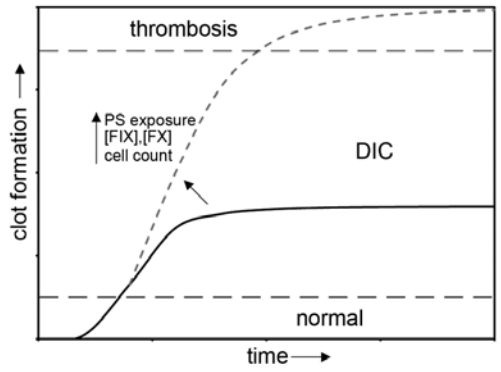


**Figure 8.3. Schema of coagulation initiation by procoagulant leukemia cells. Initiation times and clot growth rates were demonstrated to shorten at increased cell counts and by elevated PS exposure stemming from exposure to cytotoxic drugs.**

### 8.3 Characterization of coagulation enzymes on the migration of cancer cells

Drug-resistant metastatic disease accounts for over 90% of mortality in cancer. TF is

overexpressed in many tumors and TF overexpression is associated with metastatic potential (Kasthuri et al. 2009). We observed in this thesis that active TF expression drives the procoagulant phenotype of AML and malignant epithelial cell lines. Several epidemiological studies have observed that anticoagulation is associated with improved survival in cancer, which may be due to decreased metastasis of cells from the primary tumor site (Kuderer et al. 2007). Taken together, anticoagulation has potential for anti-metastatic effects as well as antithrombotic effects in patients with cancer. Whether coagulation drives metastasis, or if anticoagulation is a suitable approach to combat cancer remains ill defined.

In addition to driving procoagulant activity, TF initiates intercellular signaling through protease activated receptors (PARs). Four isoforms of PARs have been identified in humans, known as PAR1-PAR4. TF-dependent signaling has been observed to convey metastatic behavior to some cancer cells and anti-metastatic properties to other cancer cell lines. Some of the discrepancies in these observations may be due to the use of purified systems to investigate the TF-dependent signaling in cancer cells. TF•FVIIa has been shown to activate PAR2, while thrombin can activate PAR1 and PAR 4. Recently, APC-mediated signaling through PAR1, PAR3 and PAR4 has been demonstrated (Madhusudhan et al. 2012; Mosnier et al. 2012). Moreover, APC and FIIa can activate the same receptor, but bias the signaling towards different signaling cascades (Mosnier et al. 2012). Taken together, TF•FVIIa, FXa, FIXa, FIXa, FIIa, FXIIIa and APC may be generated on the surface of a procoagulant cancer cell in plasma. The effect of coagulation enzymes on PAR-signaling in regulating cancer cell physiology is ill defined.

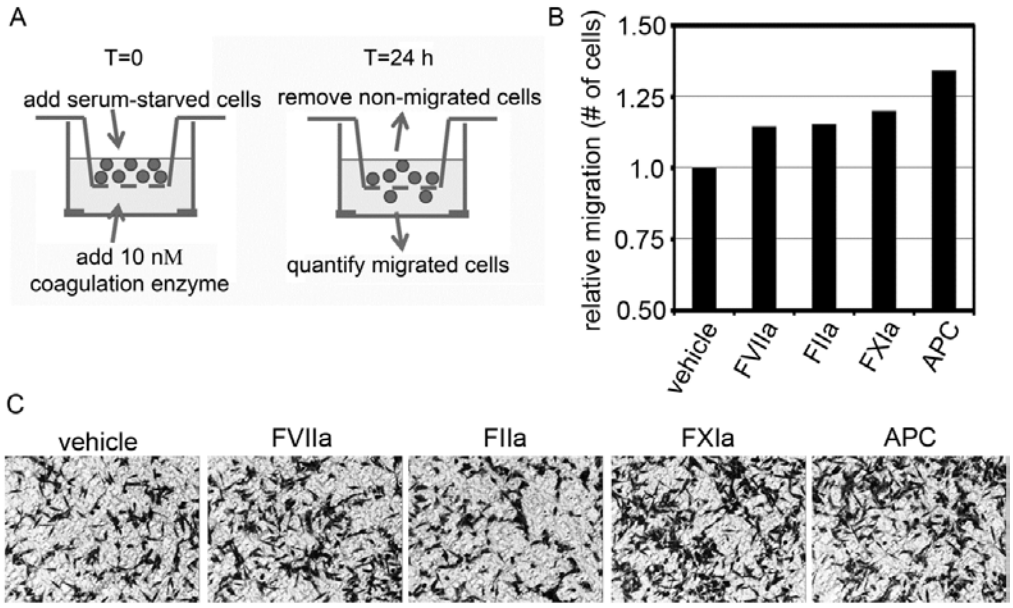


**Figure 8.4. Transwell migration assay to evaluate the effects of coagulation enzymes on the migratory phenotype of cancer cells. (A) General protocol to assess coagulation enzyme effect on cell migration. (B) Quantification of the number of migrated cells. (C) Bright field microscopy of the migrated cells adherent to the transwell membrane following a 24 hour exposure to 10 nM of indicated coagulation enzyme.**

Transwell migration assays may be utilized to elucidate mechanisms by which PAR signaling modulates cancer cell metastatic phenotypes. The transwell assay protocol is illustrated in Figure 8.4A. My preliminary results utilizing purified coagulation enzymes and the metastatic breast cancer cell line, MDA-MB-231, show that equimolar concentrations of FVIIa, FXa, FXIa and APC enhance the migration of cancer cells as compared to vehicle (serum free cell media; Figure 8.4B,C).

Future studies will utilize transwell assays to evaluate concomitant exposure to two or more enzymes, in varied concentrations, and in the presence of anticoagulants, antibodies or inhibitors to TF or PS in order to elucidate the role of coagulation enzyme activation of the PAR signaling cascade in regulating cancer cell migration.

# References


Ahmad, S. S., F. S. London, et al. (2003). "The assembly of the factor X-activating complex on activated human platelets." J Thromb Haemost 1(1): 48-59.

Akl, E. A., N. Labedi, et al. (2011). "Anticoagulation for the long-term treatment of venous thromboembolism in patients with cancer." Cochrane Database Syst Rev(6): CD006650.

Akl, E. A., S. R. Vasireddi, et al. (2011). "Oral anticoagulation in patients with cancer who have no therapeutic or prophylactic indication for anticoagulation." Cochrane Database Syst Rev(12): CD006466.

Allard, W. J., J. Matera, et al. (2004). "Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases." Clin Cancer Res 10(20): 6897-904.

Ambrus, J. L., C. M. Ambrus, et al. (1975). "Causes of death in cancer patients." J Med 6(1): 61-4.

Andree, H. A., P. B. Contino, et al. (1994). "Transport rate limited catalysis on macroscopic surfaces: the activation of factor X in a continuous flow enzyme reactor." Biochemistry 33(14): 4368-74.

Ansell, J., J. Hirsh, et al. (2008). "Pharmacology and management of the vitamin K antagonists: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition)." Chest 133(6 Suppl): 160S-198S.

Aras, O., A. Shet, et al. (2004). "Induction of microparticle- and cell-associated intravascular tissue factor in human endotoxemia." Blood 103(12): 4545-53.

Ashworth, T. R. (1869). "A case of cancer in which cells similar to those in the tumours were seen in the blood after death." Australian Medical Journal 14: 146-7.

Auger, J. M., M. J. Kuijpers, et al. (2005). "Adhesion of human and mouse platelets to collagen under shear: a unifying model." Faseb J 19(7): 825-7.

Auwerda, J. J., Y. Yuana, et al. (2011). "Microparticle-associated tissue factor activity and venous thrombosis in multiple myeloma." Thromb Haemost 105(1): 14-20.

Awtry, E. H. and J. Loscalzo (2000). "Aspirin." Circulation 101(10): 1206-18.

Ay, C., D. Dunkler, et al. (2011). "Prediction of venous thromboembolism in patients with cancer by measuring thrombin generation: results from the Vienna Cancer and Thrombosis Study." J Clin Oncol 29(15): 2099-103.

Bach, R. R. and C. F. Moldow (1997). "Mechanism of tissue factor activation on HL-60 cells." Blood 89(9): 3270-6.

Baron, J. A., G. Gridley, et al. (1998). "Venous thromboembolism and cancer." Lancet 351(9109): 1077-80.

Barrowcliffe, T. W., P. Fabregas, et al. (2002). "Procoagulant activity of T lymphoblastoid cells due to exposure of negatively charged phospholipid." Thromb Haemost 87(3): 442-9.

Baugh, R. J., G. J. Broze, Jr., et al. (1998). "Regulation of extrinsic pathway factor Xa formation by tissue factor pathway inhibitor." J Biol Chem 273(8): 4378-86.

Baumgartner, H. R. (1973). "The role of blood flow in platelet adhesion, fibrin deposition, and formation of mural thrombi." Microvasc Res 5(2): 167-79.

Berny-Lang, M. A., J. E. Aslan, et al. (2011). "Promotion of experimental thrombus formation by the procoagulant activity of breast cancer cells." Phys Biol 8(1): 015014.

Berny, M. A., I. C. Munnix, et al. (2010). "Spatial distribution of factor Xa, thrombin, and fibrin(ogen) on thrombi at venous shear." PLoS One 5(4): e10415.

Berny, M. A., I. A. Patel, et al. (2010). "Rational Design of an *Ex Vivo* Model of Thrombosis." Cellular and Molecular Bioengineering 3(2): 187-189.

Bevers, E. M., P. Comfurius, et al. (1982). "The nature of the binding for prothrombinase at the platelet surface as revealed by lipolytic enzymes." Eur J Biochem 122(1): 81-5.

Bevers, E. M., P. Comfurius, et al. (1983). "Changes in membrane phospholipid distribution during platelet activation." Biochim Biophys Acta 736(1): 57-66.

Blom, J. W., C. J. Doggen, et al. (2005). "Malignancies, prothrombotic mutations, and the risk of venous thrombosis." Jama 293(6): 715-22.

Blom, J. W., S. Osanto, et al. (2004). "The risk of a venous thrombotic event in lung cancer patients: higher risk for adenocarcinoma than squamous cell carcinoma." J Thromb Haemost 2(10): 1760-5.

Blomback, B., B. Hessel, et al. (1978). "A two-step fibrinogen--fibrin transition in blood coagulation." Nature 275(5680): 501-5.

Bock, P. E. (1992). "Active-site-selective labeling of blood coagulation proteinases with fluorescence probes by the use of thioester peptide chloromethyl ketones. II. Properties of thrombin derivatives as reporters of prothrombin fragment 2 binding and specificity of the labeling approach for other proteinases." J Biol Chem 267(21): 14974-81.

Boland, M. P., S. J. Foster, et al. (1997). "Daunorubicin activates NFkappaB and induces kappaB-dependent gene expression in HL-60 promyelocytic and Jurkat T lymphoma cells." J Biol Chem 272(20): 12952-60.

Boles, J. C., J. C. Williams, et al. (2012). "Anthracycline treatment of the human monocytic leukemia cell line THP-1 increases phosphatidylserine exposure and tissue factor activity." Thromb Res 129(2): 197-203.

Boltzmann, L. (1894). "Zur integration der diffusionsgleichung bei variablen diffusion koeffizienten." Annalen der Physik and Chemie 53: 959-964.

Bose, R., M. Verheij, et al. (1995). "Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals." Cell 82(3): 405-14.

Bouillard, J. and S. Bouillard (1823). "De l'Obliteration des veines et de son influence sur la formation des hydropisies partielles: consideration sur la hydropisies passive et general." Arch. Gen. Med 1: 188-204.

Breccia, M., G. Avvisati, et al. (2007). "Occurrence of thrombotic events in acute promyelocytic leukemia correlates with consistent immunophenotypic and molecular features." Leukemia 21(1): 79-83.

Brummel-Ziedins, K. E., R. L. Pouliot, et al. (2004). "Thrombin generation: phenotypic quantitation." J Thromb Haemost 2(2): 281-8.

Carson, S. D., S. J. Pirruccello, et al. (1990). "Tissue factor antigen and activity are not expressed on the surface of intact cells isolated from an acute promyelocytic leukemia patient." Thromb Res 59(1): 159-70.

Ceelie, H., R. M. Bertina, et al. (2001). "Polymorphisms in the prothrombin gene and their association with plasma prothrombin levels." Thromb Haemost 85(6): 1066-70.

Chew, H. K., A. M. Davies, et al. (2008). "The incidence of venous thromboembolism among patients with primary lung cancer." J Thromb Haemost 6(4): 601-8.

Chew, H. K., T. Wun, et al. (2006). "Incidence of venous thromboembolism and its effect on survival among patients with common cancers." Arch Intern Med 166(4): 458-64.

Chou, J., N. Mackman, et al. (2004). "Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation." Blood 104(10): 3190-7.

Chuang, Y. J., R. Swanson, et al. (2001). "Heparin enhances the specificity of antithrombin for thrombin and factor Xa independent of the reactive center loop sequence. Evidence for an exosite determinant of factor Xa specificity in heparin-activated antithrombin." J Biol Chem 276(18): 14961-71.

Cohen, S. J., C. J. Punt, et al. (2008). "Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer." J Clin Oncol 26(19): 3213-21.

Conkling, P. R., C. S. Greenberg, et al. (1988). "Tumor necrosis factor induces tissue factor-like activity in human leukemia cell line U937 and peripheral blood monocytes." Blood 72(1): 128-33.

Connor, J., C. Bucana, et al. (1989). "Differentiation-dependent expression of phosphatidylserine in mammalian plasma membranes: quantitative assessment of outer-leaflet lipid by prothrombinase complex formation." Proc Natl Acad Sci U S A 86(9): 3184-8.

Contino, P. B., H. A. Andree, et al. (1994). "Flow dependence of factor X activation by tissue factor-factor VIIa." J Physiol Pharmacol 45(1): 81-90.

Crawley, J. T. and D. A. Lane (2008). "The haemostatic role of tissue factor pathway inhibitor." Arterioscler Thromb Vasc Biol 28(2): 233-42.

Cristofanilli, M., G. T. Budd, et al. (2004). "Circulating tumor cells, disease progression, and survival in metastatic breast cancer." N Engl J Med 351(8): 781-91.

Cvirn, G., H. J. Gruber, et al. (2007). "High availability of intravascular tissue factor in neonates." J Pediatr Hematol Oncol 29(5): 279-83.

Dachary-Prigent, J., J. M. Pasquet, et al. (1995). "Calcium involvement in aminophospholipid exposure and microparticle formation during platelet activation: a study using Ca2+-ATPase inhibitors." Biochemistry 34(36): 11625-34.

Danila, D. C., G. Heller, et al. (2007). "Circulating tumor cell number and prognosis in progressive castration-resistant prostate cancer." Clin Cancer Res 13(23): 7053-8.

de Bono, J. S., H. I. Scher, et al. (2008). "Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer." Clin Cancer Res 14(19): 6302-9.

De Stefano, V., F. Sora, et al. (2005). "The risk of thrombosis in patients with acute leukemia: occurrence of thrombosis at diagnosis and during treatment." J Thromb Haemost 3(9): 1985-92.

Dielis, A. W., E. Castoldi, et al. (2008). "Coagulation factors and the protein C system as determinants of thrombin generation in a normal population." J Thromb Haemost 6(1): 125-31.

Dimopoulos, M. A. and V. Eleutherakis-Papaiakovou (2004). "Adverse effects of thalidomide administration in patients with neoplastic diseases." Am J Med 117(7): 508-15.

Donati, M. B. and R. Lorenzet (2012). "Thrombosis and cancer: 40 years of research." Thromb Res 129(3): 348-52.

Dong, J. F., M. C. Berndt, et al. (2001). "Ristocetin-dependent, but not botrocetin-dependent, binding of von Willebrand factor to the platelet glycoprotein Ib-IX-V complex correlates with shear-dependent interactions." Blood 97(1): 162-8.

Elice, F., F. Rodeghiero, et al. (2009). "Thrombosis associated with angiogenesis inhibitors." Best Pract Res Clin Haematol 22(1): 115-28.

Elliott, J. I., A. Surprenant, et al. (2005). "Membrane phosphatidylserine distribution as a non-apoptotic signalling mechanism in lymphocytes." Nat Cell Biol 7(8): 808-16.

Engelmann, B. and S. Massberg (2013). "Thrombosis as an intravascular effector of innate immunity." Nat Rev Immunol 13(1): 34-45.

EPIC (1994). "Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. The EPIC Investigation." N Engl J Med 330(14): 956-61.

Epstein, D. J., P. W. Bergum, et al. (1984). "Radioimmunoassays for protein C and factor X. Plasma antigen levels in abnormal hemostatic states." Am J Clin Pathol 82(5): 573-81.

Esmon, C. T. (1979). "The subunit structure of thrombin-activated factor V. Isolation of activated factor V, separation of subunits, and reconstitution of biological activity." J Biol Chem 254(3): 964-73.

Esmon, C. T. and W. G. Owen (1981). "Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C." Proc Natl Acad Sci U S A 78(4): 2249-52.

Fair, D. S. (1983). "Quantitation of factor VII in the plasma of normal and warfarin-treated individuals by radioimmunoassay." Blood 62(4): 784-91.

Falanga, A., R. Consonni, et al. (1994). "Cancer procoagulant in the human promyelocytic cell line NB4 and its modulation by all-trans-retinoic acid." Leukemia 8(1): 156-9.

Falanga, A., L. Iacoviello, et al. (1995). "Loss of blast cell procoagulant activity and improvement of hemostatic variables in patients with acute promyelocytic leukemia administered all-trans-retinoic acid." Blood 86(3): 1072-81.

Falanga, A. and M. Marchetti (2009). "Venous thromboembolism in the hematologic malignancies." J Clin Oncol 27(29): 4848-57.

Falanga, A. and F. R. Rickles (2007). "Management of Thrombohemorrhagic Syndromes (THS) in hematologic malignancies." Hematology Am Soc Hematol Educ Program: 165-71.

Falati, S., Q. Liu, et al. (2003). "Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin." J Exp Med 197(11): 1585-98.

Ferry, J. D., S. Shulman, et al. (1952). "The conversion of fibrinogen to fibrin. IX. Further flow birefringence studies on inhibited clotting systems." Arch Biochem Biophys 39(2): 387-94.

Forman, S. D. and Y. Nemerson (1986). "Membrane-dependent coagulation reaction is independent of the concentration of phospholipid-bound substrate: fluid phase factor X regulates the extrinsic system." Proc Natl Acad Sci U S A 83(13): 4675-9.

Fulcher, C. A., J. E. Gardiner, et al. (1984). "Proteolytic inactivation of human factor VIII procoagulant protein by activated human protein C and its analogy with factor V." Blood 63(2): 486-9.

Furie, B. and B. C. Furie (2008). "Mechanisms of thrombus formation." N Engl J Med 359(9): 938-49.

Gailani, D. and G. J. Broze, Jr. (1991). "Factor XI activation in a revised model of blood coagulation." Science 253(5022): 909-12.

Gallagher, R., S. Collins, et al. (1979). "Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia." Blood 54(3): 713-33.

Gemmell, C. H., V. T. Turitto, et al. (1988). "Flow as a regulator of the activation of factor X by tissue factor." Blood 72(4): 1404-6.

Giesen, P. L., U. Rauch, et al. (1999). "Blood-borne tissue factor: another view of thrombosis." Proc Natl Acad Sci U S A 96(5): 2311-5.

Giesen, P. L., G. M. Willems, et al. (1991). "Production of thrombin by the prothrombinase complex is regulated by membrane-mediated transport of prothrombin." J Biol Chem 266(3): 1379-82.

Gil-Bernabe, A. M., S. Ferjancic, et al. (2012). "Recruitment of monocytes/macrophages by tissue factor-mediated coagulation is essential for metastatic cell survival and premetastatic niche establishment in mice." Blood 119(13): 3164-75.

Gladhaug, A. and H. Prydz (1970). "Purification of the coagulation factors VII and X from human serum. Some properties of factor VII." Biochim Biophys Acta 215(1): 105-11.

Gleghorn, J. P., E. D. Pratt, et al. (2010). "Capture of circulating tumor cells from whole blood of prostate cancer patients using geometrically enhanced differential immunocapture (GEDI) and a prostate-specific antibody." Lab Chip 10(1): 27-9.

Goth, S. R. and R. S. Stephens (2001). "Rapid, transient phosphatidylserine externalization induced in host cells by infection with Chlamydia spp." Infect Immun 69(2): 1109-19.

Hathcock, J. J. (2006). "Flow effects on coagulation and thrombosis." Arterioscler Thromb Vasc Biol 26(8): 1729-37.

Hayashi, T., G. Honda, et al. (1992). "An atherogenic stimulus homocysteine inhibits cofactor activity of thrombomodulin and enhances thrombomodulin expression in human umbilical vein endothelial cells." Blood 79(11): 2930-6.

Hollopeter, G., H. M. Jantzen, et al. (2001). "Identification of the platelet ADP receptor targeted by antithrombotic drugs." Nature 409(6817): 202-7.

Howard, P. R., E. G. Bovill, et al. (1994). "Factor VII antigen levels in a healthy blood donor population." Thromb Haemost 72(1): 21-7.

Hoyer, L., W. Wyshock, et al., Eds. (1994). Hemostasis and Thrombosis: basic principles and clinical practice. Philadelphia, J.B. Lippincott Company.

Hoyer, L., W. Wyshock, et al. (1994). Hemostasis and Thrombosis: basic principles and clinical practice. Philadelphia, J.B. Lippincott Company.

Hoyer, L. W. and N. C. Trabold (1981). "The effect of thrombin on human factor VIII. Cleavage of the factor VIII procoagulant protein during activation." J Lab Clin Med 97(1): 50-64.

Hrachovinova, I., B. Cambien, et al. (2003). "Interaction of P-selectin and PSGL-1 generates microparticles that correct hemostasis in a mouse model of hemophilia A." Nat Med 9(8): 1020-5.

Iodice, S., S. Gandini, et al. (2008). "Venous thromboembolic events and organ-specific occult cancers: a review and meta-analysis." J Thromb Haemost 6(5): 781-8.

Itakura, A., N. G. Verbout, et al. (2011). "Activated factor XI inhibits chemotaxis of polymorphonuclear leukocytes." J Leukoc Biol 90(5): 923-7.

Jungi, T. W. (1990). "A turbidimetric assay in an ELISA reader for the determination of mononuclear phagocyte procoagulant activity." J Immunol Methods 133(1): 21-9.

Kamphuisen, P. W., F. R. Rosendaal, et al. (2000). "Factor V antigen levels and venous thrombosis: risk profile, interaction with factor V leiden, and relation with factor VIII antigen levels." Arterioscler Thromb Vasc Biol 20(5): 1382-6.

Kane, W. H. and E. W. Davie (1988). "Blood coagulation factors V and VIII: structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders." Blood 71(3): 539-55.

Karnicki, K., W. G. Owen, et al. (2002). "Factors contributing to individual propensity for arterial thrombosis." Arterioscler Thromb Vasc Biol 22(9): 1495-9.

Kasthuri, R. S., M. B. Taubman, et al. (2009). "Role of tissue factor in cancer." J Clin Oncol 27(29): 4834-8.

Khorana, A. A. and G. C. Connolly (2009). "Assessing risk of venous thromboembolism in the patient with cancer." J Clin Oncol 27(29): 4839-47.

Khorana, A. A., C. W. Francis, et al. (2007). "Frequency, risk factors, and trends for venous thromboembolism among hospitalized cancer patients." Cancer 110(10): 2339-46.

Khorana, A. A., C. W. Francis, et al. (2007). "Thromboembolism is a leading cause of death in cancer patients receiving outpatient chemotherapy." J Thromb Haemost 5(3): 632-4.

Khorana, A. A., C. W. Francis, et al. (2008). "Plasma tissue factor may be predictive of venous thromboembolism in pancreatic cancer." J Thromb Haemost 6(11): 1983-5.

Khorana, A. A., N. M. Kuderer, et al. (2008). "Development and validation of a predictive model for chemotherapy-associated thrombosis." Blood 111(10): 4902-7.

Kisiel, W., W. M. Canfield, et al. (1977). "Anticoagulant properties of bovine plasma protein C following activation by thrombin." Biochemistry 16(26): 5824-31.

Kobayashi, T. and K. J. Laidler (1974). "Theory of the kinetics of reactions catalyzed by enzymes attached to the interior surfaces of tubes." Biotechnol Bioeng 16(1): 99-118.

Ku, G. H., R. H. White, et al. (2009). "Venous thromboembolism in patients with acute leukemia: incidence, risk factors, and effect on survival." Blood 113(17): 3911-7.

Kuderer, N. M., A. A. Khorana, et al. (2007). "A meta-analysis and systematic review of the efficacy and safety of anticoagulants as cancer treatment: impact on survival and bleeding complications." Cancer 110(5): 1149-61.

Kunzelmann-Marche, C., N. Satta, et al. (2000). "The influence exerted by a restricted phospholipid microenvironment on the expression of tissue factor activity at the cell plasma membrane surface." Thromb Haemost 83(2): 282-9.

Langer, F., A. Amirkhosravi, et al. (2004). "An in vitro study on the mechanisms of coagulation activation in acute myelogenous leukemia (AML): role of tissue factor regulation by cytotoxic drugs and GM-CSF." Thromb Haemost 92(5): 1136-46.

Langer, F., B. Spath, et al. (2008). "Tissue factor procoagulant activity of plasma microparticles in patients with cancer-associated disseminated intravascular coagulation." Ann Hematol 87(6): 451-7.

Lanotte, M., V. Martin-Thouvenin, et al. (1991). "NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3)." Blood 77(5): 1080-6.

Lawson, J. H. and K. G. Mann (1991). "Cooperative activation of human factor IX by the human extrinsic pathway of blood coagulation." J Biol Chem 266(17): 11317-27.

Lee, A. M., G. W. Tormoen, et al. (2012). "Modeling and simulation of procoagulant circulating tumor cells in flow." Front Oncol 2: 108.

Lee, B. J., J. H. Kim, et al. (2011). "Tissue factor is involved in retinoblastoma cell proliferation via both the Akt and extracellular signal-regulated kinase pathways." Oncol Rep 26(3): 665-70.

Legnani, C., B. Cosmi, et al. (2003). "Venous thromboembolism, oral contraceptives and high prothrombin levels." J Thromb Haemost 1(1): 112-7.

Lerner, R. G., R. Goldstein, et al. (1971). "Stimulation of human leukocyte thromboplastic activity by endotoxin." Proc Soc Exp Biol Med 138(1): 145-8.

Liebman, H. A. and D. I. Feinstein (2003). "Thrombosis in patients with paroxysmal noctural hemoglobinuria is associated with markedly elevated plasma levels of leukocyte-derived tissue factor." Thromb Res 111(4-5): 235-8.

Lipowsky, H. H. and B. W. Zweifach (1977). "Methods for the simultaneous measurement of pressure differentials and flow in single unbranched vessels of the microcirculation for rheological studies." Microvasc Res 14(3): 345-61.

Liu, Y., P. Jiang, et al. (2011). "Tissue factor-activated coagulation cascade in the tumor microenvironment is critical for tumor progression and an effective target for therapy." Cancer Res 71(20): 6492-502.

Lloyd-Jones, D., R. J. Adams, et al. (2010). "Heart disease and stroke statistics--2010 update: a report from the American Heart Association." Circulation 121(7): e46-e215.

Lopez-Pedrera, C., N. Barbarroja, et al. (2006). "Tissue factor as an effector of angiogenesis and tumor progression in hematological malignancies." Leukemia 20(8): 1331-40.

Lu, G., G. J. Broze, Jr., et al. (2004). "Formation of factors IXa and Xa by the extrinsic pathway: differential regulation by tissue factor pathway inhibitor and antithrombin III." J Biol Chem 279(17): 17241-9.

Lupu, C., A. D. Westmuckett, et al. (2005). "Tissue factor-dependent coagulation is preferentially up-regulated within arterial branching areas in a baboon model of Escherichia coli sepsis." Am J Pathol 167(4): 1161-72.

Ma, Z., T. Zhang, et al. (2011). "Tissue factor-factor VIIa complex induces epithelial ovarian cancer cell invasion and metastasis through a monocytes-dependent mechanism." Int J Gynecol Cancer 21(4): 616-24.

Mackman, N. (2009). "The many faces of tissue factor." J Thromb Haemost 7 Suppl 1: 136-9.

Mackman, N. (2009). "The role of tissue factor and factor VIIa in hemostasis." Anesth Analg 108(5): 1447-52.

Madhusudhan, T., H. Wang, et al. (2012). "Cytoprotective signaling by activated protein C requires protease-activated receptor-3 in podocytes." Blood 119(3): 874-83.

Mandala, M., S. Barni, et al. (2010). "Acquired and inherited risk factors for developing venous thromboembolism in cancer patients receiving adjuvant chemotherapy: a prospective trial." Ann Oncol 21(4): 871-6.

Manly, D. A., J. Wang, et al. (2010). "Increased microparticle tissue factor activity in cancer patients with Venous Thromboembolism." Thromb Res 125(6): 511-2.

Mann, K. G. (2003). "Thrombin formation." Chest 124(3 Suppl): 4S-10S.

Mann, K. G., S. Butenas, et al. (2003). "The dynamics of thrombin formation." Arterioscler Thromb Vasc Biol 23(1): 17-25.

Marchetti, M., E. Diani, et al. (2012). "Characterization of the thrombin generation potential of leukemic and solid tumor cells by calibrated automated thrombography." Haematologica 97(8): 1173-80.

Marcus, A. and M. Zucker (1965). Physiology of Blood Platelets. New York, NY, Grune & Stratton.

Marrinucci, D., K. Bethel, et al. (2012). "Fluid biopsy in patients with metastatic prostate, pancreatic and breast cancers." Phys Biol 9(1): 016003.

Martin, S. J., C. P. Reutelingsperger, et al. (1995). "Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl." J Exp Med 182(5): 1545-56.

Maynard, J. R., C. A. Heckman, et al. (1975). "Association of tissue factor activity with the surface of cultured cells." J Clin Invest 55(4): 814-24.

McGee, M. P., L. C. Li, et al. (1992). "Diffusion control in blood coagulation. Activation of factor X by factors IXa/VIIIa assembled on human monocyte membranes." J Biol Chem 267(34): 24333-9.

Melillo, L., E. Grandone, et al. (2007). "Symptomatic venous thromboembolism and thrombophilic status in adult acute leukemia: a single-center experience of 114 patients at diagnosis." Acta Haematol 117(4): 215-20.

Menell, J. S., G. M. Cesarman, et al. (1999). "Annexin II and bleeding in acute promyelocytic leukemia." N Engl J Med 340(13): 994-1004.

Meyer, J. S. and M. Boxer (1973). "Leukemic cellular thrombi in pulmonary blood vessels. Subleukemic myelogenous leukemia following chloramphenicol-induced aplastic anemia." Cancer 32(3): 712-21.

Mohren, M., I. Markmann, et al. (2006). "Increased risk of venous thromboembolism in patients with acute leukaemia." Br J Cancer 94(2): 200-2.

Moosbauer, C., E. Morgenstern, et al. (2007). "Eosinophils are a major intravascular location for tissue factor storage and exposure." Blood 109(3): 995-1002.

Morris, D. R., Y. Ding, et al. (2006). "Protease-activated receptor-2 is essential for factor VIIa and Xa-induced signaling, migration, and invasion of breast cancer cells." Cancer Res 66(1): 307-14.

Mosnier, L. O., R. K. Sinha, et al. (2012). "Biased agonism of protease-activated receptor 1 by activated protein C caused by noncanonical cleavage at Arg46." Blood 120(26): 5237-46.

Nadir, Y., T. Katz, et al. (2005). "Hemostatic balance on the surface of leukemic cells: the role of tissue factor and urokinase plasminogen activator receptor." Haematologica 90(11): 1549-56.

Nagrath, S., L. V. Sequist, et al. (2007). "Isolation of rare circulating tumour cells in cancer patients by microchip technology." Nature 450(7173): 1235-9.

Neeves, K. B., D. A. Illing, et al. (2010). "Thrombin flux and wall shear rate regulate fibrin fiber deposition state during polymerization under flow." Biophys J 98(7): 1344-52.

Nemerson, Y. (1968). "The phospholipid requirement of tissue factor in blood coagulation." J Clin Invest 47(1): 72-80.

Nishiguchi, T., T. Kobayashi, et al. (1999). "Upregulation of thrombomodulin antigen levels in U937 cells by combined stimulation with estradiol-17beta and vitamin K2 (menaquinone 4)." Semin Thromb Hemost 25(5): 509-17.

Noble, S. and J. Pasi (2010). "Epidemiology and pathophysiology of cancer-associated thrombosis." Br J Cancer 102 Suppl 1: S2-9.

Oehadian, A., M. Iqbal, et al. (2009). "Deep vein thrombosis in acute myelogenous leukemia." Acta Med Indones 41(4): 200-4.

Offermanns, S. (2006). "Activation of platelet function through G protein-coupled receptors." Circ Res 99(12): 1293-304.

Okorie, U. M., W. S. Denney, et al. (2008). "Determination of surface tissue factor thresholds that trigger coagulation at venous and arterial shear rates: amplification of 100 fM circulating tissue factor requires flow." Blood 111(7): 3507-13.

Osterud, B. and S. I. Rapaport (1977). "Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation." Proc Natl Acad Sci U S A 74(12): 5260-4.

Panes, O., V. Matus, et al. (2007). "Human platelets synthesize and express functional tissue factor." Blood 109(12): 5242-50.

Panizzi, P., R. Friedrich, et al. (2006). "Novel fluorescent prothrombin analogs as probes of staphylocoagulase-prothrombin interactions." J Biol Chem 281(2): 1169-78.

Panteleev, M. A., M. V. Ovanesov, et al. (2006). "Spatial propagation and localization of blood coagulation are regulated by intrinsic and protein C pathways, respectively." Biophys J 90(5): 1489-500.

Paul, C. C., M. Tolbert, et al. (1993). "Cooperative effects of interleukin-3 (IL-3), IL-5, and granulocyte-macrophage colony-stimulating factor: a new myeloid cell line inducible to eosinophils." Blood 81(5): 1193-9.

Pawlinski, R., J. G. Wang, et al. (2010). "Hematopoietic and nonhematopoietic cell tissue factor activates the coagulation cascade in endotoxemic mice." Blood 116(5): 806-14.

Pickering, W., E. Gray, et al. (2008). "Effects of apoptosis and lipid peroxidation on T-lymphoblastoid phospholipid-dependent procoagulant activity." J Thromb Haemost 6(7): 1122-30.

Pickering, W., E. Gray, et al. (2004). "Characterization of the cell-surface procoagulant activity of T-lymphoblastoid cell lines." J Thromb Haemost 2(3): 459-67.

Poitevin, S., E. Cochery-Nouvellon, et al. (2007). "Monocyte IL-10 produced in response to lipopolysaccharide modulates thrombin generation by inhibiting tissue factor expression and release of active tissue factor-bound microparticles." Thromb Haemost 97(4): 598-607.

Pratt, C. W. and D. M. Monroe (1992). "Microplate coagulation assays." Biotechniques 13(3): 430-3.

Radomski, M. W., R. M. Palmer, et al. (1987). "Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium." Lancet 2(8567): 1057-8.

Ratnoff, O. D. and B. Bennett (1973). "The genetics of hereditary disorders of blood coagulation." Science 179(4080): 1291-8.

Rickles, F. R. and R. L. Edwards (1983). "Activation of blood coagulation in cancer: Trousseau's syndrome revisited." Blood 62(1): 14-31.

Rickles, F. R. and M. N. Levine (2001). "Epidemiology of thrombosis in cancer." Acta Haematol 106(1-2): 6-12.

Ritis, K., M. Doumas, et al. (2006). "A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways." J Immunol 177(7): 4794-802.

Roehrig, S., A. Straub, et al. (2005). "Discovery of the novel antithrombotic agent 5-chloro-N-({(5S)-2-oxo-3- [4-(3-oxomorpholin-4-yl)phenyl]-1,3-oxazolidin-5-yl}methyl)thiophene- 2-carboxamide (BAY 59-7939): an oral, direct factor Xa inhibitor." J Med Chem 48(19): 5900-8.

Rosendaal, F. R. and P. H. Reitsma (2009). "Genetics of venous thrombosis." J Thromb Haemost 7 Suppl 1: 301-4.

Saito, T., T. Koyama, et al. (1996). "Anticoagulant effects of retinoic acids on leukemia cells." Blood 87(2): 657-65.

Salomon, O., D. M. Steinberg, et al. (2008). "Reduced incidence of ischemic stroke in patients with severe factor XI deficiency." Blood 111(8): 4113-7.

Sampson-Johannes, A., W. Wang, et al. (1996). "Colonization of human lung grafts in SCID-hu mice by human colon carcinoma cells." Int J Cancer 65(6): 864-9.

Savage, B., E. Saldivar, et al. (1996). "Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor." Cell 84(2): 289-97.

Scharf, R. E. and W. Schneider (1990). "Relationship of thrombin generation to peripheral blast cell count in patients with acute myeloblastic leukemia (AML)." Eur J Haematol 44(5): 273-6.

Shaw, A. W., V. S. Pureza, et al. (2007). "The local phospholipid environment modulates the activation of blood clotting." J Biol Chem 282(9): 6556-63.

Shearer, M. J. (1990). "Vitamin K and vitamin K-dependent proteins." Br J Haematol 75(2): 156-62.

Shen, F., R. R. Pompano, et al. (2009). "Confinement regulates complex biochemical networks: initiation of blood clotting by "diffusion acting"." Biophys J 97(8): 2137-45.

Shu, F., H. Kobayashi, et al. (2000). "Activated protein C suppresses tissue factor expression on U937 cells in the endothelial protein C receptor-dependent manner." FEBS Letters 477(3): 208-212.

Siebenlist, K. R., D. A. Meh, et al. (2001). "Protransglutaminase (factor XIII) mediated crosslinking of fibrinogen and fibrin." Thromb Haemost 86(5): 1221-8.

Sorensen, H. T., L. Mellemkjaer, et al. (2000). "Prognosis of cancers associated with venous thromboembolism." N Engl J Med 343(25): 1846-50.

Sorensen, H. T., L. Mellemkjaer, et al. (1998). "The risk of a diagnosis of cancer after primary deep venous thrombosis or pulmonary embolism." N Engl J Med 338(17): 1169-73.

Staindl, O. (1979). "The healing of wounds and scar formation under the influence of a tissue adhesion system with fibrinogen, thrombin, and coagulation factor XIII." Arch Otorhinolaryngol 222(4): 241-5.

Stenflo, J. (1972). "Vitamin K and the biosynthesis of prothrombin. II. Structural comparison of normal and dicoumarol-induced bovine prothrombin." J Biol Chem 247(24): 8167-75.

Stoffel, N., C. Rysler, et al. (2010). "Leukocyte count and risk of thrombosis in patients undergoing haematopoietic stem cell transplantation or intensive chemotherapy." Thromb Haemost 103(6): 1228-32.

Sunnerhagen, M., S. Forsen, et al. (1995). "Structure of the Ca(2+)-free Gla domain sheds light on membrane binding of blood coagulation proteins." Nat Struct Biol 2(6): 504-9.

Suomela, H. (1976). "Human coagulation factor IX. Isolation and characterization." Eur J Biochem 71(1): 145-54.

Svensson, P. J. and B. Dahlback (1994). "Resistance to activated protein C as a basis for venous thrombosis." N Engl J Med 330(8): 517-22.

Sweeney, J. D. and L. A. Hoernig (1993). "Age-dependent effect on the level of factor IX." Am J Clin Pathol 99(6): 687-8.

Tafur, A. J., H. Kalsi, et al. (2011). "The association of active cancer with venous thromboembolism location: a population-based study." Mayo Clin Proc 86(1): 25-30.

Takemori, N., K. Hirai, et al. (1993). "Disseminated intravascular coagulation in a patient with acute myeloid leukemia. Ultrastructural evidence of hypercoagulation in bone marrow." Am J Clin Pathol 99(6): 695-701.

Tanaka, M. and H. Yamanishi (1993). "The expression of tissue factor antigen and activity on the surface of leukemic cells." Leuk Res 17(2): 103-11.

Tesselaar, M. E., F. P. Romijn, et al. (2007). "Microparticle-associated tissue factor activity: a link between cancer and thrombosis?" J Thromb Haemost 5(3): 520-7.

Thaler, J., C. Ay, et al. (2011). "Circulating procoagulant microparticles in cancer patients." Ann Hematol 90(4): 447-53.

Thiagarajan, P. and J. F. Tait (1991). "Collagen-induced exposure of anionic phospholipid in platelets and platelet-derived microparticles." J Biol Chem 266(36): 24302-7.

Tian, M., Y. Wan, et al. (2011). "Depletion of tissue factor suppresses hepatic metastasis and tumor growth in colorectal cancer via the downregulation of MMPs and the induction of autophagy and apoptosis." Cancer Biol Ther 12(10): 896-907.

Tormoen, G. W., S. Rugonyi, et al. (2011). "The role of carrier number on the procoagulant activity of tissue factor in blood and plasma." Phys Biol 8(6): 066005.

Tracy, P. B., L. L. Eide, et al. (1985). "Human prothrombinase complex assembly and function on isolated peripheral blood cell populations." J Biol Chem 260(4): 2119-24.

Trousseau, A. (1865). "Phlegmasia alba dolens." Clinique Medicale de l'Hotel-Dieu de Paris (Paris, France: The Syndenham Society): 654-712.

Tucker, E. I., U. M. Marzec, et al. (2009). "Prevention of vascular graft occlusion and thrombus-associated thrombin generation by inhibition of factor XI." Blood 113(4): 936-44.

Utsugi, T., A. J. Schroit, et al. (1991). "Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes." Cancer Res 51(11): 3062-6.

Van Dam-Mieras, M. C., C. A. Bruggeman, et al. (1987). "Induction of endothelial cell procoagulant activity by cytomegalovirus infection." Thromb Res 47(1): 69-75.

von Ahsen, N., P. Lewczuk, et al. (2000). "Prothrombin activity and concentration in healthy subjects with and without the prothrombin G20210A mutation." Thromb Res 99(6): 549-56.

Wagner, C. L., M. A. Mascelli, et al. (1996). "Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets." Blood 88(3): 907-14.

Walker, F. J., P. W. Sexton, et al. (1979). "The inhibition of blood coagulation by activated Protein C through the selective inactivation of activated Factor V." Biochim Biophys Acta 571(2): 333-42.

Wallentin, L., S. Yusuf, et al. (2010). "Efficacy and safety of dabigatran compared with warfarin at different levels of international normalised ratio control for stroke prevention in atrial fibrillation: an analysis of the RE-LY trial." Lancet 376(9745): 975-83.

Wang, J., I. Weiss, et al. (2001). "Thrombogenic role of cells undergoing apoptosis." Br J Haematol 115(2): 382-91.

Welsh, J., J. D. Smith, et al. (2012). "Tissue factor expression determines tumour cell coagulation kinetics." Int J Lab Hematol 34(4): 396-402.

White-Adams, T. C., M. A. Berny, et al. (2010). "Laminin promotes coagulation and thrombus formation in a factor XII-dependent manner." J Thromb Haemost 8(6): 1295-301.

White-Adams, T. C., M. A. Berny, et al. (2009). "Identification of coagulation factor XI as a ligand for platelet apolipoprotein E receptor 2 (ApoER2)." Arterioscler Thromb Vasc Biol 29(10): 1602-7.

Whitmore, R. (1968). Rheology of the Circulation. Oxford, UK, Pergamon Press.

Xu, C., Q. Gui, et al. (2011). "Small interference RNA targeting tissue factor inhibits human lung adenocarcinoma growth in vitro and in vivo." J Exp Clin Cancer Res 30: 63.

Yan, J., K. Wang, et al. (2010). "PML/RARalpha fusion protein transactivates the tissue factor promoter through a GAGC-containing element without direct DNA association." Proc Natl Acad Sci U S A 107(8): 3716-21.

Yang, H. C. (1978). "Immunologic studies of factor IX (Christmas factor). II. Immunoradiometric assay of factor IX antigen." Br J Haematol 39(2): 215-24.

Yates, K. R., J. Welsh, et al. (2011). "Pancreatic cancer cell and microparticle procoagulant surface characterization: involvement of membrane-expressed tissue factor, phosphatidylserine and phosphatidylethanolamine." Blood Coagul Fibrinolysis 22(8): 680-7.

Zacharski, L. R., V. A. Memoli, et al. (1986). "Coagulation-cancer interaction in situ in renal cell carcinoma." Blood 68(2): 394-9.

Zacharski, L. R., A. R. Schned, et al. (1983). "Occurrence of fibrin and tissue factor antigen in human small cell carcinoma of the lung." Cancer Res 43(8): 3963-8.

Zhang, H., E. C. Lowenberg, et al. (2010). "Inhibition of the intrinsic coagulation pathway factor XI by antisense oligonucleotides: a novel antithrombotic strategy with lowered bleeding risk." Blood 116(22): 4684-92.

Zhang, R. D., I. J. Fidler, et al. (1991). "Relative malignant potential of human breast carcinoma cell lines established from pleural effusions and a brain metastasis." Invasion Metastasis 11(4): 204-15.

Zhou, H., H. Hu, et al. (2008). "The expression and the functional roles of tissue factor and protease-activated receptor-2 on SW620 cells." Oncol Rep 20(5): 1069-76.

Zhou, J., J. Shi, et al. (2010). "Phosphatidylserine exposure and procoagulant activity in acute promyelocytic leukemia." J Thromb Haemost 8(4): 773-82.

Ziegler, S., W. R. Sperr, et al. (2005). "Symptomatic venous thromboembolism in acute leukemia. Incidence, risk factors, and impact on prognosis." Thromb Res 115(1-2): 59-64.

Zwicker, J. I., H. A. Liebman, et al. (2009). "Tumor-derived tissue factor-bearing microparticles are associated with venous thromboembolic events in malignancy." Clin Cancer Res 15(22): 6830-40.

## Biographical Sketch

Garth William Tormoen was born on August 4, 1980 in Wisconsin Rapids, Wisconsin to Lynne and Terrence Tormoen. On March 25, 2008, he was married to Ann Marie Bozung. On March 1, 2011, Ann and Garth's son Graham Sheldon was born in Portland, Oregon.

Garth attended Port Edwards High School, and upon graduation, enrolled in Michigan Technological University (MTU). As an undergraduate, he joined the lab of Dr. Jaroslaw Drelich. Garth was a member of an undergraduate research team that was awarded the TMS Student Design Competition Award for a project to develop novel lead-free solder formulations that was sponsored by Industrial Business Machines (IBM®). In June of 2002, he received his Bachelor of Science degree in Materials Science and Engineering.

Garth continued his education as a graduate student at MTU under the guidance of Dr. Drelich. He performed research aimed at developing a novel technique utilizing Atomic Force Microscopy (AFM) to measure surface free energy of nanoscale materials. He spent the summer of 2003 at Argonne National Lab in the laboratory of Dr. Jeff Eastman. In June of 2004, he received his Master of Science degree in Materials Science and Engineering from MTU.

Garth spent the summer of 2004 backpacking along the Appalachian Trail with his older brother Craig. His journey began at Springer Mountain, Georgia and finished at Mount Katadhin, Maine. Of note, Garth spotted seven black bears, one bobcat, thirteen Eastern Diamondback rattlesnakes, two Copperhead snakes and Kevin Bacon while on the trail.

Garth moved to San Antonio, Texas in December of 2004 where he was hired as an engineer at Southwest Research Institute (SwRI®). Garth was mentored by Dr. C. Sean Brossia and Dr. Narasi Sridhar, and became involved in a number of research and development projects.

Garth enrolled in the Medical Scientist Training Program at Oregon Health & Science University (OHSU) in the Fall of 2008. He joined the laboratory of Dr. Owen McCarty in the Department of Biomedical Engineering in June 2009, where his work focused on physical determinants for the procoagulant phenotype of circulating cancer cells.

During his graduate studies at OHSU, Garth was awarded an Achievement Rewards for College Scientists scholarship, a Young Investigator Award from the International Society on Thrombosis and Haemostasis, and the American Heart Association WSA Joel Drillings Award for Excellence in Cardiovascular Research. Garth has received research funding through an American Heart Association predoctoral fellowship. Garth has presented his research in peer-reviewed journals and at conferences in the U.S. and Asia. Current publications are listed below:

**Publications**


1. Beach ER, **Tormoen GW**, Drelich J, Han R. "Pull-off force measurements between rough surfaces by atomic force microscopy". *J Colloid Interface Sci*. 2002; 247: 84-99.

2. Beach ER, **Tormoen G**, Drelich J. "Pull-off forces measured between hexadecanethiol self-assembled monolayers in air using an atomic force microscope: analysis of surface free energy". *J Adhesion Sci Technol*. 2002; 16: 845-68.

3. Drelich J, **Tormoen GW**, Beach ER. "Determination of solid surface tension from particle-substrate pull-off forces measured with the atomic force microscope". *J Colloid Interface Sci*. 2004; 280: 484-97.

4. **Tormoen GW,** Drelich J, Beach ER. "Analysis of atomic force microscope pull-off forces for gold surfaces portraying nanoscale roughness and specific chemical functionality". *J Adhesion Sci Technol*. 2004; 18: 1-18.

5. **Tormoen GW,** Drelich J. "Deformation of soft colloidal probes during AFM pull-off force measurements: elimination of nano-roughness effects". *J Adhesion Sci Technol*. 2005; 19: 181-98.

6. **Tormoen GW,** Drelich J, Nalaskowski J. "A distribution of AFM pull-off forces for glass microspheres on a symmetrically structured rough surface". *J Adhesion Sci Technol*. 2005; 19: 215-34.

7. **Tormoen G,** Burkett J, Dante JF, Sridhar N. "Monitoring the adsorption of volatile corrosion inhibitors in real time with surface-enhanced raman spectroscopy". *Corrosion*. 2006; 62: 1082-91.

8. Anderko A, Sridhar N, Jakob MA, **Tormoen G**. "A general model for the repassivation potential as a function of multiple aqueous species. 2. Effect of oxyanions on localized corrosion of Fe-Ni-Cr-Mo-W-N alloys". *Corrosion Science*. 2008; 50: 3629-47.

9. Sridhar N, **Tormoen G**, Hackney S, Anderko A. “Effect of Aging Treatments on the Repassivation Potential of Duplex Stainless Steel S32205”. *Corrosion*. 2009; 65: 650-62.

10. **Tormoen G,** Sridhar N, Anderko A. “Localised corrosion of heat treated alloys Part 1 - repassivation potential of alloy 600 as a function of solution chemistry and thermal aging”. *Corrosion Engineering, Science and Technology*. 2010; 45: 155-62. Awarded the 2011 Institute of Metals, Materials and Mining Guy Bengough award for a paper published by the Institute which makes an outstanding contribution to the subject of corrosion and degradation of all types of materials and their control.

11. Anderko A, Sridhar N, **Tormoen G**. “Localised corrosion of heat-treated alloys Part II Predicting grain boundary microchemistry and its effect on repassivation potential”. *Corrosion Engineering, Science and Technology*. 2010; 45: 204-23.

12. McCarty OJ, Conley RB, Shentu W, **Tormoen GW**, Zha D, Xie A, Qi Y, Zhao Y, Carr C, Belcik T, Keene DR, de Groot PG, Lindner JR. “Molecular imaging of activated von Willebrand factor to detect high-risk atherosclerotic phenotype”. *JACC Cardiovasc Imaging*. 2010; 3: 947-55.

13. Berny-Lang MA, Aslan JE, **Tormoen GW**, Patel IA, Bock PE, Gruber A, McCarty OJ, “Promotion of experimental thrombus formation by the procoagulant activity of breast cancer cells”. *Phys Biol*. 2011; 8(1) 015014 (1-7).

14. Aslan JE, **Tormoen GW**, Loren CP, Pang J, McCarty OJ, “S6K1 and mTOR regulate Rac1-driven platelet activation and aggregation”. *Blood*, 2011; 118(11): 3129-36.

15. **Tormoen GW**, Rugonyi S, Gruber A, McCarty OJ, “Role of carrier number on the procoagulant activity of tissue factor in blood and plasma”. *Phys Biol*, 2011; 8: 066005(1-7).

16. Lee AM, **Tormoen GW**, Kanso E, McCarty OJ and Newton PK (2012) Modeling and simulation of procoagulant circulating tumor cells in flow. *Front. Oncol.* 2012; 2: 108(1-9).

17. **Tormoen GW**, Haley KM, Levine RL, McCarty OJ. Do circulating tumor cells play a role in coagulation and thrombosis? *Front. Oncol.* 2012; 2: 115 (1-5).

18. **Tormoen GW**, Cianchetti FA, Bock PE and McCarty OJ (2012) Development of coagulation factor probes for the identification of procoagulant circulating tumor cells. *Front. Oncol.* 2012; 2: 110 (1-12).

19. Larson MK, **Tormoen GW**, Patel IA, Hjelmen CE, Ensz NM, McComas LS, McCarty OJ. Exogenous modification of platelet membranes with the omega-3 fatty acids EPA and DHA reduces platelet procoagulant activity and thrombus formation. *American Journal of Physiology: Cell Physiology*: 2013; 304(3): C273-9.

20. Liu Y, Davidson BP, Yue Q, Belcik T, Xie A, Inaba Y, McCarty OJ, **Tormoen GW**, Zhao Y, Ruggeri ZM, Kaufmann BA, Lindner JR. Molecular imaging of inflammation and platelet adhesion in advanced atherosclerosis effects of antioxidant therapy with NADPH oxidase inhibition. *Circ: Cardiovasc. Imaging*. 2013; 6(1): 74-82.

21. PS-OC Cell-Line Project Team and the PS-OC Network. A physical sciences network characterization of nonmalignant and metastatic cells. *Sci. Rep*. 2013; 3: 1449.

22. **Tormoen GW**, Khader A, Gruber A, and McCarty OJ. Physiological levels of blood coagulation factors IX and X control coagulation kinetics in an *in vitro* model of circulating tissue factor. *Phys Biol*. 2013; 10(3): 036003.

23. Aslan JE, Itakura A, Haley KM, **Tormoen GW**, Loren CP, Baker SM, Pang J, Chernoff J, McCarty OJ. p21-activated kinase (PAK) signaling coordinates GPVI-mediated platelet aggregation, lamellipodia formation and aggregate stability under shear. *Arterioscler Thromb & Vasc Biol*. 2013 (In Press).

24. Colace TV, **Tormoen GW**, McCarty OJ, Diamond SL. Microfluidics and coagulation biology. *Ann.Rev. Biomed. Eng.* 2013; 283: 283-303.

**Presentations**

1. Buckmaster KL, Dziedzic JJ, Masters MA, Poquette BD, **Tormoen GW**, Swenson D, Henderson DW, Gosselin T, Kang SK, Shih DY and Puttlitz K. “The Effects of Minor Zn Alloying Additions on the Solidification Behavior and Microstructure of Near-Eutectic SN-Ag-Cu Solder Joints”, TMS 2003 Fall Meeting, Chicago, IL (NOV 2003).

2. **Tormoen G** and Drelich J, “Roughness Considerations for Adhesion Characteristics at the Nanoscale”, SME Annual Meeting, Denver, CO (FEB 2004).

3. **Tormoen G**, Dante J and Sridhar N, “Correlation of In-Situ VCI Adsorption Monitoring with Real-Time Corrosion Rate Measurements” NACE International CORROSION Meeting, Nashville, TN (MAR 2007).

4. **Tormoen GW**, Berny MA, Aslan JE and McCarty OJ, “Characterization of the Procoagulant Activity of Tumor Cells”, Physical Sciences-Oncology Center TSRI site visit, La Jolla, CA (OCT, 2010).

5. **Tormoen GW**, Gruber A and McCarty OJ, “Procoagulant Activity of Intravascular Tissue Factor is Dependent on Carrier Burden”, XXIIIrd Congress of the International Society on Thrombosis and Haemostasis, Kyoto, JP (JUL, 2011).

6. **Tormoen GW** and McCarty OJ, “Regulatory mechanisms of the procoagulant activity of circulating tumor cells”, Physical Sciences-Oncology Center TSRI site visit, La Jolla, CA (OCT, 2011).

7. **Tormoen GW**, Gruber A and McCarty OJ, "Spatial Separation of Tissue Factor-Carriers Modulates Procoagulant Activity of Circulating Tissue Factor", Annual Meeting of the American Society for Hematology, San Diego, CA (DEC, 2011).

8. **Tormoen GW** and McCarty OJ, "Characterizing the role of blood coagulation factors on cancer cell migration", 3rd Annual NCI Physical Sciences – Oncology Center Conference, Tampa, FL (APR, 2012).

9. **Tormoen GW** and McCarty OJ, "Characterizing the role for coagulation factor concentrations on susceptibility to CTC-induced coagulation, 3rd Annual NCI Physical Sciences – Oncology Center Conference, Tampa, FL (APR, 2012).

10. **Tormoen GW**, Cianchetti F, McCarty OJ, "Development of Coagulation Factor Probes for the Identification of Procoagulant Cancer Cells", Physical Sciences – Oncology Center TSRI site visit, La Jolla, CA (OCT, 2012).

11. **Tormoen GW**, Cianchetti F, McCarty OJ, "Development of Coagulation Factor Probes for the Identification of Procoagulant Cancer Cells", OHSU Knight Cancer Institute Annual Retreat, Skamania, WA (OCT, 2012).